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(Class XXX)

"CHARACTERIZATION OF MOLECULAR MECHANISMS DRIVING EPIGENETIC CONVERSION AND PHENOTYPE SWITCH OF FIBROBLASTS INTO INSULIN SECRETING CELLS"

Doctoral Thesis

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"You don't have to see the whole staircase,

just take the first step."

(Martin Luther King Jr.)

"Because sometimes,

even if you know how something's going to end,

that doesn't mean you can't enjoy the ride."

(Ted Mosby)

INDEX

ENGLISH ABSTRACT

Epigenetic conversion is a powerful technique that allows a mature somatic cell to switch into a different and alternative functional phenotype. The result is acquired without any transgenic modification, nor the acquisition of a stable and irreversible pluripotent state, making this approach very valuable for regenerative medicine. The protocol is robust, reproducible and ensures good functional efficiency, however, cells obtained are not completely mature and the optimal scale up conditions are needed for clinical translation.

Aim of the present PhD project was to investigate whether the use of ambient conditions that try to closely mimic the physiological milieu, and limit the differences between *in vitro* and *in vivo* situations, may generate terminally differentiated cells and boost efficiency. To this purpose, physiological oxygen and different glucose concentrations were tested in order to assess cell responses and conversion ability in the different environments. In parallel, the use of three-dimensional (3D) culture systems was investigated, with the specific aim to study the impact of stiffness on epigenetic conversion and the acquisition of a functional, mature phenotype. The data obtained suggest that genetic background has a profound effect on the response to oxygen during the differentiation process and that conversion efficiency is strictly dependent on the glucose concentrations applied at cell isolation from the original tissue. 3D culture systems that match the stiffness typical of the original organ were able to increase differentiation and favored the acquisition of a mature pancreatic phenotype, distinctive of terminally differentiated cells. Last but not least, key molecular informations deriving from the ongoing gene editing experiments are expected to further clarify and substantiate the data obtained. Altogether, the information derived in this PhD project may find useful applications in order to design the best *in vitro* conditions and obtain a powerful scale-up protocol for pre-clinical studies and regenerative medicine of diabetes.

ITALIAN ABSTRACT

La conversione epigenetica è una tecnica promettente che consente ad una cellula somatica matura di passare ad un fenotipo funzionale diverso ed alternativo rispetto a quello di origine. Questo risultato viene perseguito senza alcuna modificazione transgenica e senza l'acquisizione di uno stato di pluripotenza stabile e irreversibile, caratteristiche che rendono questo approccio molto prezioso per la medicina rigenerativa. Il protocollo di conversione epigenetica è robusto, riproducibile e assicura una buona efficienza ed il conseguimento di un fenotipo funzionale. Tuttavia, le cellule ottenute non sono completamente mature e differenziate ed è necessario identificare le migliori condizioni per realizzare uno "scale-up" che permetta l'applicazione in studi preclinici. Lo scopo del presente progetto di dottorato è stato quello di individuare condizioni di coltura fisiologiche, limitando le differenze tra l'ambiente *in vitro* e quello *in vivo*, al fine di aumentare l'efficienza del processo di differenziamento. Più precisamente, sono state testate concentrazioni fisiologiche di ossigeno e di glucosio per poter valutare l'efficienza di conversione cellulare nei diversi ambienti. Parallelamente, è stato valutato l'uso di sistemi di coltura tridimensionale (3D), con lo scopo di studiare il loro impatto sull'efficienza di conversione e sull'acquisizione di un fenotipo funzionale e maturo. I dati ottenuti suggeriscono che il "background genetico" ha un effetto significativo sulla risposta cellulare alle diverse condizioni di ossigeno durante il processo di differenziamento. D'altro canto, l'efficienza di conversione è risultata strettamente dipendente dalle concentrazioni di glucosio utilizzate durante l'isolamento delle cellule dal tessuto di origine. Inoltre, l'utilizzo di sistemi di coltura 3D, che riflettono la rigidità e l'elasticità proprie dell'organo *in vivo*, ha dimostrato un effetto positivo per l'acquisizione di un fenotipo pancreatico maturo, tipico delle cellule terminalmente differenziate. Infine, le informazioni molecolari ottenute dagli esperimenti di "genome editing" (ancora in corso) dovrebbero ulteriormente chiarire e corroborare i dati ottenuti.

Complessivamente, i risultati di questa tesi possono fornire informazioni utili sia per la comprensione dei meccanismi di base che regolano la crescita e il differenziamento cellulare, così come per la messa a punto di un protocollo di "scale-up" da utilizzare nella realizzazione di studi preclinici finalizzati alla medicina rigenerativa del diabete.

CHAPTER 1:
INTRODUCTION

1. INTRODUCTION

1.1 Diabetes

The diabetes cases are increasing worldwide due to population growth, aging, urbanization and increasing prevalence of obesity and physical inactivity. Diabetes represents one of the most highly widespread disease. Indeed, it is estimated that the total number of diabetes cases will rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004).

There are three main typology of diabetes:

- Type 1 Diabetes
- Type 2 Diabetes
- Gestational Diabetes

Type 1 Diabetes (T1D) used to be called juvenile-onset diabetes. It is mainly caused by an autoimmune reaction where the body's immune system attacks insulin producing cells (β cells), destroying them. The development of T1D involves several years of a "pre-diabetic" state associated with gradual worsening in glucose regulation. T1D in usually associated with other common autoimmune disease such as celiac disease, Addison's and thyroid disorders (Barker, 2006). People affected by T1D need daily insulin injections in order to control their blood glucose level. If people with T1D do not have access to insulin, they will die. Moreover, diabetes is an important risk factor for several disease like atherosclerosis, vascular damage including non-occlusive macroangiopathy (angiopathy affecting large blood vessels) and microangiopathy (angiopathy affecting small blood vessels). T1D can also involve severe damages as blindness, heart attacks, renal failure and even amputation (Kota et al., 2013).

Type 2 Diabetes (T2D) accounts for at least 90% of all diabetes cases; it is used to be called non-insulin dependent diabetes or adult-onset diabetes and it is characterized by insulin resistance and relative insulin deficiency (Kota et al., 2013). People affected by T2D can often initially manage their condition through exercise and appropriate diet (W.-X. Li et al., 2016). T2D is recognized as a progressive disorder, which means that it is associated with decreasing pancreatic function over time. Early recognition of the disease is very important in the clinical management of the disorder because, depending

on the stage, effective control may require lifestyle modification such as: oral agent therapy, oral agents combined with insulin, or insulin alone (Cefalu, 2006). Type 2 diabetes is also a risk factor for blindness, cardiac and vascular complication, and renal failure (W.-X. Li et al., 2016).

Gestational Diabetes (GDM) is a diabetes form consisting of high blood glucose levels during pregnancy. GDM usually disappears after pregnancy but women and their children with GDM have major probability to develop Type 2 diabetes later in life.

1.2 Therapies for diabetes

Except for the exogenous administration of insulin, which can mimic the effects produced by the endogenous hormone, at present, there are no definitive therapies for patients suffering from T1D. However, in some patients this therapy does not allow to achieve and maintain an adequate glycemic control. In these patients, the administration can be replaced by an insulin pump that allows the administration of insulin in a continuous manner and in variable doses according to the necessity.

This therapy is generally effective, but patients can be affected by episodes of hypoglycemia, which cause a number of serious side effects. The lack of sugar determines problems in brain functionality, which, in turn, leads to blurred vision, dizziness, headache, concentration difficulties and other neurological symptoms. Hypoglycemia also triggers the release of hormones, such as epinephrine and norepinephrine that cause tachycardia, tremor, hunger, sweating and anxiety. It is not possible to completely eliminate these complications of insulin administration, nor this therapy leads to a cure of the disease, therefore it is considered only palliative (DeWitt & Hirsch, 2003).

Pancreas transplant may represent a valid alternative solution to treat the most serious form of diabetes. The pancreas transplant can be carried out alone or in association with a kidney transplant. An obvious limitation to this intervention appears to be the large number of healthy pancreas needed to cure the many diabetics in the world. In addition, patients must be in good clinical conditions, in order to be able to face the surgery (Ludwig & Kersting, 2013).

Another possibility, characterized by an easier implantation technique, is represented by the transplantation of pancreatic islets. This allows to obtain a normal balance of sugar metabolism without the continuous need of insulin administration. However, this

therapy requires two or three donors for each receiver in order to obtain satisfactory results. Moreover, the processes of isolation and purification to obtain the pancreatic islets are still not optimal and can damage the islets (Schenker & Viebahn, 2009).

The greatest complication in islet or pancreas transplantation is the immune rejection of the transplanted organ that is recognized and attacked by the patient's immune system. To avoid this type of reaction an immunosuppressive therapy is required. It begins during surgery and must continue for life. This will prevent organ rejection by controlling the activity of the immune system but, at the same time, it exposes the patients to a high risk of infection (Malaise et al., 2008).

Considering the huge limitations that characterize the currently available therapies described above, researchers have been studying alternative methods to treat this disease for years. Currently the focus is heavily concentrated on the study of cell therapy that uses new cells to repair or substitute the damaged tissues (Kirchstein & Ruth, 2001).

1.3 Stem cells

A stem cell is an undifferentiated cell able to self-replicate for indefinite periods, often throughout the life of the organism. Under specific conditions, or signals, stem cells can differentiate into one or more cell types of the organism.

1.3.1 Adult Stem Cells

Adult Stem Cells persist for the whole lifetime and fulfill the functions of replacing cells that are eliminated following their natural turnover or traumatic events. These cells can be isolated from different adult tissues.

Research on Adult Stem Cells has attracted much interest for their abilities to divide indefinitely and to generate all cell types specific of the organ from which they originate, potentially regenerating the entire organ from few cells. Unlike embryonic stem cells, the isolation of Adult Stem Cells does not require the destruction of an embryo, therefore their use in research and therapy is not ethically controversial (Bhatia, 2007). Adult Stem Cells are found in specific niches placed in several tissues such as muscles, brain, heart, epithelium and others. However, a limitation for the use of Adult Stem Cells is that their isolation may be impractical as, for example, in the case of neural cells from a patient's brain (Choumerianou et al., 2008). Other Adult Stem Cells, such as those isolated from the muscle, are difficult to expand in culture. Another issue

is that the cells multipotency is difficult to be maintained *in vitro* (Westerman et al., 2010).

The bone marrow and the umbilical cord are a source for both Hematopoietic Stem Cells (HSCs), and Mesenchymal Stem Cells (MSCs). These are amongst the most widely studied stem cells because they are able to differentiate into several cell types of the mesenchymal lineage, such as chondrocytes, osteoclasts and adipocytes. Recently, it has been demonstrated that MSCs can differentiate also into neuronal cells and muscle cells if cultivated under specific conditions (Jackson et al., 2007).

In vitro differentiation of MSCs into pancreatic islets was attempted by several groups (Zhang & Dou, 2014; Kadam et al., 2012; Phadnis et al., 2011; Bhartiya, 2016). Gopurappilly et al. (2013) used pancreas isolated MSCs to differentiate into islets. Fetal islets can be expanded in culture to obtain MSCs (Joglekar et al., 2009). To evaluate the ability of cord blood mononuclear cells to differentiate into islets, Parekh et al., (2009) used cord blood samples concluding that a sub-set of 'pancreas committed cells' existed and increased after mice underwent partial pancreatectomy. Overall, however, all these attempts have remained inefficient since MSCs originate from mesoderm whereas β cells from endoderm and this concept remains controversial. Although phenotypic differentiation of MSCs into islets are reported, functional ability of the differentiated islet-like structures has not been demonstrated. MSCs have also been injected directly in the pancreas and being niche providing cells, helping, through several mechanisms, to alleviate diabetes symptoms like nephropathy, neuropathy, diabetic foot, *etc*. However, the effect of MSCs injection appears to be due to a generalized "niche effect" rather than to a real regeneration (Hashemian et al., 2015).

1.3.1.1 Pancreatic Stem Cells

Unlike other tissue-specific stem cells, Pancreatic Stem Cells (PSCs) were proposed only relatively recently (Peck et al., 2000) and their presence and origin has been hotly debated (Dor et al., 2004). Beta cells are regenerated during obesity, partial pancreatectomy and pregnancy and the observed regenerations led to the birth of the PSCs concept (Bonner-Weir et al., 2002). The existence of PSCs is also suggested by the continuous islets regeneration activity following transplantation (Ryan et al., 2002, 2005) and this suggests that PSCs and/or functional β cells are capable of self-duplication.

Further studies should determine the biological potential and the molecular signature of these self-renewing cells. Increasing evidence shows that insulin gene expression is not a β cells exclusively functional marker since it has been detected in multiple cell types mainly responsible for development of mature islet cells, like PDX1+ and Ngn3+ progenitors cells (Jiang et al., 2010). Therefore, insulin-expressing cells represent developmentally heterogeneous populations. It has been reported that a few Ngn3+ cells in the developing pancreas co-express insulin in mouse (Hara et al., 2006). Consistently with this observation, some NGN3+ cells co-express insulin also in human fetal pancreas between 10 and 21 weeks (Lyttle et al., 2008). Moreover, the insulin-expressing cells in the developing pancreas gave rise to other islet cell types in addition to β cells (Alpert et al., 1988). So, basically, insulin gene expression is not an exclusive marker of functional β cells. Transplantation studies or lineage-tracing, performed using marker cells labelled under the control of other mature β-cell specific genes promoters, will be important to confirm the self-duplication of functional β cells.

In vitro evidence has indicated that "pluripotent PSC" may be present in all three major pancreas compartments, i.e. islets, acinar tissue and ductal epithelium (Zulewski et al., 2001; Seaberg et al., 2004; Peck et al., 2000; Cornelius et al., 1997; Suzuki et al., 2002). This evidence comes from studies of both rodent and human pancreas. For example, a potential PSC candidate has been purified by flow cytometry in the developing and adult mouse pancreas. These cells are identified by expression of the receptor for hepatocyte growth factor, c-Met, and absence of blood cell surface markers such as Flk-1, TER119, CD45 and c-Kit. These cells can differentiate *in vitro* into multiple pancreatic lineage cells from individual cells, whereas, following transplantation *in vivo*, are able to give rise to pancreatic acinar and endocrine cells (Suzuki et al., 2002).

Anyway, the molecular characteristics and the *in vivo* localization of these c-met expressing cells are largely unknown and clonogenesis at the single cell level has not been definitively proved.

Since fetal mouse pancreatic cells are believed to be a rich source for potential PSC their differentiation and proliferation has been studied. Results indicate that bone morphogenetic proteins promote the development of pancreatic cystic epithelial colonies, containing β cells and pancreatic precursors (Jiang et al., 2002; Jiang & Harrison, 2005a). This process is similar to islet cells' delamination taking place during duct formation *in vivo*. Moreover, colony formation can be stimulated by various isoforms of epidermal growth factors (Jiang & Harrison, 2005b). These data indicate that various growth factors' families can modulate fate changes of pancreas precursor/stem cells, like other stem/progenitor systems. However, currently, these colony-forming cells can be considered precursors rather than stem cells because *in vitro* self-renewal has not been established yet.

In conclusion, current data from literature indicate that a pancreatic stem cell population has not been identified and, therefore, at present, cannot be considered as a viable candidate for a regenerative medicine approach.

1.3.2 Embryonic Stem Cells (ESCs)

When Adult stem cells are not available for different reasons, an alternative approach is represented by Embryonic Stem Cells (ESCs), which are derived from the Inner Cell Mass (ICM) of a blastocyst. They are capable of unlimited and undifferentiated proliferation *in vitro* (Evans & Kaufman, 1981). In particular, ESCs are defined by the capacity for repeated generation of two classes of progeny: daughter cells with equivalent proliferative and developmental potential and daughters specified for differentiation. ESCs have an indefinite proliferative life span, and long-lived subclones obtained by single-cell expansion retain pluripotency (Martello & Smith, 2014). This undifferentiated state is maintained by several factors. Firstable, the cytokine leukemia inhibitory factor (LIF), an interleukin 6 class cytokine (IL-6) that affects cell growth by inhibiting differentiation (K. Onishi & Zandstra, 2015). In particular, soon after the initial derivation of mouse (m) ESCs it was discovered that their *in vitro* propagation required the activity of LIF (Smith et al., 1988; Williams et al., 1988), drived by activating the Janus kinase-signal transducer and activator of transcription 3 (JAK-

STAT3) signaling pathway (Boeuf et al., 1997; Niwa et al., 1998). JAK-STAT signaling is mediated primarily through the IL-6 family that signal via either non-signaling α-receptors or as signaling receptors. LIF, for instance, first binds to its signaling receptor, LIF-R (Gearing et al., 1991), and recruits another signaling receptor, glycoprotein 130 (GP130), to form a heterodimer that mediates downstream signal transduction. Upon dimerization, the signaling receptors recruit and phosphorylate JAKs (JAK1, JAK2, JAK3 and Tyk2) which phosphorylate STAT3. This cascade culminates in the dimerization of phosphorylated STAT3 (pSTAT3), its translocation to the nucleus and the direct regulation of the transcription of a wide range of genes, included the JAK-STAT inhibitor suppressor of cytokine signaling 3 (SOCS3) (Naka et al., 1997; Starr et al., 1997). Furthermore, inhibition of STAT3 is also independently mediated through protein inhibitor of activated STAT3 (PIAS3) (Chung et al., 1997). On the other hand, JAK-STAT signaling also the transcription of STAT3, JAK1, GP130 and LIF-R (Davey et al., 2007; He et al., 2005). These autoregulatory aspects of JAK-STAT signaling are capable of cycling between dormancy and activity in response to specific developmental cues and to external stimuli postnatally.

However, LIF-STAT3 pathway is not sufficient. In fact, *in vitro*, if serum is removed from ESCs, they continue to proliferate but progressively loose ES cell features and differentiate over five to six days, mostly into neural precursors and neurons (Ying et al., 2003a). The implication is that serum provides an additional signal to LIF that is required to fully suppress differentiation, in particular to the neural lineage. Bone morphogenetic proteins (BMPs) are potent antagonists of neural specification in vertebrate embryos and they can replace serum and sustain self-renewal in combination with LIF (Ying et al., 2003b). This effect of BMP had been elusive because BMP alone promotes nonneural differentiation (Malaguti et al., 2013; Wiles & Johansson, 1999), and the self-renewal action is apparent only in the presence of LIF.

Moreover, the fibroblast growth factor (FGF) pathway is operative in ESC cultures and, in particular, ESCs produce appreciable amounts of FGF4. Although initially considered as a potential autocrine self-renewal stimulus, characterization of ESCs deleted for FGF4 highlighted a role in differentiation (Wilder et al., 1997). Another important factor, the glycogen synthase kinase-3 (GSK3), was also implicated as an antagonist of ESCs self-renewal (Doble et al., 2007; Sato et al., 2004). Inhibition/deletion of GSK3 combined with LIF allowed efficient ES cell self-renewal.

GSK3 is a negative regulator of many basic cellular processes, restraining several intracellular signaling pathways (Doble & Woodgett, 2003), most notably canonical Wnt/β-catenin (Clevers, 2006). In particular, Wnt signaling and Wnt proteins are important for the maintenance of stem cells of various lineages (Nusse et al., 2008). The Wnts comprise a large family of protein ligands that affect several processes such as embryonic induction, generation of cell polarity and the specification of cell fate (Logan & Nusse, 2004). In stem cell biology, the interactions between Wnts and membrane are likely of great importance in understanding how niches control stem cell fate, commonly thought to be in close cell to cell configurations. In particular, several studies, mostly coming from mouse ESCs research, demonstrated that Wnt signaling components are involved in ESCs control (Pereira, et al., 2006; Hochedlinger et al., 2005; Kielman et al., 2002).

