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Human induced pluripotent stem cells as a source of insulin-producing cells for cell therapy of diabetes

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CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

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Data Firma

ABSTRACT

BACKGROUND

New sources of insulin-secreting cells are strongly required for the cure of diabetes. Recent successes in differentiating embryonic stem cells, in combination with the discovery that it is possible to derive human induced pluripotent stem cells (iPSC) from somatic cells, have raised the possibility that patient-specific β cells might be derived from patients through cell reprogramming and differentiation.

AIMS

In this study, we aimed to obtain insulin-producing cells from human iPSC and test their ability to secrete insulin *in vivo*.

METHODS:

Human iPSC, derived from both fetal and adult fibroblasts, were differentiated *in vitro* into pancreas-committed cells and their ability to secrete insulin was measured. iPSC-derived cells at two different stages of differentiation (posterior foregut and endocrine cells) were transplanted into immunodeficient mice to test their ability to engraft, differentiate and secrete insulin.

RESULTS:

IPSC were shown to differentiate into insulin-producing cells *in vitro*, following the stages of pancreatic organogenesis. At the end of the differentiation, the production of INSULIN mRNA was highly increased and up to 14% of the cell population became insulin-positive. Terminally differentiated cells also produced C-peptide *in vitro* in both basal and stimulated conditions. *In vivo*, mice transplanted with pancreatic cells secreted human C-peptide in response to glucose stimulus, but transplanted cells were observed to lose insulin secretion capacity during the time. At histological evaluation, the grafts were composed of a mixed population of cells containing mature pancreatic cells, but also pluripotent cells and rare neuronal cells.

CONCLUSION:

These data overall suggest that human iPSC have the potential to generate insulin-producing cells and that these differentiated cells can engraft and secrete insulin *in vivo.*

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I. ACRONYMS AND ABBREVIATIONS

iPSC: induced Pluripotent Stem Cells

WHO: World Health organization

T1D: Type 1 Diabetes

HbA1c: Glycated Hemoglobin

CITR: Collaborative Islet Transplant Registry

GH: Growth Hormone

GLP-1: Glucagon-like Peptide 1

HGF: Hepatocyte Growth Factor

Pdx-1: Pancreatic and duodenal homeobox 1

Gal: Galactose α1,3Galactose

PERV: Porcine Endogenous Retrovirus

ESC: Embryonic Stem Cells

HSC: Hematopoietic Stem Cells

MSC: Mesenchymal Stem Cells

GFP: Green Fluorescence Protein

FGF-β: Fibroblast Growth Factor-β

EGF: Endothelial Growth Factor

PKC: Protein Kinase C

TGFβ: Transforming Growth Factor β

MEFs: Murine Fetal Fibroblasts

bFGF: basic Fibroblast Growth Factor

AMD: Age-related Macular Degeneration

HLA: Human Leucocyte Antigen

FBS: Fetal Bovine Serum

DE: Definitive Endoderm

PG: Primitive Gut Tube

CYC: KAAD-Cyclopamine

PF: Posterior Foregut

GLP-1: Glucagon-like peptide-1

DAPT: N-[N-(3,5-Difluorophenacetyl)-L-alanyl] -S-phenylglycine t-butylester

NGN3: Neurogenin3

EN: Hormone-expressing Endocrine

IGF-1: Insulin-like growth factor 1

HGF: Hepatocyte growth factor

IL V: Indolactam V

EB: Embryoid Bodies

AU: Arbitrary Units

SeV: Sendai Virus

ME: Mesendoderm

PE: Pancreatic Endoderm

BM: Basal Medium

BSA: Bovine Serum Albumin

KGF: Keratinocyte Growth Factor

ITS: Insulin-Transferrin-Selenium

PdBU: Phorbol 12,13-dibutyrate

T_3: L-3,3',5-Triiodothyronine

ddPCR: Droplet Digital PCR

DAVID: Database for Annotation, Visualization and Integrated Discovery

FDR: False Discovery Rate

FACS: Fluorescence-activated cell sorting

ELISA: Enzyme-Linked Immunosorbent Assay

OGTT: Oral Glucose Tolerance Test

AP: Alkaline Phosphatase

FC: Fold Change

SEM: Standard Error of the Mean

IBMX: phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine

KCl: potassium chloride

SSC: Side Scatter

APC: Allophycocyanin

PE: phycoerythrin

MODY: Maturity onset diabetes of the young

II. INTRODUCTION

1. Type 1 Diabetes

In 2014 The World Health Organization (WHO) estimates that there were 387 million people suffering from diabetes worldwide, a number that is predicted to increase to 592 million by 2035 (Whiting et al., 2011). Approximately 10% of these cases are of type 1 diabetes (T1D) (Stanekzai et al., 2012), a disease characterized by an absolute deficiency of insulin-producing pancreatic β-cells caused by an autoimmune-mediated destruction.

Administration of exogenous insulin, regular blood glucose monitoring and dietary restrictions are the fundamental means of treating hyperglycemia in all patients with T1D. Although life-saving, insulin therapy does not restore the physiological regulation of blood glucose and is not able to prevent either the dangerous states of hypoglycemia or long-term complications like ketoacidosis, kidney failure, cardiovascular diseases, neuropathy and retinopathy (van Belle et al., 2011). Despite new technologies like slow-release insulin or insulin pumps have been developed in the last years and have substantially improved glycemic control as well as the quality of life of diabetic patients (Saudek et al., 2013), a fail-safe physiological regulation of systemic blood glucose levels remains challenging. The only possible definitive cure for this disease consists in providing a new β cell source capable of performing two essential functions: assessing blood sugar levels and secrete insulin in a glucose-dependent manner.

2. β cell replacement with β cells

2.1 Allogeneic adult cells

At present, only pancreas or islet transplantation offer an alternative treatment option through restoration of the physiological response to changes in blood glucose levels. Whole pancreas transplantation is very effective in achieving insulin independence and in maintaining long-term physiological glycemic control: currently, graft survival for pancreas transplants alone is 82% at 1 year and 58% at 5 years, and these numbers are increased to 89% and 71% respectively for dual pancreas and kidney transplants (Redfield et al., 2015). However, because of the significant morbidity associated with this major surgery, this therapeutic approach is almost exclusively limited to diabetic

patients who also suffer for end-stage renal disease undergoing a simultaneous kidney transplant (Ichii & Ricordi, 2009). By contrast, islet transplantation requires a minimally invasive surgical procedure in which islet preparations are infused into the recipient liver through the portal vein, using a percutaneous radiologic procedure (Venturini et al., 2005). A functional transplant in a T1D patient can eliminate hypoglycemic episodes, correct glycated hemoglobin (HbA1c), reduce or reverse risk of secondary complications associated with the disease and, in the best cases, lead to insulin independence achievement (Barton et al., 2012). The first attempt of an islets transplant as a treatment for diabetes could be considered that of Dr. Watson-Williams and Dr. Harshant in 1893, when they transplanted small fragments of a sheep pancreas into a young patient with diabetic ketoacidosis who died after few days (Williams et al., 1894). In this case, no attempt to purify the islets was performed. The first evidence of the effectiveness of islet isolation and transplantation was reported only in 1972 by Ballinger and Lacy in chemically induced diabetic rats (Ballinger & Lacy, 1972), with Kemp et al. establishing the liver as the most suitable site for islet implantation (Kemp et al., 1973). Five years later the first islet infusion in human was performed, with azathioprine and corticosteroid as immunosuppressive drugs (Najarian et al., 1977). Since then, many efforts and significant progress have been achieved in the field of islet transplantation, in terms of human islets isolation (Ricordi et al., 1988), immunosuppression strategies (Oberholzer et al., 2000; Hering et al., 1994) and optimal number of transplanted islets per kilograms of body weight (Secchi et al., 1997). The knowledge deriving from these experiences led to the appearance in 2000 of the "Edmonton protocol", that was then adopted by all the centers of islet transplantation in the world; that year indeed Shapiro et al. published a success rate of 100% at 1 year in seven out of seven consecutive T1D patients, introducing several novelties to the procedures, above all the use of a glucocorticoid-free immunosuppressive regimen (Shapiro et al., 2000). Since then, the outcomes and safety of human islet allografts have steadily improved through the past few years. As recently reported by the Collaborative Islet Transplant Registry (CITR), insulin independence at 3 years after transplant improved from 27% in the early era (1999–2002) to 37% in the middle phase (2003–2006) and to 44% in the most recent era (2007–2010) (Barton et al., 2012). Moreover, five independent centers (Edmonton, Minnesota, Geneva, Milan and Lille)

are reporting a 5-year rate of insulin independence exceeding 50% (Shapiro et al., 2011), closely matching the results of pancreas-alone transplantation from the International Pancreas Transplant Registry. Islet transplantation is currently being fully reimbursed under non-research, clinical care streams in several countries including Canada, United Kingdom, Sweden and the Nordic Network, Swiss and Australia. At present however, islet transplantation is far from being a standard procedure because of two main problems: the need for lifelong immunosuppression (with multiple adverse side-effects) and the lack of pancreases from heart-beating brain-dead donors (the only suitable source of human islets for clinical use). For these reasons, islet transplantation is presently restricted to diabetic patients showing unexplained metabolic instability despite carefully monitored insulin therapy, complicated by recurrent hypoglycemic events (Bertuzzi et al., 2007).

In this scenario, a novel strategy to address the problem of how to reconstitute pancreatic endocrine function in diabetic patients is clearly needed. Many approaches are currently being studied intensively, in particular β cell proliferation/regeneration, xenotransplantation and differentiation of pluripotent stem cells (**Figure 1**).

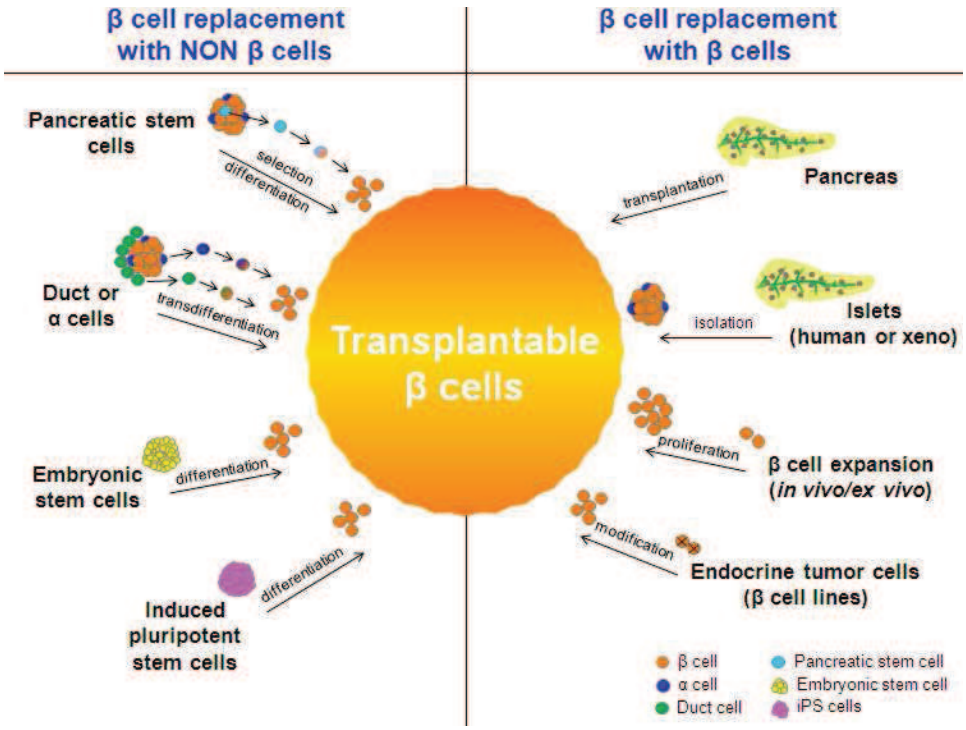

Figure 1. The most promising sources and the related strategies currently studied in order to obtain a large amount of transplantable β cells. *Pellegrini, 2013.*

2.2 Autologous cells (β cell proliferation or trans-differentiation *in vivo/ex vivo*)

Unlike blood, skin or intestine, that are tissues with a relatively rapid turnover of cells, β cells in the pancreatic islets are a quiescent population with a proliferative ratio of 0,1-0,3%/day in 1-year-old mice (Teta et al., 2005). Recent studies, however, have shown that also β cells mass is regulated dynamically and the relation between replication and apoptosis can determine the final mass (P. C. Butler et al., 2007; Lipsett et al., 2006). In human, normal expansion of the β cell mass occurs during the neonatal period, but fades early in childhood (Meier et al., 2008); in adult, β cell replication resulted increased in some physiological or pathological states, such as pregnancy (Parsons et al., 1995) or an obesity-induced insulin-resistant state (Gupta et al., 2007). Thus, the use of external agents to expand β cells *ex vivo* for transplantation purpose or to stimulate endogenous cell proliferation *in vivo* in order to increase the β cells mass in diabetic patients may be an attractive approach for β cells supplementation. In fact, β cell regeneration has been observed also in T1D patients after onset (Willcox et al., 2010) or even many years after diagnosis (Pipeleers & Ling, 1992; Keenan et al., 2010). Moreover, Dor et al. in a lineage-tracing study in mice observed a dramatic increase in β cell mitotic index following pancreatic injury such as 50~70% pancreatectomy (Dor et al., 2004) or a selective β cell genetic ablation (Nir et al., 2007). Transfection of many cell cycle regulators like cdks (cycline dependent kinases) and cyclins into rodent and human islets *ex vivo*, leads to an increase in the replication rate of β cells (Cozar-Castellano et al., 2004; Fiaschi-Taesch et al., 2010), but the prolonged expression of these molecules would increase also the risk of oncogenesis. A safer option is represented by the addition in culture of growth factors, such as growth hormone (GH), glucagon-like peptide-1 (GLP-1) or hepatocyte growth factor (HGF), that have been described to increase the replication rate of rodent β cells (Nauck et al., 1993); unfortunately, in human the elevated proliferation is associated with a loss of β cell features, like Pdx-1 or insulin expression (Parnaud et al., 2008). An *in vivo* therapy with long-acting GLP-1 analogues (exenatide or liraglutide) has been considered to have a potential for the stimulation of β cell replication in diabetic patients after proof-of-concept studies performed in patients treated with GLP-1 (Nauck et al., 1993; Rachman et al., 1997), but long-term data of the evidence of such increase in patients have yet to

be provided. In the field of β cell proliferation, a gene therapy aimed at the reversible inclusion of genes capable of immortalizing β cells has been tried as well. During the past 30 years, a number of β cell lines have been established in rodent (Gazdar et al., 1980; Hohmeier & Newgard, 2004) and many attempts have been made to generate human β cell lines from many pancreatic sources, but insulin production by these cells was extremely low or limited at few passages (Levine et al., 1995; de la Tour et al., 2001). In 2005, Narushima et al. reported the successful establishment of a functional human β cell line, NAKT-15, that looked promising for cell therapy of diabetes, but no new reports on the utility of this line have been published since 2005 (Narushima et al., 2005). In 2011 another human β cell line was established transducing human fetal pancreases with a lentiviral vector that expressed SV40LT and human telomerase reverse transcriptase (hTERT). One of the cell lines generated with this strategy, the EndoC-βH1, was further characterized and resulted able to secrete insulin in response to glucose stimulation, was stable at least for 80 passages and expressed many specific β cell markers, without any substantial expression of markers of other pancreatic cell types (Ravassard et al., 2011). In view of clinical use, a second generation of human β cell lines has been recently developed; the conditionally immortalized EndoC-βH2 cell line is based on Cre-mediated excision of the immortalizing transgenes, leading to an arrest of cell proliferation and pronounced enhancement of β cell–specific features such as insulin expression, content, and secretion (Scharfmann et al., 2014), but further studies are required to determine the actual safety of these cells.

Another completely different point of view is the theory that neogenesis and not proliferation is the mechanism responsible for β cells-mass expansion in conditions like pregnancy or obesity. A recent autopsy study on human pancreata during or after pregnancy supports this hypothesis: Butler et al. observed the presence of more new small islets rather than an increase in β cell replication, islet size or change in apoptosis (Butler et al., 2010). They also observed an increased number of insulin positive cells within ducts, indicating that duct cells can differentiate in β cells in certain conditions or that pancreatic stem/progenitor cells are localized in pancreatic ducts. In previous works putative pancreatic stem cells have been localized also in exocrine cells and endocrine islets, suggesting a widespread distribution within the pancreas and that a precise characterization of these cells still lacks (Jones et al., 2008). Experiments of 90%

pancreatectomy in rats show the substantial regenerative capacity of the adult pancreas (Dor et al., 2004; Bonner-Weir et al., 1993) and in a recent work it was demonstrated that this regeneration follows a dedifferentiation-redifferentiation paradigm, in which mature duct cells dedifferentiate to a progenitor-like state and then differentiate to form all pancreatic cell types, including β cells (Li et al., 2010). Also in this work an increased proliferation rate of the remaining β cells was observed, indicating that replication and neogenesis are not mutually exclusive and they both contribute to maintain an adequate β cell mass after birth, but there are important differences in the balance of these two pathways depending on species and age (Bonner-Weir et al., 2010). The potential of α cells as possible source of insulin-producing cells has also been explored, since these cells are preserved in diabetic patients (Gianani et al., 2011) and are the most abundant endocrine cells in islets other than β cells. Collombat and colleagues have shown that the ectopic expression of Pax4 could force mature α cell conversion to β cells, reversing chemically induced diabetes in mice (Collombat et al., 2009). In addition, Thorel et al. confirmed the differentiation potential of α cells reporting their spontaneous conversion to new functional β cells using a selective diphtheria toxin-mediated β cells ablation model (Thorel et al., 2010). Whether this plasticity might exist in human is unknown, but experiments in chemically-induced diabetes in non-human primate didn't show evidence of β cells regeneration (Saisho et al., 2011).

2.3 Xenogeneic cells

Using islets of Langerhans derived from other species seems an obvious way of providing the large amount of islets required for transplantation therapy of diabetes. Most effort in this area has been directed towards the use of pig islets for many reasons: (i) porcine pancreas as a by-product of pork production has been used for years as an exogenous source of insulin before recombinant human insulin became available, (ii) porcine islets regulate glucose levels in the same physiologic range as humans, (iii) high islets yields can be obtained with techniques similar to those for human islet isolation and (iv) pigs can be genetically modified for making their islets more suitable for human transplantation (Klymiuk et al., 2010). Recent studies in nonhuman primates reported the long-term survival of neonatal (Cardona et al., 2006) or adult (Hering et al.,

2006) porcine islets in the presence of immunosuppression therapy and/or adoptive transfer of expanded autologous regulatory T cells (Shin et al., 2015). Two main problems have however limited the use of pig islets in humans. First, the risk of an hyperacute immunologic rejection, because humans have natural preformed antibodies that react to a saccharide, Galactose α1,3Galactose (Gal) expressed on cells of lower mammals but not on cells of humans or monkeys (Galili et al., 1988): the binding of antibodies to Gal antigens results in almost an immediate complement activation, with consequent destruction of the graft. Second, the risk of zoonosis because porcine endogenous retroviral (PERV) sequences can infect several human cells *in vitro* (Patience et al., 1997; Wilson et al., 1998) and may be activated after the xenotransplant (van der Laan et al., 2000). A strategy currently studied in order to overcome the problem of the immunogenicity of pig cells consists in islets microencapsulation; the cells can be enveloped within a biocompatible membrane (often of barium alginate) and, due to the molecular weight cutoff of the capsule material, cells are isolated from the host immune system (Rayat et al., 2000). Studies in both non-human-primate (Dufrane et al., 2010) and human recipients (Elliott et al., 2007) without immunosuppressive drugs were performed and, despite promising results, whether encapsulated islets will survive and function for long periods in human is unknown. Currently, two clinical trials using encapsulated porcine islets are ongoing in New Zealand (DIABECELL®) and in Russia and their findings are expected to be published imminently. No subjects, to our knowledge, have been rendered insulin free with such approaches to date (Moore SJ, 2015). In summary, encouraging results in extending the survival and the safety of transplanted pig islets have recently been obtained, but several issues must still be addressed and this strategy is far from an ideal option.

3. β cell replacement with non β cells

Currently, many opportunities for the cell therapy of single-cell disorders like diabetes are offered by stem cell differentiation. Stem cells are, by definition, undifferentiated cells that hold both the potential to differentiate into a large variety of specialized cell type and the ability to go through numerous cycles of cells division while maintaining their undifferentiated state (self-renewal). In mammals, there are two broad types of

stem cells: Embryonic stem cells (ESC), which are isolated from the blastocysts, and adult stem cells, which are found in various tissues (Calafiore & Basta, 2015).

3.1 Adult stem cells

Adult stem cells are multipotent progenitors that, by definition, can differentiate only in certain types of specialized cells, and are deputed to the maintenance, repair and reconstitution of the tissue in which they are found. For many years these cells were considered to be able to differentiate only in cells of the tissue/organ of origin, but subsequently it has been shown that adult stem cells can trans-differentiate into cells of other tissues (Davis et al., 1987). This opened the way for the use of stem cells of different types of tissues as a source of progenitor cells potentially able to become an autologous source of insulin-secreting cells. Since the identification of pancreatic stem cells is still controversial (Dor et al., 2004; Jiang & Morahan, 2014), many studies have focused their efforts on the use of bone marrow-derived stem cells, in particular hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC), as they are easily accessible and hold a remarkable cellular plasticity. In an initial study the ability of bone marrow stem cells to localize into pancreatic islets *in vivo* and to differentiate into insulin-expressing cells was demonstrated (Ianus et al., 2003), but this has not been confirmed by other groups. For instance, using GFP transgenic mice as donors, two groups evaluated the distribution of HSC in the pancreas after bone marrow transplantation and found that none of GFP-positive cells localized in islet or around the ducts expressed insulin, even after pancreatic injury (Choi et al., 2003; Lechner et al., 2004). In humans, a study analyzed 31 human pancreata obtained at autopsy from HSC transplant recipients who had received their transplant from a donor of the opposite sex, and no contribution of HSC to endocrine pancreas was observed (Butler et al., 2007). These studies support the hypothesis that trans-differentiation of bone marrow cells is not a significant mechanism for adult pancreatic β cell renewal. In addition, one study demonstrated that cell fusion rather than differentiation lies at the root of many processes of apparent bone marrow differentiation into ectodermal or endodermal tissues (Terada et al., 2002). *In vitro* multiple strategies involving exposure to various growth factor combinations under specific culture conditions, often augmented by genetic manipulation, were explored in order to differentiate HSC into insulin-

producing cells but, at this time, consensus exists that MSC but not HSC can be induced to exhibit pancreatic properties (Ciceri & Piemonti, 2010). Several studies reported, after the treatment with defined combinations of growth factor, the appearance of insulin mRNA in cultures of MSC derived not only from bone marrow but also from adipose tissue or cord blood cells (Timper et al., 2006; Thatava et al., 2006; Sun et al., 2007; Karnieli et al., 2007; Hisanaga et al., 2008; Chang et al., 2008; Xie et al., 2009; Bhandari et al., 2011; Dave et al., 2014; Qu et al., 2014). To give an example, recently a study was published about the differentiation of MSC into β cells with a differentiation protocol of 18 days which includes the addition of FGF-β, EGF, Activin A and β-cellulin. Differentiated cells formed cell clusters some of which resembled pancreatic islet, stained positive with dithizone and were able to produce C-peptide (Czubak et al., 2014). The limits of this and of many studies published before is that, at a deeper look, none of these differentiated cells exhibit the necessary conditions to be defined as β cells: insulin secretion in response to glucose stimuli and capacity to normalize glycemia in diabetic animal models. Moreover, safety is an issue when stem cells are forcedly converted in another cell type. For instance, in a study by Tang et al. islet-like MSC-derived cells expressed multiple genes related to islet development and β cell function, produced insulin, demonstrated time-dependent glucose-stimulated insulin release, and the ability to ameliorate hyperglycemia in chemically induced diabetic mice, but, when transplanted into diabetic immunocompromised mice, differentiated cells became tumorigenic (Tang et al., 2012). Recently, multiple studies supported the differentiation capacity into insulin-producing phenotypes also of other adult extra-pancreatic stem cell populations, like cells derived from liver, intestine, spleen, brain, dermis and mouse salivary glands (Limbert et al., 2008), but these studies have not proven to be reproducible. In conclusion, many tissues offer the possibility to derive progenitor cells able to differentiate into pancreatic beta-like cells, but until now none of the sources analyzed has proved to be capable of producing clinical-grade material, because of problems related to restricted proliferative capacity, low levels of insulin expression and poor, or non-existent, insulin secretion (Jones et al., 2008).

3.2 Embryonic stem cells

Embryonic stem cells (ESC) are considered to be the most promising source of cells for cell/organ replacement therapies due to their virtually endless proliferative capacity and their potential to differentiate into cells of all the three embryonic germ layers (pluripotency). Therefore, the development of ESC lines from the inner cell mass of early stage mouse embryos (Evans & Kaufman, 1981; Martin, 1981) and, 17 years later, human embryos (Thomson et al., 1998) offered the potential to generate any specialized cell type in large quantities, including insulin-producing cells. Initially Soria et al. observed that undifferentiated ESC are able to naturally express insulin, so they tried to select these cells using a technique called "cell-trapping" (transfecting the cells with a chimeric construct which couples insulin gene with a gene that confers drug resistance) and directed their differentiation by modulating culture conditions to obtain a β cell-like phenotype. The cells generated were able to secrete insulin *in vitro* in response to various stimuli but not to glucose (Soria et al., 2000). One year later another group developed an alternative approach, which consists in generating insulin-positive cells from murine Nestin-positive ESC colonies; Nestin is expressed in developing neurons, and islet and neural cells share phenotypic similarities (Zulewski et al., 2001). This type of approach led to the formation of cells capable of co-expressing all kinds of hormones produced by the cells in the pancreatic islets, but the final insulin content was very low (Lumelsky et al., 2001). Further progress has been made after the identification of the developmental cues that could induce ESC to replicate key aspects of the segregation of specific germ layers, as occurs during gastrulation in the normal embryo; since the pancreas is an endoderm-derived tissue, an important turning point was the induction of differentiation of ESC first into mesendoderm (progenitor of both endoderm and mesoderm), and subsequently into definitive endoderm (distinct from the extraembryonic visceral endoderm, which appears earlier and does not contribute to adult organ structures) (D'Amour et al., 2005). A major step forward came when the same group continued in a stepwise fashion to identify culture conditions and developmental signals that induce pancreatic organogenesis *in vivo,* in order to drive *in vitro* the further differentiation of human ES cell-derived definitive endoderm cells through subsequent stages on the desired path: posterior foregut, pancreatic endoderm, progenitors of endocrine pancreas and, finally, hormone-producing endocrine cells.