Therefore, ESCs are a possible source of cells for many regenerative medicine applications, including diabetes. The use of ESCs in cell therapy is conceptually simple and involves their differentiation into pancreatic progenitors for transplantation. However, the pancreatic progenitors derived from ESCs needs to be packed in immuno-isolating capsules prior to the subcutaneous transplantation, to avoid life-long immuno-suppressive therapy because they would be exogenous to the patient. These encapsulated cells (expected to mature into islets on transplantation) will have the ability to secrete appropriate amount of insulin in a glucose-responsive manner over a period of time. This would represent a more physiological approach compared to daily insulin injections and are expected to remain functional over a long time (Bhartiya, 2016). Jiang et al. observed that about 30% of transplanted mice showed reduction in hyperglycemia on transplanting insulin positive cells (obtained by ESCs differentiation), for over six months (Jiang et al., 2007). Thus, proof of concept for use of human ES cells for diabetes was established but, however, the process remains highly inefficient.

Moreover, ESCs are difficult to control since, once transplanted *in vivo*, they can form tumors. This issue represents a huge limitation in their use for therapy. Furthermore, their derivation involves several ethical and legal issues (Wobus, 2001).

1.3.2.1 ESCs pluripotency factors

The mainteinance of pluripotency in ESCs is regulated by several factors.

First of all, the preeminent pluripotency factor is the POU-domain transcription factor Oct4 (*Pou5f1*). This was the first transcription factor identified and characterized as a regulator of pluripotency (Okamoto et al. 1990, Schöler et al., 1990). Oct4 is expressed in oocytes and early embryos and it is maintained exclusively in the germ cell lineage (Martello & Smith, 2014). *In vitro* Oct4 is found only in embryonal carcinoma (EC), ES and embryonal germ (EG) cells. Upon deletion of Oct4, pluripotency fails to become established in the embryo and, in particular, ICM cells lose their identity and differentiate into trophectoderm (Nichols et al., 1998), which is not a common fate for ES cells (Niwa et al., 2000). However, forced expression of Oct4 does not consolidate or enhance ESCs self-renewal. On the other hand, even modest overexpression precipitates differentiation (Niwa et al., 2000). Conversely, previous works showed that reduced levels of Oct4 impaired ESCs differentiation, without affecting self-renewal (Karwacki-Neisius et al., 2013; Radzisheuskaya et al., 2013).

Moreover, the SRY-box transcription factor Sox2 is also essential for ESCs self-renewal. As a matter of fact, Sox2 inactivation in ESCs results in trophoblast formation, as in Oct4 deletion (Masui et al., 2007). In particular, it was demonstrated that Sox2 is an Oct4 partner (Ambrosetti et al., 1997) and it physically interacts with Oct4 protein (Pardo et al., 2010; van den Berg et al., 2010). Sox2 binds DNA together with Oct4 at Oct/Sox elements (Chen et al., 2008) and positively regulates Oct4 transcription (Masui et al., 2007). However, Sox2 is much more broadly expressed than Oct4, and, in addition to the pre- and post-implantation epiblast, it is expressed in trophectoderm (Keramari et al., 2010) and later on by all neuroectodermal cells and in several endodermal and epithelial tissues. Furthermore, overexpression of Sox2 predisposes ESCs to differentiation (Kopp et al., 2008; Zhao et al., 2004), suggesting that, like Oct4, Sox2 expression levels should be constrained for efficient self-renewal.

Then, a coregulated target identified later is the gene encoding Nanog, a classic pluripotency-maintaining factor. Nanog is a homeodomain-containing transcription factor and its expression, *in vivo,* is more restricted to the naive pluripotency compartment compared to Oct4 and Sox2 (Silva et al., 2009), although it is reexpressed exclusively in the early egg cylinder and is present in primordial germ cells (PGCs). In addition, the forced expression of Nanog in ESCs confers the ability to self-renew in the

absence of LIF (Chambers et al., 2003). On the other hand, loss of Nanog destabilizes pluripotent cells both *in vitro* and *in vivo*, as measured by impaired colony forming capacity (Chambers et al., 2007) of "Nanog null ESCs" and the failure of "Nanog null embryos" to generate the epiblast (Mutsui et al., 2003; Silva et al., 2009).

Definitely, Oct4, Sox2 and Nanog have been shown to cross-regulate each other (Figure 1) and consequently are proposed to form a core triad that maintains the pluripotent state (Young, 2011). However, it is not sufficient to explain the observed properties of ES cells. As a matter of fact, a number of transcription factors specific to naïve epiblast and ground state ESCs have been identified. Among these factors, Esrrb, Klf4, Klf2, and Tbx3 have been functionally implicated in ESCs self-renewal through forced expression studies (Nichols & Smith, 2012). These transcription factors are interconnected each other and with Oct4, Sox2, and Nanog (Chen et al., 2008; Marson et al., 2008). Like Nanog, their deletion can be tolerated by ESCs, although self-renewal is heavily compromised.
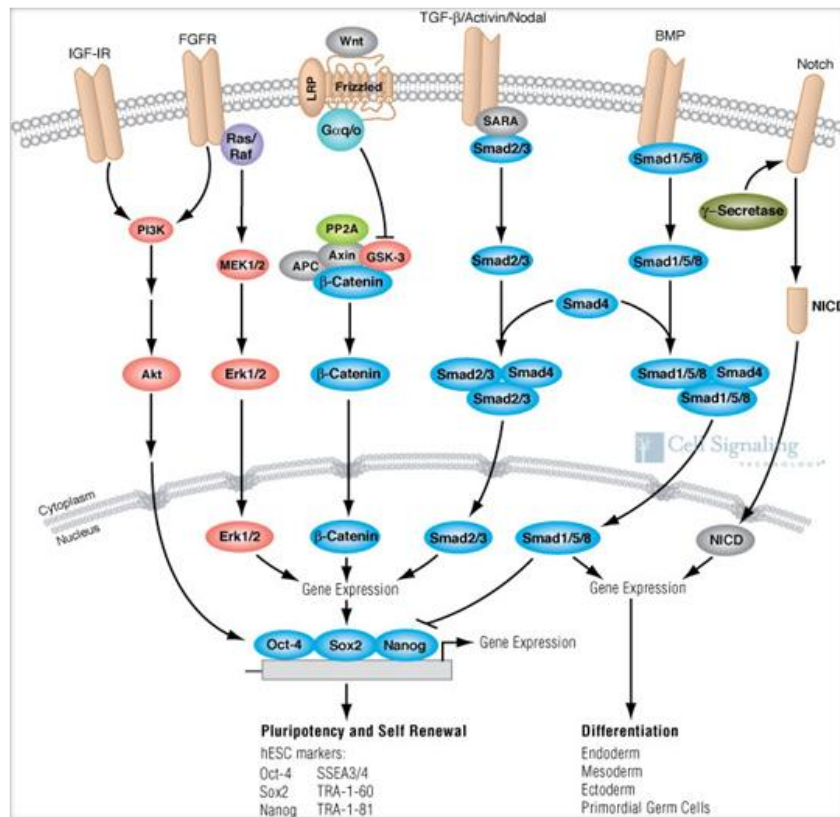
Figure 1: combinatorial signaling pathways involved in maintaining ESC pluripotency.
(Cell signaling)

1.3.3 Induced Pluripotent Stem cells (iPSCs)

The ethical difficulties related to the use of human embryos, as well as the problem of tissue rejection following transplantation in patients, can be circumvented by the generation of pluripotent cells directly from the patient's own cells. It has been demonstrated that the factors that play an important role in the maintenance of ESCs identity also play an essential role in the induction of pluripotency in somatic cells. This is achieved by the retroviral transfection of four genes, responsible for the maintenance of ESC identity, into adult somatic cells. The introduction of Oct4, Klf4, Sox2 and v-myc avian myelocytomatosis viral oncogene homolog (c-MYC) (Meissner et al., 2007; Okita et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006), ensure the transformation of any adult somatic cells in ESC-like cells, that are known as Induced Pluripotent Stem Cells (iPSCs) (Figure 2) (Takahashi & Yamanaka, 2006).

Resulting iPSCs can be transcriptionally, epigenetically and functionally equivalent to ESCs (Okita et al., 2007; Takahashi & Yamanaka, 2006; Wernig et al., 2007). Reprogramming provides an alternative system to gauge the relative contribution of transcription factors controlling the naïve pluripotent state. In particular, transcription factors associated with the ESCs state have been extensively implicated in reprogramming. For example, epiblast stem cells (EpiSCs), which already express Oct4 and Sox2, can be efficiently converted to naïve iPS cells forcing the expression of Nanog, Klf4, Klf2, Esrrb, Tfcp2l1, Tbx3 or Gbx2 (Festuccia et al., 2012; Guo et al., 2009; Hall et al., 2009; Martello et al., 2013; Silva et al., 2009; Ye et al., 2013) or by hyperactivation of Stat3 (Onishi et al., 2014; Yang et al., 2010).

Definitely, the potential of iPSCs is enormous but, however, many obstacles remain before their medical and pharmaceutical applications can be fully realized. In fact, their ability of unlimited self-renewal and the ability to differentiate into all body cell types (Ezashi et al., 2009; Esteban et al., 2009) constitute a limitation for their use in regenerative medicine, since the attenuation of proliferative and differentiation controls may increase the risk of a neoplastic transformation after transplantation. Moreover, the requirement of permanent integration of viral vectors (retroviruses or lentiviruses), into the host genome to generate iPSCs (Yamanaka, 2007), poses a severe limit to their current therapeutic use (Okita et al., 2007). These problems have stimulated the development of several protocols for a virus-free iPSC derivation (Zhou et al., 2009; Okita et al. 2008) but, at present, these approaches are generally more technical

demanding and less efficient (Lengner, 2010). Therefore, overall, the use of pluripotent stem cells derived, either from an embryo or from the genetic manipulation of a somatic cell, is still characterized by several problems that drastically limit their use in the cure of diabetes, as well as of any other disease. However, finding new sources of β-cells remains at the forefront of goals in diabetes research. Several alternative strategies to generate β-cells are currently being pursued and one of the most promising is epigenetic conversion.
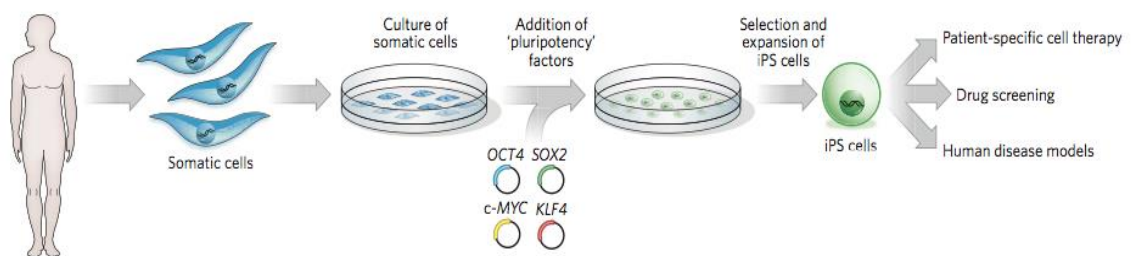
Figure 2: iPSCs creation

(Yamanaka & Blau, 2010)

1.4 Pancreas organogenesis

1.4.1 Pancreas structure

Pancreas is a elongated and flattened soft gland, 12-20 cm in length in human and it is covered with a thin connective tissue. The pancreas head is on the right side and lies within the duodenum curvature. In the rear abdomen is possible to identify neck, body and tail of the pancreas that lies obliquely, with the tail extending as far as the gastric surface of the spleen (Longnecker, 2014). Pancreas is composed by peculiar structures called lobules, which are connected by connective tissue septa containing blood vessels, nerves, lymphatic and excretory ducts (Figure 3).
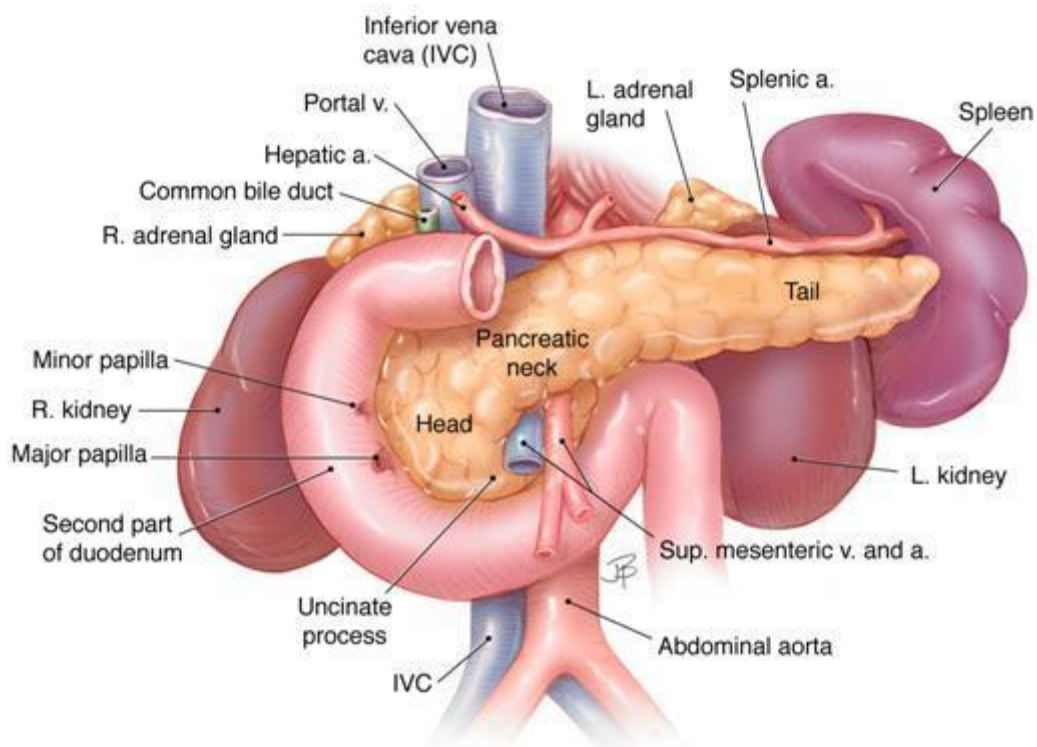
Figure 3: anatomic relationships of the pancreas with surrounding organs and structures (Longnecker, 2014)

Pancreas is composed by two portions:

- *Exocrine pancreas*, the portion that produce and secrete digestive enzymes into the duodenum. This includes acinar and duct cells with associated connective tissue, nerves and vessels. The exocrine components comprise more than 95% of the pancreatic mass (Longnecker, 2014). Moreover, exocrine pancreas has numerous secretory (zymogen) granules, containing several digestive enzymes, including amylases, lipases, proteases and nucleases. All these enzymes are secreted in the digestive tract (Slack, 1995).
- *Endocrine pancreas*, the portion that make and secrete insulin, glucagon, somatostatin and pancreatic polypeptide into the blood (the islets). Islets are compact spheroidal clusters embedded in the exocrine tissue, comprising 1-2% of pancreatic mass (Longnecker, 2014).

There are four principal types of endocrine cells (Figure 4):
- α (or A or A2) cells secreting Glucagon;
- β (or B) cells secreting Insulin, C peptide, Islet amylod polipeptide (IAPP or amylin) and Gamma-aminobutyric acid (GABA);
- δ (or D or A1) cells producing Somatostatin (SS);
- ε (or E) cells which secrete Ghrelin;
- PP (or F) cells which secrete pancreatic polypeptide (PP).
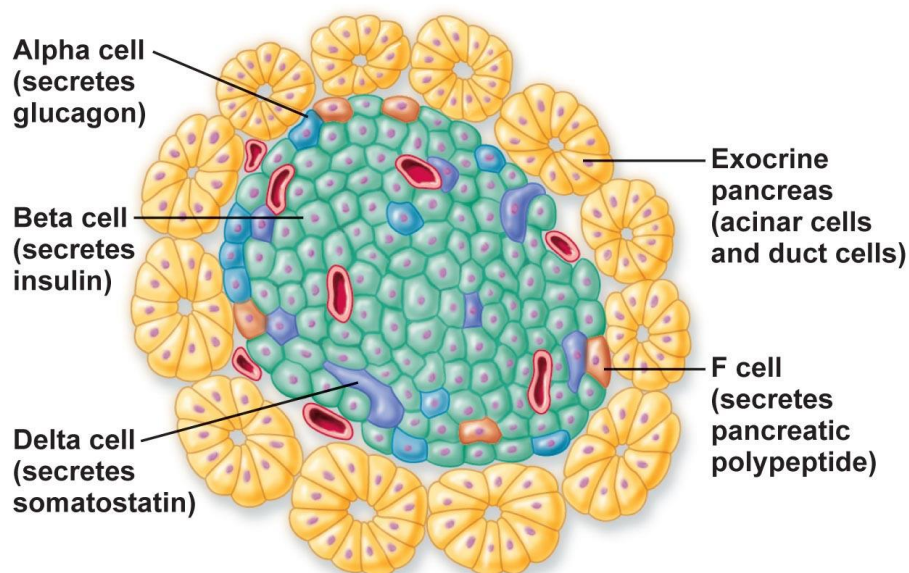
Figure 4: exocrine and endocrine pancreatic cells.

1.4.2 Overview of pancreas development

1.4.2.1 Mouse pancreas development

Pancreas is a compound gland derived from the endoderm. Prior to organogenesis, the gut endoderm becomes grossly regionalized into distinct organ fields by a series of anteroposterior and dorsoventral patterning events (Pan & Wright, 2011). Then, such events are usually drived by extrinsic signals from adjacent mesodermal derivatives, as well as by intrinsic events regulated by specific factors expressed within the endodermal cells themselves. In particular, during gastrulation, the dorsal pancreatic region first receives inductive signals from the mesoderm, then, permissive signals from the nearby notochord and, finally, proliferative signals from the pancreas mesenchyme (Figure 5A). On the other hand, the ventral pancreas patterning is regulated by distinct sets of signals from the lateral plate mesoderm, cardiac mesoderm, and septum transversum, as visible in figure 5B (Pan & Wright, 2011).
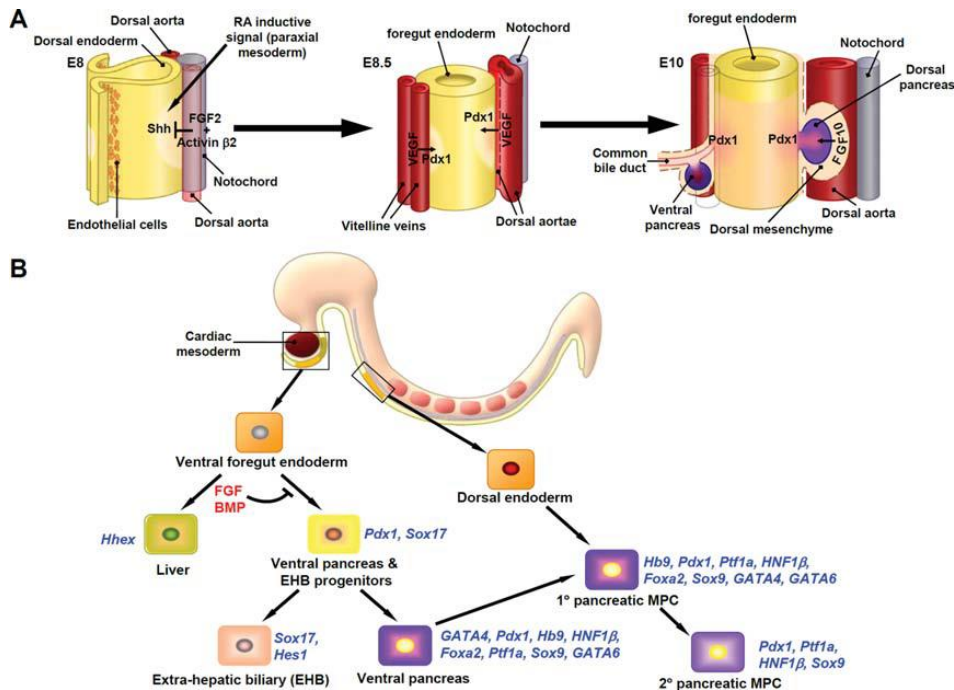
Figure 5: early pancreas development: anteroposterior and dorsoventral patterning events.