With their five-steps differentiation protocol the Baetge group (from Novocell, Inc., a preclinical stage stem cell engineering company focused on diabetes, that in 2010 changed its name into ViaCyte, Inc) succeeded to obtain from human ESC about 7% of cells that expressed high levels of proinsulin that was processed, albeit inefficiently, to insulin and C-peptide. Insulin secretion was not responsive to glucose levels, but could be increased by other compounds known to act on β-cells in fetal pancreas, which also respond poorly to glucose (D'Amour et al., 2006). Two other groups, using different culture conditions, confirmed that ESC are able to differentiate in insulin-producing cells, albeit with a lower efficiency (W. Jiang et al., 2007; J. Jiang et al., 2007). Subsequently Baetge and colleagues improved their results, optimizing their differentiation protocol and transplanting ESC-derived pancreatic progenitor cells into mice such that after three months *in vivo* the implanted cells differentiate into mature endocrine cells that can regulate blood glucose levels after diabetes induction (Kroon et al., 2008). They also identified CD142 (Tissue Factor) as a novel surface marker for the selection of pancreatic progenitor cells obtained through the differentiation of ESC; $CD142^{+}$ cells transplanted *in vivo* give rise to all the pancreatic lineages, including functional insulin-producing cells (Kelly et al., 2011). The same group recently developed a scalable and standardized system for the production of functional pancreatic progenitors from human ESC, further optimizing their differentiation protocol for the CyT49 ESC line (Schulz et al., 2012). Finally, October 29th, 2014 Viacyte announced the beginning of a Phase 1/2 clinical trial and that the first patient of this study was successfully implanted with ESC-derived insulin-producing cells delivered under the skin in a proprietary device with a selectively porous cell-impermeable membrane, called the Encaptra® drug delivery system; this device is designed to protect the implanted cells from possible immune rejection, to permanently contain the cells and prevent their distribution away from the implantation site, and to provide a platform for product vascularization. This is the first time that an embryonic stem cell-derived cell replacement therapy for diabetes is studied in human subjects, and it represents the culmination of a decade of effort by the ViaCyte team (http://viacyte.com). Meanwhile, modified or improved protocols have been established using combinations of cytokines and small molecules, such as many Fibroblast Growth Factors, Sonic hedgehog pathway inhibitors (KAAD-cyclopamine or SANT-1),

Retinoic Acid, Nicotinamide, protein kinase C (PKC) activator (Indolactam V) or TGFβ pathway inhibitors (Alk5 inhibitor, Dorsomorphin or Noggin) (Chen et al., 2009; Ameri et al., 2010; Mfopou et al., 2010; Nostro et al., 2011a; Xu et al., 2011; Rezania et al., 2012; Rezania et al., 2013; Nostro et al., 2015). Noteworthy are in particular the directed differentiation strategies reported by the research units of Melton and Kieffer (Pagliuca et al., 2014; Rezania et al., 2014). These two groups reported a novel and efficient approach to generate *in vitro* 20%–50% insulin (C-peptide)-positive cells from hESCs. Upon transplantation into immunocompromised mice, the graft (composed of endocrine and ductal cells) restored glycemia within 2 (Pagliuca et al., 2014) or 6 weeks (Rezania et al., 2014) after transplantation, a tremendous improvement compared with the 2-3 months period required after transplantation of hESC-derived pancreatic progenitors (Kroon et al., 2008). Nevertheless, the similarities and differences between β-like cells generated by all these groups remain to be elucidated by a direct comparison. Despite significant successes, three main problems still limit the use of ESC-derived insulin-producing cells. First, due to their pluripotency, undifferentiated cells give rise to teratoma formation *in vivo* and the transplantation of unselected differentiated cells would inevitably lead to tumorigenesis because of the presence of some residual undifferentiated cells (Kroon et al., 2008); several attempts have been made to identify surface markers able to select pancreatic progenitor cells (Kelly, Chan, L. A. Martinson, et al., 2011; Jiang et al., 2011) or to eliminate only pluripotent cells (Ben-David et al., 2013), but the safety of the selected cells requires further investigation. Another unsolved problem is related to the evidence that each ESC cell line has a different propensity to give rise to pancreatic cells (Osafune et al., 2008). Therefore many cell lines have to be tested (and, accordingly, the differentiation protocol must be optimized) in order to identify a set of ESC lines that could facilitate genetic matching of donor cells to patients and therefore prevent graft rejection and life-long immunosuppression. The last major problem, which greatly limits the use of ESC in many countries of the world, is the presence of ethical concerns regarding the destruction of human embryos for the production of these cell lines.

4. Induced pluripotent stem cells

4.1 Definition and characterization

Because of their self-renewal abilities and the capacity to differentiate into any cell of the body, ESC have always been considered the most promising source for cell replacement therapies; despite this, however, their controversial origin and the impossibility to perform autologous therapies, had so far limited their use in clinical practice. To overcome these obstacles and still obtain pluripotent cells, the only possible way appeared to be the reprogramming of somatic cells to a state of embryonic-like stem cells. Initially, the reprogramming process of somatic cells has proved possible only transferring the nuclear content of these cells into oocytes (Wilmut et al., 1997) or fusing them with ESC (Tada et al., 2001). The results obtained from these experiments led many researchers to believe that the oocytes and ESC contain factors able to confer characteristics of pluripotency to somatic cells. The group of Yamanaka and colleagues in particular speculated that the elements capable of cover this important role in reprogramming induction were those involved in the maintenance of the undifferentiated state of ESC; to identify these factors they selected 24 candidate genes encoding for proteins that play an important role in maintaining cell identity of ESC and in their proliferation, including transcription factors (such as Oct3/4, Sox2, Nanog) or genes that are frequently overexpressed in tumors (such as Stat3, E-Ras, C-myc or Klf4), and tested their ability to reprogram somatic cells to pluripotent cells. They first transfected murine fetal fibroblasts (MEFs) with retroviral vectors containing the genes coding for 24 proteins of interest and observed the generation of colonies of embryonic-like stem cells; later, in order to identify which of these 24 genes were necessary and sufficient to reprogram somatic cells, they monitored the formation of colonies by combining the transfection of the various factors among them. Using this strategy in 2006 Prof. Yamanaka (winner of the Nobel prize in 2012 for this discovery) and his team found a set of four genes that, when over-expressed in murine fibroblasts (both embryonic and adult), are able to reprogram these somatic cells to pluripotent cells capable of self-renewal. This four reprogramming factors (also known as “Yamanaka’s factors”) are:

- POU domain class 5 transcription factor 1 (POU5F1), also known as Octamer-binding transcription factor 4 (OCT3/4), a transcription factor important for self-renewal of undifferentiated embryonic cells;
- Krüppel-like factor 4 (KLF4), a repressor of p53, which is essential for the regulation of cell cycle;
- SRY (sex determining region Y)-box 2 (SOX2), another transcription factor important for the self-renewal of undifferentiated cells;
- Myelocytomatosis oncogene (c-MYC), a proto-oncogene crucial to drive cell proliferation and allow the self-renewal of ESC.

Transduction of murine fibroblasts with retroviral vectors containing the genes encoding for these 4 factors resulted in the formation of colonies of cells with the same morphology **(Figure 2)**, proliferation rate **(Figure 3)** and gene expression profile **(Figure 4)** of ESC; these cells were called "induced pluripotent stem cells" (iPSC).

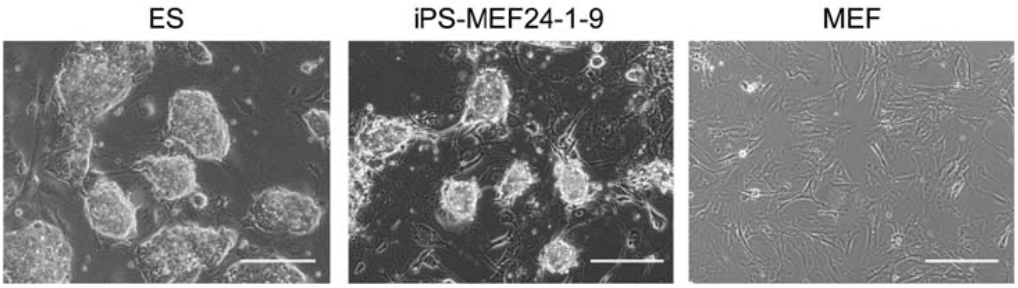

Figure 2. Morphology of an ES cell line, a colony of murine iPSC and a murine fetal fibroblasts cell line. Scale bar = 200µm. *Takahashi, 2006.*

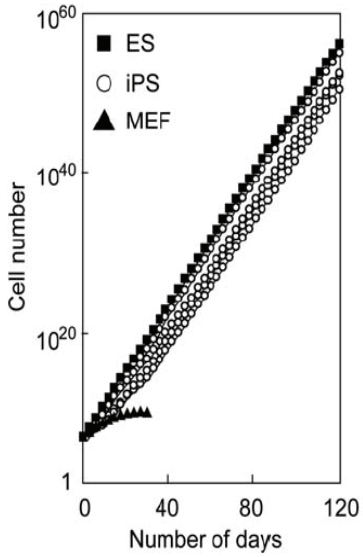

Figure 3. Growth curves of ESC, iPSC (iPS-MEF24, clones 2-1–4), and MEFs. 3 × 105 cells were passaged every 3 days into each well of six-well plates. *Takahashi, 2006.*

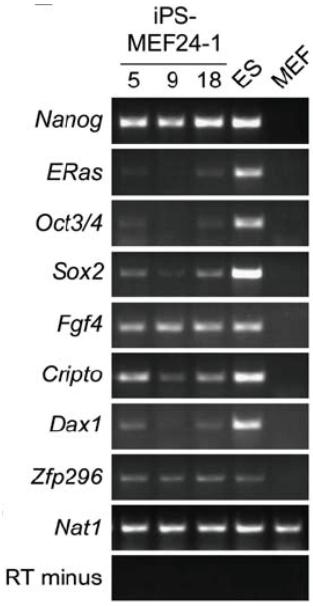

Figure 4. RT-PCR analysis of ES cell marker genes in iPSC (iPS-MEF24, clones 1-5, 1-9, and 1-18), ES cells, and MEFs. Nat1 was used as a loading control. *Takahashi, 2006.*

The iPSC lines produced are in fact, as ESC, formed by cells with a rounded shape, large nuclei and scant cytoplasm. They also showed a high proliferative rate, in fact the doubling time of these cells (17-19 hours) was equivalent to that of ESC (17 hours). Also the gene expression analysis showed the similarity of iPSC cells to ESC, as they express genes as Oct3/4, Nanog, E-Ras, Crypto, DAX1, Zfp196 and Fgf4, which are characteristic markers of undifferentiated cells.

It was also evaluated the pluripotency of the iPSC lines produced through two different types of studies:

- First, it was assessed the ability of these cells to form teratomas into immunodeficient animals by subcutaneous injection of cells; histological examination revealed that the iPSC lines generated were able, even after numerous passages *in vitro*, to give rise to tumors composed of cells derived from all three embryonic germ layers **(Figure 5)**;

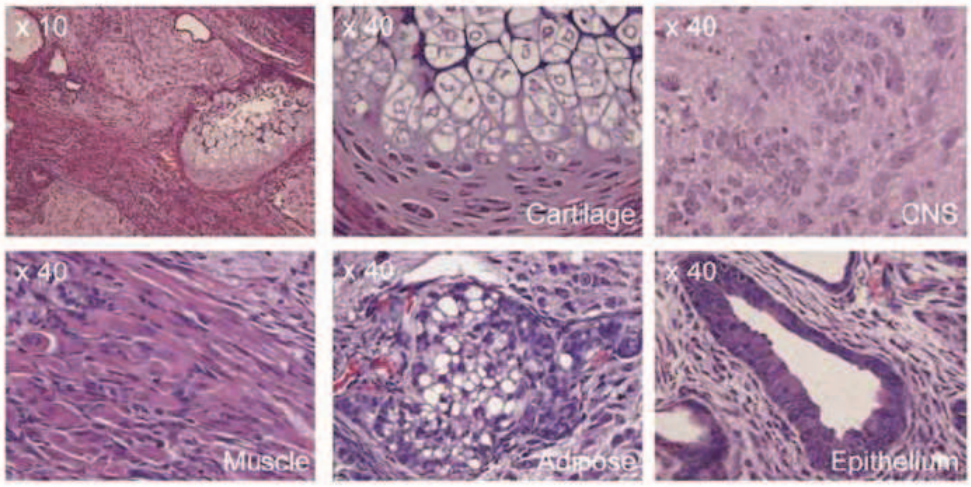

Figure 5. Histology of different tissues present in teratomas derived from the transplantation of iPSC in mice. *Takahashi, 2006.*

- It was also evaluated the ability of these cells to differentiate *in vitro* into cells of the three embryonic germ layers. First, the formation of three-dimensional structures called "embryoid bodies" (EBs), a critical step to examine the potential of differentiation of ESC, was induced and then these clusters were grown in suspension condition to allow spontaneous differentiation. After a few days of culture, cells stained positive for the α-smooth muscle actin (mesoderm marker), the α-fetoprotein (endodermal tissues marker) and βIII tubulin (ectoderm marker) **(Figura 6).** (Takahashi & Yamanaka, 2006).

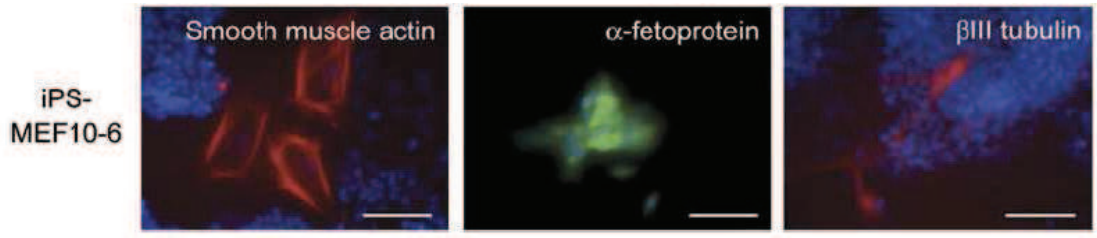

Figure 6. Immunostaining confirming *in vitro* differentiation into cells of all three germ layers. Scale bars = 100 μm. Secondary antibodies were labeled with Cy3 (red), except for α-fetoprotein in iPS-MEF10-6, labeled with Alexa 488 (green). Scale bar = 100μm. *Takahashi, 2006.*

One year later, Yamanaka's and two other groups have successfully repeated the reprogramming process using human somatic cells (Takahashi et al., 2007; Yu et al.,

2007; Park et al., 2008). Even these lines of human iPSC generated resulted comparable to ESC, as these cells showed the same morphology, the same proliferative capacity, had similar telomerase activity, a normal karyotype, expressed surface markers and genes that characterize human ESC, and were also able to form teratomas *in vivo* and to differentiate into cells of all three germ layers *in vitro*. The protocol described by Yamanaka's group for the generation of iPSC by using adult human fibroblasts requires a first step of infection with retroviral vectors containing the genes encoding for the 4 factors (Oct4, Klf4, Sox2 and C-Myc) and the growth in a medium containing 10% FBS (fetal bovine serum). After 6 days, cells are harvested using trypsin and plated on a layer of feeder cells (usually MEFs) inactivated with mitomycin C; from the next day onwards the cells are grown in the medium typically used for the culture of ESC supplemented with bFGF (basic fibroblast growth factor) to avoid spontaneous differentiation. Approximately 2-3 weeks after, flat and uniform colonies of cells similar to ESC start to appear with an overall efficiency of the reprogramming process of 0,2%; these colonies are then selected and expanded *in vitro* (Takahashi et al., 2007). Is also important to mention that Oct4, Sox2, Klf4 and C-Myc are not the only factor combination that can generate iPSC. For example, human iPSC have been derived by enforced expression of Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007), suggesting that different routes may lead to a common pluripotent ground state or, alternatively, that different transcription factors activate the same program by reinforcing each other's synthesis. Similarly, Sox2 and Klf4 have been replaced with related protein such as Sox1, Sox3 and Klf2 (Nakagawa et al., 2008). After the publication of these groundbreaking works, iPSC were created by reprogramming of somatic cells of different species, including human, mouse, rat, pig, horse and monkey (Stadtfeld & Hochedlinger, 2010; Kumar et al., 2015), demonstrating that the fundamental features of the transcriptional network governing pluripotency remain conserved during evolution. Similarly, iPSC have been derived from other somatic cell populations **(Figure 7)**, such as keratinocytes, neural cells, stomach and liver cells, melanocytes, terminally differentiated lymphocytes (Singh et al., 2015) and also from pancreatic β cells (Stadtfeld et al., 2008), further underscoring the universality of the reprogramming process.

Type of cells	Reprogramming factors	References
Fibroblast	OSKM	Takahashi and Yamanaka, 2006a
	OSLN	Yu et al., 2007
Keratinocytes	OSKM	Aasen et al., 2008
Cord blood endothelial cells	OSLN	Haase et al., 2009
Cord blood stem cells	OSKM	Ye et al., 2009
Neural stem cells	O	Kim et al., 2009a
Melanocytes	OSKM	Utikal et al., 2009
Amniotic cells	OSKM	Li et al., 2009
Adipose derived stem cells	OSKM	Sugii et al., 2010
Hepatocytes	OSKM	Liu et al., 2010
Circulating T cells	OSKM	Seki et al., 2010
Astrocytes	OSKM	Ruiz et al., 2010
Peripheral blood	OSKM	Kunisato et al., 2011
Kidney mesangial cells	OSKM	Song et al., 2011
Urine cells	OS	Zhou et al., 2012

Figure 7. Different cell sources and different combinations of reprogramming factors have been used by different groups for reprogramming to iPSCs (O- Oct4; S- Sox2; K- Klf4; M- c-Myc; N- Nanog, L-Lin28). *Singh, 2015.*

Studies of this type have raised an important issue, that is the need to verify whether if iPSC generated from different cell types are biologically and functionally similar. In some studies substantial differences in the propensity to form tumors *in vivo* or in the persistence of gene expression characteristic of the cell of origin were observed between iPSC derived from different types of somatic cells (Ben-David & Benvenisty, 2011). In 2010 Hochedlinger's group compared gene expression level, epigenetic profile and differentiation abilities of four lines of iPSC derived from different types of somatic murine cells; they observed that iPSC reprogrammed from cells of different nature have a different transcriptional and epigenetic pattern, which allows to differentiate in the cell type of origin with more efficiency than in other cell types. They suggest that early-passage iPSC retain a transient epigenetic memory of their somatic cells of origin, but that continue passaging of the cells largely attenuates these differences and that the cell lines become almost indistinguishable from each other after about ten splits (Polo et al., 2010). It should therefore to be taken into account that the somatic cells used for reprogramming are crucial, at least in the first steps, for the differentiation abilities of iPSC, since they still retain a epigenetic memory. Theoretically, to obtain iPSC differentiation with high efficiency, it would be more appropriate to reprogram cells at

least derived from the same germ layer and begin the differentiation process within a few passage from the reprogramming process. This strategy, however, holds issues that should not be underestimated, as in a recent work Batada and his group studied the number of CNVs (Copy Number Variations, genomic alterations in the number of copies of one or more regions of DNA) which are generated during the reprogramming process by comparing them to ESC and fibroblasts used for the production of these iPSC lines. They demonstrated that the median number of *de novo* CNVs that are generated from the reprogramming process are almost twice compared to ESC or fibroblasts, and that this number is not affected either by the presence or absence of C-Myc or by the reprogramming strategy. The interesting element, however, is that the number of these CNVs decreases drastically, reaching levels comparable to ESC, during the passages of the cell lines, indicating that the most aberrant cells are eliminated by natural selection (Hussein et al., 2011). In conclusion, iPSC immediately after reprogramming can be differentiated with more efficiency because of their epigenetic and transcriptional memory, but are more genetically unstable, and consequently less safe.

4.2 Safety issues

The main problem of iPSC, which currently still preclude their use in humans is related to their intrinsic characteristic: as pluripotent cells, like ESC, also iPSC determine the formation of tumors when transplanted into immunodeficient animals. In addition, other problems caused by the reprogramming process itself, as the use for transfection of integrating virus like retroviruses, may cause insertional mutagenesis, interfere with gene transcription and induce tumors formation. Yamanaka and colleagues in fact demonstrated that after pluripotency establishment, the transgenes derived from retrovirus are silenced, but that each iPSC clone contained from three to six retroviral integrations for each factors, which may increase the risk of tumorigenesis (Takahashi et al., 2007). Another issue is related to the use of C-Myc, a well-known proto-oncogene, as reprogramming factor, because it can lead to the generation of neoplastic formations. To overcome these obstacles, various strategies have been developed:

- First, it was decided to eliminate the oncogene C-Myc from the set of genes required for reprogramming. It was in fact demonstrated that one of the causes of

tumor development was due to the reactivation of the C-Myc retrovirus (Okita et al., 2007). The same group of Yamanaka was able to achieve this goal by changing their reprogramming protocol doubling the time of exposure (from 1 to 2 weeks) to the drugs used to select the transfected cells. With this method modification they obtained an absolute lower number of iPSC colonies, but the obtained colonies were more similar to ESC colonies in terms of morphology and gene expression profile, indicating that without C-Myc the reprogramming process is more specific, even if less efficient and slower. The C-Myc free iPSC lines generated were less tumorigenic as compared to cells derived from the reprogramming performed using all 4 factors, since none of the 26 mice chimera (generated by implanting the cells of interest into blastocysts which are then transplanted into the uterus of pseudo-pregnant mothers) developed tumor at 100 days from birth (Nakagawa et al., 2008).

- To try to further improve the safety of iPSC, several reprogramming strategies (summarized in **Table 1**) which did not include the use of retroviral vectors have been developed. At first were tested inducible lentiviral vectors, whose expression can be controlled by administration of the inert drug doxycycline, decreasing the risks related to the continuous transgenes expression and allowing the selection only of fully reprogrammed cells, since cells that are dependent on exogenous factors expression readily stop proliferating upon doxycycline withdrawal (Brambrink et al., 2008). In addition, infection of different types of somatic cells is more efficient with lentiviral compared to retroviral vectors, and lentiviruses can express polycistronic cassettes encoding all four factors simultaneously, thereby increasing the efficiency of reprogramming process (Carey et al., 2009). Alternative to integrating retro or lentiviruses, non-integrating strategies have been tested for reprogramming, and can be divided into three categories:
    - Those that use vectors that do not integrate into the host genome: the first integration-free iPSC were generated using adenoviral vectors that allow a transient, high-level expression of exogenous genes without integrating into the host genome (Okita et al., 2008; Stadtfeld et al., 2008). The adenoviral vectors contained Oct4, Sox2, Klf4 and C-Myc genes and were delivered repeatedly to maintain transgene expression for up to twelve days, resulting in the generation of iPSC without evidence of gene integration and demonstrating the feasibility

of nuclear reprogramming without permanent genetic alterations. Another non-integrating virus is represented by the Sendai virus system; Sendai virus (SeV) vectors replicate in the form of negative-sense single-stranded RNA in the cytoplasm of infected cells, which do not go through a DNA synthesis nor integrate into the host genome, allowing an efficient production of iPSC and later on elimination of the viral vector (Fusaki et al., 2009). Also self-replicating episomal vectors for reprogramming of somatic cells were described (Yu et al., 2009). After spontaneous loss of the episome by proliferating cells, iPSC completely free of vector and transgene sequences are generated, but the reprogramming efficiency in human fibroblasts is particularly low (about three to six iPSC colonies per 10^6 input cells). Also polycistronic minicircle DNA vectors, produced by the recombinatorial elimination of the bacterial backbone of the original plasmids, were used for the generation of virus-free iPSC (Jia et al., 2010), but with an overall reprogramming efficiency of ~0.005% .

- Those that use integrating vectors that can be subsequently removed from the genome: reprogramming efficiency with non-integrating methods is quite low, therefore several laboratories have developed integration-dependent gene delivery vectors with incorporated, at the ends of the genes of interest, loxP sites that can be subsequently excised from the host genome by the transient expression of Cre recombinase (Kaji et al., 2009; Soldner et al., 2009). It remains however to be assessed whether short vector sequences, which inevitably remain into host cell DNA after excision, affect cellular function. The recent development of hyperactive transposase enzymes makes transposon systems an interesting alternative to viral based methods, especially because they are able to carry large DNA cargo into cells. Transposons are mobile genetic elements that can be introduced and removed into host genome by transient expression of the transposase (Woltjen et al., 2009; Yusa et al., 2009). The commonly employed piggyBac and Sleeping Beauty (Kues et al., 2013; Talluri et al., 2014) transposon systems hold a low error rate due to a seamless excision, but require characterization of integration sites in iPSC before and after reprogramming.

- Those that do not use nucleic acid-based vectors: successful reprogramming has been achieved also without the use of viral or plasmid vectors at all. At first, delivery of the reprogramming factors as proteins seemed an obvious alternative. In 2009 transgene-free iPSC were produced with repeated supplementations of recombinant proteins of reprogramming factors, but with a low reprogramming efficiencies and a high costs for repeated treatments with protein factors (Zhou et al., 2009). Another group proposed the use of small molecules instead of transcription factors (Ichida et al., 2009): in this study Sox2 transcription factor was substituted by a small molecule that inhibits transforming growth factor-beta (TGFβ) signaling and this inhibition promotes the completion of reprogramming through induction of the transcription factor Nanog. The most recent trend in the field of non-viral iPSC generation is reprogramming by RNA molecules. Recently, modified mRNAs encoding Yamanaka's reprogramming factors were employed to generate iPSC with high efficiency (2-4%) without compromising genomic integrity (Warren et al., 2010). Moreover, it was shown that also microRNA (miRNA) expression is sufficient to induce pluripotency. Two independent groups reported iPSC generation by delivery of miR302/367 cluster or miR200c, miR302, and miR369 (Anokye-Danso et al., 2011; Miyoshi et al., 2011). These miRNA-derived iPSC were indistinguishable from conventionally generated iPSC and have the advantage of avoiding transduction of proto-oncogenic transcription factors.

Strategies	Reprogramming	Efficiency	References
Integrating	Retrovirus	0,01-0,5%	Takahashi et al., 2006
	Inducibile Lentivirus	0,1-1%	Brambrink et al., 2008
Excisable	Floxed Lentivirus	0,1-1%	Kaji et al., 2009 Soldner et al., 2009
	Transposon (piggyBac or Sleeping Beauty)	0,01%	Woltjen et al. 2009 Yusa et al. 2009
Non-integrating	Adenovirus	0,001%	Okita at al., 2008 Stadtfled et al., 2008
	Sendai virus	0,1%	Fusaki et al., 2009
	Episomal vectors	0,001%	Yu et al., 2009
	Minicircle DNA	0,005%	Jia et al., 2010
DNA-free	Small molecules	0,05%	Ichida et al., 2009
	Proteins	0,001%	Zhou et al., 2009
	Synthetic mRNA	2-4%	Warren et al., 2010
	MicroRNA	~10%	Anokye-Danso et al., 2011 Miyoshi et al., 2011

Table 1. Different delivery methods for transfer of reprogramming factors for iPSC generation.

All these new strategies allowed to successfully execute the reprogramming of somatic cells into iPSC without integration into genomic DNA of the cell, thereby drastically decreasing the tumorigenicity risk without altering the pluripotency potential of the reprogrammed cells.

4.3 Current hurdles in advancing personalized iPSC

The discovery of iPSC led to many more studies in the pluripotent arena, including the developing of "disease-in-a-dish" models for drug-screening platforms, the generation of disease-specific iPSC lines to study the pathophysiology of diseases, and creating personalized therapies for autologous stem cell transplantation (Matsa et al., 2014). In fact these cells can be derived from the somatic cells of each individual and can be used for autologous cell replacement therapies, theoretically avoiding the administration of immunosuppressive drugs. Murine or human iPSC have been successfully differentiated into many specialized cell types like neurons (Wernig et al., 2008), hematopoietic cells (Raya et al., 2009) or cardiomyocytes (J. Zhang et al., 2009) and the differentiated cells

may also be used to study *in vitro* cell types that are difficult to access or with limited proliferative capacity. A further advantage is that iPSC can also be obtained through the reprogramming of somatic cells of patients suffering from various diseases, including T1D (Maehr et al., 2009); the use of iPSC thus could allow to **(Figure 8)**:

- Study *in vitro* the molecular and genetic causes of the disease;
- Perform screening of molecules in order to identify new drugs for the cure of the disease of interest;
- Perform autologous or allogeneic transplants of differentiated cells (if necessary corrected *ex vivo* by homologous recombination or gene therapy) to cure the disease.

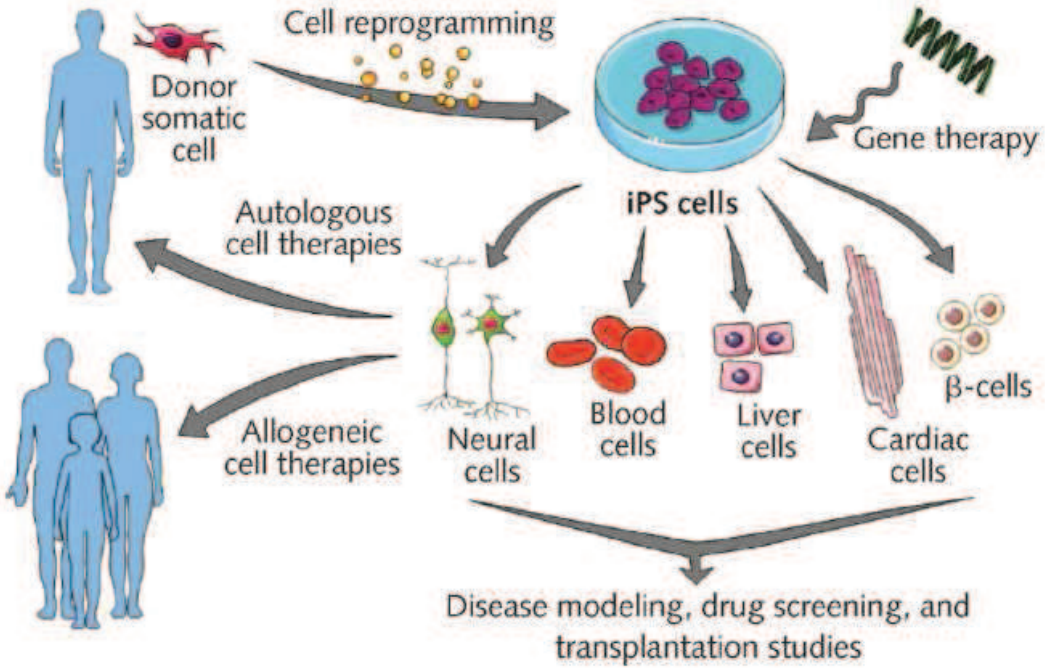

Figure 8. Summary outline of the potential use of iPSC. *Power, 2011.*

Therefore iPSC hold a huge potential, both in terms of cell replacement therapy for the possible treatment of many diseases and for the creation of *in vitro* models for the study of diseases or for drugs screening. In September 2014 the first pioneering clinical trial using autologous iPSC for the treatment of an AMD (age-related macular degeneration) patient was launched by Masayo Takahashi in Japan. After the transplantation of the first patient with his own iPSC-derived retinal pigment epithelial cells the trial was

stopped in July 2015 because the iPSC of the second enrolled patient did not pass a genomic validation step (6 mutations that were not present in the original somatic cells were found). At present then, many barriers preclude the use of iPSC in the clinical practice and some obstacles still need to be addressed:

- The generation of reprogramming strategies able to generate lines of iPSC with high safety and efficiency, without viral integration or genomic alterations;
- The optimization of efficient and reliable *in vitro* differentiation protocols into the target therapeutic cell of interest
- The post-transplantation efficacy, determined by the integration, maturation, survival and function of implanted cells to induce a therapeutically detectable effect, have to be further addressed.
- The prospective removal (for example, before transplantation) of tumorigenic cells would provide the highest level of safety while reducing the need for post-transplantation surveillance. In this context, new cell markers and methods that allow to select fully differentiated cells alone or to eliminate pluripotent cells are strongly required (Lee et al., 2013).
- Currently the culture media for human pluripotent stem cells contain animal-derived components that could pose a hurdle for therapeutic use in humans. However, most of the issues have been solved by recent technological innovations such as use of animal-free culture media and reagents. Hence, we expect that, in the near future, clinical-grade iPSCs will be generated and differentiated under conditions that correspond to good manufacturing practices (GMPs) (Neofytou et al., 2015).
- Last but not least, nowadays the preparation of autologous iPSC from each patient carries a high medical cost, and reprogramming, characterization and subsequent differentiation of each cell line requires several months (Ohnuki & Takahashi, 2015), limiting the suitability of this personalized iPSC derivative approach. Strategies for the generation of iPSC with low costs and short times are therefore strongly required.