(Pan & Wright, 2011)

In particular, pancreas development is clearly first evident at Embryonic day 9.5 (E9.5) in mouse, when the dorsal foregut endoderm thickens and evaginates bulging into the surrounding mesenchyme. This is followed at E10 by the emergence of the anlage of the ventral pancreas bile duct, from the ventral foregut endoderm. Specifically, rodent pancreas formation has been classically divided into two overlapping waves of development. The first one, occurring between E9.5–E12.5, is characterized by a broad morphogenetic change of the pancreatic epithelium (Pan & Wright, 2011). In particular, during this first transition, is possible to observe an active proliferation of pancreatic progenitors that generate a stratified epithelium. Then, the formation of multiple microlumens (and their subsequent coalescence) give rise to the first differentiated endocrine cells in the dorsal bud, mainly glucagon-producing cells (Herrera, 2000; Kesavan et al., 2009; Villasenor, Chong, Henkemeyer, & Cleaver, 2010). Subsequently, around E11.5, the gut tube begins to coil, allowing the conjoining of dorsal and ventral buds into a single organ. Then, at E12.5, the densely packed epithelium starts to undergo active plexus remodeling, continued epithelial expansion and production of more plexus, sending finger-like protrusions into the mesenchyme and over the entire organ (Figure 6). Concurrently, compartmentalization of the ''protodifferentiated'' pancreatic epithelium commences, with the segregation of the epithelium into distinct ''tip'' and ''trunk'' domains. While the tip domains contain multipotential pancreatic cells (MPC), which are later destined to change into acinar-fated progenitors, the adjacent trunk epithelial region consists of an endocrine-duct bipotential progenitor pool (Zhou et al., 2007) (Figure 6).

Starting at E13.5, the epithelium undergoes a striking morphogenetic event called the ''secondary transition'', characterized by a massive differentiation wave towards the three main pancreatic lineages. Firstly, acinar cells derive from the extending tip epithelium and continue to undergo active proliferation to increase their number (Pan & Wright, 2011), producing a great numbers of acini throughout the organ (Figure 6). Secondly, endocrine cells become committed from the trunk epithelial region, going through a not well defined epithelial exit process that involve an epithelial-to-mesenchymal transition (EMT) (Rukstalis & Habener, 2007). Thus, endocrine cells organize themselves into clustered endocrine islets, often located nearby to their parent ducts.

After the secondary transition, at E16.5, the epithelium expands further, largely driven by acinar proliferation. In particular, the competence of the trunk epithelium to give rise to endocrine cells of various types changes according to the developmental stage (Johansson et al., 2007). Finally, during late gestation and in the first few weeks of postnatal life, endocrine cells start to coalesce and round up into mature islets. In the mouse, β-cells form the islet core surrounded by a coat composed by α, δ and PP-cells, whereas the very minor ε-cell population is dispersed throughout the islet. About 80% of the islet cell mass present at birth is generated by the proliferation and differentiation of endocrine progenitors, with the other 20% coming from islet cell proliferation (Bouwens & Rooman, 2005).
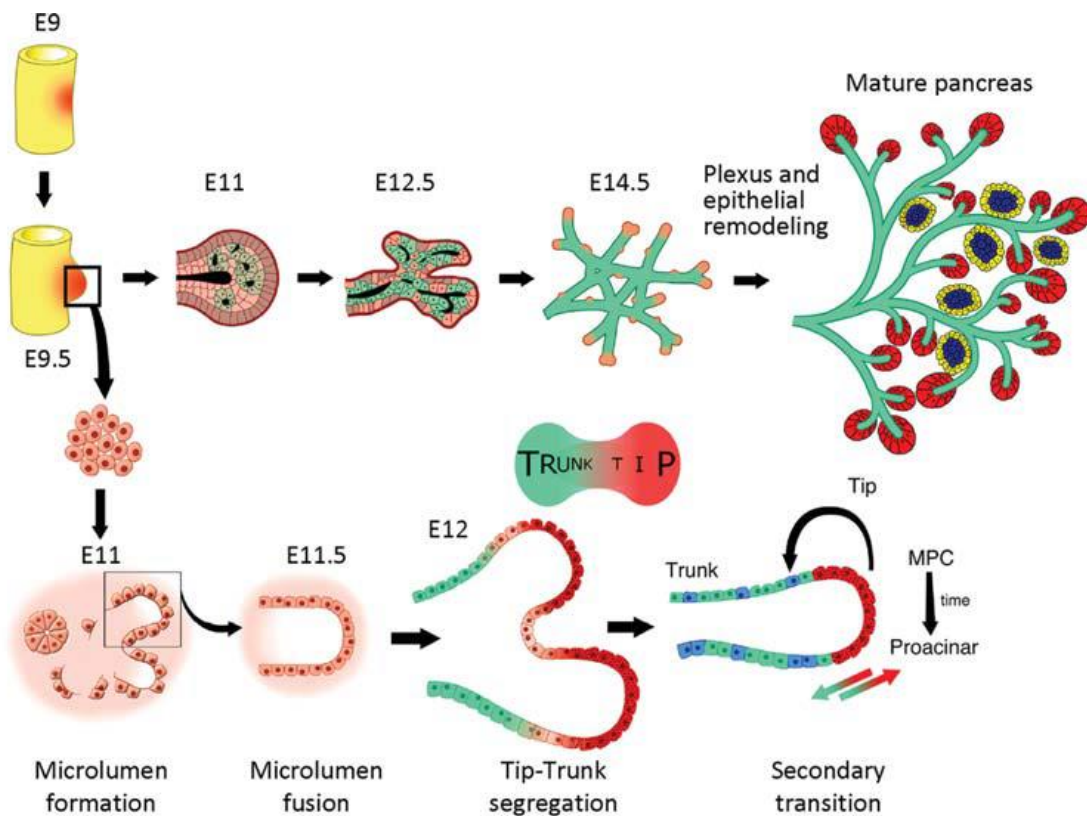
Figure 6: mouse pancreas development.
(Pan & Wright, 2011)

1.4.2.2 Human pancreas development

A wealth of data and reviews exist on mammals pancreas development, primarily mice and other vertebrates, but, in contrast, human pancreatic development has been less reviewed (Jennings et al., 2015). It seems obvious that a detailed understanding of human model could be crucial for learning the best methods of *in vitro* directed differentiation of hES/iPS cells towards functional β-cells.

Despite ethical constraints on procurement of human fetal tissue, pioneering studies from Scharfmann's group provide precious informations on human pancreas organogenesin, including early differentiation and proliferation of endocrine cell types (Polak et al., 2000), *ex vivo* analysis of endocrine and exocrine differentiation in human fetal pancreas grafted under the kidney capsule (Castaing et al., 2001; Castaing et al., 2005), establishment of *in vitro* culture system for human fetal pancreas (Ye et al., 2005) and, finally, *in vivo* lineage tracing system for labeling β-cells (Scharfmann et al., 2008). In general, human pancreas organogenesis largely mimics the mouse process. In particular, dorsal and ventral pancreas budding is first evident at 26–35 dpc (day post coitum) and their fusion occurs around 6 weeks of gestation (G6w) (Pan & Wright, 2011). Specifically, the dorsal bud produces most of the head, body and tail of the mature pancreas, whereas the ventral bud contributes to the inferior part of the head of the organ (Piper et al., 2004; Polak et al., 2000; Slack, 1995). In contrast to the typical mouse pancreatic development characterized by two overlapping waves, in human pancreatic development there may be a single, extended transition. As a matter of fact, it is difficult to assume that there is an equivalent of these transitions, in terms of morphology or any broad changes in endocrine-specific transcription regulator expression (Sarkar et al., 2008). Furthermore, there are important functional differences between the human ''mixed-islet'' architecture and the canonical core (β-cell) and mantle (other endocrine) structure that is found in mice. Islet-like clusters appear from approximately G11w in human pancreas. Unlike the mixed-islet architecture observed in human adult pancreas, the aggregated insulin and glucagon-expressing cells in the human fetal islets (around G14– 16w) seem to be arranged similarly to the mouse adult islet (Jeon et al, 2009). However, at G19w, it is proposed that a transient separation of the peripheral α and δ-cells occurs, away from the β-cell core, to form homogeneously mono-hormone-producing clusters. Presumably, the mono-hormone islets reintegrate amongst each other after G22w, and there is significant intermixing to generate the adult

islet architecture (Jeon et al., 2009). Moreover, there are also comparative studies showing the differential distribution of endocrine cells amongst the various regions of the human and mouse pancreas. In particular, greater numbers of PP cells are located in the human head region, with more α-cells and β-cells in the neck, body and tail regions (Brissova et al., 2005). Finally, human islets contain 50% β-cells, 40% α-cells, 10% δ-cells and a few PP cells, while mouse islets are composed by 75% β-cells, 20% α-cells, and 5% other endocrine cells (Brissova et al., 2005).

The importance of these differences is still unclear, and any relevance to endocrine cell function and glucose/energy metabolism requires further detailed analysis.

1.4.3 Transcriptional regulation of endocrine pancreatic cells development

During embryogenesis, pancreas development occurs following several differentiation stages, as visible in Figure 7.
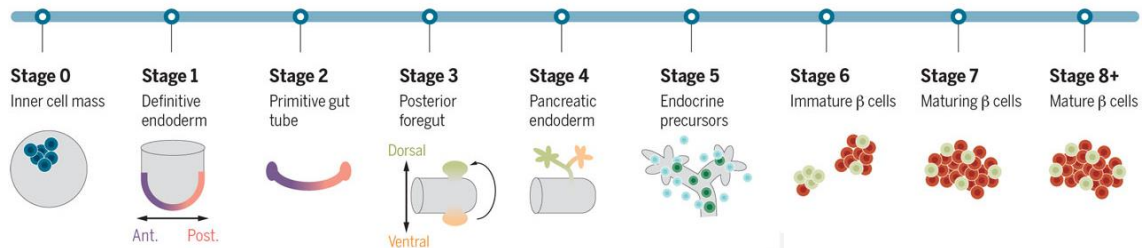
Figure 7: *in vivo* **endocrine pancreatic development stages.**
(Bruin et al., 2015)

Along this process, a number of transcription factors are activated at each stage (Figure 8), as described below.
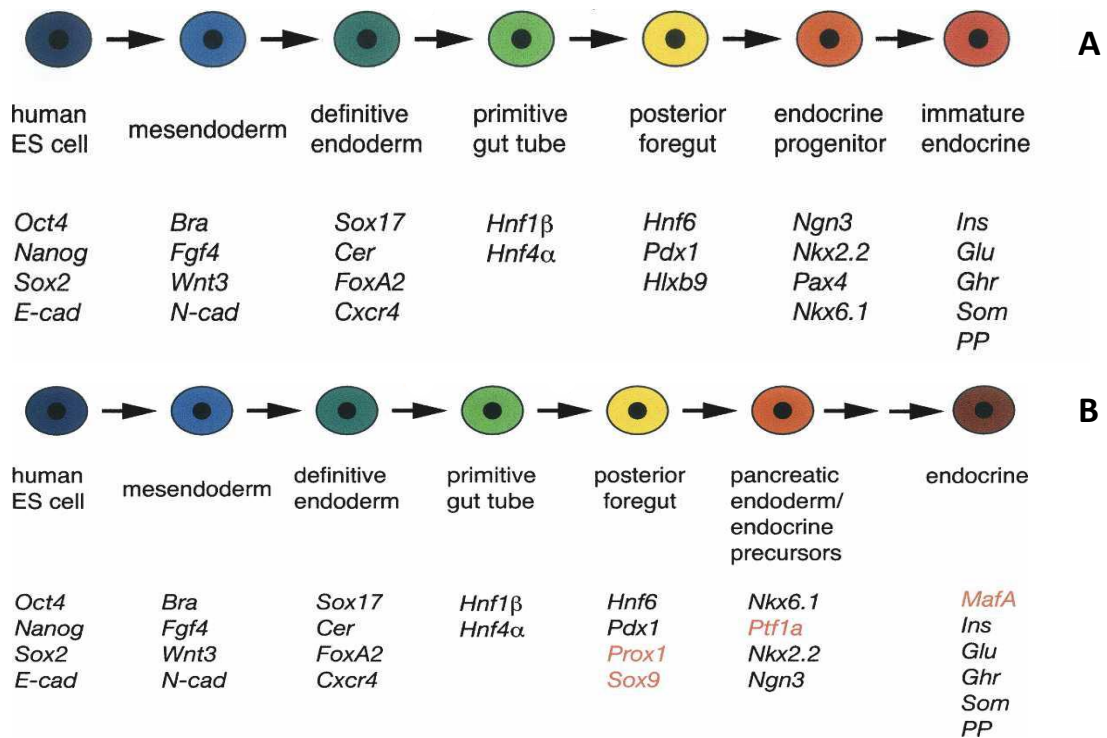
Figure 8: differentiation of pancreatic endocrine cells from human ES cells. Transcription factors expressed at each stage proposed by D'Amour et al. (2006) (A) and Kroon et al. (2008) (B).

1.4.3.1 Definitive endorderm transcription factors

The pancreas develops from the definitive endoderm (DE) germ layer, which is generated during the gastrulation stage of embryogenesis (Tam et al., 1993).

Among all transcription factors expressed during this stage, two of these are the most studied:

Forkhead box A2 (FOXA2)

During early pancreatic development, the FOXA2 transcription factor is consistently expressed from week 4 forward, as revealed by recent studies carried out on human fetal pancreas (Jennings et al., 2013; Jeon et al., 2009; Lyttle et al., 2008). This expression profile is similar to broad mouse FOXA2 expression throughout pancreatic development. Furthermore, FOXA2 persists in all mature pancreatic cell types of both mice and humans (Cano et al., 2014; Pan & Wright, 2011).

SRY (sex determining region Y)-box 17 (SOX17)

In contrast to FOXA2, expression of SOX17 is observed immediately before 4 weeks in humans and is then excluded from pancreatic cells about 1 week later, similar to the down-regulation of SOX17 during mouse pancreatic development (Rachel E. Jennings et al., 2013; Piper et al., 2004). Studies in mice have indicated that, although early SOX17 expression is necessary for endoderm formation, it later represses the pancreatic fate (Spence et al., 2009).

1.4.3.2 Primitive gut tube transcription factors

DE initially consists of a flat sheet of cells that has anterior-posterior pattern information (Conrad et al., 2014). Then, this peculiar structure forms a primitive gut tube, along which the domains for various endoderm organ primordia are specified (Wells & Melton, 1999). In particular, during the primitive gut tube formation, the expression of two important factors was detected:

Hepatocyte nuclear factor 1 homeobox b (HNF1b)

A high level of HNF1b expression begins as early as 7 weeks in humans, persisting throughout pancreatic development (Jeon et al., 2009). Interestingly, heterozygous loss-of-function HNF1b mutations (termed MODY5) result in diabetes in humans but only homozygous mutations produced diabetes in mice (Horikawa et al., 1997). This could be due to a potentiated single wave of human endocrine differentiation versus the two

phases observed in rodents, rendering human cells more sensitive to HNF1b dosage (Cano et al., 2013).

Hepatocyte nuclear factor 4α (HNF4α)

In rodents, HNF4α is nuclear hormone expressed in the primitive endoderm at E4.5, in the gut endoderm at E8.5, in the pancreatic epithelium at E9.5 and, postnatally, in both endocrine and exocrine cells (Duncan et al., 1994; Nammo et al., 2008). Similar expression pattern occurs in human.

1.4.3.3 Posterior foregut transcription factors

Then, pancreas develops from the posterior foregut, emerging as buds from the dorsal and ventral sides of the gut tube. At this early stage, formation of the pancreatic anlage depends on retinoid signaling and on inhibition of hedgehog signaling (Lau et al., 2006; Stafford et al., 2004).

The developing organ mainly express the following transcriptional factors:

Hepatocyte nuclear factor (HNF6)

Recent sudies demonstrated that HNF6 is consistently expressed in 7–21 weeks human pancreas aged (Jeon et al., 2009; Lyttle et al., 2008). In parallel, mouse HNF6 expression is visible starting from the E8.5, with broad expression throughout development, directing endocrine allocation until just before birth when it becomes restricted to alfa and acinar cells (Zhang et al., 2009).

Pancreatic and duodenal homeobox 1 (PDX1)

Also known as insulin promoter factor 1 (IPF1), PDX1 has been studied for its role throughout all phases of pancreatic development. PDX1 is largely expressed at around 4 weeks with a high level of expression being restricted later to adult human β cells (Rachel E. Jennings et al., 2013; Lyttle et al., 2008). Based on the staging of the surrounding tissue morphology, PDX1 appears slightly later in human development than in mice. In humans, its expression is only evident after the notochord and aorta separation from the dorsal foregut (Rachel E. Jennings et al., 2013; Jeon et al., 2009). On the other hand, in the mouse, PDX1 expression compares in the pre-pancreatic endoderm, around E8.5 (Ahlgren et al., 1996). Mouse lineage-tracing studies demonstrated that PDX1+ cells mark progenitors of all the mature pancreatic cell types including endocrine, acinar and ductal cells (Gu et al. , 2002).

SRY (sex determining region Y)-box 9 (SOX9)

SOX9 is found in PDX1+ cells in early human and mouse pancreas by about 4 weeks and E9, respectively, and is then excluded from mature endocrine cells (Cano et al., 2013; Rachel E. Jennings et al., 2013; Pan & Wright, 2011). Furthermore, in mice, SOX9 is necessary for the maintenance of multipotent progenitor populations (Rachel E. Jennings et al., 2013; McDonald et al., 2012; Pan & Wright, 2011; Piper et al., 2002).

1.4.3.4 Pancreatic endoderm and endocrine precursors transcription factors

The next phase of pancreas development is endocrine cell specification, which occurs through inhibition of Notch signaling in the pancreatic epithelium, allowing expression of the pro-endocrine genes (Ahlgren et al., 1996).

Neurogenin 3 (NGN3)

NGN3 is expressed in all endocrine progenitors (Gu et al., 2002), initiating a cascade of transcription-factor expression, driving endocrine cell differentiation. Coincident with SOX9 loss, in pancreatic epithelial cells, endocrine cell specification initiates with NGN3 expression, in both human and mouse models (Gradwohl et al., 2000; Jennings et al., 2013; Lyttle et al., 2008). In human, NGN3 is seen as early as 8 weeks and becomes more highly expressed at around 11 weeks. Then, expression declines to only low levels at 19 weeks (Capito et al., 2013; Gradwohl et al., 2000; Rachel E. Jennings et al., 2013; Jeon et al., 2009). Later induction of human transcription factors near week 15 (ISL1, NEUROD1, MAFB, NKX2.2 and PAX6) indicates that NGN3 expression precedes the expression of these factors that are implicated in late endocrine cell differentiation (Jeon et al., 2009).

NK6 homeobox 1 (NKX6.1).

Human NKX6.1 is expressed in early multipotent pancreatic progenitors after 4 weeks, once SOX17 is excluded from the pancreatic buds (Rachel E. Jennings et al., 2013). Then, its expression becomes restricted to β cells by 14–16 weeks (Brissova et al., 2005; Rachel E. Jennings et al., 2013). Similarly, early rodent NKX6.1 expression is broad, then gradually becomes beta cell specific (Jennings et al., 2013; Sander et al., 2000).

Paired box gene 4 (PAX4)

Human PAX4 expression is evident by 9 weeks in whole fetal pancreatic mRNA analysis (Jeon et al., 2009). Although its spatial pattern has yet to be reported in humans, PAX4 is found in mouse endocrine progenitors and later in β cell precursors, as a regulator of β cell commitment (Sosa-Pineda et al., 1997).

Paired box gene 6 (PAX6)

PAX6 is induced by 14–16 weeks in human and is then maintained in all adult islet cells (Ahlqvist et al., 2012; Lyttle et al., 2008). This is similar to the known PAX6 expression pattern in mice (Sander et al., 1997).

Neurogenic differentiation factor 1 (NEUROD1)

NEUROD1 is expressed at week 15 and is then found in all endocrine cell types of adult islets (Rachel E. Jennings et al., 2013; Jeon et al., 2009; Lyttle et al., 2008). However, NEUROD1 expression occurs relatively earlier in mouse development – by E10.5 – but is similarly restricted to the endocrine compartment (Gu et al., 2010; Naya et al., 1995).

V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB)

Unlike in mice, where β cell MAFB expression diminishes postnatally, in humans MAFB increases from 7 to 21 weeks and, then, remains in mature α and β cells (Dai et al., 2012; Hang & Stein, 2011; Jeon et al., 2009). Sustained MAFB expression may have functional implications in β cell development and identity. Indeed, severe reductions in MAFB levels were found in human T2DM islet α and β cells, suggesting a role in their functional maintenance (S. Guo, Dai, Guo, & Taylor, 2013).

NK2 homeobox 2 (NKX2.2)

Another key difference between mice and humans is seen with NKX2.2 expression (Rachel E. Jennings et al., 2013). Indeed, in human, its expression first appears at 8 weeks with later increased transcription by 14–16 weeks, whereas, in rodents, NKX2.2 expression is observed earlier around E9.5 (Rachel E. Jennings et al., 2013; Lyttle et al., 2008; Sussel et al., 1998). Only later, rodent NKX2.2 being restricted to β cellls and a subset of α and PP cells (Rachel E. Jennings et al., 2013; Lyttle et al., 2008; Sussel et al., 1998).

Insulin gene enhancer protein ISL-1 (ISL1).

ISL1, also called ISLET1, appears to be required for pancreatic development, in both human and mouse pancreas development (Shimomura et al., 2000). In particular, in humans, its expression has been observed at 8–10 weeks fetal pancreas and, then, transcription gradually increases from mid-gestation (Jeon et al., 2009; Lyttle et al., 2008). This is similar in mice, where ISL1 is first expressed broadly at E9 and, then, it is maintained in the mature hormone+ endocrine cells (Ahlgren et al., 1996).

Pancreas transcription factor 1A (PTF1A)

PTF1A expression is barely detectable until mid-gestation in whole human fetal pancreas, presumably due to its enriched expression at that timepoint in acinar cells. It is better characterized in mice, with broad expression at E9 in dorsal and ventral pancreatic buds that is later restricted to acinar cells only (Jeon et al., 2009; Obata et al., 2001).

1.4.3.5 Endocrine cell differentiation and maturation transcription factors

Pancreatic hormone expression first occurs about 8 weeks into human gestation, with the onset of Insulin+ cells, which become more abundant by week 9 when Glucagon+ cells also appear (Jeon et al., 2009; Polak et al., 2000). On the other hand, in rodents, two waves of endocrine development have been observed. In detail, a first wave starting from about E9.5–12.5 is characterized by Insulin and Glucagon coexpressing cells, whereas the second wave, from about E12.5 to birth, produces endocrine cells that will populate mature islets (Herrera, 2000).

As mentioned above, the mature islets comprise five endocrine cell types: α, β, δ, PP and ε cells, which produce the hormones glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin respectively. The mechanisms that control the specification of these endocrine cell types from NGN3-expressing progenitors are not well understood (Conrad et al., 2014). Once formed, the hormone-expressing endocrine cells undergo further differentiation to a mature functional state, which, for a β-cell for example, involves the ability to release Insulin in response to elevated glucose concentrations.

1.5 Epigenetic cell conversion

1.5.1 Epigenetic mechanisms

In a complex multicellular organism, all cells have the same genome. However, in the body, there are many different cell types, obtained through the adoption of a specific specialization in several tissues. This is the result of cell differentiation processes that are regulated by the expression of several genes, responsible for a specific phenotype (Brevini et al., 2015). In particular, gene expression is regulated by extrinsinc and intrinsic factors (Swain et al., 2002): the first ones include environmental cues that can originate from the organism's ambient (e.g. temperature and oxygen) or from other cells within the organism (e.g. small molecules, secreted proteins). In contrast, intrinsic regulation takes place through the cell's own machinery that chemically modifies the DNA. These changes are used to be called epigenetic modifications since they do not alter the primary DNA sequence, but, instead, affect gene expression by changing the accessibility of genes to transcription factors, in either a positive or negative manner.

Two main mechanisms are involved in these regulatory processes: DNA methylation and histone modifications (Goldberg et al., 2007). DNA methylation is a biochemical process where a methyl group (CH_3) is added to the cytosine or adenine DNA nucleotides. The covalent addition of a CH_3 group at the 5-carbon of the cytosine ring is controlled by a specific family of enzymes: the DNA methyltransferases (DNMTs). In particular, DNMT3a and DNMT3b are required for the establishment of de novo DNA methylation patterns (Okano et al., 1999), whereas DNMT1 appears to be responsible for their maintenance (Takeshita et al., 2011).

The second mechanism involved in transcriptional regulation is histone modification. Histones are subject to a complex and dynamic set of covalent modifications, including acetylation, methylation, phosphorylation, SUMOylation, citrullination, ADP ribosylation and ubiquitination (Spivakov & Fisher, 2007). Binding of different molecules to the histone tail allows or inhibit transcription factors and other proteins to access the DNA.

During the embryo/fetus development, all cells undergo a further specification process characterized by differential gene expression and epigenetic restrictions that gradually limits cell potency to a more limited phenotype-related expression pattern, producing specialized committed populations (Waddington Conrad Hal, 1957). The acquisition of

epigenetic marks culminates with the fixation of a distinct lineage fate that has been considered stable and potentially irreversible for many years (Figure 9).

1.5.2 Reversal of cell fate

Mature cells of an adult organism acquire a differentiated state through a specification process that takes place during development of the embryo/fetus. This process is characterized by differential gene expression and epigenetic restrictions that gradually limit cell differentiation potency (Hemberger et al., 2009) to a more limited phenotype-related expression pattern, resulting in a progressive restriction in cell options (Zhou and Melton 2008) and finally producing highly specialized committed populations.

This final state is achieved and maintained through the epigenetic mechanisms that regulate gene expression (Li et al., 2012). Furthermore, because this series of events is extremely stable, a complete reversal of cell fate requires a wide reprogramming process that makes it inefficient and prone to errors (Plath and Lowry, 2011). Indeed, although cellular differentiation is usually unidirectional *in vivo*, it can be reversed *in vitro* (De Carvalho et al., 2011). In particular, terminally differentiated somatic cells can be reprogrammed using defined factors to generate induced pluripotent stem cells (iPSCs). Cell reprogramming requires higher levels of gene expression than those needed once the pluripotent state is reached, and equivalent to what is defined as 'activation energy' (Hemberger et al., 2009). This reflects the need to initiate epigenetic reprogramming events by using retroviral vectors carrying the transcription factors needed to reach the pluripotent state (Oct4, KLF4, SOX2 and c-MYC). Retroviral vectors integrate in the host genome and force ectopic overexpression of these reprogramming factors, resulting in reactivation of endogenous genes and regaining a developmental potency, comparable to that of ESCs. Unfortunately, as reported previously in this thesis, this approach suffers from severe limitations that prevent its possible use in regenerative medicine (Yamanaka, 2009).

To bypass these troubles, alternative gene factor delivery systems have been proposed, including non-integrating adenoviruses (Stadtfeld et al., 2014), plasmid transfection (Okita et al., 2008), a doxycycline-inducible excisable piggyBac (PB) transposon system (Woltjen et al., 2009) and non-integrating episomal vectors (Kaji et al., 2009). Nonetheless, other concerns associated with the risk of tumours onset remain unsolved.

Indeed, iPS cells display low differentiation efficiency that rarely exceeds 30%, leaving mature cells mixed with undifferentiated cells (Cohen and Melton, 2011).

Furthermore, the acquisition of a stable pluripotent state is not physiological and appears to be difficult to handle. To bypass this problem, a new strategy, termed 'transdifferentiation', has been introduced. This technique consists of direct conversion of one fully differentiated adult cell type into another without an intermediate pluripotent state, but rather through simultaneous downregulation of one genetic program and upregulation of the new one (Jopling et al., 2011). However, all these promising approaches involve the use of retrovirus for the overexpression of one or more specific transcription factors (Cohen and Melton, 2011), leading to several limitations and making cells unsuitable for use in cell therapy and regenerative medicine.

1.5.3 Epigenetic direct conversion

In recent years several protocols that avoid the use of virally or non-virally introduced exogenous factors have been developed. Recently, a novel technique was reported by Lim et al. (Lim et al., 2011) for isolating ESCs from mammalian preimplantation embryos by altering the epigenotype of embryonic explants with 5-aza-cytidine (5-aza-CR). These findings have opened the way to new approaches in which small molecules and, more recently, epigenetic modifiers are used to directly convert cells from one type into another.

Recently, in the Laboratory of Biomedical Embryology UNISTEM where I carried out my Ph.D project, alternative protocols have been developed in order to directly convert an adult mature cell into another differentiated cell type, avoiding a stable pluripotent state and the related limitations (Brevini et al., 2016; Pennarossa et al., 2016; Brevini et al., 2014; Pennarossa et al., 2014; Pennarossa et al., 2013; Harris et al., 2011). This approach is based on the concept that, among the different mechanisms involved in lineage commitment and differentiation, DNA methylation plays an essential role, both during early embryonic development and cell lineage specification. Based on this idea, we investigated and demonstrated that brief exposure to a demethylating agent can push cells to a less committed state, increasing their plasticity (Brevini et al., 2016; Brevini et al., 2014; Pennarossa et al., 2014; Pennarossa et al., 2013). To this end, we selected 5-aza-cytidine (5-aza-CR), a well-characterized DNMT inhibitor used previously to

'boost' progenitor cell differentiation (Galvez et al., 2008; Lefebvre et al., 2010; Naeem et al., 2013). This drug is a chemical analogue of cytosine and it is known to be a direct inhibitor of methyltransferase activity at low doses, as well as of methylation in newly synthesized DNA. These features give 5-aza-CR the ability to induce DNA hypomethylation, because the molecule substitutes for cytosine into DNA, blocking DNMT function (Stresemann and Lyko, 2008). On these bases, it has been demonstrated that exposing terminally differentiated cells to the demethylating agent 5-aza-CR induces a transient phase of high plasticity. This technique avoids a stable pluripotent state, is highly efficient, is applicable to different species and does not involve the use of viral vectors (Brevini et al., 2016; Pennarossa et al., 2014; Pennarossa et al., 2013). In particular, we demonstrated that adult skin fibroblasts exposed to 5-aza-CR for 18 h changed their phenotype, exhibiting reduced dimensions, increased nuclear volume and highly decondensed chromatin (Brevini et al., 2016; Brevini et al., 2015; Pennarossa et al., 2014; Pennarossa et al., 2013). These morphological features are distinctive of a highly permissive state, with cells containing more loosely packed chromatin than their differentiated counterparts to maintain genes in a potentially open state and prepare them for future expression (Tamada et al., 2006). These morphological modifications were accompanied by a specific and consistent gene regulatory response, which highlights the acquisition of increased plasticity. These observations indicate that, in response to an epigenetic modifier (e.g. 5-aza-CR), cells are pushed into a brief and transient 'highly permissive state'. Once they have entered into this higher plasticity window, they can easily be directed towards a different phenotype exposing them to specific differentiation stimuli (Brevini et al., 2016; Brevini et al., 2014; Pennarossa et al., 2014; Pennarossa et al., 2013). More specifically, in the diabetes care context, we were able to convert fibroblasts towards endodermic lineage commitment using a three-step induction protocol that allows cells to transit from the early pancreatic differentiation stage to mature endocrine cells, expressing the main pancreatic hormones (Brevini et al., 2016; Pennarossa et al., 2014; Pennarossa et al., 2013). Firstly, the use of Activin A was able to drive 5-aza-CR treated fibroblasts towards the endodermic lineage commitment. Subsequently, stimulating cells with a combination of Activin A and Retinoic Acid lead cells into the early pancreatic differentiation stage. These changes are underlined by an evolving pattern of gene expression that begins with the expression of endoderm (SOX17, FOXA2) and primitive gut tube (HNF4) markers.

Then, further differentiation towards the pancreatic lineage was induced by B27 supplement, basic fibroblast growth factor (bFGF), and insulin transferrin selenium (ITS). This resulted in the rearrangement of cells, with the formation of large three-dimensional spherical structures that tended to detach and float freely in the culture medium, reminiscent of *in vitro*-cultured pancreatic islets. These morphological changes were accompanied by the activation of transcription for advanced pancreatic genes, namely NKX6.1, MAFA, ISL1, PAX6, NEUROD, PCSK1, and PCSK2, and, most importantly, the converted cells expressed hormone and glucose sensor genes characteristic of mature endocrine pancreatic cells (Somatostatin, Insulin, Glucagon, Pancreatic Polypeptide and Glucokinase) (Brevini et al., 2016; Pennarossa et al., 2014; Pennarossa et al., 2013). The newly acquired phenotype supported a profound and functional change in the epigenetic converted cells. Moreover, changes in the culture environment, such as stimulation with hyperglycemic medium, were able to trigger the active release of Insulin in cell supernatants, showing a dynamic response similar to pancreatic β-cells, in which changes in ambient glucose represent the primary and physiological stimulus for insulin secretion.

Converted cell functionality was also demonstrated *in vivo* after injection into immunodeficient severe combined immunodeficiency (SCID) mice whose β-cells had been selectively destroyed with streptozotocin. Cell transplantation restored normal glycemic levels in these diabetic mice and stably maintained them (Pennarossa et al., 2013). The possibility to convert a skin cell into one that produces pancreatic hormones in a simple and safe way, suggests a great potential of this approach for the treatment of diabetes.

Therefore, this method opens the possibility to obtain any cell type using different induction protocols, starting from cells treated with 5-aza-CR. For example, in a different study, human granulosa cells exposed to 5-aza-CR were addressed toward muscular differentiation. The starting cell population showed significant morphological and structural changes, resulting in cells that exhibit muscular phenotype (Brevini et al., 2014). These results confirm the efficiency of the epigenetic conversion protocol.

However, the cells obtained through the epigenetic conversion are not fully mature and terminally differentiated *in vitro* and there is the need to optimize these protocol in order to improve cell differentiation and, at the same time, closely mimic β-cells *in vivo* environment.
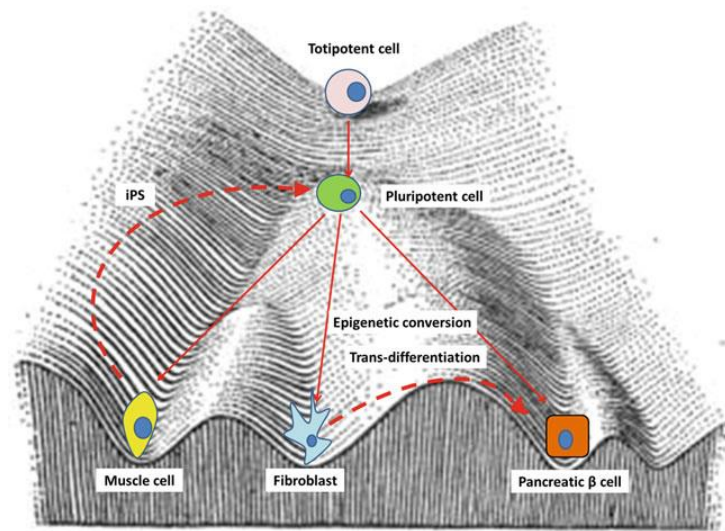
Figure 9: **from totipotent to terminally differentiated cells and back: a multi-directional process.**

(Pennarossa et al., 2016)

1.6 Physiological environment aspects

Regenerative medicine represents a promising approach for a wide variety of diseases, diabetes included, and its main goal is to maximize functionality of cells.

For this purpose, one strategy is to improve *in vitro* conditions in order to mimic as closer as possible the *in vivo* environment.

1.6.1 The role of oxygen

In mammals embryonic development takes place in low oxygen conditions where Hypoxia-inducible factors (HIF) activate genes responsible for cell morphogenesis (Dunwoodie, 2009).

Hypoxia regulates gene expression to alter cell homeostatic functions on normal or cancer cells. A fundamental component of the low oxygen response is the activation of the hypoxia-inducible factor 1 (HIF-1) (Semenza, 2014), together with the synergistic or antagonistic effects of the tumor suppressor protein P53, retinoblastoma protein (Rb) and the interplay between check-point control factors on cell cycle regulation for pro-survival or pro-death state (Figure 10) (Sermeus & Michiels, 2011). However, the mechanisms responsible for this state transition remain obscured and it seems to be related to the cell types and depends on the severity of the hypoxic condition.

Anyway, in uterine environment oxygen levels are usually included in a range between 1% and 5% (0.5-30 mmHg) (Fraker et al., 2007). In murine embryos, cells in hypoxia conditions (oxygen ≤2%) are very spread (Lee et al., 2001; Pringle et al., 2007), until around mid-gestation where the maternal blood is interfaced to the embryonal one. From that moment onward, cells in hypoxic state are still present in specific embryo's regions, including heart, skeleton and intestine. Moreover, hypoxic regions are also present in extra-embryonic tissues such as the yolk sac, allantois and placenta (Lee et al., 2001). In particular, the yolk sac develops in hypoxic conditions from the earliest stages of gestation. This phenomenon suggests the presence of a particular relation between blood vessel formation process (angiogenesis) and low oxygen condition. Indeed, several studies have demonstrated the presence of hypoxic conditions in several organs such as brain, heart and somites where angiogenesis is essential for proper blood supply (Noden, 1989). The hypothesis that hypoxia is maintained during embryogenesis and that it represents an important stimulus for embryonic angiogenesis is supported by previous studies where HIF-1a knockout mice showed defects in neural development, in

addition to the lack of erythropoiesis, vasodilation and angiogenesis induction (Maltepe et al., 1997). Hypoxia affects the proliferation and differentiation of various stem cell and progenitor cell populations (Bruick, 2003), deeply affecting stem cells development, promoting the differentiation of some cell type or inhibiting others. This phenomenon was originally described in the Neural Crest Stem Cell cultures where hypoxic condition (5% oxygen) was able to increase cell proliferation, compared to the same cells maintained in high oxygen (20%) (Bruick, 2003). Furthermore, it was demonstrated that in Central Nervous System precursor cells a hypoxic condition promotes cell proliferation, reducing cell death. In contrast, low oxygen concentrations inhibit adipocytes differentiation (Yun et al., 2002). Several studies performed on HSCs showed a hypoxic situation in the bone marrow where these cells reside in adult mammals (Cipolleschi et al., 1993; Parmar et al., 2007). Moreover, recent studies have shown that HSCs grow better in 1,5% of oxygen and they are able to repopulate the hematopoietic organ in immune-compromised recipient mice (Danet et al., 2003). Despite these results, the exact location of HSCs in the bone marrow is still controversial and it was supposed that these cells require different oxygen concentrations, depending on where their niche is placed (Simon and Keith, 2008).