At present therefore, the idea of generating autologous iPSC lines cells as a source of transplantable cells, is not feasible in practical and economic terms for all the millions of patients who would benefit from this type of approach. The overall feasibility of the

iPSC-based therapeutic approach will be determined essentially by whether the technical, financial and temporal issues can be adequately resolved. With the extensive amount of research currently being conducted in the iPSC field, it is plausible to consider that these feasibility issues will be adequately addressed in due course (Byrne, 2013). Meanwhile, many groups are working on the possibility of using allogeneic HLA (Human Leucocyte Antigen)-matched iPSC lines for transplantation purpose. Experience with solid organ and bone marrow transplantation has been used to help estimating the scale of iPSC banking that would be required to provide adequately matched tissues in a population; a study conducted on Japanese population estimate that 50 homozygous iPSC lines would provide a haplotype match for 90.7% of individuals (Nakatsuji et al., 2008). These findings were confirmed in a subsequent study, which showed that 150 homozygous cell lines could provide a haplotype match for 93% of the population of the United Kingdom (Taylor et al., 2012). The generation of these iPSC lines would potentially be useful for the transplantation of a large number of patients, greatly reducing the need for immunosuppressive drugs. Another group has instead shown that generating a master cell bank for more diverse populations would be far more challenging using a probabilistic model to estimate the rate of haplotype matching of a carefully selected homozygous HLA-type iPSC bank for a North American population. According to their estimates a bank comprising 100 iPSC lines with the most frequent HLA in each population would leave out only 22% of the European Americans, but 37% of the Asians, 48% of the Hispanics, and 55% of the African Americans, indicating that an allogeneic cell bank in genetically homogenous countries like Japan or Iceland could be a viable option, but a similar bank in US would require a large-scale concerted worldwide collaboration (Gourraud et al., 2012). Finally, it should be noted that even highly matched cells could still trigger rejection. Although substantial debate exists within this field (Zhao et al., 2011), the current evidence supports the hypothesis that autologous iPSC-derived grafts are not strongly immunogenic. Recent studies using syngeneic mouse models demonstrated that transplanted iPSC-derived embryoid bodies, skin and bone marrow tissues engrafted efficiently with almost no signs of rejection (Araki et al., 2013; Guha et al., 2013). However, it would still be very difficult to predict problems with human iPSC based on mouse-to-mouse studies and also these problems must be addressed as soon as possible,

as medical communities are starting to build tissue banks that could offer groundbreaking treatments (Neofytou et al., 2015).

4.4 Potential of iPSC in T1D

Recent successes in differentiating ESC into insulin-producing cells, in combination with the discovery that pluripotent cells can be obtained through somatic cells reprograming, have raised the possibility that a sufficient amount of patient-specific β cells might be derived from patients through cell reprogramming and differentiation. At present several scientific works reported successful differentiation of ESC into cells capable of secreting insulin and, as mentioned previously, the pioneer in this field was the Viacyte group. Fundamental was their contribution to the identification of the growth factors able to promote the differentiation of pluripotent cells into definitive endoderm cells (D'Amour et al., 2005). The endoderm is the germ layer that gives rise, as well as epithelial cells of the respiratory and digestive systems, thyroid gland, thymus and liver, also to the pancreas and the pancreatic β cells of the islets of Langerhans. Genetic analyses in murine animal models have shown that disruption of either the WNT or TGFβ signaling pathways prevents formation of the primitive streak, the mesoderm and the definitive endoderm (Conlon et al., 1994; Lowe et al., 2001). In addition, high expression of Nodal, a member of the TGFβ superfamily, is essential for specification of endoderm during gastrulation in mice, while low levels lead to mesoderm formation (Lowe et al., 2001; Vincent et al., 2003). Although Nodal is an attractive candidate for inducing definitive endoderm differentiation of ESC *in vitro*, a source of highly active protein is not readily available. However, another TGFβ family member, Activin, binds the same Nodal receptors, triggering similar intracellular signaling events (de Caestecker, 2004), and therefore can be used to mimic Nodal activity *in vitro*. In this study Baetge and his team were able to demonstrate that the addition of Activin A to the culture media in the absence of FBS leads to the specification of ESC into about 80% of definitive endoderm cells (SOX17, CXCR4 and FOXA2 positive) (D'Amour et al., 2005). One year later the same group optimized this protocol reducing the days of treatment with Activin A (from 5 to 3 days) and adding in culture also the protein Wnt3a for the first 1-2 days, increasing the efficiency of differentiation into definitive endoderm cells; this initial specification has proved to be

crucial for an efficient production of cells capable of expressing pancreatic hormones (D'Amour et al., 2006). The final goal was in fact to obtain insulin-secreting cells and to try to this aim they looked for chemokines and growth factors that could favor the formation of β cells *in vitro,* mimicking pancreatic organogenesis which occurs during embryo formation; in this study they analyzed many protocols with different combinations of molecules, different concentration and time of administration. What they achieved with this kind of approach is a 5-stages protocol **(Figure 9)** in which the production of β-like cells is obtained through the sequential specification of human ESC into:

- Definitive Endoderm (DE) through the addition of Activin A and Wnt3a in the absence of FBS (2-4 days);
- Primitive Gut Tube (PG) through the stimulus given by FGF 10 and KAAD-cyclopamine (CYC), the removal of Activin A and the presence of 2% FBS (2-4 days). The growth factor FGF10 is usually produced by mesenchymal cells and is important for the growth and differentiation of the pancreatic epithelium. Fundamental is also the contribution of CYC, an inhibitor of hedgehog signaling, since the inhibition of the signaling within this pathway is fundamental for pancreatic specification;
- Posterior Foregut (PF) adding to the culture medium containing FGF10 and CYC also Retinoic Acid (2-4 days); it rapidly induces the expression of Pdx1, which is a transcription factor necessary for the development and maturation of pancreatic β cells;
- Pancreatic Endoderm (PE) through the addition for 2-3 days of two molecules:
    - Exendin-4: analog of GLP-1 (glucagon-like peptide-1), which by binding to GLP-1R receptor leads to the activation of intracellular pathways that increase β cell mass and insulin gene expression;
    - DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl] -S-phenylglycine t-butylester) an inhibitor of Notch pathway: the decrease of the signal within this pathway is essential to allow the expression of Neurogenin3 (Ngn3), a protein produced by the endocrine progenitors and necessary for the development of the pancreatic endocrine cells. The production of Ngn3 in

turn allows the expression of two important transcription factors Nkx2.2 and Nkx6.1, which control the differentiation into endocrine cells.

- Hormone-expressing Endocrine cells (EN) through the administration of Exendin-4 and two growth factors, IGF-1 (Insulin-like growth factor 1) and HGF (Hepatocyte growth factor) for 3 or more days.

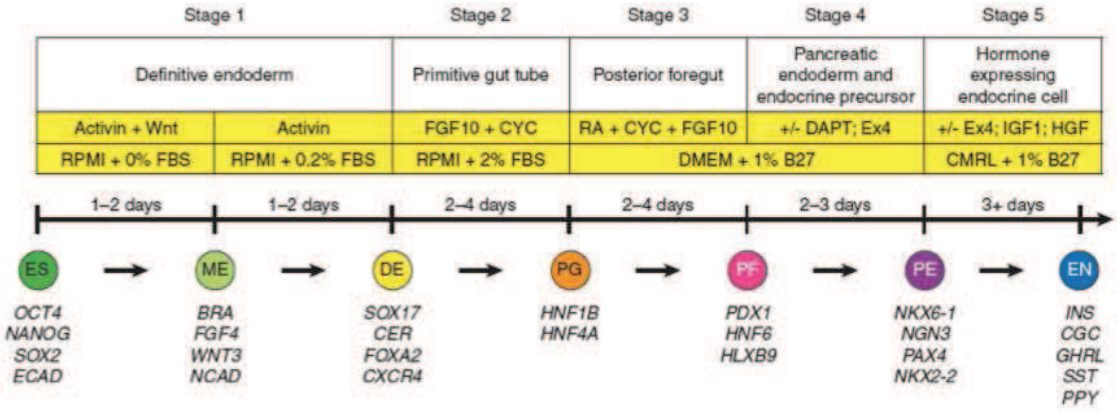

Figure 9. Scheme of differentiation procedure and protein expression for some key markers of pancreatic differentiation. The differentiation protocol is divided into five stages and the growth factors, medium and range of duration for each stage are shown. This protocol orchestrates differentiation through five identifiable endodermal intermediates en route to production of hormone-expressing endocrine cells. Several markers characteristic of each cell population are listed. CYC, KAAD-cyclopamine; RA, all-trans retinoic acid; DAPT, γ-secretase inhibitor; Ex4, exendin-4; ES, hES cell; ME, mesendoderm; DE, definitive endoderm; PG, primitive gut tube; PF, posterior foregut endoderm; PE, pancreatic endoderm and endocrine precursor; EN, hormone-expressing endocrine cells. *D'Amour, 2006.*

With this 5 stages protocol that goes *in vitro* through the steps that determine the generation of pancreatic organogenesis, D'Amour and colleagues were able to obtain cells expressing markers typical of each stage of differentiation and, after about 15 days of culture, up to 7.3% of insulin-positive cells **(Figure 10)**, even if they are not responsive to glucose (D'Amour et al., 2006).

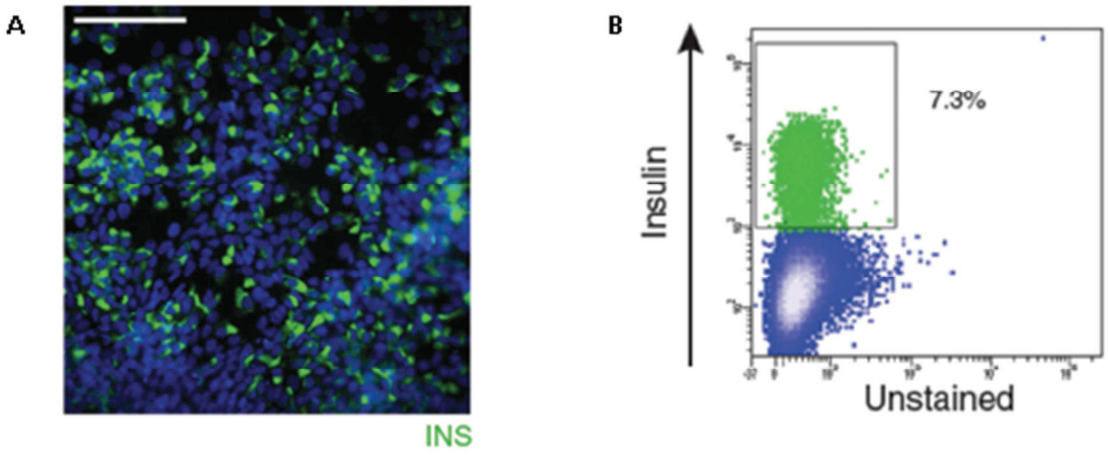

Figure 10. As demonstrated by immunofluorescence (A) and flow cytometry analysis (B) the cells resulting from the differentiation of ESC are able to produce insulin. A: DAPI (blue) for nuclei staining, stairs bar = 100µm. *D'Amour, 2006.*

After the publication of this important work, many other groups have focused on the possibility to generate cells capable of producing insulin from ESC and numerous protocols of differentiation that include the use of different molecules and growth conditions were created. Two studies published in 2007 described protocols for the differentiation of ESC into insulin-producing cells in conditions of total absence of both serum and feeder cells and, in particular, with the growth in suspension condition after the specification into pancreatic progenitor cells. The total efficiency of the production of insulin-positive cells was about 2-8% (J. Jiang et al., 2007) or 15% (W. Jiang et al., 2007). Jiang W et al. also transplanted the ESC-derived insulin-producing cells into chemically induced diabetic mice and 30% of the animals reverted the hyperglycemic status; however no human C-peptide was detected in the serum of the mice, even after glucose stimulus (W. Jiang et al., 2007). In 2009 Melton's group performed a screening of 5000 molecules in order to identify which one were able to increase the efficiency of the differentiation of human ESC into pancreatic-derived cells capable of expressing the transcription factor Pdx1. With this strategy they were able to identify a molecule, (-) - Indolactam V (IL V), which is able to increase the percentage of Pdx1 positive cells and, consequently, the amount of cells differentiating into pancreatic cells. IL V, acting in synergy with FGF10, allows the activation of PKC (protein kinase C) signaling, that regulates differentiation, proliferation and cell survival. Pdx1 positive cells were obtained by treatment with Activin A, Wnt3a, FGF10, CYC and, finally, administration of IL V; the cells were further differentiated *in vitro* using bFGF and nicotinamide,

didn't generate insulin-secreting cells with an efficiency comparable to those obtained by Viacyte group (Chen et al., 2009). A common element in all of these studies is the observation that all the ESC lines tested were able to differentiate into pancreatic β-like cells, but not with the same efficiency, making necessary the optimization of the various differentiation protocols for each cell line (D'Amour et al., 2006; Chen et al., 2009). Several strategies to differentiate ESC into cells capable of producing insulin have been described and, after the generation of human iPSC in 2007, these differentiation protocols were tested also on these induced pluripotent cells. The first paper that reported successful differentiation of human iPSC into insulin-secreting cells dates back to 2008, when the group of Zhang adapted the four-step differentiation protocol developed for ESC from Jiang J and colleagues (J. Jiang et al., 2007) and obtained for the first time β-like cells *in vitro* from reprogrammed human fibroblasts. Unfortunately, the efficiency of differentiation process was very low and the total C-peptide content was significantly lower compared to adult β cells (Tateishi et al., 2008). Subsequent studies focused on the culture conditions in order to increase the efficiency of differentiation of the iPSC into insulin-secreting cells; for example in 2010 the group led by Yupo Ma applied a protocol which allowed the differentiation of murine ESC in pancreatic β cells (Schroeder et al., 2006) to iPSC derived from adult mouse fibroblasts. This differentiation protocol requires three phases: the formation of EBs through the growth in suspension conditions, the transfer of EBs in adhesion to allow the spontaneous differentiation of the cells and, finally, the addition to the culture medium of insulin, laminin and nicotinamide, to allow pancreatic specification. With this differentiation protocol Alipio et al. were able to obtain from murine iPSC up to 50% of cells capable of secreting insulin in response to glucose stimulus and, if transplanted into diabetic mice, these cells were capable to restore normoglycemia (Alipio et al., 2010). This experiment, however, was performed only on six mice and remains to be confirmed if the same differentiation protocol could have the same efficiency in differentiating human iPSC. One year later a group reported the differentiation of human iPSC into insulin-secreting cells responsive to glucose using another differentiation protocol; it requires the culture in the absence of feeder cells and the addition, compared to Viacyte protocol, of two molecules, IL V (as suggested by Melton group) (Chen et al., 2009) and GLP-1 (instead of Exendin 4). The differentiation

efficiency was very low, as only 1.29% of insulin positive cells were obtained, and their ability to secrete insulin *in vivo* has not been verified. This group also reported the observation that the different efficiency of iPSC lines to differentiate into β-like cells depends not only on genetic and epigenetic characteristics of the cells used for the reprogramming process, as they found variations among iPSC clones derived also from the same tissue of origin. This suggests that probably a different number of copies of pluripotency genes integrated, or different levels of silencing/reactivation of these genes in the various clones are able to influence the differentiation capacity of iPSC (Thatava et al., 2011). Encouraging results have been reported by other several *in vitro* studies that used protocols mimicking the mechanism of *in vivo* pancreas development to guide the differentiation of iPSC into β-like cells (Zhang et al., 2009; Nostro et al., 2011; Kunisada et al., 2012; Hua et al., 2013), but with a lower efficiency compared to ESC. Moreover, none of these focused on the potential of these cells to engraft and secrete insulin *in vivo*. This is of particular relevance because *in vivo* engraftment could represent a critical step in implementing pluripotent stem cells differentiation into insulin-producing cells, as described for ESC (Kroon et al., 2008). Insulin-producing cells, although with low efficiency, were also generated with iPSC derived from the reprogramming of fibroblasts of two diabetic patients (Maehr et al., 2009), opening the way not only to autologous cell replacement therapy of T1D, but also to *in vitro* modeling of this disease. As mentioned previously, iPSC were also generated using pancreatic β cells as somatic cells for the reprograming, both of murine (Stadtfeld et al., 2008) and human origin (Bar-Nur et al., 2011). Ban-Nur and colleagues reprogrammed human pancreatic β cells and afterwards re-differentiated them into insulin-secreting cells; in this study the efficiency of differentiation was higher using β cell-derived iPSC compared to ESC or iPSC generated through the reprogramming of other somatic cell types. These observations further confirmed the theory that iPSC retain epigenetic memory of the somatic cell of origin even after the reprogramming process; in particular, it was shown that β-cell derived iPSC have an open chromatin structure in the regions coding for key genes that allow the definition of β cells, and a methylation pattern that differs from that of all other iPSC lines derived from other somatic cells (Bar-Nur et al., 2011). However this type of approach makes impracticable the possibility to perform a patient-specific β cell replacement therapy, because in diabetic

patients the β cells have been destroyed by autoimmune assault. In the last few months two important groups described for the first time that pancreatic cells derived from the differentiation of pluripotent stem cells (both embryonic and induced) are capable to revert diabetes in mice (Rezania et al., 2014; Pagliuca et al., 2014). The main difference between these outstanding works consist in the timing of reversion of the disease; Rezania et al. in fact transplanted cells that exhibit key markers of mature pancreatic beta cells, but that are able to secrete insulin only after a 40 days period of further maturation *in vivo* (Rezania et al., 2014). The group of Melton instead reported a scalable differentiation protocol that can generate millions of glucose-responsive β cells that secrete insulin shortly after transplantation. This 4-5 weeks *in vitro* differentiation protocol involves a combination of sequential culture steps using factors that affect signaling in numerous pathways, including signaling by WNT, Activin, Hedgehog, TGFβ, retinoic acid and γ-secretase inhibitors and leads to the generation of ~50% of C-peptide and Nxk6.1 double positive cells from both ESC and iPSC **(Figure 11)** (Pagliuca et al., 2014).

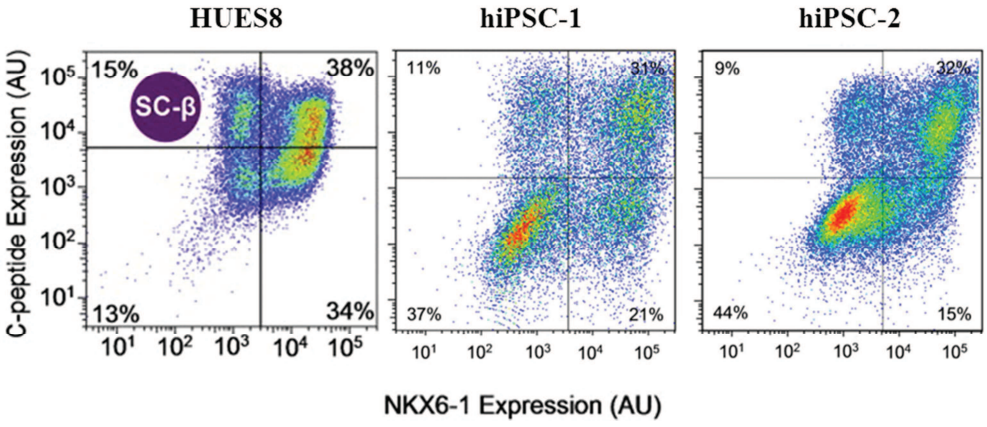

Figure 11. Representative flow cytometry dot plots and population percentages of cells stained for C-peptide and NKX6-1 in HUES8 (ESC line) and two lines of iPSC. AU = arbitrary units. *Pagliuca, 2014.*

In conclusion, iPSC offer great hope for cell replacement therapy for diabetes, but many efforts still need to be done in order to make both processes of reprogramming and differentiation safer and more efficient.

III. AIM OF THE PROJECT

Currently, the only possible strategy able to restore a physiological condition of normoglycemia in patients with T1D is constituted by β cell replacement therapy, in particular the transplantation of pancreas *in toto* or purified pancreatic islets. The shortage of organ donors and the need of a life-long immunosuppression therapy however, strongly limits this approach only to a small number of patients. Is therefore fundamental to look for an alternative, unlimited sources of cells capable of secreting insulin in response to glucose stimuli. Many research groups focused on the possibility of differentiate ESC *in vitro* into β-like cells, but this type of approach, although successful, it is still inefficient and limited by ethical problems. The revolutionary discovery of the possibility to reprogram somatic cells to an embryonic-like state through the expression of four genes (Oct4, Sox2, Klf4 and C-Myc) made in 2006 by Yamanaka's group overcame some of these obstacles and opened the way for an autologous stem cells-based therapy. The overall aim of this project is to try to obtain an unlimited source of pancreatic β cells from human iPSC in order to overcome the lack of organ donors and to get closer to an autologous β cells replacement therapy for diabetic patients. To this purpose in particular we plan to:

1) Generate iPSC lines through the reprogramming of human fibroblasts.
2) Identify a protocol that allows iPSC differentiation into insulin-producing cells *in vitro* with high efficiency.
3) Use iPSC differentiation process as a tool to study pancreatic organogenesis *in vitro*.
4) Test the ability of iPSC-derived pancreatic cells to differentiate and secrete insulin *in vivo* in a murine model.

IV. MATERIALS AND METHODS

1. iPSC generation from human skin fibroblasts

1.1 iPSC generation with retroviral vectors and characterization

The human iPSC lines used in this study are the result of a collaboration with the research group of Dr. Vania Broccoli (San Raffaele Scientific Institute, Stem Cells and Neurogenesis Unit). Human fetal fibroblasts (IMR90 cell line, ATCC, catalogue number CCL-186) were reprogrammed to iPSC as previously reported (Takahashi et al., 2007) in Broccoli's laboratory. Briefly, fibroblasts were infected twice (once every 24 hours) with retroviruses expressing the transcription factors OCT4, SOX2 and KLF4 and the day after second infection cells were plated on mitotically Mitomycin C (Sigma-Aldrich) inactivated murine embryonic fibroblasts (MEFs) layer on Matrigel (BD)-coated dishes and maintained in ESC medium. Approximately after 6 weeks after infection iPSC colonies started to appear and were manually picked using a stereomicroscope (Leica). Cells were maintained at 37°C 5% CO_2. Human iPSC clones were characterized for pluripotency (gene and protein expression analysis of markers of pluripotency such as alkaline phosphatase, *NANOG, OCT4, SOX2, DPPA4, FGF4, REX1, TDGF1, DPPA2, GDF3, hTERT*). To further corroborate the pluripotency of reprogrammed iPSC was verify *in vitro* the ability to differentiate towards cells of the three germ layers; in details, iPSC were cultured in ESC supplemented with 20% FBS for up to 20 days and then tested by immunofluorescence with corresponding markers: Sox17 (clone 245013, R&D) for endoderm, Smooth Muscle Actin (SMA, clone 1A4 Abcam) for mesoderm and Neuronal Class III Beta Tubulin (clone Tuj1, Covance) and Nestin (clone MAB353, Millipore) for ectoderm. To corroborate their pluripotency, $1x10^6$ iPSCs (2 clones for each reprogrammed line) were subcutaneously injected into immunodeficient mice (6 week old SCID female mice, Charles Rivers) to test their ability to generate teratomas. After 2-3 months mice displayed subcutaneous masses that were dissected and paraffin-embedded. An histological evaluation of the explanted tissue by hematoxylin and eosin staining was performed to confirm the presence of teratoma. Differentiation experiments were performed on two clones (number 1 and 5).

1.2 iPSC generation with non-integrating Sendai virus vectors and characterization

The human integration-free iPSC (SeV-iPSC) line used in this study is the result of a collaboration with the research group of Dr. Gianvito Martino (San Raffaele Scientific Institute, Neuroimmunology Unit). Human skin fibroblasts derived from an healthy donor dermal biopsy explant were grown to confluence and infected with viral particles mixture expressing the transcription factors OCT4, SOX2, KLF4 and cMYC in CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Invitrogen) following manufacturer's instructions. On day 7 infected human fibroblasts have been seeded on MEF layer in human ESC medium for 6-8 weeks. When iPSCs colonies started to appear, they were manually picked using a stereomicroscope (Leica) and transferred in 48 multiwell plate matrigel ES (BD) coated and kept in culture with mTeSR1 medium (StemCell Technology). Cells were maintained at 37°C 5% CO_2. To assess that viral genome has been eliminated from the culture, RT–PCR was performed on purified RNA from iPSC clones using recommended set of primers for Sendai Oct4, Sox2, Klf4 and cMYC (see Cytotune kit guidelines). Further, SeV-iPSC clones were characterized for pluripotency by RT-PCR gene expression analysis using the endo pluripotency markers for NANOG, OCT4, SOX2. Sev-iPSC clone 5 was characterized to assess pluripotency by immunofluorescence using the following Antibodies: rabbit anti-Nanog (1:150, Abcam); mouse anti-OCT4 (1:50, Santa Cruz Biotechnology); mouse IgM anti-TRA 1-60 (1:100, Millipore); mouse IgM anti-TRA 1-81 (1:100, Millipore); mouse anti-SSEA4 (1:100, Millipore); mouse anti-SSEA3 (1:100, Millipore). As secondary antibody: AlexaFluor 488/546 conjugated goat anti-mouse IgG (1:1000); AlexaFluor 488/564 conjugated goat anti rabbit- IgG (1:1000); AlexaFluor 647 conjugated goat anti-Rat IgM (1:1000). Nuclei were stained with DAPI. Imaging was performed using a Leica TCS SP5 confocal microscope. To corroborate the pluripotency of reprogrammed SeV-iPSC, the ability to differentiate into cells of the three germ layers was assessed *in vitro* culturing iPSC as EB and analyzed 4 days after by immunofluorescence with corresponding markers: Neuronal Class III Beta Tubulin (clone Tuj1, Covance) for ectoderm, cytokeratin 8-18 (Ck818, Clone EP17/EP30, Dako) for endoderm, Smooth Muscle Actin (aSMA1, clone 1A4, A2547 Sigma) for mesoderm. Further, the pluripotency into cells of the three germ layers was assessed also *in vivo* through a

teratoma formation assay. Tumors were removed and paraffin-embedded tissue sections were stained with hematoxylin and eosin to confirm the presence of teratoma.

2. iPSC differentiation into insulin-producing cells

2.1 Differentiation protocol 1

Human iPSCs were differentiated into insulin-producing cells following Viacyte differentiation protocol with slight modifications (D'Amour et al., 2006). The differentiation schedule consists of 6 stages with different culture conditions: (i) stage 1, mesendoderm (ME) formation: RPMI 1640 (Lonza) supplemented with 100 ng/ml Activin A and 25 ng/ml Wnt3a (R&D Systems) for 2 days; (ii) stage 2, definitive endoderm (DE) formation: RPMI supplemented with 0.2% FBS (Lonza) and 100 ng/ml Activin A for 2 days; (iii) stage 3, primitive gut tube (PG) formation: RPMI supplemented with 2% FBS, 50ng/ml of Fibroblast Growth Factor-10 (FGF-10, R&D Systems) and 0.2 μM KAAD-cyclopamine (CYC, Calbiochem) for 2 days; (iv) stage 4, posterior foregut (PF) formation: DMEM (Lonza) supplemented with 1% B27 (Invitrogen), 50 ng/ml FGF-10, 0.2 μM CYC and 2 μM Retinoic Acid (Sigma Aldrich) for 4 days, replacing with fresh medium on the second day; (v) stage 5, pancreatic endoderm (PE) formation: DMEM supplemented with 1% B27, 1 μM N-[N-(3,5-Difluorophenacetyl) L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma Aldrich) and 50 ng/ml Exendin-4 (AnaSpec) for 3 days; (vi) stage 6, hormone expressing endocrine cells (EN) formation: CMRL-1066 (Connaught Medical Research Laboratories, Mediatech) supplemented with 1% B27, 50 ng/ml Exendin-4, 50 ng/ml Insulin Growth Factor- 1 (IGF-1, Sigma Aldrich) and 50 ng/ml Hepatocyte Growth Factor (HGF, Peprotech) for 5 days, replacing with fresh medium on the second day. Cells were incubated at 37° C with 5% CO_2 and maintained in culture in adhesion in 6-wells plates (Costar) until stage 4 and in 6-wells Ultra Low Attachment Plates (Corning) in suspension condition for stage 5 and 6. The modifications introduced are: (i) 300 nM Indolactam V (Alexis Biochemicals) (Chen et al., 2009) was added to the culture during stages 4 and (ii) cells were detached with 4 mg/ml collagenase IV (Gibco) and re-seeded in Ultra Low Attachment Plates for suspension culture between stages 4 and 5. The differentiation schedule is shown in **Table 2**.

Stage of differentiation	Days of culture	Medium	Supplements
Stage 1: Mesendoderm	2	RPMI 1640 (Lonza)	100 ng/ml Activin A (R&D system) 25 ng/ml Wnt3A (R&D system)
Stage 2: Definitive Endoderm	2	RPMI 1640 (Lonza)	100 ng/ml Activin A (R&D system) 0,2% FBS (Lonza)
Stage 3: Primitive gut tube	2	RPMI 1640 (Lonza)	2% FBS (Lonza) 50 ng/ml FGF-10 (R&D system) 0,2μM CYC (Calbiochem)
Stage 4: Posterior Foregut	4 (fresh medium after 2 days)	DMEM (Lonza)	1% B27 (Invitrogen) 50 ng/ml FGF-10 (R&D system) 0,2μM CYC (Calbiochem) 2μM Retinoic Acid (Sigma Aldrich)
Stage 5: Pancreatic endoderm	3 (culture in suspension)	DMEM (Lonza)	1% B27 (Invitrogen) 1μM DAPT (Sigma Aldrich) 50 ng/ml Exendin-4 (AnaSpec) 300 nM Indolactam V (Alexis Biochemical)
Stage 6: Endocrine cells	4-5 (culture in suspension)	CMRL-1066 (Connaught Medical Research Laboratories, Mediatech)	1% B27 (Invitrogen) 50 ng/ml Exendin-4 (AnaSpec) 50 ng/ml IGF-1 (Sigma Aldrich) 50 ng/ml HGF (Peprotech)

Table 2: Protocol of differentiation of hiPSC into insulin producing cells. *Pellegrini, 2015*.

Cells were imaged during the differentiation steps under an inverted microscope (Leica DMIRE2) equipped with a digital camera (Leica DC300Fx).

2.2 Differentiation protocol 2

Human SeV-iPSC were differentiated into insulin-producing cells following Melton differentiation protocol with some modifications (Pagliuca et al., 2014). The differentiation schedule consists of 5 stages with different culture conditions:

- Stage 1, definitive endoderm (DE) formation: STEMdiff™ Definitive Endoderm Kit (Stemcell technologies) for 4 days;
- Stage 2, primitive gut tube (PG) formation: from this stage on the medium (called Basal Medium, BM) used is MCDB131 (Gibco) supplemented with 8mM D-Glucose, 1.23 g/L $NaHCO_3$, 0.25 mM Vitamin C, 2% Bovine Serum Albumin (BSA, Sigma Aldrich), 1% Pen/Strep and 1% L-Glutamine (Lonza).

  For differentiation BM is supplemented with 50ng/ml of Keratinocyte Growth Factor (KGF, Peprotech) and 1:50000 Insulin-Transferrin-Selenium (ITS, Gibco) for 3 days;

- Stage 3, posterior foregut (PF) formation: BM supplemented with 50 ng/ml KGF, 0.25µM SANT1, 2µM Retinoic Acid, 200nM LDN193189 (only the first day) (Sigma Aldrich), 500nM Phorbol 12,13-dibutyrate (PdBU, Millipore), 1:200 ITS for 2 days;
- Stage 4, pancreatic endoderm (PE) formation: BM supplemented with 50 ng/ml KGF, 0.25µM SANT1, 100nM Retinoic Acid and 1:200 ITS for 4 days;
- Stage 5, hormone expressing endocrine cells (EN) formation: MB supplemented with 12mM D-Glucose, 0,52 g/L NaHCO3, 10µg/ml Heparin (Sigma Aldrich), 0.25µM SANT1, 100nM Retinoic Acid, , 1µM γ-secretase inhibitor XXI (Millipore), 10 µM Alk5 Inhibitor II (Enzo Life Science), 1µM L-3,3',5-Triiodothyronine (T_3, Sigma Aldrich), 20 ng/ml Betacellulin (R&D) and 1:200 ITS for 4 days.

Cells were incubated at 37° C with 5% CO2 and maintained in culture in adhesion in 6-wells plates (Costar) until stage 4 and in 6-wells Ultra Low Attachment Plates (Corning) in suspension condition for stage 4 and 5. Cells were imaged during the differentiation steps under an inverted microscope (Leica DMIRE2) equipped with a digital camera (Leica DC300Fx).

3. Molecular analysis of iPSC differentiation

3.1 RNA extraction and retro-transcription

In order to assess gene expression during differentiation of iPSC, the undifferentiated cells and those derived from each of the 4 stages of differentiation (**Figure 12**) that we established (DE, PF, PE and EN), were washed with PBS, resuspended in 600µl of lysis buffer (mirVana isolation kit, Ambion) and frozen at -80° C until RNA extraction.

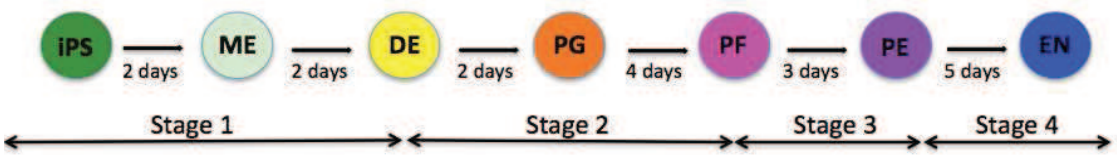

Figure 12. Schematic representation of the steps of *in vitro* differentiation of iPSC into insulin-producing cells and of the 4 stages whose final products have been analyzed to verify gene expression during pancreatic specification.

Total RNA was extracted with mirVana Isolation Kit following manufacturer's instructions. The quality and quantity of RNA extracted were measured by run at 100 volts for 30 minutes on a 1.5% agarose gel and spectrophotometer lecture (Epoch instrument, BioTek, Gen5 analysis software). For RT-PCR, after DNAse (Invitrogen) treatment, RNA was retrotranscribed in a 20-µl reaction volume containing 1–5 µg of total RNA and SuperScript III RT, according to the manufacturer's instructions (Invitrogen).

3.2 Taqman Real-Time PCR

PCR runs and fluorescence detection were carried out in a 7900 Real-Time PCR System (Applied Biosystems) at the following temperature conditions: 50° C for 2 minutes, 95° C for 10 minutes and 50 cycles of 95° C for 15 seconds and 60° C for 1 minute. Each sample was analyzed in duplicate with predesigned gene-specific primer and probe sets from TaqMan Gene Expression Assays (Applied Biosystems) for the following genes: Oct4 (Hs00742896_s1), Nanog (Hs02387400_g1), Sox17 (Hs00751752_s1), Foxa2 (Hs00232764_m1), Hnf1b (Hs00172123_m1), Pdx1 (Ha00195591_m1), Ngn3 (Hs00360700_g1), Nkx2.2 (Hs00159616_m1), Nkx6.1 (Hs00232355_m1), Ins (Hs00356618_m1), Gcg (Hs00174967_m1) and Glk (Hs00175951_m1). Normalized gene expression levels are reported with the highest expression set to 1 and all others relative to this or expressed as fold changes over glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) expression ($2^{-\Delta Ct}$ method).

3.3 Droplet Digital PCR (ddPCR)

Droplet Digital PCR (ddPCR) was used to measure NANOG, SOX17, HNF1b, NGN3 and INS absolute gene expression during differentiation. ddPCR was performed on a QX100 ddPCR system (Bio-Rad) using TaqMan Gene Expression Assays. A total of 50ng of cDNA were used to set-up 3 replicate ddPCR reactions; these were emulsified in a QX100 droplet generator (Bio-Rad), transferred to 96 well plates and subjected to thermal cycling on a T100 instrument (Bio-Rad), according to the manufacturer's instructions. After amplification, plates were read and individual sample droplets analyzed on a Bio-Rad QX100 droplet reader. The number of gene copies/ng of equivalent RNA was determined using the QuantaSoft v1.2.10 software, applying a

correction based on the Poisson distribution to the counted number of droplets positive for the different time points.

3.4 MicroRNAs expression profiling and validation

Analysis of microRNAs was performed in collaboration with the research group of Dr. Francesco Dotta (Diabetes Unit, Department of Medicine, Surgery and Neuroscience, University of Siena). MicroRNAs expression profiling was performed using Taqman Human MicroRNA Array Cards Panel A v2.1 + Panel B v.3.0 (Life Technologies) which allowed us to evaluate the expression of 768 microRNAs. MicroRNAs were reverse-transcribed using Megaplex Human microRNA RT primers pool A v2.1 and pool B v3.0 (Lifetechnologies). A total of 500ng of total RNA/each pool was used for reaction. Then, 9 µl of synthesized cDNA were loaded in Taqman Human MicroRNA Array Cards following manufacturer instructions. ViiA7 Real Time PCR instrument was used to perform Taqman Array Cards reaction runs.

Real-Time PCR for single microRNA expression levels validation was performed using microRNA specific TaqMan MGB probe (Lifetechnologies) and TaqMan Universal Master Mix II in duplicate in a VIIA7 Real Time PCR instrument (Applied Biosystem) following manufacturer's guidelines. The following Taqman microRNA expression assays were used: hsa-miR-9- ID:000583, hsa-miR-9#- ID:002231, hsa-miR-10a- ID:000387, hsa-miR-31- ID:002279, hsa-miR-99a- ID:000435, hsa-miR-124a- ID:001182, hsa-miR-127- ID:000452, hsa-miR-135a- ID:000460, hsa-miR-138- ID:002284, hsa-miR-143- ID:002249, hsa-miR-149- ID:002255, hsa-miR-211- ID:000514, hsa-miR-224- ID:002099, hsa-miR-302c#- ID:000534, hsa-miR-342-3p- ID:002260, hsa-miR-373- ID:000561, hsa-miR-375- ID:000564, hsa-miR-518b- ID:001156, hsa-miR-520c-3p- ID:002400. MicroRNA expression levels were normalized to the internal controls smallRNAs RNU48 and RNU6.

3.5 Gene ontology classification analysis

MicroRNA target genes prediction analysis was performed by Dr. Dotta's research group employing the online algorithm Targetscan (Release 6.2) and applying specific cutoff parameters: at least 2 target sites within target gene 3'UTR sequences or, when just 1-site type prediction was present, a total context score <0.40. Functional

classification of putative microRNA target genes according to Targetscan 6.2 analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatic Resources 6.7 as previously described (Huang, Sherman, & Lempicki, 2009). Briefly, predicted target genes of upregulated or downregulated microRNAs during differentiation stages were taken into consideration and their official gene name retrieved from NCBI databank (http://www.ncbi.nlm.nih.gov/gene/). Gene ontology classification term "Biological Process" (GOTERM_BP_ALL) was used to search for target genes classification categories. FDR (False Discovery Rate) corrected (Benjaminy-Hoechberg) p-values <0.05 were used to further select potential categories of interest.

3.6 Data and statistical analysis

For gene expression data, a nonparametric test (Mann–Whitney) was used to compare groups, and a 2-tailed *P* value less than 0.05 or 0.01 was considered significant. Analysis of data was performed using the SPSS statistical package for Windows (SPSS Inc.). For miRNA expression the data were analyzed using the Expression Suite Software 1.0.3 (Lifetechnologies) and GraphPad 5.1 software by applying the 2-ΔCt or 2-ΔΔCt method and differentially expressed miRNAs were identified using a fold change cutoff <0.1 (downregulation) and >10.0 (upregulation). Student t-test was performed on normalized ΔCT values in order to select significantly differentially expressed microRNAs ($p<0.02$).

4. Cytofluorimetric analysis of iPSC differentiation

For cytofluorimetric analysis human iPSC during the defined steps of *in vitro* differentiation were dispersed into single-cell suspension by incubation in 0,25% trypsin (BioWhittaker, CambrexBio Science) at 37°C for 5 minutes; cells were then collected and washed twice with PBS (Lonza). Cells were stained with Live/Dead (Molecular Probes, lifetechnologies) to exclude dead cells from the analysis. Intra cellular staining required cell fixation and permeabilization (Cytofix/Cytoperm, BD Bioscience). Cells were then washed with blocking buffer (PBS+2g/L Bovine Serum Albumin, BSA, Sigma) and stained using the following monoclonal antibodies (mAbs): 813-70 FITC anti-Ssea-4 (BD Bioscience); 44716 PE anti-Cxcr4 (R&D); 182410 APC

anti-insulin (R&D); 199017 PE anti-Glut2 (R&D); CLB/TF-5 FITC anti-CD142 (Novus Biologicals); R11-560 PE anti-Nkx6.1 (BD Bioscience). Analysis was carried out on a FACS Canto flow cytometer (BD Bioscience) using FACS Diva software (BD Bioscience). Results were analyzed with FCS Express 4 (De Novo software) and expressed as the mean percentage of positive cells and standard deviation (SD) from multiple experiments.

5. C-peptide content and release assays

Human C-peptide levels in culture supernatants at every differentiation stage were measured using ELISA kit following manufacturer's instructions (Mercodia). C-peptide release in response to stimuli was measured by incubating *in vitro* terminally differentiated iPSCs in Krebs–Ringer solution [25 mMNaCl, 5 mMKCl, 1 mM CaCl2, 24 mM NaHCO3, 1 mM MgCl2, 10 mM HEPES, 0.1 % (wt/vol) BSA (Sigma)]. Initial 1-h incubation was considered a wash; the medium was discarded, and this was followed by a 1-h incubation in basal medium containing 0.5 mM D-glucose (Sigma) and then a 1-h incubation in the stimulation condition [5, 11, 20 and 27 mM D-glucose (+ or - 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Gibco) and 30 mM KCl (Sigma)]. C-peptide release was then measured by C-peptide ELISA kit. The fold stimulation was calculated for each culture by dividing the C-peptide concentration in the stimulation supernatant by the C-peptide concentration in the basal supernatant.

6. Transplantation of differentiated iPSC under the kidney capsule of NOD/SCID mice and evaluation of graft function

To assess the ability of iPSC to differentiate into pancreatic cells and to secrete insulin *in vivo*, a total of 4–5 × 10^6 differentiated human iPSC at the stage of posterior foregut or of endocrine cells were transplanted under the kidney capsule of immunodeficient NOD/SCID mice (male, 8 weeks old, Charles River). At least 3 mice for each time point were transplanted. For transplantation, the animals were anesthetized by intraperitoneal injection of 2,2,2-Tribromoethanol (20 mg/ml, Sigma), an incision was made to allow the exposure of the left kidney, subsequently a small hole on the surface of the renal capsule was performed and the cells were infused through the use of a P-50 catheter. Then the incision was cauterized, the kidney repositioned and subcutaneous

tissue and skin were sutured. Blood sugar levels were measured every week post-transplantation with Ascensia Confirm Glucometer (Bayer). An oral glucose tolerance test (OGTT) was performed at 1, 4 and 12 weeks after transplantation to evaluate the function of the transplanted cells. OGTT was initiated after a 4-h fast; mice were given glucose (1 g/kg) by oral gavage. Blood samples were collected at 0, 10, 30 and 60 min after glucose administration and were used to determine glucose concentrations. Serum C-peptide was measured by ELISA assay (Ultrasensitive C-peptide ELISA kit, Mercodia). Care of animals was in accordance with institutional guidelines.

7. Immunohistochemical analysis of cell engraftment and evaluation of graft composition

For morphological investigations, recipient mice were sacrificed at 1, 4 and 12 weeks after transplantation by cervical dislocation. Kidneys were explanted and fixed in 10% buffered formalin (Sigma) and processed routinely for histology. Briefly, the organs were then embedded in paraffin and cut with microtome (Leica RM 2035). The sections placed on glass slides were treated with Bio-clear (Bio-Optica) for 30 minutes to eliminate the residual paraffin. Slides were then rehydrated with decreasing concentrations (from 99% to 70%) of alcohol until distilled water. The immunohistochemical evaluation required an initial unmasking specific for each antibody and subsequently the incubation of the sections with the primary antibodies (listed in **Table 3**) for 1 hour at room temperature. The peroxidase-antiperoxidase immunohistochemistry method (Labvision, Thermo Scientific) was used for detection.

Antigen	Clone	Supplier	Dilution
SOX2	polyclonal	R&D	1:100
CK 8/18	NCL-5D3	Santa Cruz	1:400
GFAP	polyclonal	Dako	1:800
PDX1	EPR3358(2)	Epitomics	1:5000
KI67	SP60	Bio-Optica	1:100
INS	polyclonal	Dako	1:50
GCG	polyclonal	Novocastra	1:200
SYP	27G12	Novocastra	1:200
CHGA	LK2H10	BioGenex	1:600

Table 3: Antibodies used for hystochemical analysis of transplanted hiPSCs-derived cells

The sections were counterstained with hematoxylin (Bio-Optica) and analyzed with a color camera, which allows scanning and digitalization of the slide by multiple vertical scans with 40× magnification (AperioScanscope, Leica) and acquired with the SpectrumTM Plus software.

V. RESULTS

1. Generation and characterization of iPSC reprogrammed with retroviral vectors

Human lung fetal fibroblast IMR90 cell line was infected with OCT4-, SOX2- and KLF4-expressing retroviruses and cultured in human ESC medium. After 6–8 weeks, ESC-like colonies of iPSC appeared and the clones #1 and #5 were further characterized and used in this study. Reprogrammed cells exhibited the typical morphology of pluripotent cells (**Figure 13, A-B**) and expressed the pluripotency-related proteins alkaline phosphatase (AP), OCT4 and NANOG (**Figure 13, C-J**).

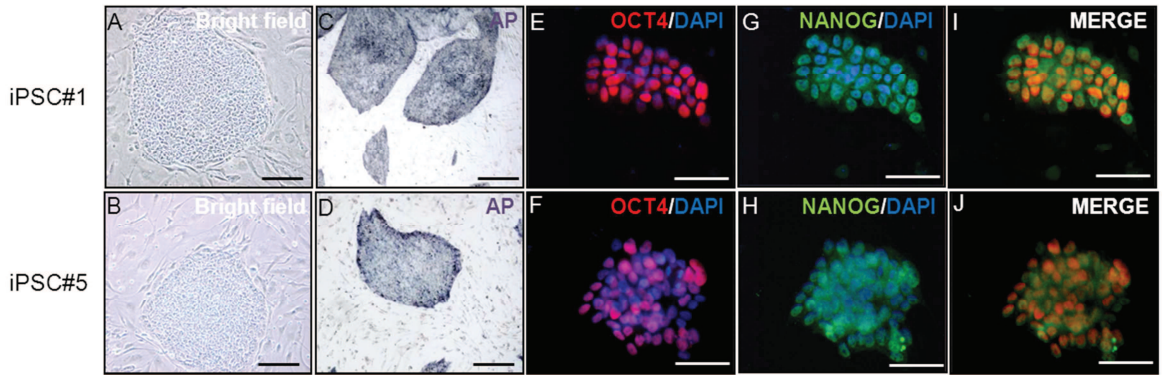

Figure 13. Two lines of iPSC reprogrammed from fetal fibroblasts (clones 1 and 5) were characterized in terms of pluripotency. A-B. Morphology of iPSC colonies at the end of the reprogramming process; C-D. Staining for alkaline phosphatase activity; scale bar50 µm. E-J. Staining for OCT4 (red) and NANOG (green) expression by immunofluorescence; nuclei evidenced by DAPI staining (blue); scale bar 25 µm.

Molecular analysis by RT-PCR on both iPSC clones revealed the expression of the pluripotency related genes Oct4, Sox2, Dppa4, Fgf4, Rex1, Tdgf1, Dppa2, Gdf3 and hTert, that were not expressed in IMR90 fibroblasts before reprogramming (**Figure 14**).

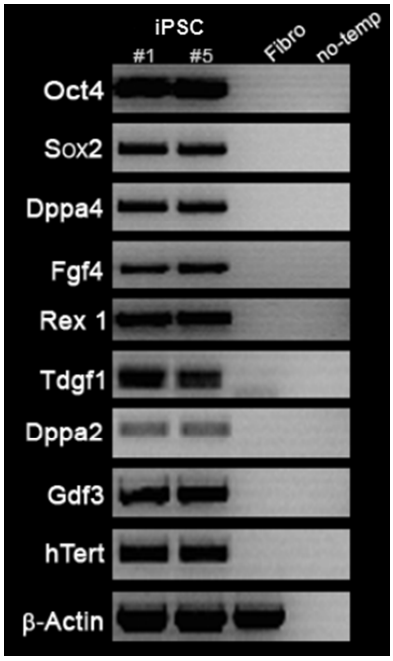

Figure 14. Expression by RT-PCR of pluripotency genes Oct4, Sox2, Dppa4, Fgf4, Rex1, Tdgf1, Dppa2, Gdf3 and hTert in iPSC clone 1 and 5 and in fibroblasts before reprogramming. Beta actin was used as positive control gene. No-temp: negative control with no cDNA template.

The ability of the reprogrammed cells to give rise to cells of all three embryonic germ layers *in vitro* was also tested to assess their pluripotency. After the formation of Embryoid Bodies (EB) and the growth on gelatine coated plates at a high percentage of FBS, iPSC were able to spontaneously differentiate into cells of the three germ layers, as shown by the expression of marker typical of endoderm (SOX17), mesoderm (SMA) or neuroectoderm (TUJ1 and NESTIN) cells (**Figure 15**).

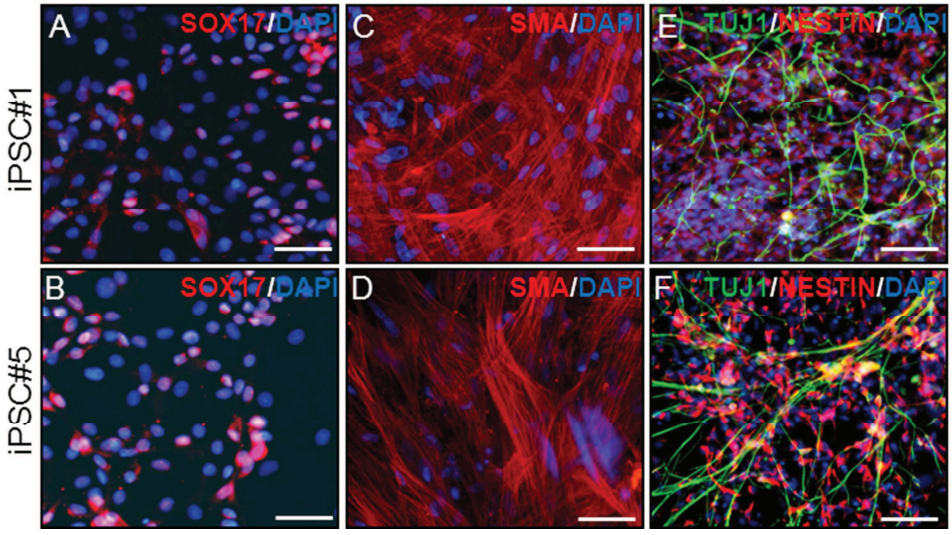

Figure 15. Assessment of pluripotency *in vitro*. A-B: Staining for the endodermal marker SOX17 (red); C-D: Staining for the mesodermal marker Smooth Muscle Actin (SMA, red); scale bar 25 µm. E-F: Staining for the ectodermal markers Tuj1 (green) and Nestin (red); scale bar 50 µm. Nuclei evidenced by DAPI staining (blue).

We also tested the capacity of the reprogrammed cells to give rise to all three embryonic germ layers *in vivo* through a teratoma formation assay. After injection into nude immunodeficient mice, both human iPSC lines were able to spontaneously differentiate into cells of different tissues (**Figure16**).

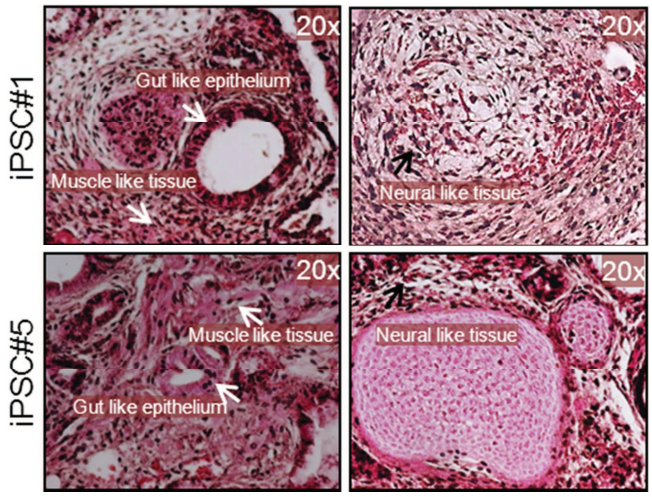

Figure 16. Assessment of pluripotency in vivo. Staining with hematoxylin/eosin of teratomas generated by iPSC injected in immunodeficient mice: gut, neural and muscle-like tissues are present and indicated by arrows; magnification ×20.

2. Differentiation of iPSC into insulin-producing cells *in vitro*

Human iPSC generated with retroviral infection of fetal fibroblasts were cultured with a modified version of a protocol described for the differentiation of ESC into pancreatic β cells (D'Amour et al., 2006). This protocol was developed after a comparative evaluation of the protocol described by Viacyte group with or without the supplementation of Indolactam V (Chen et al., 2009) and the culture in suspension conditions (J. Jiang et al., 2007).

To verify the actual differentiation of human iPSC during the various stages of pancreatic specification (**Figure 17**) we evaluated the expression levels of the following genes:

- OCT4 and NANOG: characteristic markers of undifferentiated cells, used to

assess both the real pluripotency of the iPSC used and to ensure the loss of the expression of these genes during differentiation;

- SOX17 and FOXA2: characteristic markers of the definitive endoderm (DE). Their expression during the first steps of the differentiation determines the transition from pluripotent cells to DE cells;
- HNF1b and PDX1: typical markers of posterior foregut (PF) cells. Their expression is fundamental for the regulation of the development of embryonic pancreas. PDX1 in particular, is a key transcription factor for the activation of insulin, glucokinase and glucose transporter type 2 (GLUT2) gene transcription.
- NGN3 and NKX2.2: transcription factors typically expressed by pancreatic endoderm (PE) cells and that regulate β cells function.
- INS: characteristic marker of the pancreatic endocrine β cells.

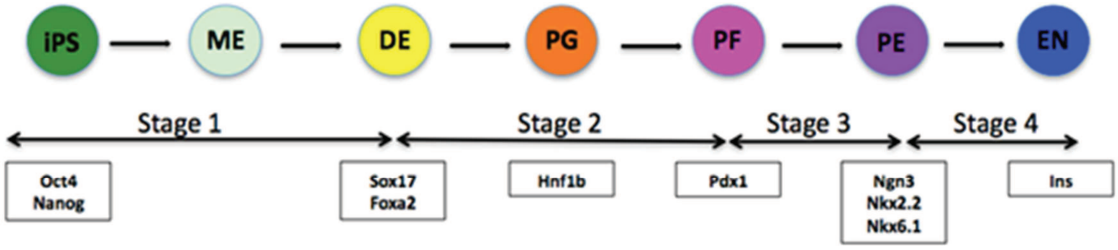

Figure 17. Schematic representation of the stages of differentiation of iPSC into insulin-secreting cells, the 4 stages that have been analyzed and the genes examined to verify the gene expression of the cells during pancreatic specification at the end of each stage.

Gene expression levels are expressed as Fold Change (FC) compared to ΔCt of undifferentiated iPSC. The ΔCt of the 7 different replicates of iPSC used in this study, expressed as mean ± standard error, are: Oct4: 10.6 ± 0.4; Nanog: 6.7 ± 0.5; Sox17: 10.5 ± 0.5; Foxa2: 9.1 ± 0.9; Hnf1b: 10.5 ± 0.7; Pdx1: 26.8 ± 2.1; Ngn3: 18.8 ± 1.3; Nkx2.2: 19.2 ± 2.1; Ins: 20,4 ± 1.4.