Finally, low oxygen promotes ESCs proliferation. In particular, this phenomenon was observed for the first time in bovine blastocysts. In detail, it was demonstrated that in hypoxic conditions ICM significantly increase its development and efficiency, compared to a blastocyst maintained in high oxygen concentration (Harvey et al., 2004). Furthermore, a subsequent work has shown that even the human ESCs proliferate better in hypoxia (3-5%) (Ezashi et al., 2005). Therefore, these results suggest the existence of a general link between the hypoxia state and the maintenance of a pluripotent state, but the cellular mechanisms involved are still unclear.

As a consequence, hypoxia is today routinely adopted in several protocols for different stem cell populations culture in order to improve both cell proliferation and functional efficiency (Rajan et al., 2003).

Definitely, oxygen tension plays an important role during several cell differentiation processes.
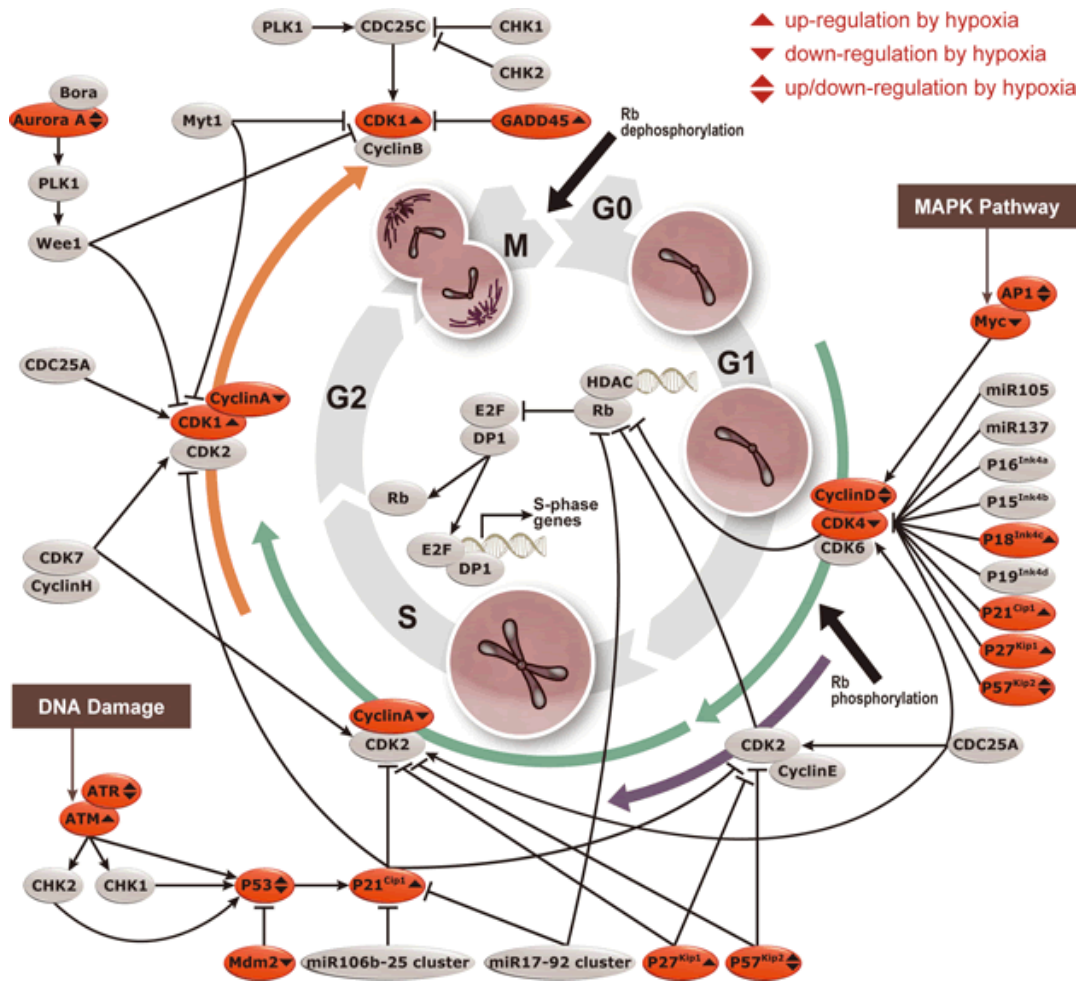
Figure 10: hypoxia regulation of cell cycle.

(www.genetex.com)

1.6.2 The role of glucose

In addition to oxygen tension, another important environment aspect to be addressed for therapeutic approach is represented by the use of the correct and physiological glucose concentrations. Currently, in the regenerative medicine applications, including our protocols, most of the culture media used contain high and unphysiological glucose levels.

Several studies displayed that glucose, as a preferred carbon source, regulates a large number of genes in many cell types, especially in liver and pancreatic β cells (Girard et al., 1997; Vaulont et al., 2000). Also, high glucose has been demonstrated to promote adipogenic differentiation of muscle derived stem cells (Aguiari et al., 2008). Glucose concentration has been indicated to be a crucial factor in regulating the differentiation, although this activity depends on the cell type considered. For example, high glucose has been demonstrated to promote adipogenic differentiation of muscle derived stem cells (Aguiari et al., 2008). On the other hand, another study has demonstrated that neural stem cells highly differentiated into a higher variety of neurons and astrocytes in low glucose concentrations (Horie et al., 2004). Furthermore, it has been shown that differentiation of mouse ES cells by embryoid bodies (EBs) based protocols does not need high level of sugar while ES cell differentiation in monolayer conditions depends on glucose concentration (Mochizuki et al., 2011). Also, physiological low glucose concentration promotes EB-based differentiation of ES cells toward neuronal lineage (Mochizuki et al., 2011). Similarly, another study has demonstrated that neural stem cells highly differentiated into a higher variety of neurons and astrocytes at the presence of low glucose concentration (Kazuyuki, 2004).

Moreover, high glucose has been shown to cause abnormalities during embryogenesis by reducing inositol levels (one of the principal second messenger of insulin action) and increasing oxidative stress (Wentzel et al., 2001).

Hyperglycemia stimulates signal transduction of Angiotensin II (ANG II) through the JAK/STAT pathway leading to an increase in stem cell proliferation (Kim and Han, 2008). Another study has suggested that high glucose stimulates PI3-K/Akt and MAPKs pathways which results in increased embryonic stem cell proliferation (Kim et al., 2006). Also, hyperglycemia can stimulate calcification of vascular smooth muscle cells (Chen et al., 2006).

Moreover, some researchers have shown that reduction in glucose concentrations led to decreased apoptosis and an increased rate of MSCs proliferation (Stolzing et al., 2006). *In vitro* studies have shown that α and β cells are highly influenced by the presence of glucose which can regulate endocrine development. One of these demonstrate that glucose plays a major and specific role in pancreatic endocrine cell development. In particular, using an *in vitro* model where both acinar and endocrine cells develop in culture from rat embryonic pancreas, it was demonstrated that glucose does not have a main effect on pancreatic acinar cell development but, on the other hand, it is crucial for both alpha and beta cells development (Guillemain et al., 2007). Moreover, this study demonstrated that glucose controls endocrine cells development by regulating specific steps of pancreatic endocrine cell differentiation and not by controlling cell proliferation (Guillemain et al., 2007).

These observations led us to apply physiological glucose concentrations during the epigenetic conversion of fibroblasts into insulin secreting cells, in order to closely represents the *in vivo* milieu. As a matter of fact, in our standard protocols, differentiation procedure was performed by applying an unphysiological condition of high glucose concentration (17,5 mM). Besides, at the end of the differentiation process, cells were usually stimulated with hyperglycemic medium (20 mM) in order to test their efficiency, in terms of insulin release in response to glucose variations. In this way, cells are able to release insulin but this condition is not compatible with the physiological glucose levels. As a matter of fact, in healthy physiological condition, blood glucose is tightly maintaining from 5,5 mM for fasting and it can rise up to 7,0-8,5 mM after eating (Ceriello & Colagiuri, 2008). Furthermore, when these cells were transplanted into streptozotocin (STZ)-induced diabetic mice, the hyperglycemia is normalized within a week. During this week, mice suffer the hyperglycemia state and cells failed to immediately respond secreting insulin. Failure of an immediate response to release insulin may reflect on different glucose concentrations during cell conversion *in vitro*, compared to physiological glucose conditions *in vivo*.

This potential weakness might limit cell therapy approach.

1.6.3 The role of three-dimensional (3-D) environment

Tissues are viscoelastic structures, composed by cells embedded in a complex microenvironment. Most of the cells are attached by anchor specific sites called focal adhesions, which bind them to neighboring cells and to surrounding extracellular matrix (ECM). In particular, ECM shows a specific and defined elasticity, normally represented by the elastic modulus (Young's modulus), which varies from 0.1 kPa in the brain to several hundred MPa in the calcified bone (Engler et al., 2006; Samani et al., 2003). On the contrary, *in vitro* cell culture is usually carried out on rigid polystyrene or glass surfaces characterized by thousands MPa stiffness.

Traditionally, tissue culture has been dominated by growing cells as monolayers. While these two dimensional (2-D) systems are well documented and have enabled approaches to understanding individual cellular phenomena, they lack the ability to reproduce the morphology, three dimensional (3-D) architecture and some biochemical features of cells typical of the original tissue. The 3-D environment offers the possibility to explore the potential mechanisms that underline the process of tissue formation (migration, proliferation, adhesion, differentiation and apoptosis) under conditions that emulate an *in vivo* environment. In addition, the 3-D environment enables cells to form cell-cell and cell-matrix interaction and also facilitates biological responses that might not be observable on 2-D substrates. For example, the collective cell migration, force generation and tissue folding that occurs during gastrulation, the angiogenic sprouting of blood vessels and the migration of cancerous cells through stroma and into lymphatics during metastasis, are all cases of higher-order cell processes that are inherently 3-D (Baker & Chen, 2012). Moreover, several studies demonstrated a direct effect of substrate rigidity on lineage commitment and cell differentiation (Engler et al., 2006; Evans et al., 2009; Huebsch et al., 2010; Gilbert et al., 2010; Hu et al., 2011; Nam et al. 2011; Viale-Bouroncle et al., 2011). In particular, the use of surface, which matches the stiffness of native tissues, significantly influences cell behavior and might be fundamental for a number of specific cellular functions (Schellenberg et al., 2014). As a matter of fact, several studies demonstrated the positive effect of more physiological culture surfaces. For instance, the use of substrates approximating to the elastic moduli of brain (0.1 kPa), pancreas (1.2 kPa), cartilage (3 kPa), muscle (8 to 17 kPa) and bone tissue (40 kPa), could directly drive stem cells to differentiate into

neurogenic, pancreatic, chondrogenic, myogenic and osteogenic lineages, respectively (Engler et al., 2006; Narayanan et al., 2014; Wang et al., 2014).

One important contribution for the "closer-to-*in vivo*" behaviour of cells when grown as 3-D cultures is the matrices and scaffolds that are used for obtaining such cultures (Ravi et al., 2016). More than 100 types of matrices and scaffolds are being used at present. The most commonly used scaffolds are agarose, collagen, fibronectin, gelatin, laminin, and vitronectin. Type I collagen matrix is used commonly in 3-D culture system. These composites mimic the native extracellular matrix by porosity, fibrosity, permeability and mechanical stability (Geckil et al., 2010).

Among all existing matrices, hydrogels have gained wide popularity as useful substrates for studying cellular mechano-transduction due to their ability to mimic salient extracellular matrix characteristics including mechanics, water content and the facilitation of cell adhesion. These reticulated structures possess high water contents, facilitate transport of oxygen, nutrients and waste, as well as transport of soluble factors (Nguyen & West, 2002). These hydrogels can be designed to support specific cell types growth and function by either trapping cells in an artificial ECM (Jongpaiboonkit et al., 2008) or allowing cells to migrate from the surface to the interior of the gel (Topman et al., 2013).

Furthermore, an ulterior novel 3-D culture system, namely Liquid Marble (LM) microbioreactor, was recently developed. LM, first described by Aussillous & Quéré (2001), consists of a drop of liquid encapsulated by hydrophobic powder particles. These particles are able to adhere to the surface of the liquid drop, isolating the liquid core from the supporting surface and allowing gas exchange between the interior liquid and the surrounding environment (Ledda et al., 2016). The coating material acts as a confined space which is non-adhesive and allows the cells to freely interact with each other.

The resulting LM are found to behave like a soft solid, and show dramatically reduced adhesion to a solid surface (Figure 11) (Aussillous & Quéré, 2001).

Figure 11: liquid marble drop.
(Aussillous & Quéré, 2001)

In the last few years, a number of works showed that LM support the growth of living microorganisms (Tian et al., 2013), tumor spheroids (Arbatan et al., 2012), fibroblasts, red blood cells (Serrano et al., 2015), embryonic stem cells (Sarvi et al., 2015) and the maturation of oocytes (Ledda et al., 2016).

These observations led us to perform the epigenetic conversion of fibroblasts into insulin secreting cells using two different 3-D culture systems: Polytetrafluoroethylene micro-bioreactor (PTFE; Sigma) and Polyacrylamide hydrogels (PAA; Cell Guidance)

1.7 Genome editing

Programmable DNA nucleases are recent and powerful tools for precision genome editing. Zinc Finger Nucleases (ZFNs) (Gaj et al., 2013; Hockemeyer et al., 2009; Y. G. Kim & Chandrasegaran, 1994), the Transcription Activator-Like Effector Nucleases (TALENs) (Boch et al., 2009; Bogdanove & Voytas, 2011; Hockemeyer et al., 2012), and especially at the moment, the Clustered Regularly Interspaced Short Palindromic Repeats-associated nuclease (CRISPR/Cas) (Cong et al., 2013; Haurwitz et al., 2010; Mali et al., 2013; J. Zhou et al., 2016) represent the most promising techniques to introduce sitespecific modifications in endogenous genoma of living cells and organisms.

In origin, the study of natural DNA repair pathways in bacteria and yeast, as well as the mechanisms of DNA recombination (Mansour et al., 1988; Rong, 2000; Scherer & Davis, 1979; Smithies et al., 1985), revealed that cells have endogenous machinery to repair double-strand DNA breaks (DSBs) that would otherwise be lethal (Choulika et al., 1995; Plessis et al., 1992; Rouet et al., 1994; Rudin et al., 1989). Thus, methods for introducing precise breaks in the DNA were recognized as a efficient strategy for targeted genomic engineering.

1.7.1 ZFNs

The first reprogrammable DNA nucleases used were the ZFNs, proteins comprising two domains: a DNA-binding zinc-finger protein (ZFP) domain and a nuclease domain derived from the Fok1 restriction enzyme.

In detail, zinc fingers are specific protein motifs capable of DNA binding, whose sequence specificity can be predetermined. Each zinc finger, consisting of approximately 30 amino acids in a conserved ββα configuration (Beerli & Barbas, 2002), recognizes 3-4 nucleotides, and, by assembling three or four suitable zinc finger motifs, a sequence-specific DNA-binding domain can be created (Papaioannou et al., 2012). ZFNs are created by joining the DNA-binding region to the catalytic domain of the Fok1 endonuclease. Fok1 nuclease activity requires dimerization and, so, the customized ZFNs function in pairs. As shown in figure 12, the zinc finger-binding domain brings two Fok1 units together over the target sequence, inducing Fok1 dimerization and target sequence cleavage (Papaioannou et al., 2012).
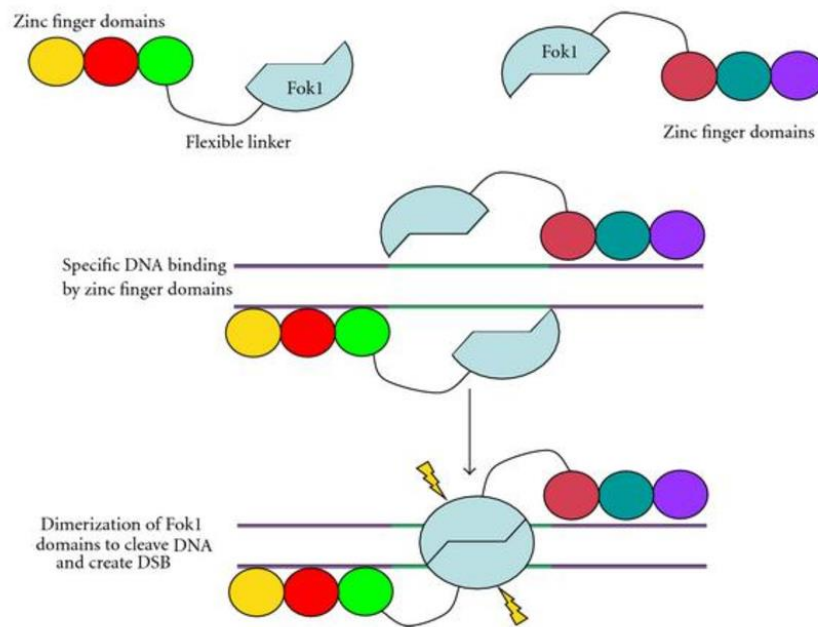
Figure 12: basic structure and design of a zinc finger nuclease (ZFN).
(Papaioannou et al., 2012)

Recently, a huge number of ZFN gene targeting successes has been reported, including the generation of gene-knockout rats and manipulation of human embryonic or induced pluripotent stem cells (Jensen et al., 2011; Rahman et al., 2011; Carroll, 2008; M. Porteus, 2007; M. H. Porteus, 2006;). However, one problem related to the use of ZFNs technique is related to the genotoxicity due to off-target cleavages that may involve and disrupt other genes (Mussolino & Cathomen, 2011), although improved screening and designs procedures are helping to solve this issue (Pruett-Miller et al., 2009; Sander et al., 2011). Furthermore, a second concern is that ZFNs can recleave a repaired site, although this can be minimized and controlled by inserting a few silent mutations into the donor DNA template in order to impair subsequent ZFN binding (Isalan, 2011). Definitely, although ZFNs are effective genome editing tools, they were not widely adopted because of the difficulty inherent in validating and designing such proteins for a specific DNA locus of interest.

1.7.2 TALENs

TALEN proteins have three characteristic domains: a nuclear localization domain, a nuclease domain derived from the Fok1 endonuclease (similar to ZFNs) and a DNA binding domain consisting of various numbers of tandem 34 aminoacids repeats (Y. Zhou et al., 2016) (Figure 13). Specifically, in the tandem array, each repeat is identical except for the two residues located at position 12 and 13, known as the repeat-variable di-residue (RVD), which defines the DNA binding specificity using an ''RVD-DNA'' codon (Boch et al., 2009). TALENs are similar to ZNFs since they use DNA binding motifs to direct the same nuclease to cleave the genome at a specific locus, but, instead of recognizing DNA triplets, each domain recognizes a single nucleotide, thanks to RVDs (Moscou & Bogdanove, 2009). The relationship between the preferred binding site of a TALE and its successive RVDs constitute a simple code, with each repeat specific for its targeted base (Boch et al., 2009; Moscou & Bogdanove, 2009). In particular, RVDs, namely NI, NG, HD and NN/NK selectively bound adenine (A), thymine (T), cytosine (C) and guanine (G), respectively (Cong et al., 2013; Mali et al., 2013; Miller et al., 2011; Haurwitz et al., 2010; Boch et al., 2009) (Figure 14).
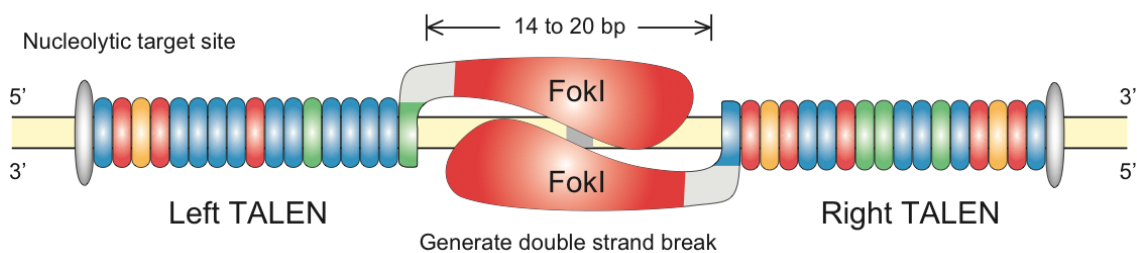
Figure 13: basic structure and design of a TALEN.

(Sanjana et al., 2012)
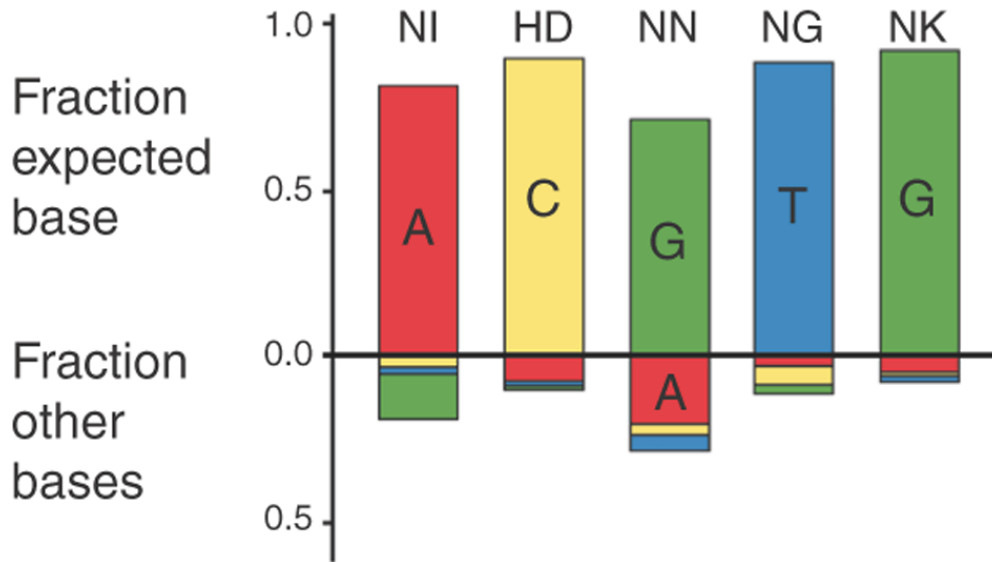
Figure 14: for each RVD (NI, HD, NN, NG, NK), the average frequency of its preferred target base is shown above the *x* axis, whereas frequencies of the remaining bases are shown below the *x* axis. (Miller et al., 2011)

Within the few last years, the TALENs technology was adopted all around the world and its wide use has greatly promoted the publication of several studies focused on the use of gene editing in a numbers of organisms and cell types, such as hESCs and iPSCs (Hockemeyer et al., 2012), rats (Tesson et al., 2011), zebrafish (Bedell et al., 2012), pigs (Carlson et al., 2012) and rice (Li et al., 2012).

Although TALENs are easier than ZFNs to produce and validate, difficulties of protein design, synthesis and validation remained an obstacle to broad adoption of these engineered nucleases for routine use (Doudna & Charpentier, 2014).

1.7.3 CRISPR/Cas

1.7.3.1 History and biology of CRISPR-Cas systems

In the mid-2000s, a few bioinformatics and microbiology laboratories began investigating on CRISPR, series of short repeats interspaced with short sequences in the E. Coli genome, firstly described in 1987 by Japanese researchers (Ishino et al., 1987). Then, CRISPRs were also identified in archaea and bacteria (Mojica et al., 2000) and their involvement in DNA repair or gene regulation was hypothesized (Guy et al., 2004; Makarova et al., 2002). A revolutionary insight came in 2005, when it was observed that most of spacer sequences within CRISPRs derive from plasmids and virus (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Furthermore, together with the finding that Cas (CRISPR-associated) genes encode for proteins with specific nuclease and helicase domains (Bolotin et al., 2005; Haft et al., 2005; Pourcel et al., 2005; Jansen et al., 2002), CRISPR-Cas association was proposed as an adaptive defense system regulated by antisense RNAs, as memory signatures of past invasions (Makarova et al., 2006). This hypothesis was confirmed by several studies carried out on different microorganisms, such as Streptococcus thermophilus (Barrangou et al., 2007), E. coli (Brouns et al., 2012) and Staphylococcus epidermidis (Marraffini & Sontheimer, 2008). In particular, in 2014 Doudna & Charpentier illustrated the CRISPR-Cas loci composition, consisting in a CRISPR array characterized by identical repeats intercalated with DNA-targeting spacers encoding the CRISPR RNAs (crRNA) components and an operon of Cas genes that encode the Cas protein (Doudna & Charpentier, 2014).

In particular, adaptive immunity occurs in three different stages (Figure 15):

1. <u>Adaptation</u>: insertion of a short invading DNA string as a spacer sequence into the CRISPR array;
2. <u>Expression</u>: transcription of precursor crRNA (pre-crRNA) that undergoes maturation to generate individual crRNAs, composed by a specific repeat portion and by an invader targeting spacer portion;
3. <u>Interference</u>: crRNA-directed cleavage of foreign nucleic acid by Cas proteins at sites complementary to the crRNA spacer sequence.
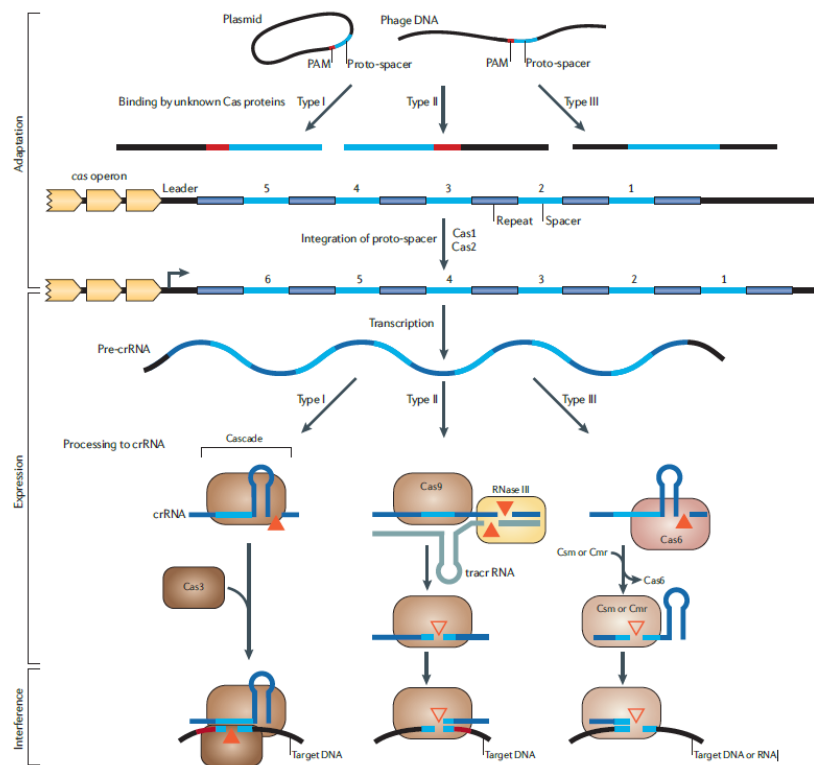
Figure 15: the three stages of CRISPR–Cas action.

(Makarova, et al., 2011b)

Within this overall theme, three CRISPR system types (I, II, III) use different mechanisms to recognize and cleavage the nucleic acid target (Makarova et al., 2011; Makarova et al., 2011b). A short sequence motif adjacent to the crRNA-targeted sequence on the invading DNA, known as the protospacer adjacent motif (PAM), plays a fundamental role in the adaptation and interference stages in both type I and type II systems (Deveau et al., 2008; Horvath et al., 2008; Mojica et al., 2005; Shah et al., 2013). Moreover, the type I and type III systems use a large complex of Cas proteins for crRNA-guided targeting (Brouns S. J. J. et al., 2012; Hatoum-Aslan et al., 2011; Haurwitz et al., 2010; Nam et al., 2012; Rouillon et al., 2013). On the other hand, the type II system requires only a single protein (Cas9) for RNA-guided DNA recognition and cleavage (Gasiunas et al., 2012; Jinek et al., 2012). For this reason, CRISPR type II system represent the most suitable and useful tool for genome engineering applications.

1.7.3.2 CRISPR type II system: CRISPR/Cas 9

The Cas9 nuclease, derived from the type II CRISPR system of Streptococcus pyogenes (SpCas9), is a universal DNA nuclease protein that can introduce double-strand DNA breaks into a genomic locus and, specifically, SpCas9 action is mediated by the complementarity of a small guide RNA (sgRNA) (Vad-Nielsen et al., 2016). This simple CRISPR/Cas9 RNA-DNA binding system has greatly simplified its design, construction and application, revolutionizing biological research in the very recent years (Chapman et al., 2015; Feng et al., 2014; Mandal et al., 2014; Platt et al., 2014; Shao et al., 2014; Ran et al., 2013). In 2011 (Deltcheva et al., 2011), a small RNA trans-encoded upstream of the type II CRISPR-Cas locus in S. pyogenes, namely trans-activating crRNA (tracrRNA), was reported to be crucial for crRNA maturation. Moreover, in 2012 (Jinek et al., 2012), the S. pyogenes CRISPR-Cas9 protein was shown to be a dual-RNA-guided DNA endonuclease that employs the tracrRNA-crRNA duplex (Deltcheva et al., 2011) to specifically cleavage DNA (Jinek et al., 2012). Furthermore, DNA target recognition requires both base pairing to the crRNA sequence and the presence of a PAM adjacent to the DNA targeted sequence (Gasiunas et al., 2012; Jinek et al., 2012) (Figure 16).

Figure 16: biology of the type II-A CRISPR-Cas system. (A) The cas gene operon with tracrRNA and the CRISPR array. (B) The natural pathway of anti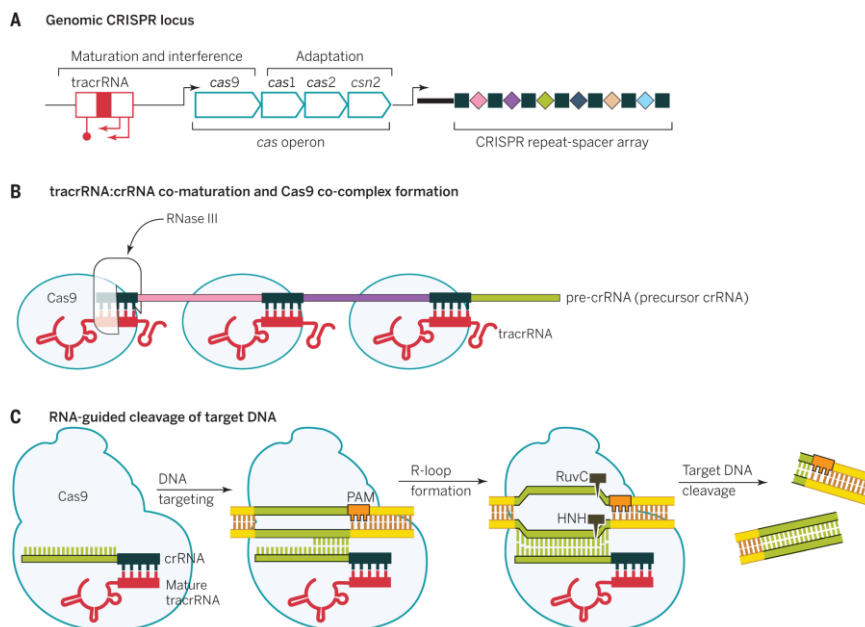viral defense involves association of Cas9 with the tracrRNA:crRNA duplexes, RNA co-processing by ribonuclease III, further trimming, R-loop formation, and target DNA cleavage. (C) DNA cleavage with the duplex tracrRNA:crRNA. (Doudna & Charpentier, 2014)

Then, the tracrRNA-crRNA duplex was engineered as a single sgRNA that maintain two critical features: the 20-nucleotide sequence at the 5′ end of the sgRNA to determine the DNA target site (by Watson-Crick base pairing) and the double-stranded structure at the 3′ side of the guide sequence that binds to Cas9 (Jinek et al., 2012) (Figure 10). This created a simple two-component system in which changes to the guide sequence (20 nucleotides in the native RNA) of the sgRNA can be used to program CRISPR-Cas9 to target and cleavage any DNA sequence of interest (Jinek et al., 2012).

In contrast to ZFNs and TALENs, which require protein engineering for each DNA target site to be modified, the CRISPR/Cas9 system requires only a change in the sgRNA sequence. For this reason, the CRISPR-Cas9 technology has been quickly and widely adopted by the scientific community to target, edit or modify a vast number of cell types and organisms genomes.

Presently, CRISPR/Cas9 genome editing system has been applied in various cell types and organisms such as plants (Feng et al., 2014; Miao et al., 2013), bacteria (Yosef at al., 2015; Jiang et al., 2013), C.Elegans (Friedland et al., 2014; Dickinson et al., 2013), Zebrafish (Hwang et al., 2013; Jao et al., 2013), mice (Platt et al., 2014; Cong et al., 2013), rats (Chapman et al., 2015; Shao et al., 2014), pigs (Zhou et al., 2015; Hai et al., 2014;), primates (Niu et al., 2014) and human cells (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013), including hiPSCs (Merkle et al., 2015) and human hematopoietic stem cells (Mandal et al., 2014).

In the present PhD project, under the guidance and supervision of prof. Yonglun Luo (Department of Biomedicine, Aarhus University, Denmark), the CRISPR/ Cas9 technique was applied to edit human and murine fibroblasts genome. In particular, the aim of these genome editing experiments was to completely delete the pluripotent related transcription factor Oct4 (Pou5f1) in human and mouse primary fibroblast cell lines, perturbing the pluripotency pathway of these cells. The scope of this alteration is to determine whether pluripotency is required for the epigenetic conversion and pancreatic induction process.

CHAPTER 2:
AIMS OF THE STUDY

2. AIMS OF THE STUDY

The present PhD project was focused on the characterization of epigenetic conversion of adult somatic cells into a different cell type. In particular, the studies here presented were aimed to the understanding of the molecular mechanisms driving epigenetic conversion and phenotype switch of adult fibroblasts into insulin secreting cells, in order to optimize our protocol for preclinical studies. To this purpose, several experiments were carried out, subjecting cells to conditions that better reflect the physiological environment, in order to improve cell conversion efficiency, maturation and differentiation, trying to mimic, as better as possible, the *in vivo* milieu.

For these reasons, aims of the present PhD project were focused on:

1. the assessment of the optimal oxygen tension to be used in culture, differentiating cells in low and physiological 5% oxygen, as well as in the standard *in vitro* culture 20%;

2. the establishment of the best glucose concentrations leading to cells that respond to glucose variations in a physiological way, converting cells in 5,5 and 8,5 mM glucose concentrations (corresponding to normoglycemia before meals and after meals respectively), as well as the standard 17,5 mM;

3. the use of 3-D systems and the evaluation of mechanosensing related responses, plating cells in Polytetrafluoroethylene micro-bioreactor (PTFE; Sigma) and on Polyacrylamide hydrogels (PAA; Cell Guidance), as well as plastic substrates as control;

4. the role of the pluripotency related gene Oct4 in the molecular mechanisms driving epigenetic conversion and phenotype switch, through the use of CRISPR/Cas9 strategy.

The elucidation of these aspects may be useful for a more functional and characterized approach to the use of epigenetic conversion for human regenerative medicine in diabetes care.

CHAPTER 3:

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Assessment of the optimal oxygen tension to be used in culture

3.1.1 Epigenetic conversion and pancreatic induction protocol

3.1.1.1 Murine skin fibroblasts cell culture

Fibroblasts were obtained from a primary culture isolated from three different mouse strains:

- **NOD** (non-obese diabetic) inbred mouse strain from non-disease stage;
- **C57 BL/6J** inbred mouse strain;
- **CD-1** outbred mouse strain.

These cells were cultured in DMEM medium (Life Technologies) supplemented with 20% (vol/vol) of fetal bovine serum (FBS, Gibco), 2 mM glutamine (Sigma) and antibiotics (Sigma) (Table 1).

PRODUCT	COMPANY	CATALOG N°	COMPOSITION (%)
DMEM High Glucose + Pyruvate	Life Technologies	41966-029	78%
Fetal Bovine Serum	Life Technologies	10108-165	20%
Antibiotic Antimycotic solution (100X)	Sigma	A5955	1%
L-Glutamine solution	Sigma	G7513	1%

Table 1: Fibroblast Culture Medium formulation.

Upon reaching the full confluence, cells were trypsinized and put into other flasks with a 1:3 ratio (twice a week). All experiments were conducted on three independent replicates of the same cell line.

3.1.1.2 Cell plating

Upon reaching the confluence, cells were trypsinized, centrifuged and resuspended in 5 ml of culture medium. From this solution, 6.6 μl were collected and loaded in a specific counting chamber (®KOVA). Based on the number of cells counted, a dilution with the culture medium was carried out in order to obtain a concentration of 300'000 cells per ml. Subsequently, cells present in this solution were plated in 4-wells plates (Nunc) on

0.1% porcine gelatin (Sigma), with a volume equal to 0.5 ml per well (150,000 cells per well).

3.1.1.3 5-Aza CR treatment

After 24 hours, cells were incubated with a solution of 1 uM of 5-aza-CR (Sigma) for 18 hours. The concentration and the exposure time were chosen in agreement with previous studies (Hattori et al., 2004).

At the end of the 18 hours exposure to this demethylating agent, cells were washed three times with PBS and incubated with High Plasticity medium (HP; Table 2) for 3 hours.

PRODUCT	COMPANY	CATALOG N°	COMPOSITION (%)
Ham's F-10 Nutrient Mix	Life Technologies	31550-023	40%
DMEM Low Glucose + Pyruvate	Life Technologies	31885-023	40%
KnockOut™ Serum Replacement	Life Technologies	10828-028	10%
Fetal Bovine Serum	Life Technologies	10108-165	5%1
Antibiotic Antimycotic Solution (100×)	Sigma	A5955	1%1
L-Glutamine solution	Sigma	G7513	1%
NUCLEOSIDE MIX (Table 3)	---	---	1%
MEM Non-Essential Amino Acids Solution	Life Technologies	11140-035	1%
2-Mercaptoethanol	Sigma	M7522	1%
ESGRO® (LIF)	Millipore	ESG1106	0,1%
Recombinant Human FGF basic (bFGF)	R&D System	233-FB-025	0,1%

Table 2: HP medium formulation.