When the protocol of differentiation proposed by D'Amour et al. in 2006 was tested on our human iPSC we obtained the following changes in gene expression compared to undifferentiated cells (Fold Change, FC = 1):

- At the end of the first stage of differentiation the down-regulation of the

expression of pluripotency genes Oct4 and Nanog (0.06 and 0.04 FC respectively) and an over-expression of the definitive endoderm marker genes Sox17 and Foxa2 (48.8 and 12.6 FC).

- At the end of the second stage the over-expression of mRNA characteristic of pancreatic progenitor cells: Hnf1b, Pdx1 and Ngn3 (134.9, 962.9 and 1772.5 FC).
- At the end of the *in vitro* differentiation process we observed no expression of the key markers and transcription factors of β cells specification Pdx1, Ngn3, Nkx2.2 and, in particular, Insulin.

We then decided to introduce two modifications to the last critical steps of *in vitro* differentiation process in order to obtain the expression of insulin and of all the others pancreatic markers, in details:

- the addition of Indolactam V after primitive gut tube (PG) specification to promote the differentiation into pancreatic endoderm cells (Chen et al., 2009);
- the detachment of cells after posterior foregut (PF) specification and the seeding of cell clusters in suspension conditions mimicking *in vitro* pancreatic islet culture, to favour β cells maturation and survival (J. Jiang et al., 2007).

Morphology, gene and protein expression of iPSC during differentiation with this modified version of the original Viacyte protocol were analyzed in seven independent experiments.

Cell morphology changed from the adherent colonies of iPSC composed of small cells with scant cytoplasm, to bigger cell aggregates composed of a central cluster of cells surrounded by cells with elongated shapes, to cluster of cells in suspension condition, as shown in **Figure 18**.

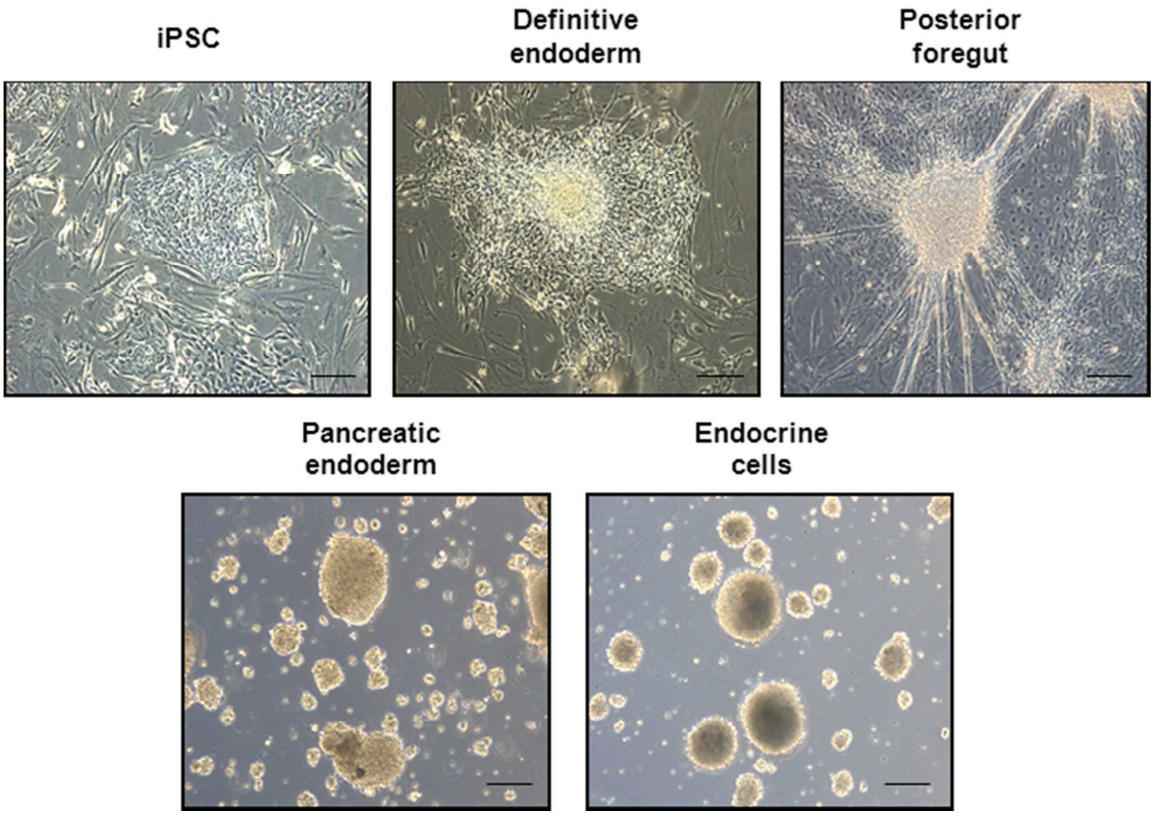

Figure 18: Representative pictures of cell morphology of iPSC during the steps of pancreatic specification, scale bar 100 µm.

We first evaluated the loss of pluripotency, that was confirmed by the downregulation of *OCT4* and *NANOG* genes and the disappearance of the surface marker Ssea-4. The loss of the undifferentiated status was accompanied by the increase in the expression of *SOX17* and *FOXA2* genes (39.25 ± 15.78 and 7.94 ± 4.16 FC over undifferentiated iPSC) and of the chemokine receptor Cxcr4, characteristic traits of the definitive endoderm stage. At the subsequent step of posterior foregut specification, the upregulation of *HNF1b* and *PDX1* genes was observed (97.14 ± 77.01, 596.34 ± 368.78 FC), while at the following differentiation step into pancreatic endoderm, *NGN3* and *NKX2.2* genes appeared in the transforming cell population (83.46 ± 80.34 and 1.62 ± 0.9 FC). Furthermore, CD142 expression, previously described as a marker of endocrine progenitor cells (Kelly et al., 2011), increased in differentiating iPSC at this time point. Finally, at the end of the differentiation process, the production of *INSULIN* mRNA was highly increased (1567.92 ± 785.1 FC) and, accordingly, analysis by flow cytometry showed the presence of insulin-positive cells at the final step of differentiation, confirming the conversion of undifferentiated iPSC into

pancreatic cells following developmental stages (**Figure 19 and 20**).

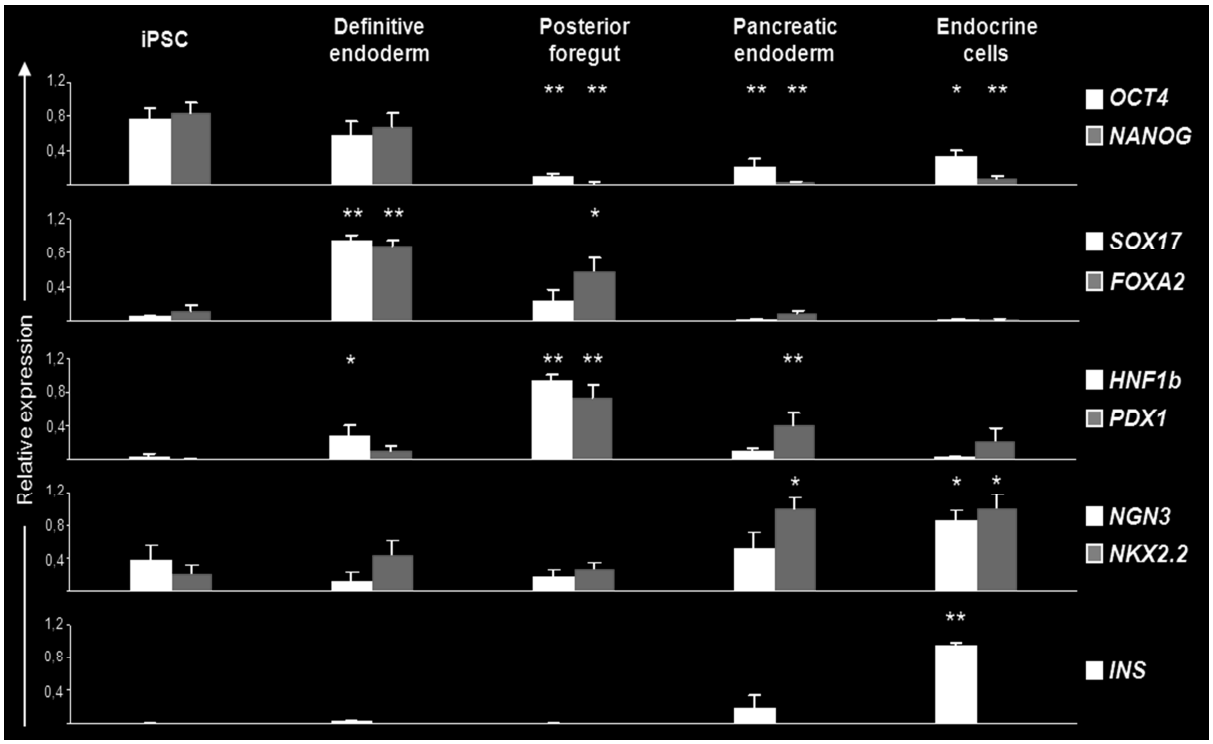

Figure 19. Gene expression analysis by Taqman of markers of pluripotency (OCT4 and NANOG), definitive endoderm (FOXA2 and SOX17), posterior foregut (HNF1b and PDX1), pancreatic endoderm (NGN3 and NKX2.2) and endocrine cells (INS). Normalized gene expression levels are reported with the highest expression set to 1 and all the others relative to this and expressed as mean + SEM of 7 experiments. *P < 0.05; **P < 0.01 compared to undifferentiated hiPSC (Mann–Whitney test). *Pellegrini, 2015.*

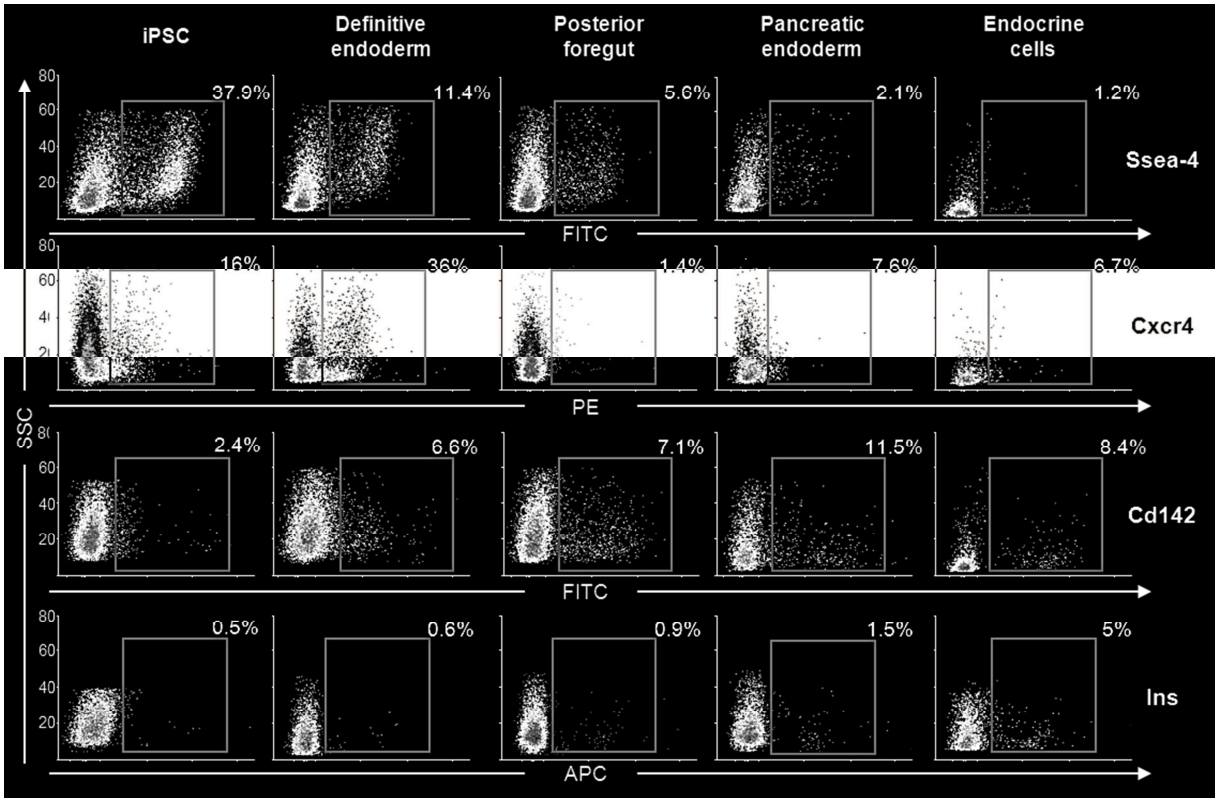

Figure 20. Protein expression analysis by flow cytometry of markers of pluripotency (Ssea 4), definitive endoderm (CXCR4), pancreatic endoderm (CD142) and endocrine cells (Ins). Gate delimitates positive events. Percentages of positive cells of a representative experiment are reported. SSC: side scatter, FITC: Fluorescein isothiocyanate, PE: phycoerythrin, APC: Allophycocyanin. *Pellegrini, 2015.*

Absolute quantification by droplet digital PCR of *NANOG*, *SOX17*, *HNF1B* and *NGN3* gene expression during differentiation corroborated these findings (**Figure 21**).

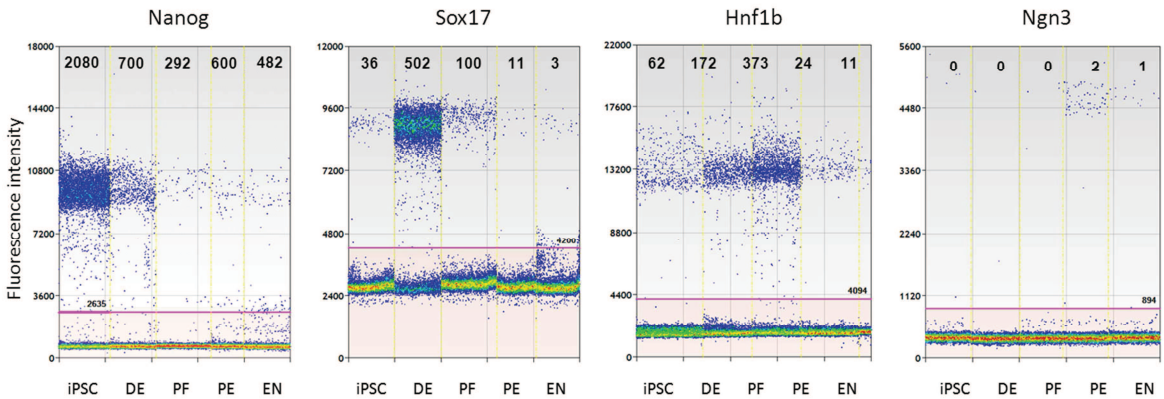

Figure 21. Quantification of NANOG, SOX17, HNF1b and NGN3 mRNAs during differentiation of iPSCs into insulin-producing cells by droplet digital PCR. Gene expression was analyzed at the steps of pluripotency (iPSC), definitive endoderm (DE), posterior foregut (PF), pancreatic endoderm (PE) and endocrine cells (EN). PCR amplification of iPSCs cDNAs was performed in an emulsion using gene

specific primers and hydrolysis probes. The number of gene specific mRNA copies in each sample, corresponding to droplets fluorescing above background level (red line), was determined after droplets acquisition and counting on a QX100 instrument (Bio-Rad) and the QuantaSoft software v1.2.10 applying a correction algorithm based on the Poisson distribution. The number of gene specific mRNA copies per nanogram of total RNA in each sample of one representative experiment is shown. *Pellegrini, 2015.*

These data refer to differentiation of iPSC derived from the reprogramming of fetal fibroblasts; however, the protocol we set up resulted successful also for the differentiation of iPSC obtained from the reprograming of fibroblasts of an healthy adult subject (**Figure 22**).

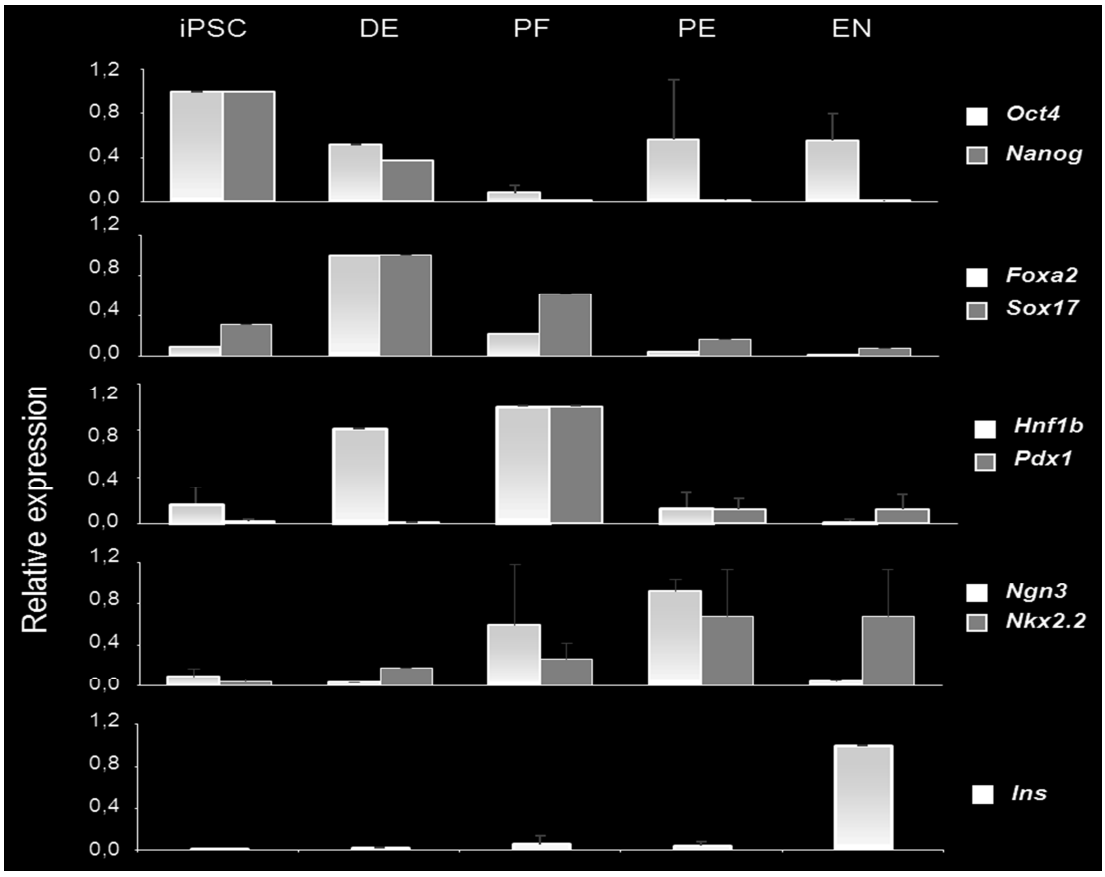

Figure 22. Gene expression of adult fibroblast-derived iPSC was analyzed during differentiation at the steps of pluripotency (iPSC), definitive endoderm (DE), posterior foregut (PF), pancreatic endoderm (PE) and endocrine cells (EN). Gene expression analysis by Taqman of markers of pluripotency (OCT4 and NANOG), definitive endoderm (FOXA2 and SOX17), posterior foregut (HNF1b and PDX1), pancreatic endoderm (NGN3 and NKX2.2) and endocrine cells (INS) is shown. Normalized gene expression levels are reported with the highest expression set to 1 and all the others relative to this. Histograms represent mean of n=2 experiments ± SEM. *Pellegrini, 2015.*

3. MicroRNAs expression profiles during iPSC differentiation into insulin-producing cells

Given the pivotal role of microRNAs in reprogramming of somatic into pluripotent stem cells (Miyoshi et al., 2011; Anokye-Danso et al., 2011), it is conceivable that this class of small RNAs may play a role also in the regulation of cell fate specification during iPSC differentiation into insulin-producing cells. Therefore, we evaluated microRNA expression profiles during the 4 main stages of endocrine pancreatic differentiation (iPSC, DE, PF and EN cells) in three independent experiments. Among 768 microRNAs analyzed, 347 resulted expressed (Ct≤35 in all samples of the same group). Firstly, we identified microRNAs specifically expressed in undifferentiated iPSC, as reported in the hierarchical heatmap clustering (**Figure 23, left panel**). Among highly expressed microRNAs (detailed zoom of hierarchical clustering analysis in **Figure 23, right panel**) we found those belonging to miR-17/92 cluster (miR-19b, miR-17, miR-20a, miR-19a) and its paralogs (miR-106a, miR-93) as well as miR-302 cluster (miR-302a, miR-302b, miR-302c and miR-367), previously reported as representative microRNA families highly expressed in ESC and iPSC (Wilson et al., 2009; Mogilyansky & Rigoutsos, 2013), thus demonstrating and confirming the pluripotent phenotype microRNA fingerprint. Indeed, microRNAs miR-19b and miR-302b showed the highest expression levels in iPSC, reporting ΔCT values of -1,18 and -0.60 respectively.

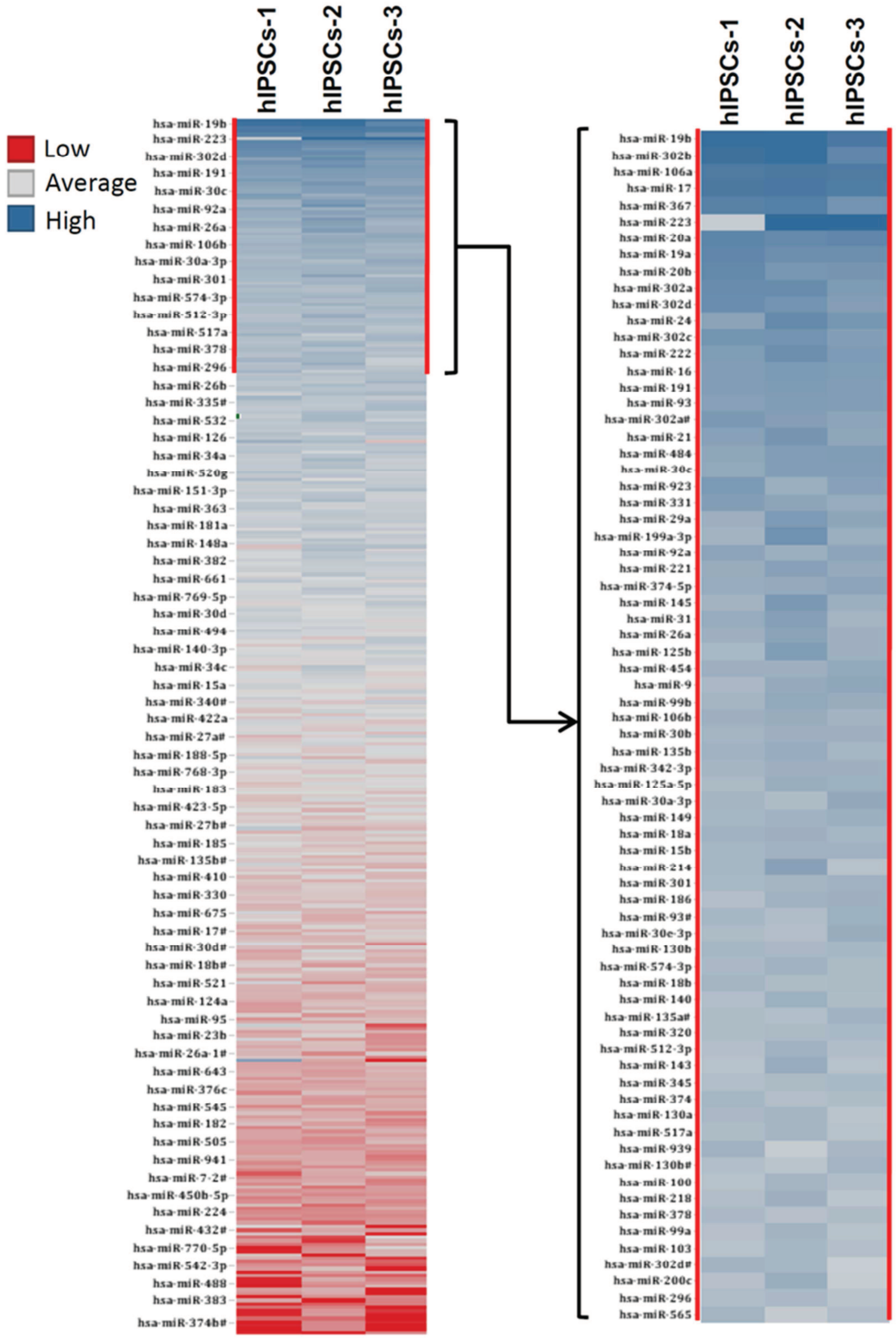

Figure 23. Total microRNA expression profiles in undifferentiated human iPSC are reported in hierarchical clustering analysis in which normalized ΔCT values of miRNAs are reported as scale colour (scale colour: blue=high expression; red=low expression) (Euclidean distance measure, correlation). Red

line highlighting upper part of hierarchical clustering represents most expressed miRNAs in iPSC. Detailed zoom of the red line highlighted hierarchical clustering is reported in the right panel.

In order to verify whether microRNAs are differentially expressed during iPSC differentiation into insulin-producing cells and which may indeed play a role in the acquisition of endocrine pancreatic phenotype, we compared the microRNA expression profiles of iPSC during the steps of differentiation respect to undifferentiated cells. Taqman array profiling analysis revealed 19 microRNAs differentially expressed ($p<0.05$) during differentiation stages vs undifferentiated iPSC. Specifically, we observed 12 microRNAs upregulated (miR-9, miR-9#, miR-375 miR-10a, miR-99a#, miR-124a, miR-135a, miR-138, miR-149, miR-211, miR-342-3p and miR-224) and 7 downregulated (miR-520c-3p, miR-302c#, miR-31, miR-127, miR-143, miR-373, miR-518b), reported in the hierarchical clustering heatmap (**Figure 24**).

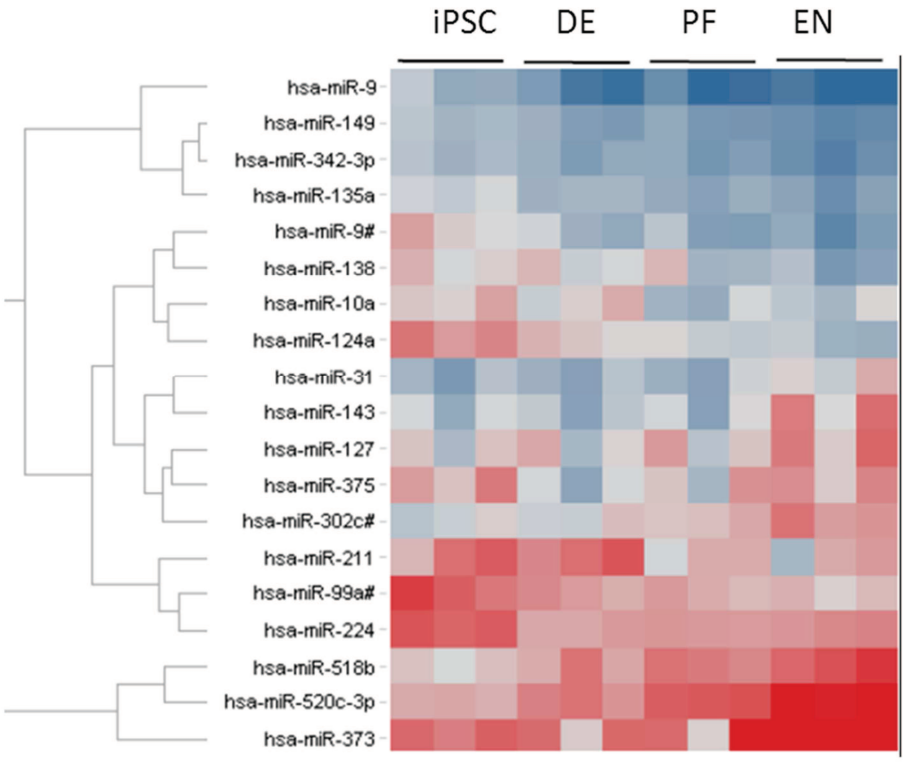

Figure 24. Hierarchical clustering analysis of differentially expressed miRNAs (fold change <0.1 and >10 and $p<0.02$, t-test): 19 miRNAs were found as differentially expressed (12 upregulated and 7 downregulated) during progressive developmental stages (DE: definitive endoderm; PF, posterior foregut; EN, endocrine pancreatic cells) compared to undifferentiated iPSC. 3 replicates for each differentiation stage are reported. MicroRNAs expression levels are reported as ΔCT values (scale colour: blue=high expression; red=low expression) (Euclidean distance measure, correlation) .

In order to confirm and validate the differential expression of those microRNAs identified through high-throughput profiling, we analyzed their expression by single assay RT-Real Time PCR. We initially validated those microRNAs, which showed an upregulation pattern during differentiation stages respect to undifferentiated cells; indeed, we confirmed the upregulation of all identified microRNAs except for miR-224 (**Figure 25**).

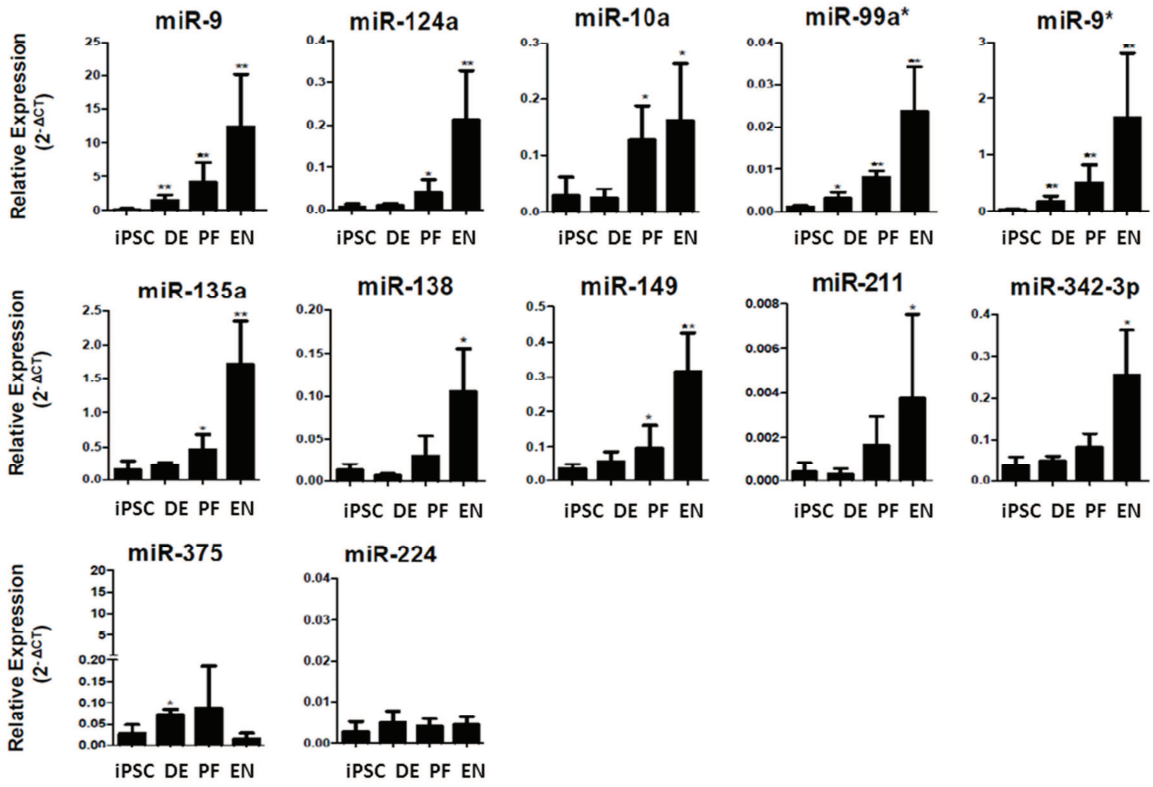

Figure 25. Differentially expressed microRNAs single assay validation through RT-Real Time PCR of upregulated microRNAs during differentiation stages. Values are reported as mean ± SEM of 2-ΔCT normalized using RNU6 and RNU48 of 3 independent experiments. * $p<0.05$, ** $p<0.001$.

Subsequently, we also validated those microRNAs which resulted downregulated during endocrine pancreatic differentiation in the initial screening. The validation process confirmed the results obtained using the profiling approach; specifically, among downregulated microRNAs we identified miR-302c#, miR-518b and miR-520c-3p which showed a progressive decrease throughout the differentiation stages. Such microRNAs have been previously associated with pluripotent phenotype (Wilson et al.,

2009; Razak et al., 2013) thus demonstrating the progressive loss of pluripotency during endocrine pancreatic differentiation (**Figure 26**).

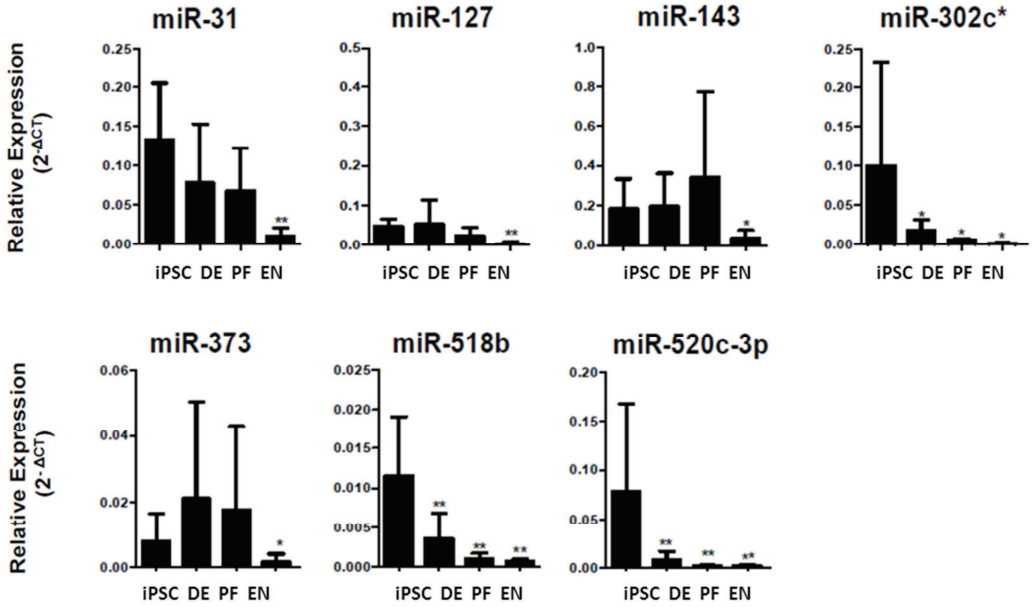

Figure 26. Differentially expressed microRNAs single assay validation through RT-Real Time PCR of downregulated microRNAs during differentiation stages. Values are reported as mean ± SEM of 2-ΔCT normalized using RNU6 and RNU48 of 3 independent experiments. * $p<0.05$, ** $p<0.001$.

In order to reveal putative functional pathways/biological processes regulated by differentially expressed microRNAs identified during iPSC differentiation into insulin-producing cells, we adopted a bioinformatic approach using two different online algorithms: Targetscan 6.2 (prediction of microRNA target genes) and DAVID 6.7 (functional classification of target genes). Firstly, we obtained the putative/predicted microRNA target genes list using Targetscan6.2, by specifically focusing on those microRNAs significantly upregulated in the analyzed stages of differentiation compared to iPSC (**Table 4**).

	GO accession ID	GO Term	No. of Genes	% of total	p-Value	Fold Enrichment
Definitive Endoderm	GO:0000902	cell morphogenesis	5	13,16	0,010154	5,7
	GO:0032989	cellular component morphogenesis	5	13,16	0,014679	5,1
	GO:0009968	negative regulation of signal transduction	4	10,53	0,015852	7,3
	GO:0010648	negative regulation of cell communication	4	10,53	0,021471	6,5
	GO:0030512	negative regulation of TGFbeta receptor signaling pathway	2	5,26	0,040189	47,4
	GO:0031018	endocrine pancreas development	2	5,26	0,040189	47,4
	GO:0045165	cell fate commitment	3	7,89	0,043987	8,7
	GO:0002064	epithelial cell development	2	5,26	0,049415	38,4
Posterior Foregut	GO:0048513	organ development	28	28,28	0,000001	2,7
	GO:0009888	tissue development	15	15,15	0,000034	3,7
	GO:0032989	cellular component morphogenesis	11	11,11	0,000119	4,6
	GO:0000902	cell morphogenesis	10	10,10	0,000260	4,7
	GO:0048468	cell development	11	11,11	0,004792	2,8
	GO:0002064	epithelial cell development	3	3,03	0,006828	23,7
	GO:0021575	hindbrain morphogenesis	3	3,03	0,008163	21,7
	GO:0007155	Cell adhesion	11	11,11	0,008472	2,6
	GO:0022610	Biological adhesion	11	11,11	0,008554	2,6
Endocrine cells	GO:0009888	tissue development	14	13,08	0,000161	3,5
	GO:0032989	cellular component morphogenesis	10	9,35	0,000627	4,1
	GO:0009653	Anatomical structure morphogenesis	18	16,82	0,000678	2,5
	GO:0000902	cell morphogenesis	9	8,41	0,001344	4,1
	GO:0009101	Glycoprotein biosynthetic process	6	5,61	0,002633	6,2
	GO:0045595	regulation of cell differentiation	10	9,35	0,002772	3,3
	GO:0048468	cell development	11	10,28	0,005215	2,8
	GO:0010558	negative regulation of macromolecule biosynthetic process	10	9,35	0,005548	3,0
	GO:0031327	negative regulation of cellular biosynthetic process	10	9,35	0,006517	2,9
	GO:0002064	Epithelial cell development	3	2,80	0,006986	23,4
	GO:0043413	Biopolymer glycosylation	5	4,67	0,007419	6,4
	GO:0006486	protein amino acid glycosylation	5	4,67	0,007419	6,4
	GO:0070085	Glycosylation	5	4,67	0,007419	6,4
	GO:0009100	Glycoprotein metabolic process	6	5,61	0,007430	4,9
	GO:0044262	Cellular carbohydrate metabolic process	8	7,48	0,008710	3,4
	GO:0045165	cell fate commitment	5	4,67	0,009855	5,9
	GO:0031016	pancreas development	3	2,80	0,018747	14,1
	GO:0006887	Exocytosis	4	3,74	0,032120	5,7

Table 4: David6.7 Gene Ontology classification analysis of upregulated microRNA target genes subdivided into differentiation stages. Functional category accession ID (GO accession ID), specific name (GO term) number of genes included (NO. of Genes), percentage of gene respect to total genes included in the analysis (% of total), significance of gene-term enrichment with a modified Fisher's Exact Test corrected with Benjamini post-hoc test (p-value) and magnitude of enrichment (Fold enrichment) for each term are reported. Grey underlined terms were considered of particular interest in that specific differentiation stage.

Interestingly, we detected several functional gene ontology (GO) categories specific for each differentiation stages. Specifically, we observed that microRNAs upregulated in

definitive endoderm stage are predicted to target several genes whose functional categories mainly belong to early stage morphogenesis function ('cell morphogenesis'- GO:0000902 p=0.010,) whereas those upregulated in posterior foregut stage putatively regulate target genes involved in tissue and organ development ('tissue development'- GO0009888 p<0.0001, 'organ development' – GO0048513 p<0.0001). Moreover, in the last stage of differentiation we detected several upregulated microRNA target genes whose functional categories can be ascribed to pancreas development and endocrine pancreatic cell functions ('pancreas development' – GO0031016 p=0.018, 'exocytosis'- GO0006887 p=0,032), thus suggesting a putative post-transcriptional control by microRNAs to those genes involved in the final stage of endocrine pancreatic differentiation. Next, we analyzed downregulated microRNAs target genes. MicroRNAs downregulated ate DE and PF stages (miR-302#, miR-518b, miR-520c-3p), having the same set of differentially expressed microRNAs, putatively regulate the same set of target genes. In this case, targeted genes mainly belong to kinase control function ('regulation of phosphorylation'- GO0042325 p=0,0012), whereas genes predicted to be targeted by downregulated microRNAs in the final differentiation stage mainly belong to the control of metabolic processes ('regulation of cellular metabolic processes'- GO031323 p=0,0007) (**Table 5**).

	GO accession ID	GO Term	No. of Genes	% of total	p-Value	Fold Enrichment
Posterior Foregut	GO:0042325	regulation of phosphorylation	6	17,6	0,0012	7,0
	GO:0051174	regulation of phosphorus metabolic process	6	17,6	0,0014	6,7
	GO:0019220	regulation of phosphate metabolic process	6	17,6	0,0014	6,7
	GO:0045859	regulation of protein kinase activity	5	14,7	0,0030	7,9
	GO:0008152	metabolic process	21	61,8	0,0068	1,5
	GO:0019222	regulation of metabolic process	13	38,2	0,0132	1,9
Endocrine cells	GO:0031323	regulation of cellular metabolic process	18	48,6	0,0007	2,2
	GO:0019222	regulation of metabolic process	18	48,6	0,0013	2,1
	GO:0060255	regulation of macromolecule metabolic process	16	43,2	0,0038	2,0
	GO:0080090	regulation of primary metabolic process	16	43,2	0,0042	2,0
	GO:0044260	cellular macromolecule metabolic process	21	56,8	0,0048	1,7
	GO:0031326	regulation of cellular biosynthetic process	14	37,8	0,0117	2,0
	GO:0009889	regulation of biosynthetic process	14	37,8	0,0123	2,0

Table 5: David 6.7 Gene Ontology classification analysis of downregulated microRNA target genes subdivided into differentiation stages. Functional category accession ID (GO accession ID), specific name (GO term) number of genes included (No. of Genes), percentage of genes respect to total genes included in the analysis (% of total), significance of gene-term enrichment with a modified Fisher's Exact Test corrected with Benjamini post-hoc test (p-value) and magnitude of enrichment (Fold enrichment) for each term are reported. Grey underlined terms were considered of utmost interest in that specific differentiation stage.

4. Terminally differentiated cells exhibit β cell characteristics

Human iPSC terminally differentiated into pancreatic endocrine cells exhibited characteristics of islet cells in terms of morphology, expression and function. Cells clustered in structure of 50-300 microns of diameter resembling pancreatic islets, some with a necrotic core (**Figure 27**).

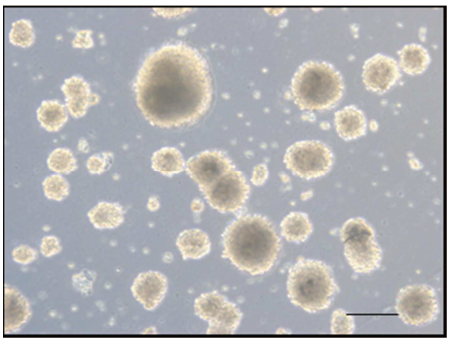

Figure 27. Cell cluster morphology, scale bar 100 µm. *Pellegrini, 2015.*

Endocrine commitment was confirmed by the increase of the absolute expression during differentiation measured through Droplet Digital PCR analysis of insulin mRNA, from 1.1 to 13.4 mRNA copies per ng of total RNA (**Figure 28**).

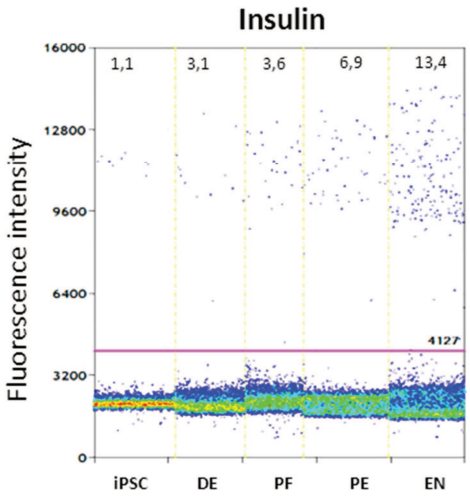

Figure 28. Quantification by droplet digital PCR of Insulin mRNAs at the steps of pluripotency (iPSC), definitive endoderm (DE), posterior foregut (PF), pancreatic endoderm (PE) and endocrine cells (EN). The number of gene specific mRNA copies per nanogram of total RNA in each sample, corresponding to droplets fluorescing above background level (red line), was determined after droplets acquisition, count on a QX100 instrument (Bio-Rad) and analysis by QuantaSoft software v1.2.10. *Pellegrini, 2015.*

Cytofluorimetric analysis confirmed the presence of insulin positive cells, in a range between 1.8 and 6.7%. At the final step of differentiation, iPSC-derived cells resulted positive for glucose transporter 2 (Glut2) (**Figure 29**).

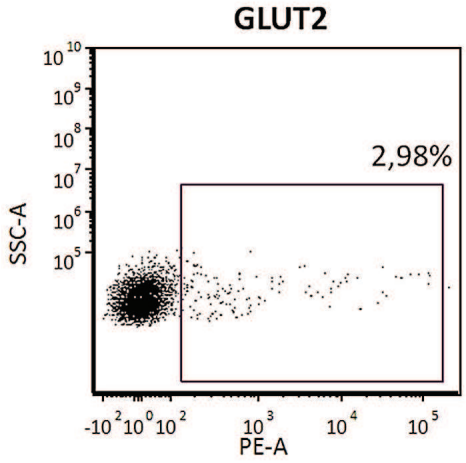

Figure 29. Protein expression analysis by flow cytometry of glucose transporter 2 (Glut2). Gate delimitates positive events. Percentage of positive cells of a representative experiment is reported. SSC: side scatter, PE: phycoerythrin. *Pellegrini, 2015.*

Moreover, the expression of the glucokinase gene, a critical enzyme involved in the glucose responsiveness of insulin release emerged in terminally differentiated cells (**Figure 30**).

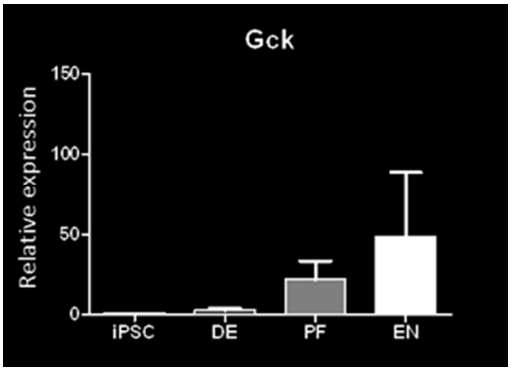

Figure 30. Expression analysis by Taqman of Glucokinase mRNA during the differentiation steps (iPSC, DE, PF, EN). Expression levels are reported as fold change compared to undifferentiated cells. *Pellegrini, 2015.*

Furthermore, at the end of the differentiation process, iPSC-derived cells secreted insulin in resting conditions, as shown by the presence of c-peptide in culture supernatants (**Figure 31**).

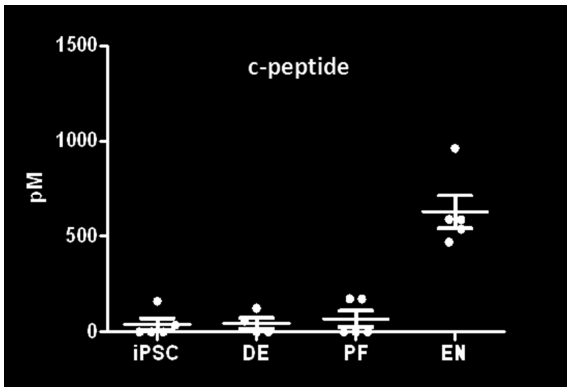

Figure 31. C-peptide level in the supernatant of iPSC during the differentiation steps (iPSC, DE, PF, EN) measured by Elisa. Graph reports single values of 5 experiments as dots and mean±SEM. *Pellegrini, 2015.*

When insulin secretion was tested in stimulated conditions, a range of concentrations of glucose *per se* was not sufficient to increase insulin release, while the combination of glucose with phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) induced a significant release of insulin; stimulation of insulin release was also obtained by direct depolarization with potassium chloride (KCl) (**Figure 32**).

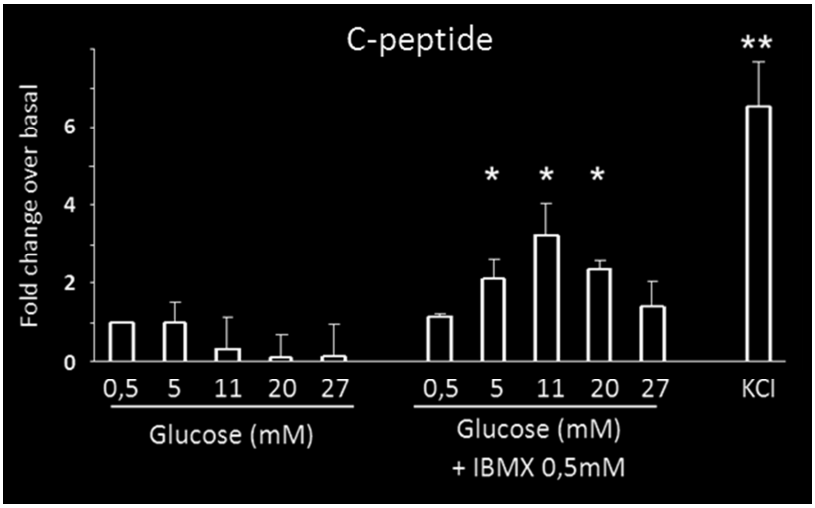

Figure 32. C-peptide secretion by terminally differentiated iPSC upon stimulation with 0,5, 5, 11, 20 and 27 mM glucose without or with 0,5 mM IBMX and KCl, measured by Elisa and reported as ratio between stimulated and basal condition. Data are shown as mean + SD of 3 experiments. *P < 0.05; **P < 0.01 compared to 0.5 mM glucose stimulus (Mann–Whitney test). *Pellegrini, 2015.*

5. iPSC-derived cells engraft and survive for short-term periods when transplanted *in vivo*

To test the capability to engraft, to survive and to release insulin *in vivo*, we transplanted terminally differentiated human iPSC under the kidney capsule of 12 immunodeficient normoglycemic NOD/SCID mice. Fasting glycemia was measured in recipient mice during 12-week follow-up. Mice remained normoglycemic during the follow-up. One week after transplantation, grafts were responsive to glucose since human C-peptide was detected in the sera after an oral glucose tolerance test, but grafts gradually lost insulin production during the time (**Figure 33**).

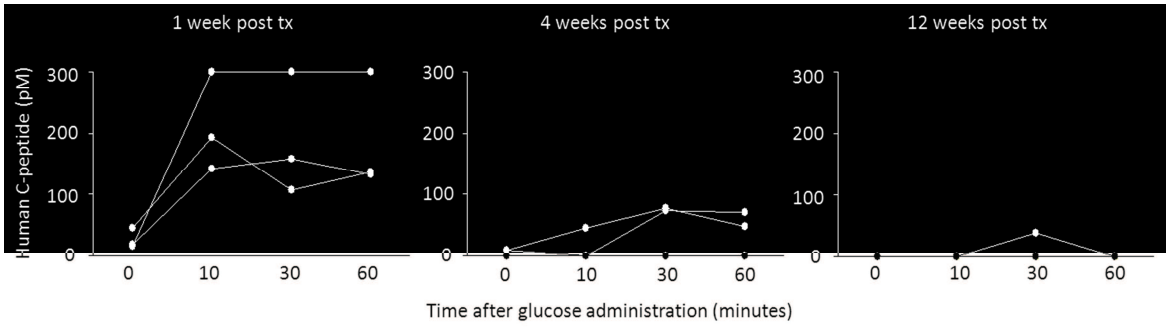

Figure 33. Functional characterization of iPSC-derived cells after transplantation in NOD/SCID mice. Analysis of graft function by human C-peptide measurement after oral glucose test tolerance in sera of mice transplanted with iPSC-derived-terminally differentiated cells. *Pellegrini, 2015.*

Since terminal differentiation of pluripotent stem cells into pancreatic hormone-secreting cells was described to occur efficiently *in vivo* (Kroon et al., 2008), we also transplanted iPSC-derived pancreatic endoderm cells. One week after transplantation, mice had significant basal levels of circulating C-peptide, but they were not responsive to glucose stimulation. Four weeks after transplantation, they acquired the ability to respond to glucose with an increase in insulin production, but, as observed for terminally differentiated iPSC transplant, transplanted cells were shown to gradually lose insulin production during the time, from the first week onwards (**Figure 34**).

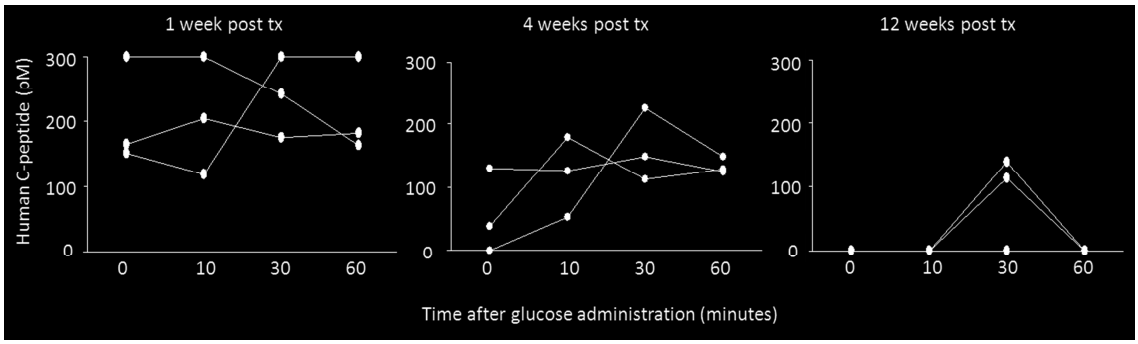

Figure 34. Functional characterization of iPSC-derived cells after transplantation in NOD/SCID mice. Analysis of graft function by human C-peptide measurement after oral glucose test tolerance in sera of mice transplanted with iPSC-derived pancreatic progenitor cells. *Pellegrini, 2015.*

In order to investigate engraftment, survival and localization of transplanted cells, we performed an immunohistochemical analysis of the graft at 1, 4 and 12 weeks after cells infusion. At 1 and 4 weeks, the grafts of mice transplanted with terminally differentiated cells showed the presence of proliferating cells (Ki67 positive cells) and pancreas-committed cells (Pdx1 positive). Significant number of insulin-positive cells was present only in the graft collected at the earliest time point of 1 week (**Figure 35**).

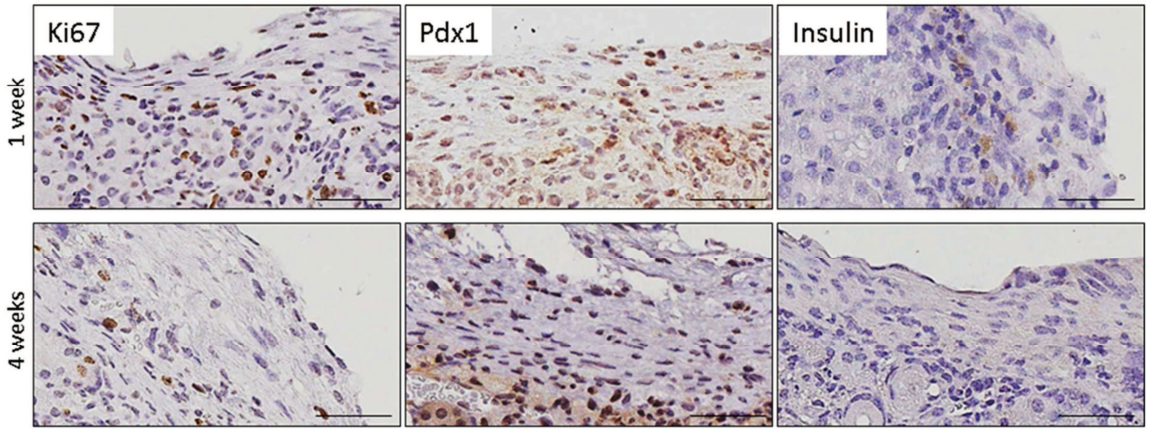

Figure 35. Immunohistochemical characterization of iPSC-derived cells after transplantation in NOD/SCID mice. Evaluation of grafts by immunohistochemistry 1 and 4 weeks after transplantation of terminally differentiated cells. Ki-67 staining was used to assess proliferation, Pdx1 and Ins as pancreatic markers. Scale bar 50 µm. *Pellegrini, 2015.*

At 12 weeks, immunohistochemical analysis of the graft showed that only few cells were present and resulted positive for Pdx1 and for glucagon, but not for insulin (**Figure 36**) and in 5 out of 6 mice the graft was not even visible.

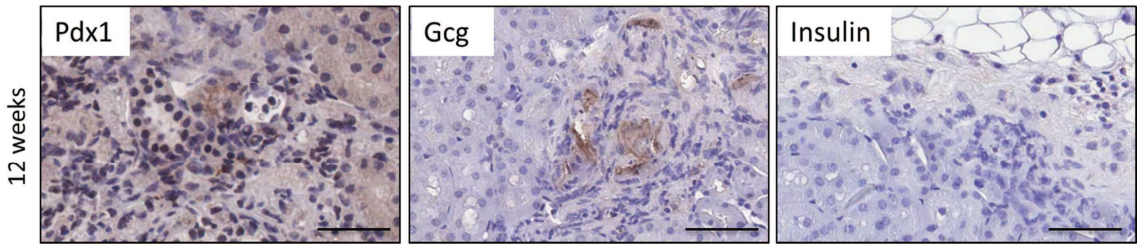

Figure 36. Immunohistochemical characterization of iPSC-derived cells after transplantation in NOD/SCID mice. Evaluation of grafts by immunohistochemistry 12 weeks after transplantation of terminally differentiated cells. Pdx1, Ins and Glucagon were used as pancreatic markers. Scale bar 50 µm.