PRODUCT	COMPANY	CATALOG N°	COMPOSITION (in 50 ml water)
Guanosine	Sigma	G6264	0.042 g
Adenosine	Sigma	A4036	0.040 g
Cytidine	Sigma	C4654	0.036 g
Uridine	Sigma	U3003	0.036 g
Thymidine	Sigma	T1895	0.012 g

Table 3: Nucleoside Mix composition.

3.1.1.4 Pancreatic induction protocol

After the incubation period in HP medium, cells were grown in specific culture media in order to induce the pancreatic differentiation. In details, cells were incubated with pancreatic medium basal supplemented with specific reagents (Table 4).

PRODUCT	COMPANY	CATALOG N°	COMPOSITION (%)
DMEM/F12	Life Technologies	11320-074	92%
B-27® Supplement Minus Vitamin A (50X)	Life Technologies	12587-010	2%
N-2 Supplement (100X)	Life Technologies	17502-048	1%
MEM Non-Essential Amino Acids Solution, 100X	Life Technologies	11140-035	1%
Antibiotic Antimycotic Solution (100×)	Sigma	A5955	1%
L-Glutamine solution	Sigma	G7513	1%
2-Mercaptoethanol	Sigma	M7522	1%
Albumin from bovine serum (BSA)**	Sigma	A3311	1%

Table 4: Pancreatic Medium Basal formulation.

In the murine model, the pancreatic induction process requires 10 days of culture and it has been developed in this way:

- *DAY 1:* pancreatic medium basal + **ACTIVIN A (1 μL/mL)**

- *DAY 2:* pancreatic medium basal + **ACTIVIN A (1 μL/mL) + RETINOIC ACID (1 μL/mL)**

- *DAYS 3-10:* pancreatic medium basal + **Insulin-Transferrin-Selenium (ITS; 10 μL/mL) + B27 supplement (20 μL/mL) + bFGF (1 μL/mL) (FINAL MEDIUM)**

3.1.2 Experimental Plans

3.1.2.1 Section 1

In order to evaluate the role of oxygen during the pancreatic differentiation process, three different mouse cell lines **(NOD; C57 BL/6J; CD-1)** were subjected to two different oxygen tensions: the standard **20%** and the physiological **5%** of oxygen following the scheme visible in Table 5.

CELL LINE	0-18 h (5-aza-CR)	18-21 h (HP)	DAY 1 (Activin A)	DAY 2 (Activin A + Retinoic acid)	DAYS 3-10 (FINAL medium)
NOD	20%	20%	20%	20%	20%
NOD	5%	5%	5%	5%	5%
C57 BL/6J	20%	20%	20%	20%	20%
C57 BL/6J	5%	5%	5%	5%	5%
CD-1	20%	20%	20%	20%	20%
CD-1	5%	5%	5%	5%	5%

Table 5: oxygen tensions used in different protocol steps (Section 1 experimental design).

To do this, cells were cultured using two different incubators:

- a traditional incubator (JOUAN 150 IGO CELLLIFE) powered by the standard 5% CO_2 in air (Figure 17);

- a low oxygen incubator (COOK V-MINC 1000) fed with a gas mixture consisting of 5% O_2, 5% CO_2 and 90% N2 (Figure 18).

Figure 17: traditional incubator.
(JOUAN 150 IGO CELLLIFE)

Figure 18: low oxygen incubator.
(COOK V-MINC 1000)

3.1.2.2 Section 2

Since NOD cells did not survive in low oxygen concentrations, it was decided to subject these cells to 5% oxygen only after performing differentiation in 20% O2, as shown in Table 6.

CELL LINE	0-18 h (5-aza-CR)	18-21 h (HP)	DAYS 1-10	DAY 11 (FINAL medium)	DAYS 12-14 (FINAL medium)
NOD	20%	20%	20%	/	/
NOD	20%	20%	20%	20%	/
NOD	20%	20%	20%	5%	/
NOD	20%	20%	20%	20%	20%
NOD	20%	20%	20%	5%	5%

Table 6: oxygen tensions used in different protocol steps (Section 2 experimental design).

3.1.3 Analysis

3.1.3.1 Cell morphology and viability

Cell morphology was evaluated using a Nikon Eclipse TE200 inverted microscope.

A cell viability test was performed using Trypan blue (ThermoFisher), following the manufactor instructions.

3.1.3.2 Immunocytochemistry

Cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma), rinsed three times in PBS and permeabilized with 0.1% and 0.4% (vol/vol) Triton X-100 (Sigma) in PBS, for 20 min. Samples were treated with blocking solution containing 5% (vol/vol) BSA and 5% (vol/vol) goat serum in PBS, for 30 min. Then, cells were incubated with anti-C-Peptide (AbCam) and anti-Oct4 antibodies (AbCam) overnight. The day after, cells were incubated with suitable secondary antibodies (Alexa Fluor® 488) for 60 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Samples were observed under a Nikon Eclipse TE200.

3.1.3.3 Cell Counting

The number of immuno-positive cells was counted in 10 randomly selected fields at 200× total magnification. A minimum of 500 cells were counted in three independent replicates. The number of positively stained cells was expressed as a percentage of the total cell counted.

3.1.3.4 Insulin release after stimulation with hyperglycemic glucose concentrations

At the end of the differentiation process, cells functional efficiency was assessed by an ELISA test, specific for mouse insulin, in order to quantify insulin release after stimulation with hyperglycemic conditions (20 mM of D-glucose). In detail, culture medium was removed, cells were rinsed with culture medium and then stimulated for 1 h with 17,5 mM D-glucose (final concentration) in Final medium without ITS. Then, media were collected, cells were rinsed with culture medium and then stimulated for 1 h with 20 mM D-glucose (final concentration) in Final medium without ITS. Glucose-dependent insulin release was assessed with Mouse Insulin ELISA Kit (Mercodia) following the manufacturer's instruction. Values were normalized against DNA content, measured using PureLink Genomic DNA Mini Kit (Invitrogen).

3.1.3.5 Statistical analysis

Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

3.2 Establishment of the best glucose concentrations leading to cells that respond to glucose variations in a physiological way

3.2.1 Epigenetic conversion and pancreatic induction protocol

3.2.1.1 Human/murine skin fibroblasts cell culture

Adult human and murine fibroblasts, obtained from fresh skin biopsies, were cultured as previously described.

3.2.1.2 Cell plating

Cells were plated with the same modalities previously described.

3.2.1.3 5-Aza CR treatment

Cells were treated with 5-Aza CR with the same modalities previously described.

3.2.1.4 Pancreatic induction protocol

After the incubation period in HP medium, cells were grown in specific culture media in order to induce the pancreatic differentiation. In details, cells were incubated with pancreatic medium basal supplemented with specific reagents (Table 4).

In the murine model, the differentiation was induced with the same modalities previously described.

By contrast, in the human model, the pancreatic induction process requires 36 days of culture and it has been developed in the following way:

- **DAYS 1-6:** pancreatic medium basal + **ACTIVIN A (1 µL/mL)**
- **DAYS 7-8:** pancreatic medium basal + **ACTIVIN A (1 µL/mL) + RETINOIC ACID (1 µL/mL)**
- **DAYS 9-36:** pancreatic medium basal + **ITS (10 µL/mL) + B27 supplement (20 µL/mL) + bFGF (1 µL/mL) (FINAL MEDIUM)**

3.2.2 Experimental Plans

3.2.2.1 Section 1

During pancreatic differentiation, human fibroblasts were cultured and differentiated in three different glucose concentrations:

- **5,5 mM** (normoglycemia)
- **8,5 mM** (normoglycemia after meals)
- **17,5 mM** (standard *in vitro* culture)

Several experimental groups were identified and cells were differentiated following the scheme visible in Table 7.

EXPERIMENTAL GROUPS	0-18 h (5-aza-CR)	DAYS 1-6 (Activin A)	DAYS 7-8 (Activin A + Retinoic Acid)	DAYS 9-36 (FINAL medium)
1	25 mM	5,5 mM	5,5 mM	5,5 mM
2	25 mM	17,5 mM	17,5 mM	5,5 mM
3	25 mM	5,5 mM	17,5 mM	5,5 mM
4	25 mM	8,5 mM	8,5 mM	8,5 mM
5	25 mM	17,5 mM	17,5 mM	8,5 mM
6	25 mM	8,5 mM	17,5 mM	8,5 mM
CTRL	25 mM	17,5 mM	17,5 mM	17,5 mM

Table 7: glucose concentrations used in different protocol steps (section 1 design).

3.2.2.2 Section 2

Since human cells did not differentiate in low glucose concentrations, it was designed a second experiment in which human cells were subjected to standard (17,5 mM) and low glucose concentrations (8,5 mM) untill day 14 of the process, in order to characterize them in the early pancreatic differentiation.

During this period, RNAs of each sample were collected every two days (T0, Post HP, Day 2, 4, 6, 8, 10, 12, 14) and the expression of 5 important genes was monitored. In particular, the expression of Vimentin (Fibroblast marker), Oct4 (Pluripotency-related gene), FOXA2 (Definitive Endoderm marker), HNF4 (Primitive Gut Tube marker) and PDX1 (Posterior Foregut marker) was monitored at each time point (Table 8).

Table 8: gene expression in Endocrine Pancreatic differentiation steps.

3.2.2.3 Section 3

The results obtained in the Experiment 2 showed that low glucose affect both the correct timing and levels of early pancreatic genes expression. Since a glucose sensitive window was identified around day 6-10 of the pancreatic induction process, corresponding to the embryonic primitive gut tube/posterior foregut stage *in vivo*, a third batch of experiments was performed.

Two experimental groups were identified:

- **GROUP A**, consisting in cells differentiated in low glucose (8,5 mM) until day 9 (corresponding to the Activin A and Retinoic Acid steps) and, then, in high glucose till the end of the process;
- **GROUP B**, consisting in cells differentiated in standard high glucose concentrations, as a control (Table 9).

EXPERIMENTAL GROUPS	0-18 h (5-aza-CR)	DAYS 1-9 (Activin A + Retinoic Acid)	DAYS 9-36 (FINAL medium)
GROUP A (LOW + HIGH GLUCOSE)	25 mM	**8,5 mM**	17,5 mM
GROUP B (CTRL)	25 mM	17,5 mM	17,5 mM

Table 9: glucose concentrations used in different protocol steps.

The expression of 6 important genes was monitored for each time point with the same modalities adopted in the previous section. In this case, Insulin expression was monitored also, in order to compare timing and expression levels of this important pancreatic marker in both experimental groups.

3.2.2.4 Section 4

Since the results obtained in the previous section did not clarify the role of glucose in the pancreatic differentiation process, it was designed a further experiment in which both human and murine skin fibroblasts were directly isolated in low and physiological glucose (5,5 mM) (Berglund et al., 2008) as well as standard glucose concentration (25 mM). Then, cells were differentiated using specific media containing two different glucose concentrations: the standard 17,5 mM and the physiological 5,5 mM (Figure 19).

In this way, four different experimental groups were obtained:

- **HG/HG** (cells isolated in high glucose, differentiated in high glucose)
- **HG/LG** (cells isolated in high glucose, differentiated in low glucose)
- **LG/LG** (cells isolated in low glucose, differentiated in low glucose)
- **LG/HG** (cells isolated in low glucose, differentiated in high glucose)
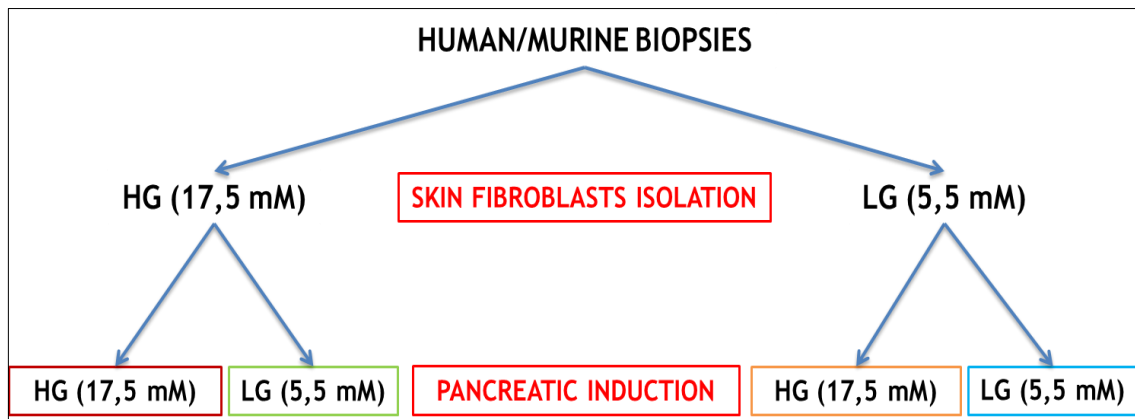

Figure 19: fibroblasts isolation/differentiation with different glucose concentrations

3.2.3 Analysis

3.2.3.1 Cell morphology

Cell morphology was evaluated using a Nikon Eclipse TE200 inverted microscope.

3.2.3.2 Immunocytochemistry

At the end of the differentiation process, cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma), permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma) in PBS and blocked with PBS containing 5% goat serum. Cells were then incubated with primary antibodies (Anti-C-Peptide and Anti-Insulin, AbCam). Subsequently, cells were incubated with secondary antibodies (Alexa Fluor) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 60 min and analyzed under a Nikon Eclipse TE200 microscope.

3.2.3.3 Cell Counting

The number of immuno-positive cells was counted in 10 randomly selected fields at 200× total magnification. A minimum of 500 cells were counted in three independent replicates. The number of positively stained cells was expressed as a percentage of the total cell counted.

3.2.3.4 Gene expression analysis

Total RNA was extracted using Trizol (Invitrogen) and DNAse I (Invitrogen) was added in Lysis solution at 1:100 concentration as indicated by manufacturer's instruction. The effective removal of genomic DNA from each RNA batch was then confirmed performing a standard PCR amplification for β-actin, using genomic DNA as positive control. Only negative samples were then reverse transcribed with Superscript™ II Reverse Transcriptase (Invitrogen). Quantitative Real-Time PCR was performed using SYBR® Green. PCR runs and fluorescence detection were carried out in a CFX CONNECT (Bio-Rad). β-actin and GAPDH were used as internal standards. Except for mouse GLUTs expression values, gene expression levels of all other samples evaluated are reported with the highest expression set to 1 and all other time points relative to this.

3.2.3.5 Insulin release after stimulation with hyperglycemic glucose concentrations

At the end of the differentiation process, cells functional efficiency was assessed by an ELISA test, as previously described.

In this case, cells were stimulated for 1 hour with the physiological 8,5 mM (blood glucose concentration after meal) as well as the standard 20 mM D-glucose in basal medium, without ITS. Glucose-dependent insulin release was assessed as previously described.

3.2.3.6 Statistical analysis

Statistical analysis was performed using Student t and ANOVA tests (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

3.3 Use of 3-D systems and evaluation of mechanosensing related responses

In this third part of the present PhD project, the epigenetic conversion of **C57 BL/6J** mouse dermal fibroblasts into insulin producing-cells was performed using two different 3-D culture systems, combining also the use of two oxygen tensions. In particular, cells were differentiated using the Polytetrafluoroethylene micro-bioreactor (PTFE; Sigma) and the Polyacrylamide (PAA; Cell Guidance) hydrogels with different stiffness (1 kPa; 4 kPa), maintained either in the standard 20% or in the more physiological 5% oxygen tensions. Standard differentiation performed on plastic substrates was carried out as a control.

3.3.1 Mouse skin fibroblasts cell culture

Adult **C57 BL/6J** mouse fibroblasts, obtained from fresh skin biopsies, were cultured as previously described.

3.3.2 Epigenetic conversion and pancreatic induction in PTFE system

3.3.2.1 5-Aza CR treatment

Firstly, hydrophobic PTFE powder bed was prepared in a 35-mm Petri dish (Figure 20a). Then, 40000 cells resuspended in 30 μl of 1 μM 5-aza-CR were dispensed over the hydrophobic PTFE powder bed. The drop obtained was gently rolled over the PTFE to fully coat it by the powder (Figure 20b). Finally, the resulting drop was transferred to a 35-mm Petri dish, placed within a bigger Petri dish that contains sterile water, to prevent evaporation (Figure 20c).
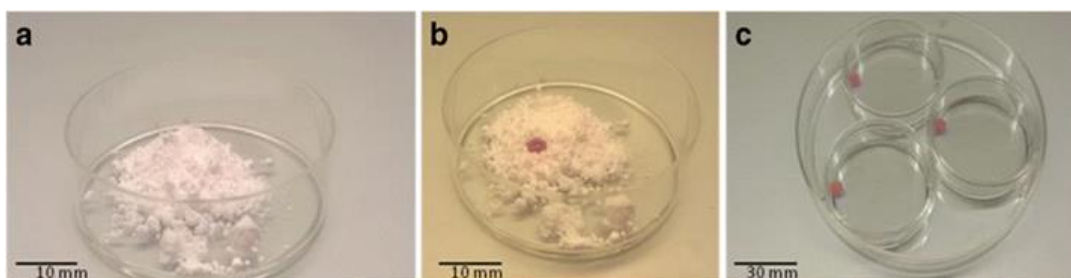

Figure 20: PTFE system preparation.

(Ledda et al., 2016)

At the end of the 18 hours exposure to the demethylating agent, drops were broken and the cell aggregates obtained were transferred in a new 35-mm Petri dish and incubated with High Plasticity medium (HP; Table 2) for 3 hours.

3.3.2.2 Pancreatic induction protocol

After the incubation period in HP medium, cells were grown in specific culture media in order to induce the pancreatic differentiation. In details, cell aggregates were transferred in new 35-mm Petri dishes and subjected to the standard pancreatic induction process, as previously described in Paragraph 3.1.1.4.

Moreover, cells were maintained either in the standard 20% or in the more physiological 5% oxygen tensions.

3.3.3 Epigenetic conversion and pancreatic induction on PAA gels

3.3.3.1 Cell plating

Mouse dermal fibroblasts were plated on two different PAA gels (750'000 cells/Petri):

- Collagen coat SOFT GEL 1 kPa (*Petrisoft^{TM} coated petri dish, Cell Guidance*);

- Collagen coat SOFT GEL 4 kPa (*Petrisoft^{TM} coated petri dish, Cell Guidance*).

Then, cells plated on both gels were differentiated in low (5%) and high (20%) oxygen tensions.

3.3.3.2 5-Aza CR treatment

Cells were treated with 5-Aza CR with the same modalities previously described.

3.3.3.3 Pancreatic induction protocol

Cells plated on both PAA gels were subjected to the pancreatic differentiation protocol, as previously described.

In summary, three different system culture (PTFE; PAA gels; PLASTIC as control) combined with the use of two different oxygen tensions (20%; 5%) were tested, as visible in Table 10:

EXP. GROUPS	0-18 h (5-Aza-CR)	18-21 h (HP)	DAY 1 (Activin A)	DAY 2 (Act. A + Ret. Acid)	DAYS 3-10 (FINAL medium)
PTFE	$20\% \ O_2$				
	$5\% \ O_2$				
PAA 1 kPa	$20\% \ O_2$				
	$5\% \ O_2$				
PAA 4 kPa	$20\% \ O_2$				
	$5\% \ O_2$				
PLASTIC	$20\% \ O_2$				
	$5\% \ O_2$				

Table 10: three different culture systems combined with the use of two different oxygen tensions.

3.3.4 Analysis

3.3.4.1 Cell morphology

Cell morphology was evaluated using a Nikon Eclipse TE200 inverted microscope.