When the infusion of pancreatic endoderm cells was analyzed, immunohistochemical analysis of the grafts at all considered time points showed that transplanted cells engrafted, survived and did not infiltrate the surrounding tissues. The grafts resulted composed of a mixed population of cells containing proliferating components (Ki67 positive), pancreatic cells (Pdx1-positive) and few scattered insulin-positive cells in the graft at 1 and 4 weeks after transplantation (**Figure 37**).

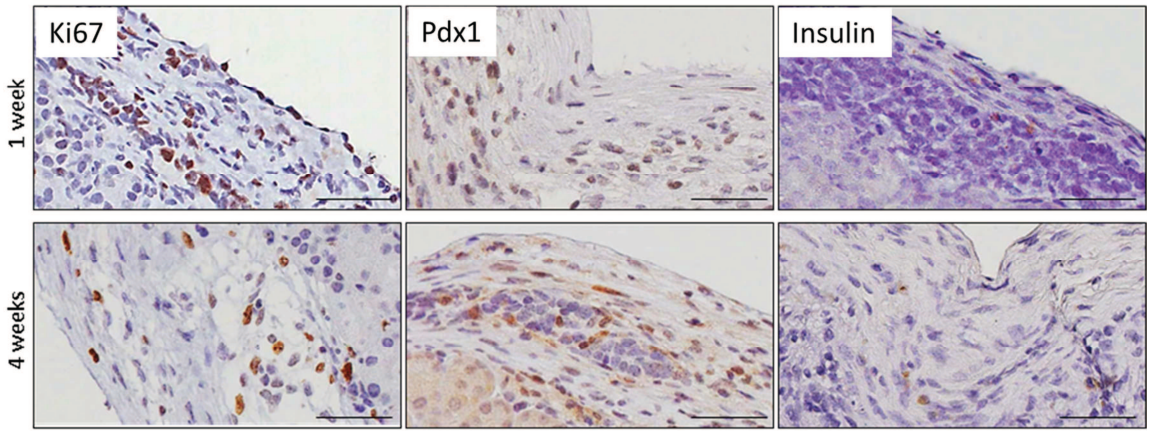

Figure 37. Immunohistochemical characterization of cells after transplantation in NOD/SCID mice. Evaluation of grafts by immunohistochemistry 1 and 4 weeks after transplantation of iPSC-derived pancreatic endoderm cells. Ki-67 staining was used to assess proliferation, Pdx1 and Ins as pancreatic markers. Scale bar 50 µm. *Pellegrini, 2015.*

At 12 weeks, all grafts were still retrievable, Ki67 and Pdx1 positive cells persisted in the graft area, while insulin-positive cells were not found (**Figure 38**).

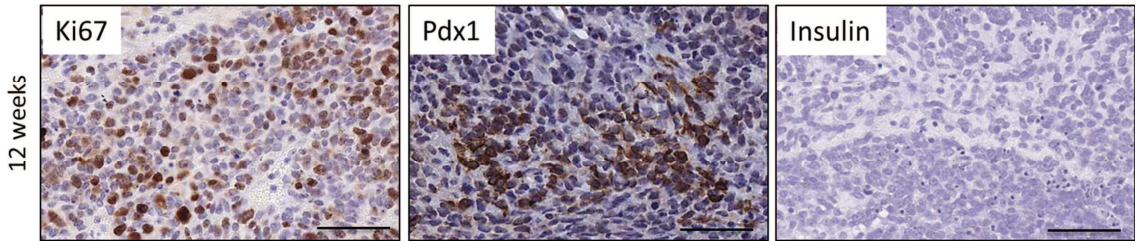

Figure 38. Immunohistochemical characterization of cells after transplantation in NOD/SCID mice. Evaluation of grafts by immunohistochemistry 12 weeks after transplantation of iPSC-derived pancreatic endoderm cells. Ki-67 staining was used to assess proliferation, Pdx1 and Ins as pancreatic markers. Scale bar 50 µm.

At all time points, both after terminally differentiated or pancreatic endoderm cell transplantation, it was possible to find cells expressing markers of pluripotency (Sox2), markers of secretory cells not specifically pancreatic (ChgA, Syp), markers of epithelial cells (Ck8-18), pancreatic endocrine cells expressing glucagon and some neuronal lineage-committed cells (Gfap positive) (**Figure 39**).

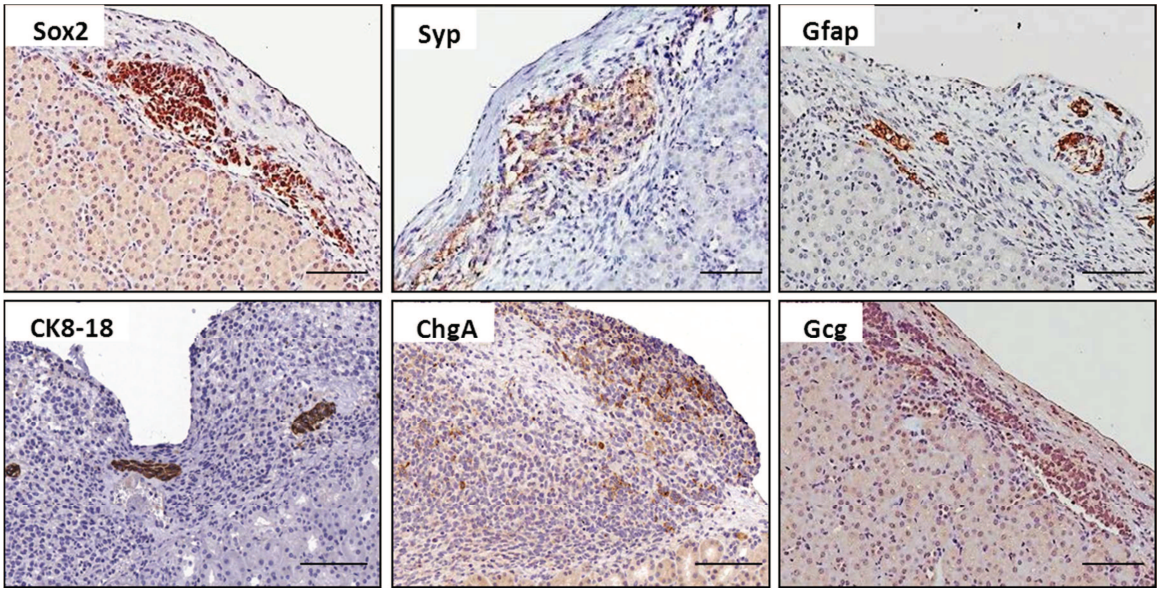

Figure 39. Immunohistochemical characterization of iPSC–derived cells after transplantation in NOD/SCID mice. Sox2 staining was used to assess pluripotency, Synaptophysin (Syp) and Chromogranin A (ChgA) for secretory cells, cytokeratin 8 and 18 (Ck8/18) as epithelial cell markers, glial fibrillary acidic protein (Gfap) to detect any neuronal cells and Glucagon (Gcg) as a marker of pancreatic endocrine cells. These images are representative for graft composition at 1, 4 and 12 weeks, after infusion of precursors or of terminally differentiated cells. Scale bar: 100µm. *Pellegrini, 2015.*

6. Generation and characterization of iPSC reprogrammed with Sendai Virus

Human iPSC were then generated using Sendai (Fusaki et al., 2009) non-integrative virus vectors containing the four Yamanaka's factors. The clone #5 obtained from the reprogramming of fibroblasts derived from a skin biopsy of a healthy adult subject was used in this study after the assessment of its pluripotency. Reprogrammed cells indeed stained positive for the pluripotency-related proteins Oct4, Nanog, Tra1-60, Tra 1-81, SSEA4 and SSEA4 (**Figure 40**).

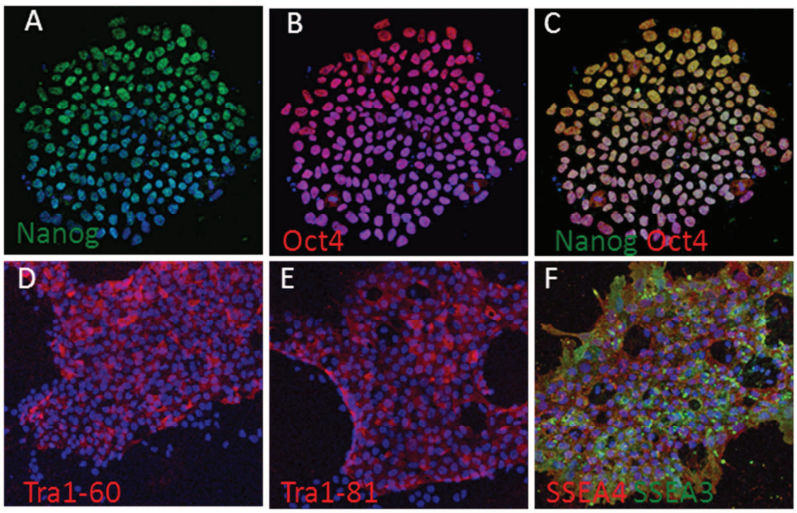

Figure 40. One line of iPSC reprogrammed from adult fibroblasts (clone 5) was characterized in terms of pluripotency. Staining for expression by immunofluorescence: A-C: OCT4 (red) and NANOG (green); D: Tra1-60 (red); E: Tra1-81 (red); F: SSEA4 (red) and SSEA3 (green); nuclei evidenced by DAPI staining (blue). Magnification ×10.

Also at molecular level Sendai virus (SeV)-iPSC expressed, as the iPSC generated with retroviral infection, all the marker genes of pluripotency like OCT4, KLF4, SOX2 and NANOG, while the uninfected fibroblasts expressed only low levels of KLF4. Analysis of fibroblasts immediately after infection with SeV revealed the expression of Sendai Virus mRNA, that was lost after pluripotency establishment (**Figure 41**).

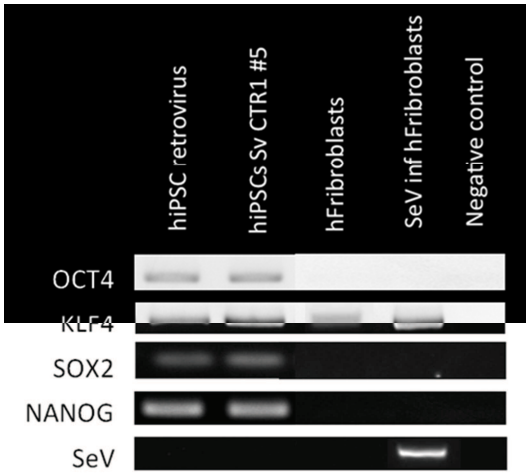

Figure 41. Expression by RT-PCR of pluripotency genes OCT4, KLF4, SOX2 and NANOG and of SeV in iPSC clone 5, iPSC generated with retroviral infection, in fibroblasts before reprogramming and fibroblasts after SeV infection. Negative control: no cDNA template.

The ability of the reprogrammed cells to give rise to cells of all three embryonic germ layers *in vitro* was also tested. After EB formation SeV-iPSC were differentiated into cells of the three germ layers, as shown by the expression of marker typical of ectoderm (bIII Tubulin), endoderm (Cytokeratin 18) and mesoderm (aSMA) (**Figure 42**).

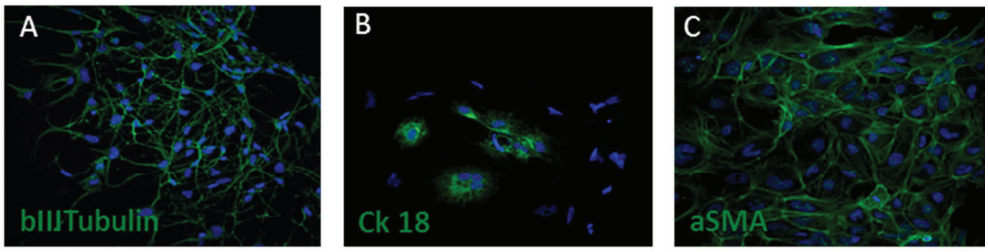

Figure 42. Assessment of pluripotency *in vitro*. A: Staining for the ectodermal markers bIIITubulin (green). B Staining for the endodermal marker Ck18 (green); C: Staining for the mesodermal marker aSMA (green). Nuclei evidenced by DAPI staining (blue). Magnification 10x.

Also the capacity of SeV-iPSC to form teratoma *in vivo* was tested. After injection into nude immunodeficient mice reprogrammed cells were able to spontaneously differentiate into cells of different tissues (**Figure 43**).

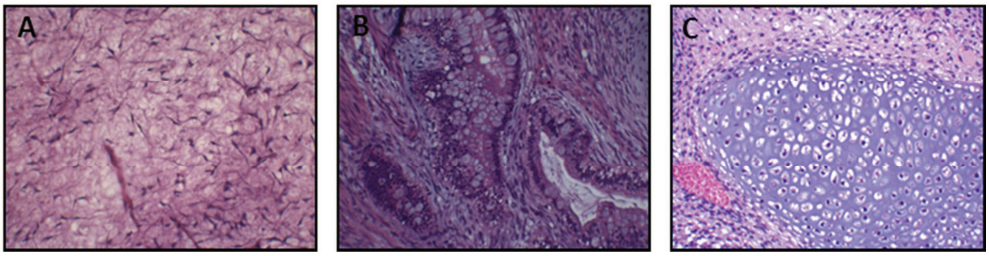

Figure 43. Assessment of pluripotency in vivo. Staining with hematoxylin/eosin of teratomas generated by SeV-iPSC injection into immunodeficient mice: gut, neural and muscle-like tissues are present. magnification ×20.

7. **Differentiation of SeV-iPSC into insulin-producing cells *in vitro***

7.1 Differentiation protocol 1

The protocol previously described for the differentiation of iPSC generated with retrovirus was tested also on SeV-iPSC. Morphology, gene and protein expression of SeV-iPSC during differentiation were analyzed in four independent experiments.

Cell morphology changed from the adherent colonies of iPSC composed of small cells with scant cytoplasm, to stellate cells, to cluster of cells in suspension condition, as shown in **Figure 44**.

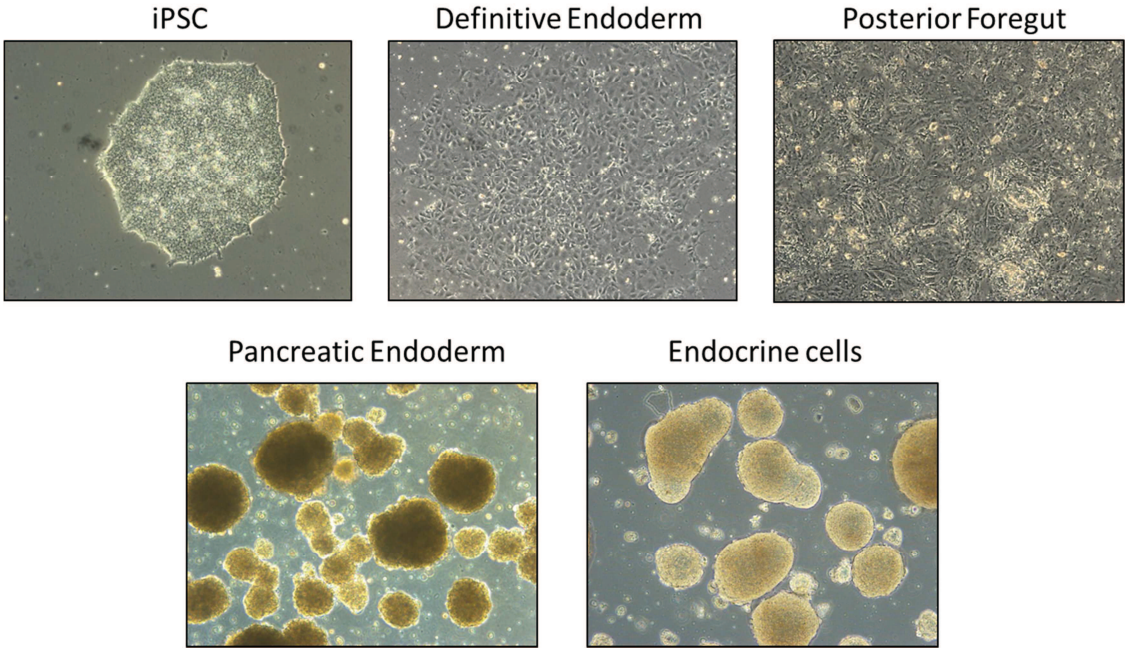

Figure 44. Representative pictures of cell morphology of SeV-iPSC during the steps of pancreatic specification, Magnification 10x.

Gene expression levels are expressed as Fold Change (FC) compared to ΔCt of undifferentiated SeV-iPSC. The ΔCt of 12 different replicates of iPSC used in this study, expressed as mean ± standard error, are: Oct4: 9.44 ± 0.1; Nanog: 5.9 ± 0.1; Sox17: 18.3 ± 0.9; Foxa2: 13.3 ± 0.2; Hnf1b: 18.1 ± 0.4; Pdx1: 28.1 ± 1.6; Nkx2.2: 18.7 ± 0.1; Nkx6.1: 16.9 ± 0.2; Ins: 30,1 ± 1.1; Gcg: 30.7 ± 0.1.

We first evaluated the loss of pluripotency, that was confirmed by the downregulation of OCT4 and NANOG genes, and the loss of the undifferentiated status was accompanied by the increase in the expression of SOX17 and FOXA2 genes (8096.7 ± 1681.4 and 973.4 ± 190.8 FC over undifferentiated iPSC) during specification into definitive endoderm cells (**Figure 45**).

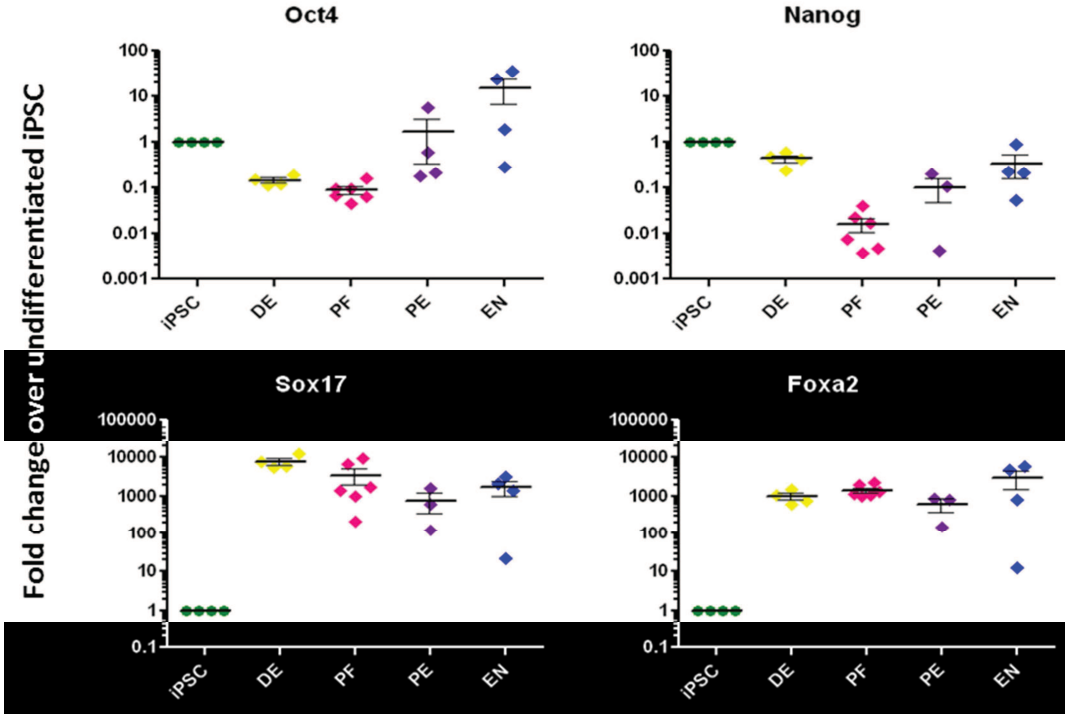

Figure 45. Gene expression analysis by Taqman of markers of pluripotency (OCT4 and NANOG) and definitive endoderm (FOXA2 and SOX17) during the stages of pancreatic differentiation. Normalized gene expression levels are reported as fold change compared to undifferentiated SeV-iPSC and expressed as mean ± SEM of 4 experiments.

At the step of posterior foregut specification, the upregulation of HNF1b and PDX1 genes (11086.5 ± 1514.9, 47811.5 ± 20399.5 FC) and their sustained expression during the subsequent steps of differentiation was observed (**Figure 46**).

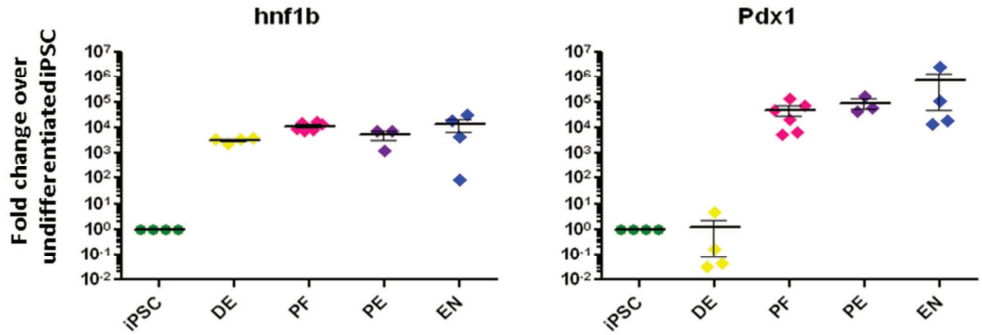

Figure 46. Gene expression analysis by Taqman of markers posterior foregut (HNF1b and PDX1) during the stages of pancreatic differentiation. Normalized gene expression levels are reported as fold change compared to undifferentiated SeV-iPSC and expressed as mean ± SEM of 4 experiments.

At the final differentiation step the overexpression of the transcription factors NNK2.2 and NKX6.1 (75.8 ± 32.1 and 7.3 ± 3.1 FC) and of the mRNA of the pancreatic hormones Insulin and Glucagon (420976.6 ± 292501.4 and 125333.7 ± 81977.4 FC) were observed (**Figure 47**).

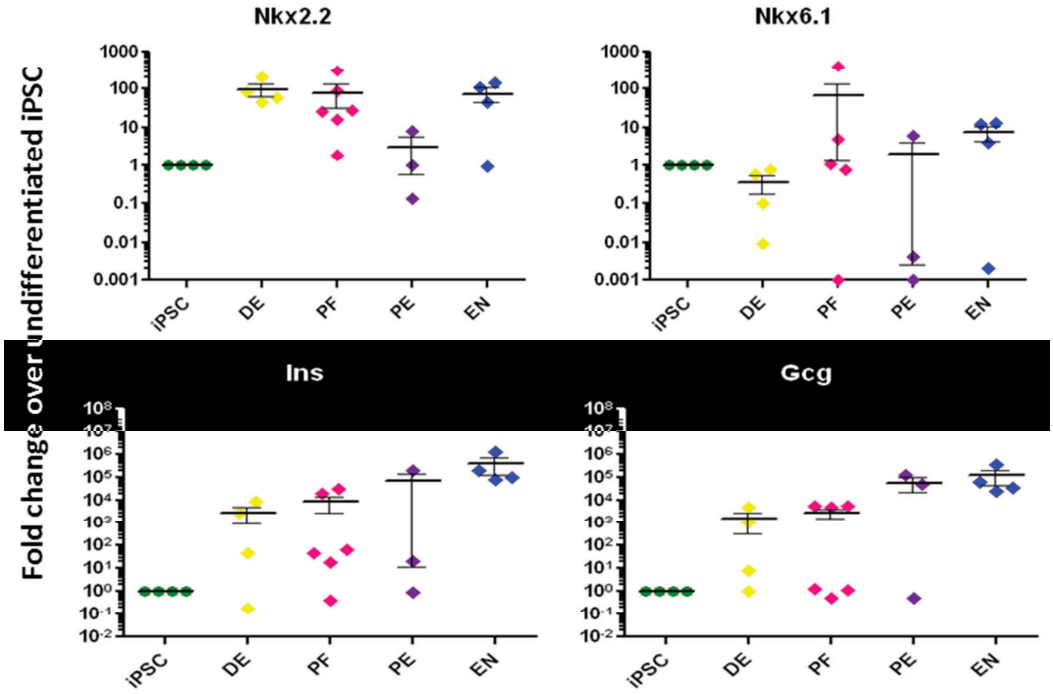

Figure 47. Gene expression analysis by Taqman of markers pancreatic endoderm (NKX2.2 and NKX6.1) and endocrine cells (INS and GCG) during the stages of pancreatic differentiation. Normalized gene expression levels are reported as fold change compared to undifferentiated SeV-iPSC and expressed as mean ± SEM of 4 experiments.

Accordingly, analysis by flow cytometry showed the disappearance of the pluripotency surface marker Ssea-4 (from 98% to 7% of positive cells) during differentiation steps, the increase of Cxcr4 positive cells at the stage of definitive endoderm (from 0,17% of iPSC to 89,44% after DE specification), and presence of few insulin (0,59%), Glut2 (0,86%) and double-positive (0,36%) cells at the final step of differentiation, confirming the conversion of undifferentiated iPSC into pancreatic cells following developmental stages although with low efficiency (**Figure 48**).

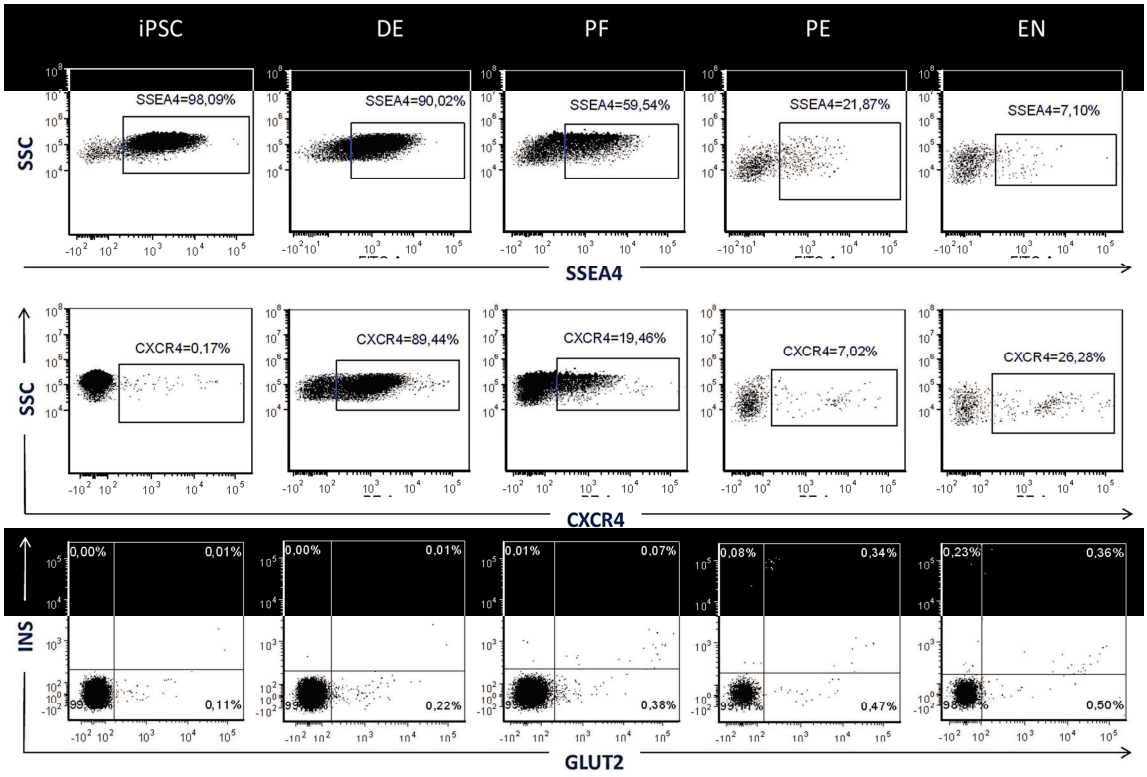

Figure 48. Protein expression analysis by flow cytometry of markers of pluripotency (Ssea 4), definitive endoderm (CXCR4) and endocrine cells (double staining for GLUT2 and Insulin). Gate delimitates positive events. Percentages of positive cells of one experiment are reported. SSC: side scatter.

7.2 Differentiation protocol 2

We optimized a new differentiation protocol for our SeV-iPSC relying on the protocol described by Melton's group for the pancreatic differentiation of pluripotent cells (Pagliuca et al., 2014). Compared to their protocol we added some modifications:

- we used the STEMdiff™ Definitive Endoderm Kit for the differentiation into definitive endoderm cells a commercial medium able to give rise to DE cells from pluripotent cells.
- we have grown iPSC in adhesion condition until the specification into posterior foregut cells as previously described.

Before the introduction of these two modification we observed a gradual and progressive death of the cells during the last step of the differentiation process and it was not possible to perform gene or protein expression analysis.

Morphology, gene and protein expression of SeV-iPSC during differentiation with this new modified protocol were analyzed in 2 independent experiments.

Cell morphology changed from the adherent colonies of iPSC composed of small cells with scant cytoplasm, to bigger cell aggregates composed of a central cluster of cells surrounded by cells with elongated shapes, to cluster of cells in suspension condition, as shown in **Figure 49**.

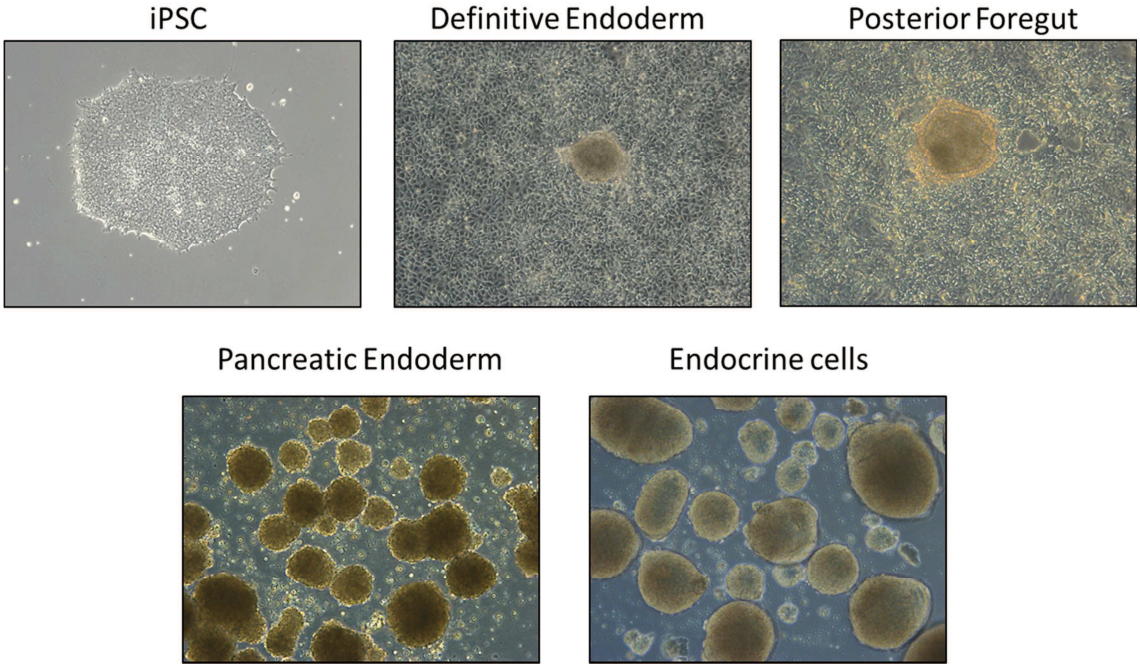

Figure 49. Representative pictures of cell morphology of SeV-iPSC during the steps of pancreatic specification, Magnification 10x.

Also with this protocol we observed the loss of pluripotency (downregulation of OCT4 and NANOG genes) and the increase in the expression of SOX17 and FOXA2 genes (7547.7 ± 3655.5 and 1159 ± 899.1 FC over undifferentiated iPSC) during specification into definitive endoderm cells (**Figure 50**).

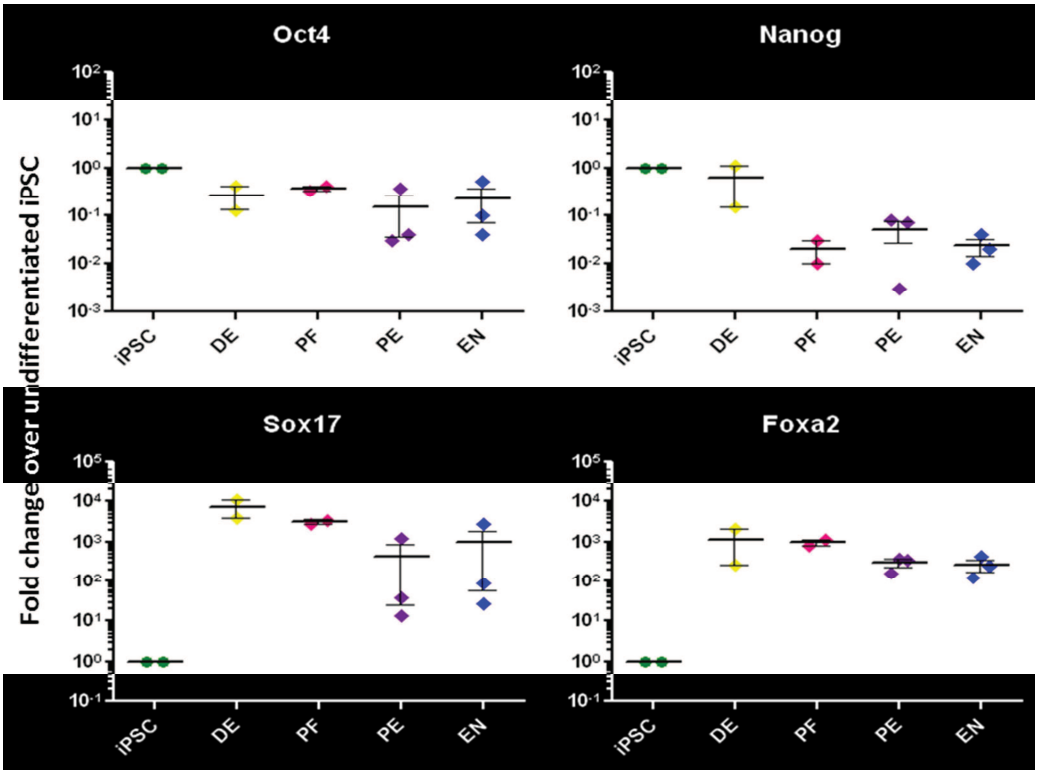

Figure 50. Gene expression analysis by Taqman of markers of pluripotency (OCT4 and NANOG) and definitive endoderm (FOXA2 and SOX17) during the stages of pancreatic differentiation. Normalized gene expression levels are reported as fold change compared to undifferentiated SeV-iPSC and expressed as mean ± SEM of 2 experiments.

At the step of posterior foregut specification, the upregulation of HNF1b and PDX1 genes (27282.8 ± 830.9, 1797153.7 ± 225679.6 FC) and their sustained expression during the subsequent steps of differentiation was observed (**Figure 51**).

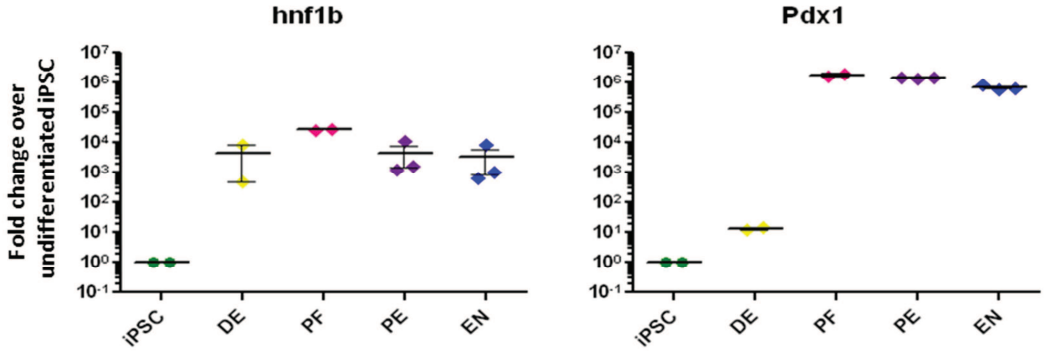

Figure 51. Gene expression analysis by Taqman of markers posterior foregut (HNF1b and PDX1) during the stages of pancreatic differentiation. Normalized gene expression levels are reported as fold change compared to undifferentiated SeV-iPSC and expressed as mean ± SEM of 2 experiments.

At the final differentiation step the overexpression of the transcription factors NNK2.2 and NKX6.1 (3653 ± 453.7 and 477.4 ± 47.3 FC) and of the mRNA of the pancreatic hormone Insulin and Glucagon (12319377.1 ± 6234648.6 and 2905349 ± 1504064.4 FC) were observed (**Figure 52**).

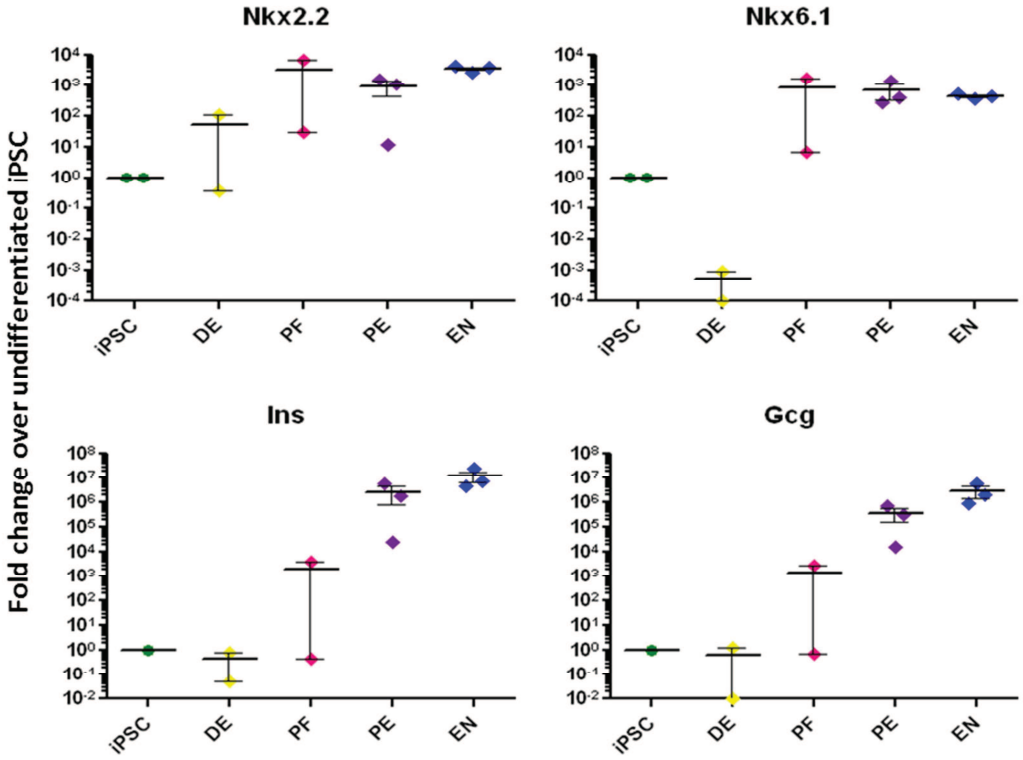

Figure 52. Gene expression analysis by Taqman of markers pancreatic endoderm (NKX2.2 and NKX6.1) and endocrine cells (INS and GCG) during the stages of pancreatic differentiation. Normalized gene expression levels are reported as fold change compared to undifferentiated SeV-iPSC and expressed as mean ± SEM of 2 experiments.

Finally, analysis by flow cytometry of cells differentiated in vitro until the last stage confirmed the presence of insulin (14.23%) and Nkx6.1 (46.09%) positive cells (**Figure 53**).

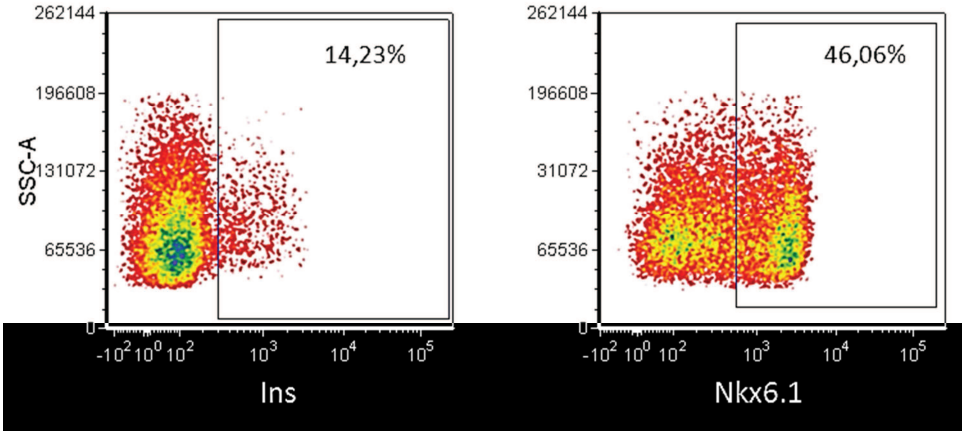

Figure 53. Protein expression analysis by flow cytometry of Insulin and Nkx6.1. Gate delimitates positive events. Percentage of positive cells of one experiment is reported. SSC: side scatter.

Insulin positive cells were detected also by immunohistochemical analysis performed on the cytospinned terminally differentiated cells (**Figure 54**).

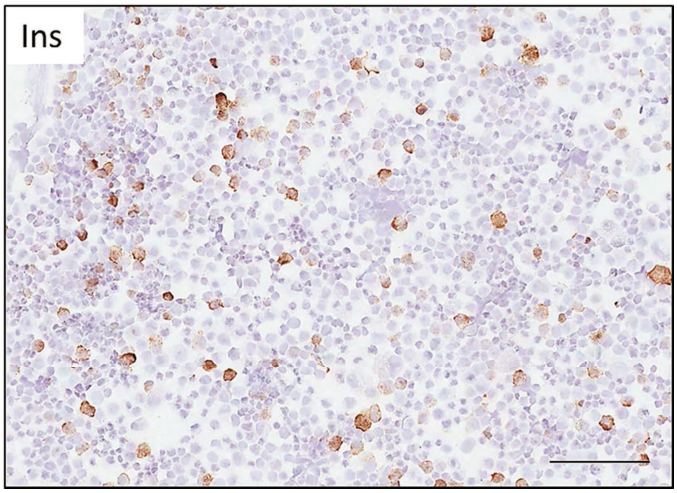

Figure 54. Immunohistochemical characterization of Sev-iPSC-derived pancreatic endocrine cells differentiated *in vitro*. Insulin was used as β cell markers. Scale bar 100µm.

Furthermore, at the end of the differentiation process, SeV-iPSC-derived cells secreted insulin in resting conditions, as shown by the presence of c-peptide in culture supernatants (**Figure 55**).

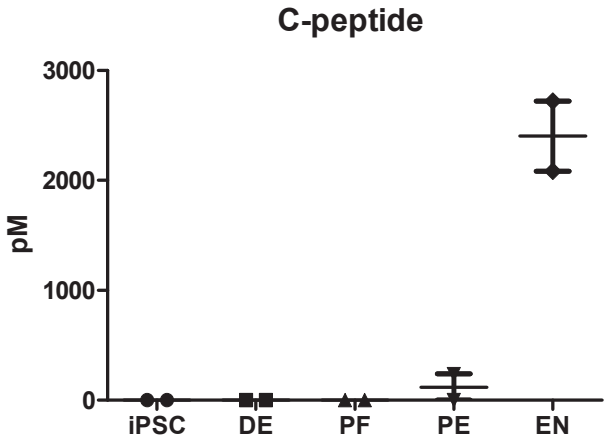

Figure 55. C-peptide level in the supernatant of iPSC during the differentiation steps (iPSC, DE, PF, PE, EN) measured by Elisa. Graph reports single values of 2 experiments as dots and mean±SEM.

When insulin secretion was tested after glucose stimulus (in the presence of IBMX) a significant release of c-peptide was observed; stimulation of insulin release was also obtained by direct depolarization with potassium chloride (KCl) (**Figure 56**).

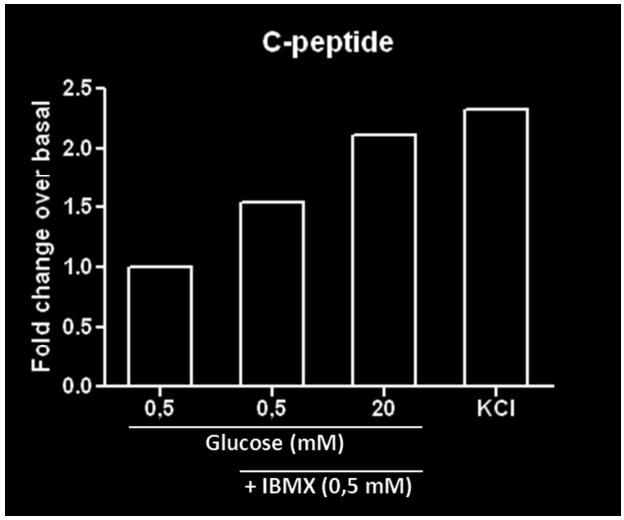

Figure 56. C-peptide secretion by terminally differentiated SeV-iPSC upon stimulation with 0,5 (without or with 0.5mM IBMX) and 20 mM glucose (+0.5mM IBMX) and KCl measured by Elisa and reported as ratio between stimulated and basal (0.5 mM glucose) condition.

VI. DISCUSSION

Many efforts in the field of diabetes research have been put on differentiating pluripotent stem cells to find an unlimited new source of pancreatic β cells. The overall aim of this project is to try to obtain an unlimited source of insulin producing cells from human iPSC in order to overcome the lack of organ donors and to get closer to an autologous β cells replacement therapy for diabetic patients. In this study, we reported a method for promoting the differentiation of iPSC into insulin-producing cells and we transplanted these differentiated pancreatic cells *in vivo*. The results obtained showed that human iPSC have the potential to generate functional insulin-producing cells *in vitro*, undergoing defined steps resembling pancreas organogenesis, and that differentiated cells can engraft and survive for short periods when transplanted *in vivo*. These data provide useful suggestions for the study of a potential use of induced pluripotent stem cells in the field of β cell replacement for diabetes treatment.

For the differentiation of our human iPSC reprogrammed with retroviral vectors containing the 4 Yamanaka's factors, we selected as a backbone the most efficient protocol for *in vitro* differentiation of ESC (D'Amour et al., 2006). We added slight modifications to this protocol in order to obtain an efficient differentiation of iPSC into the pancreatic lineage. With the addiction of the PCK activator Indolactam V (Chen et al., 2009) and the culture in suspension condition mimicking *in vitro* pancreatic islet culture (J. Jiang et al., 2007), the highest differentiation efficiency reached with this iPSC cell line was approximately 7% of insulin-positive cells, measured by flow cytometry. Gene expression data, both by Taqman and by Digital PCR, confirmed that terminally differentiated cells underwent all the steps of pancreatic organogenesis, expressing all the keys genes and transcription factors characteristic of each stage of differentiation. In particular, we observed a downregulation of the pluripotency markers Oct4, Nanog and SSEA-4 during pancreatic differentiation, although Oct4 expression was detected also during the last stage of *in vitro* differentiation; this observation is consistent with a study that reported Oct4 expression in pancreatic islet of healthy subjects (Iki & Pour, 2006). During specification to definitive endoderm cells, we detected a significant upregulation of the genes Sox17 and Foxa2 and of the microRNA miR375 (a key regulator of pancreatic development) (Lahmy et al., 2013), and an

increase in the percentage of Cxcr4 positive cells, confirming the differentiation of iPSC into DE cells. At the subsequent step of posterior foregut specification, the upregulation of Hnf1b and especially of the fundamental transcription factor Pdx1 was observed, while at the following differentiation step into pancreatic endoderm, also the genes Ngn3 and Nkx2.2, essential for β cell specification, resulted more expressed. Finally, at the end of the differentiation process, insulin mRNA expression resulted highly increased. In addition to insulin expression, other characteristics are needed to establish that iPSC have indeed generated functional β cells. Here, the final cell product presents distinctive features of mature β cells as the expression insulin, Glut2, Glucokinase, and of the microRNAs miR9 and miR-124a, two non-coding RNAs which have been described to play an active role in regulating insulin secretion (Baroukh & Van Obberghen, 2009; Ramachandran et al., 2011). Differentiated cells also showed the ability to secrete C-peptide in basal and stimulated conditions. Our *in vitro* results are in line and extend the few previous publications on human iPSC differentiation in β cell (Tateishi et al., 2008; D. Zhang et al., 2009; Nostro et al., 2011; Thatava et al., 2011; Kunisada et al., 2012; Hakim et al., 2014). The differentiation of iPSC to insulin-producing cells with a process able to mimic every stage of pancreatic organogenesis represents also a powerful tool for the *in vitro* study of pancreas development. In the present study, we reported the characterization of microRNAs expression profiles during differentiation of iPSC into insulin-producing cells in order to discover which microRNAs regulate β cells specification. During *in vitro* differentiation we detected the progressive upregulation of 11 microRNAs and downregulation of 7 microRNAs. The concept that microRNAs operate in concert in well-defined gene networks (Herranz & Cohen, 2010), prompted us to analyze the functional classification of predicted target genes belonging to differentially expressed microRNAs grouped into ‘upregulated’ or ‘downregulated’ in each differentiation stages respect to undifferentiated iPSC. Indeed, target genes analysis showed that microRNAs synergistically regulate cohorts of genes that participate in similar processes. Specifically, ontological analysis of microRNAs target genes revealed that those upregulated in DE and PF stages are predicted to regulate several genes whose functions can be ascribed to the control of tissue development or cell morphogenesis. MicroRNAs upregulated in the final stage of differentiation are instead predicted to target several genes involved in pancreas

development and exocytosis control. These data demonstrate that several microRNAs are upregulated during differentiation, in order to progressively control specific target genes involved in developmental processes or endocrine-pancreatic cell fate specification. For downregulated microRNAs instead, bioinformatic analysis revealed that most of their target genes belong to 'regulation of phosphorylation' (DE and PF stages) while 'regulation of metabolic processes' was the most significant category in the final stage. Interestingly, it has been recently reported that during differentiation of pluripotent stem cells, more than 50% of phospho-sites are regulated, providing evidence of the involvement of protein phosphorylation as a mechanism of cell fate specification (Van Hoof et al., 2009). In this context, the downregulation of such microRNAs may allow on one side the expression of those genes involved in phosphorylation processes and, on the other, facilitate the expression of those involved in metabolic processes, thus favoring the endocrine-pancreatic phenotype specification during differentiation. These results could therefore lead to the identification of markers of novel pathways and/or individual targets for possible pharmacological interventions able to increase the efficiency of the differentiation process.

This is the one of the first studies in which human iPSC-derived pancreatic cells are transplanted *in vivo* in animal models. One recent paper reported C-peptide production after *in vivo* infusion of human iPSC differentiated to β cells, but the study was mainly focused on the correction of glucokinase deficiency in iPSC derived from MODY patients (Hua et al., 2013). Other previous *in vivo* experiences were limited to mouse iPSC, showing that transplantation of differentiated cells are able to correct hyperglycemia in models of type 1 and 2 diabetes (Alipio et al., 2010; Jeon et al., 2012). Only very recently two groups reported for the first time that pancreatic cells derived from the differentiation of pluripotent cells (both ESC and iPSC) were able to revert diabetes in mice within few weeks (Rezania et al., 2014; Pagliuca et al., 2014). In our study, we transplanted human iPSC-derived cells both at the stage of endocrine (INSULIN positive cells) and of pancreatic endoderm cells (PDX1 positive cells). In the first case, cells engrafted and we were able to find them in the graft, although only after a short period of observation, while after 3 months the infused cells had almost completely disappeared. The graft resulted composed not only of pancreatic lineage cells (expressing Pdx1, insulin and glucagon), but also of pluripotent and of other

lineages cells. Significant number of insulin-positive cells was found only in grafts retrieved 1 week after infusion and not after 4 and 12 weeks. Accordingly, mice responded to oral glucose stimulus by releasing human C-peptide from the graft only 1 week after transplantation. Borrowing the experience from ESC (Kroon et al., 2008), we also transplanted iPSC-derived pancreatic endoderm cells: precursors survived and engrafted, but, as for terminally differentiated cells, at the longest time of follow-up, we were not able to find insulin-positive cells and mice did not secrete human C-peptide. Also in this setting, the graft we retrieved was characterized by a mixed population of pancreatic, neuronal and undifferentiated cells. We hypothesize that insulin-positive cells may have been replaced by undifferentiated cells in proliferation or have lost their pancreatic commitment. Besides, also the degree of neo-vascularization of the graft may have influenced insulin-positive cell survival, since it is well known that islet vasculature provides critical instructive signals necessary for β cell differentiation and survival (Reinert et al., 2013). Finally, we can speculate that the infusion of a mixed population of cells, reflected by a heterogeneous graft, could decrease the efficacy of insulin production, increase tumorigenicity and negatively impact on differentiated cell survival. To improve safety and avoid risk of tumorigenesis, it would be necessary to select only cells of pancreatic lineage. Several attempts have been made to look for new surface markers able to select pancreatic progenitors (Jiang et al., 2011; Kelly, Chan, L. A. Martinson, et al., 2011) or to get rid of unwanted pluripotent cells (Ben-David et al., 2013), but the safety of the selected cells still requires further investigation. Besides, some transcriptional factors or genes such as Pdx1, Ngn3 or Insulin could be used as selection markers, but they have intracellular or intranuclear expression; therefore, standard sorting strategies cannot be applied. Very recently, however, novel methods for cell selection based on fluorescent nanoparticles capable of detecting mRNA targets inside living cells have been developed (Lahm et al., 2015). These tools would allow separation of cells at different stages of maturation with significant reduction of contaminating cells.

To try to increase the safety of iPSC and to get closer to an eventual clinical traslation of this type of approach, we moved to the use of iPSC generated through infection of Yamanaka's factors with Sendai virus, a RNA-based virus able to reprogram somatic cells with a relatively high efficiency (0,1%) avoiding transgenes integration into the

host genome (Fusaki et al., 2009). We first tested our *in vitro* differentiation protocol on one clone of SeV-iPSC and also with this cell line we obtained the differentiation into insulin-producing cells following all the steps of pancreatic organogenesis. Compared to retrovirus-generated iPSC, we observed a higher expression of all the the key genes and transcription factors characteristic of each stage of differentiation, including insulin (1567,9±785.1 FC compared to 420976.6±292501.4 FC over undifferentiated iPSC), but the total number of insulin positive cells detected was lower. These differences in the efficiency of differentiation are reported also in the literature, and can be ascribed to the variety of protocols but also to the characteristics of the pluripotent cells used, which are different in terms of source, reprogramming methods and culture conditions in every single experience. In fact, also in the ESC field, it has been published that different cell lines show different propensity to give rise to pancreatic cells (Osafune et al., 2008). We then tested a newly described differentiation protocol able to differentiate pluripotent cells to β-like cells with high efficiency (Pagliuca et al., 2014) on SeV-iPSC, but we observed a gradual cell death during the last steps of the differentiation process. We then decided to add some modifications to the published protocol: the use of a commercial kit for a standardized and efficient differentiation into definitive endoderm cells and the growth in suspension conditions during the last steps of *in vitro* differentiation as previously described. The protocol defined by Pagliuca et al., involves, compared to the protocol of the Viacyte group, the use of different molecules but able to affect the same pathways (KGF instead of FGF-10, the sonic hedgehog inhibitor SANT-1 instead of CYC, the PCK activator PdbU instead of Indolactam V, the Notch inhibitor XXI instead of DAPT) and the addition of new factors able to increase Pdx1 and insulin expression, like TGFβ and BMP pathways inhibitors (Alk5i and LDN193189), Thyroid hormone T_3 or Betacellulin. With our new modified differentiation protocol, we obtain again an overexpression of all the keys genes and transcription factors characteristic of each stage of differentiation, but also, at the end of the differentiation process, an high percentage of insulin positive cells (14,23%) measured by flow cytometry. Terminally differentiated cells were able to secrete C-peptide both in basal and stimulated condition, indicating that iPSC-derived pancreatic cells are able to secrete insulin in response to glucose.

Taken together, our data therefore confirm that: (i) iPSC can differentiate into insulin-producing cells, (ii) pluripotent cell specification goes through the developmental stages of the embryo, (iii) the efficiency of the process, in terms of percentage of insulin-positive cells at the final step, is comparable to that of ESC, (iv) human iPSC-derived pancreatic cells can engraft and survive *in vivo* at least for a short time. These data overall suggest that human iPSC have the potential to generate insulin-producing cells, that these differentiated cells can engraft and secrete insulin in mouse models, but a significant increase in differentiation efficiency and/or a strategy of selection of target cells before infusion is strongly needed. Moreover, also an efficient, low-cost and integration-free reprogramming strategy in GMP condition is fundamental for the translation to the clinical practice of an ideal personalized cell therapy for the cure of diabetes with pluripotent stem cells.

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