3.3.4.2 Insulin release after stimulation with hyperglycemic glucose concentrations

At the end of the differentiation process, cells functional efficiency was assessed by an ELISA test, with the same modalities described above.

3.3.4.3 Gene expression analysis

Insulin and Ki67 gene expression was measured as previously described.

3.3.4.4 Immunocytochemistry

Cells growth in Petrisoft (1 kPa and 4 kPa both) were deposited onto glass slides by Cytospin centrifugation (Cytospin 4, Thermo Scientific). Then, samples were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma) and permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma) in PBS, for 20 min. Samples were treated with blocking solution containing 5% (vol/vol) BSA and 5% (vol/vol) goat serum in PBS, for 30 min. They

were then incubated with primary antibodies (anti-Glucagon, anti-Somatostatin and anti- Insulin; Abcam).Subsequently, cells were incubated with secondary antibodies (Alexa Fluor) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 60 min and analysed under a Nikon Eclipse TE200 microscope.

3.3.4.5 Image analysis

Image analysis were performed in cells differentiated on 1 kPa gel in low oxygen. In particular, Insulin,Glucagon and Somatostatin fluorescence intensity analysis were carried out along an arbitrarily set diameter-like axis, through the ImageJ software, in order to estimate the mono-hormonality acquired by cells.

3.3.4.6 Statistical analysis

Statistical analysis was performed using ANOVA test (SPSS 19.1; IBM). Data were presented as mean ± standard deviation (SD). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

3.4 The role of the pluripotency related gene Oct4 in the molecular mechanisms driving epigenetic conversion and phenotype switch

3.4.1 Section 1

The experiments performed in this section were carried out under the guidance and supervision of prof. Yonglun Luo, at the Department of Biomedicine, Aarhus University, Denmark.

3.4.1.1 Designing sgRNA oligos for the all-in-one CRISPR/Cas9 system

Human and murine Oct4 (Pou5f1) sequences were analyzed with online designing software (Zifit and Cas-OFFinder) to identify the best site where to direct the Cas9 nuclease in order to obtain the complete excision of the gene. Once the most promising human and murine sequences have been identified, four sgRNAs were designed respectively to target the human and murine Oct4 gene, with two sgRNAs (denoted as T1 and T2) targeting the first coding exon and another two sgRNAs (denoted as T3 and T4) targeting the last coding exon.

3.4.1.2 sgRNAs generation

To generate the CRISPR sgRNA vector, synthesized sgRNA spacer oligos were annealed *in vitro* and cloned in the all-in-one CRISPR/Cas9 vector (PX330_pSPCas9(BB)-2A-Puro). Then, the Oct4 CRISPR/Cas9 vectors were transformed into E.Coli competent cells. Restriction enzyme digestion and Sanger sequencing were performed to verify all Oct4 CRISPR/Cas9 vectors. For *in vitro* CRISPR/Cas9 functional assay, the dual-fluorescent reporter system (C-Check), recently established by professor Luo's group (Zhou et al., 2016), was applied. This system allows the quantification and selection of the most efficient CRISPR sgRNAs. For C-Check assay, we have transfected the Oct4 C-Check vector with each of the CRISPR/Cas9 sgRNAs into Human Embryonic Kidney cells 293 (HEK293T).

Then, green and red fluorescence were analyzed by fluorescent microscopy and flow cytometry analyses 48 hours after transfections.

Based on the C-Check based *in vitro* function assay, we selected the most efficiency CRISPR pair T1 or T2 paired with T3 or T4. Genotyping of the Oct4 complete

disrupted region was carried out by screening PCR using primers centering the T1-T4 target sites. With the complete disruption, approximately 10k genomic region was deleted.

3.4.1.3 Oct4 KO fibroblasts generation

To generate the Oct4 KO fibroblasts, *in vitro* validated CRISPR/Cas9 vectors were transfected into the cells using a Lipofectamine Transfection kit (Invitrogen) and their effectiveness was tested.

Then, two procedures have been applied to generate the Oct4 KO cells:

1. Puromycin selection, dissolving 1:1000 of Puromycin (Invitrogen) in the cell culture medium;

2. FACS-based sorting.

3.4.1.4 Screening PCR

Independent clones were derived to generate pure cell lines and DNA extraction and amplification have been performed to genotype correct Oct4 KO clones.

In particular, genotyping of the Oct4 complete disrupted sequence was carried out by screening PCR using primers centering the T2-T3 target sites in human cells and T2-T4 target sites in mouse.

In order to detect human and mouse correct KO clones, a first screening PCR was performed amplifying the whole Oct4 genomic region. In detail, two primers binding the Oct4 sequence upstream and downstream were designed, as visible in the following picture (Figure 21).
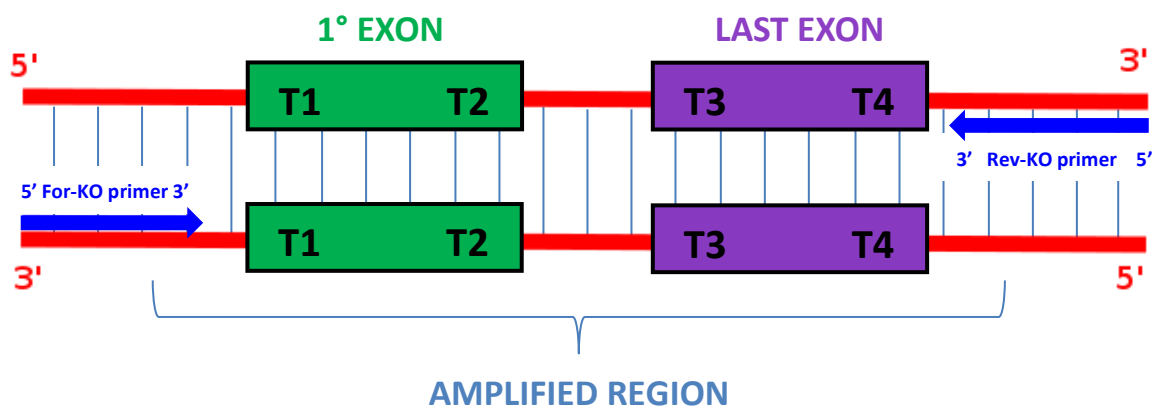

Figure 21: amplification of whole Oct4 genomic region scheme.

71

Then, after traditional PCR followed by gel electrophoresis, two different bands were expected:

- the first one with a higher DNA molecular size (~ 10k bp) corresponding to Wild Type (WT) cells where the deletion did not occur;
- the second band with a lower DNA molecular size (~ 400 bp), corresponding to the correct Oct4 knockout clones.

Furthermore, to confirm the presence of KO clones, a second PCR screening was performed. In particular, to detect the deletion of T2-T4 region in mouse cells, T3-Forward and Reverse-KO primers were used (Figure 22).
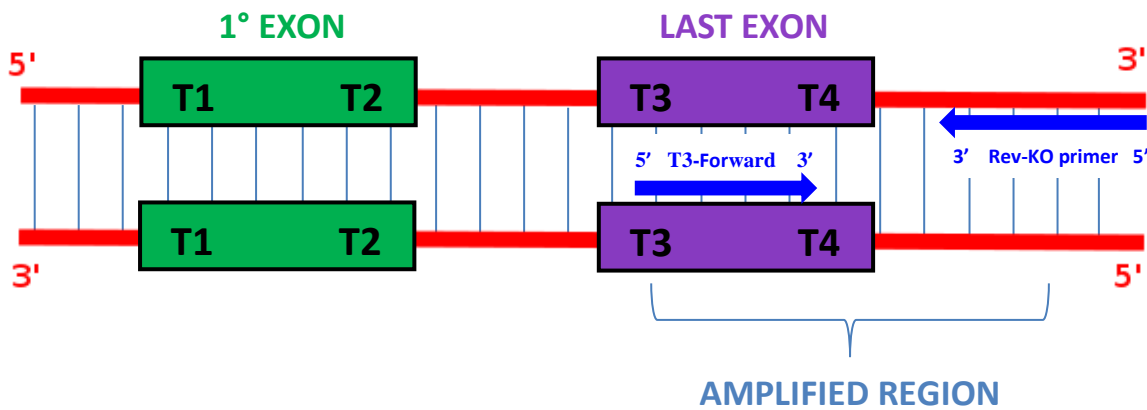

Figure 22: amplification of T3–downstream mouse Oct4 genomic region scheme.

On the other hand, to verify the deletion of T2-T3 region in human cells, T2-Forward and Reverse-KO primers were used (Figure 23).
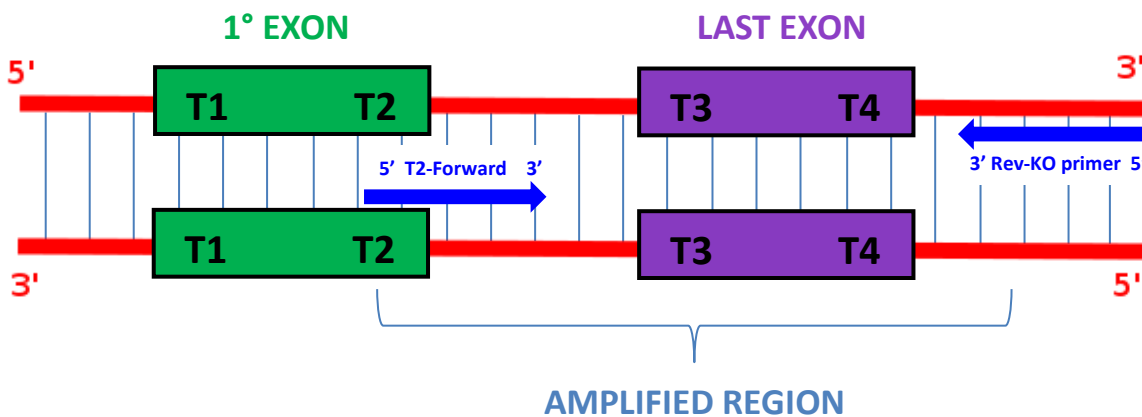

Figure 23: amplification of T2–downstream human Oct4 genomic region scheme.

3.4.1.5 5-aza-CR treatment

Both Knockout (KO) and Wild Type (WT) cells were treated with 5-Aza CR with the same modalities previously described.

3.4.1.6 Immunocytochemistry analysis

At the end of the 5-aza-CR treatment, both KO and WT cells were fixed in 4% (wt/vol) paraformaldehyde in PBS (Sigma), permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma) in PBS and blocked with PBS containing 5% goat serum. Cells were then incubated with primary antibody Anti-Oct4 (Abcam). Subsequently, cells were incubated with secondary antibody (Alexa Fluor) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 60 min and analyzed under a Nikon Eclipse TE200 microscope.

3.4.1.7 Pancreatic induction

Both KO and WT cells were subjected to the standard pancreatic induction protocol, with the same modalities previously described.

3.4.2 Section 2

3.4.2.1 Designing sgRNA oligos for the all-in-one CRISPR/Cas9 system

Human and murine Oct4 (Pou5f1) sequences were analyzed and sgRNA were designed as previously described.

3.4.2.2 sgRNAs generation

sgRNAs were generated with the same modalities previously described.

3.4.2.3 Oct4 KO fibroblasts generation

To generate the Oct4 KO fibroblasts, *in vitro* validated CRISPR/Cas9 vectors were transfected into the cells using a Lipofectamine Transfection kit (Invitrogen) and their effectiveness was tested, as previously mentioned. In this case, in both mouse and human plasmids, all four gRNAs (T1-T2-T3-T4) were inserted in order to improve the sequence deletion. Moreover, GFP and Puromycin expression cassettes were inserted as well.

Furthermore, fibroblasts were plated and tranfected on plastic and on 1 kPa PAA gel as well, in order to test whether a more physiological matrix could improve the transfection efficiency.

Then, to generate the Oct4 KO cells, Puromycin selection has been applied, as previously described.

CHAPTER 4:
RESULTS

4. RESULTS

4.1 Assessment of the optimal oxygen tension to be used in culture

4.1.1 Section 1

4.1.1.1 Cell morphology and viability

During differentiation, three murine cell lines (NOD; C57 BL/6J; CD-1) were cultured either in the standard *in vitro* culture 20% of oxygen or in the lower and physiological 5% of oxygen. Our results showed that NOD cells, which are physiologically predisposed to the onset of diabetes, differentiate in 20% of oxygen (Figure 24) but not in low oxygen (Figure 25) and they died after three days of culture. On the other hand, C57 BL/6J and CD-1 cells remain viable during the differentiation process and they were able to differentiate into insulin secreting cells in both oxygen levels (Figures 26-29).
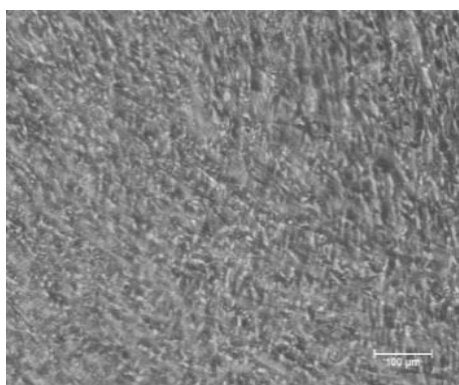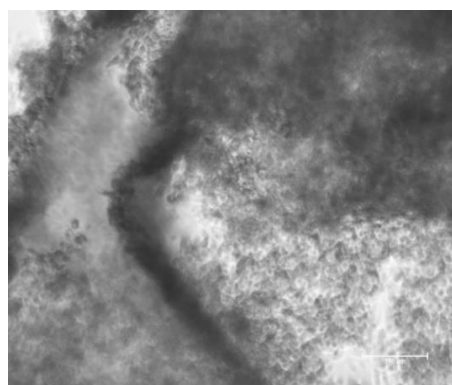

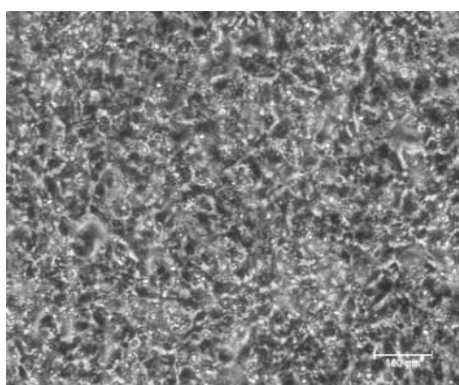**Figure 24:** NOD 20% O_2, day 10.

Figure 25: NOD 5% O_2, day 3.

Figure 26: C57BL/6J 20% O_2, day 10.
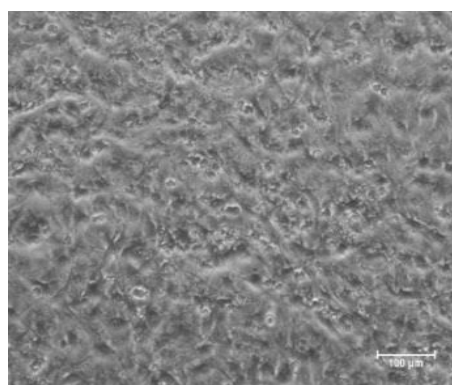
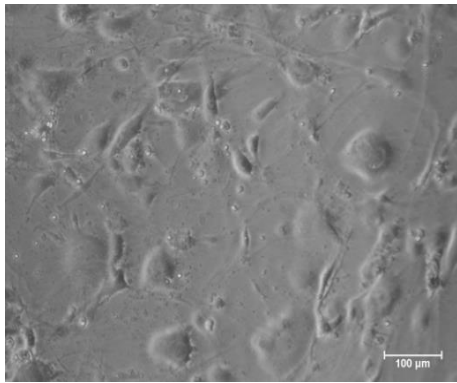**Figure 27:** C57BL/6J 5% O_2, day 10.
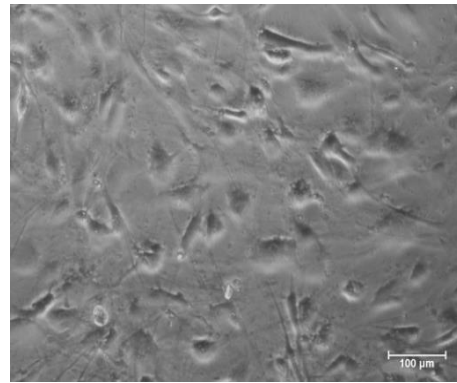
Figure 28: CD-1 20% O$_2$, day 10.

Figure 29: CD-1 5% O$_2$, day 10.

In particular, **NOD** cells maintained in hypoxic conditions showed a very low cell viability percentage, more precisely equal to 5,2% ± 1,21%. In contrast, NOD cells cultured in 20% of oxygen showed a viability percentage equal to 88,14% ± 5,76%. On the other hand, **C57 BL/6J** and **CD-1** cells did not show viability differences when subjected to both oxygen tensions (Table 11; Histogram 1).

EXP. GROUPS	CELL VIABILITY (%)
NOD 20% O$_2$	88,14 ± 5,76 (a)
NOD 5% O$_2$	5,2 ± 1,21 (b)
C57 BL/6J 20% O$_2$	89,56 ± 4,5 (a)
C57 BL/6J 5% O$_2$	88,75 ± 5,37 (a)
CD-1 20% O$_2$	87,15 ± 4,13 (a)
CD-1 5% O$_2$	88,01 ± 5,04 (a)

**Table 11: cell viability percentage in different experimental groups
(cell viability percentage is expressed as mean value ± SD).
Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).**
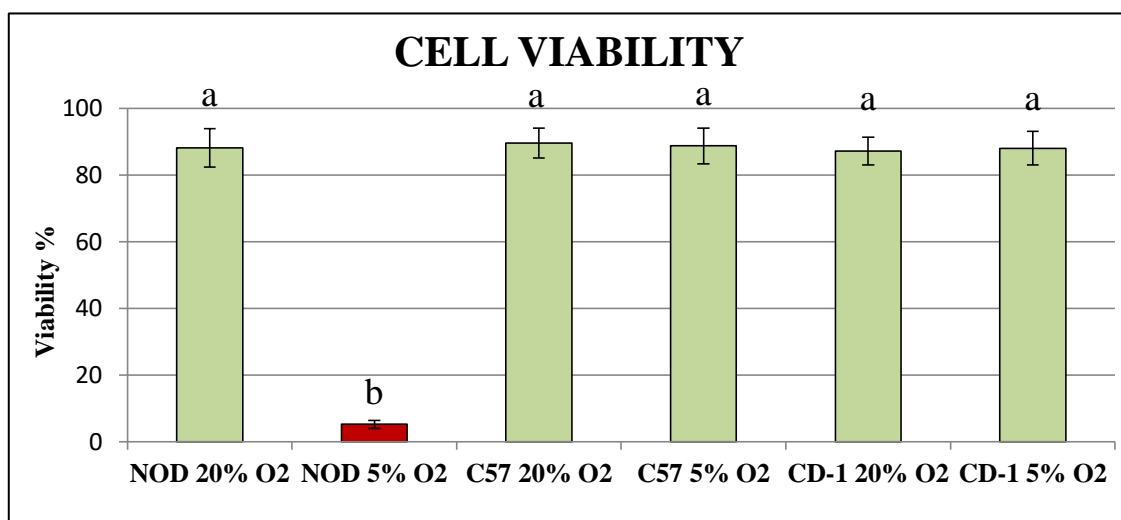
**Histogram 1: cell viability percentage in different experimental groups
(viability is expressed as mean value ± SD).
Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).**

4.1.1.2 Immunocytochemistry

4.1.1.2.1 Oct4 immune-localization

Immunostaining analysis for Oct4 performed after 5-azacytidine treatment, showed the presence of this important pluripotent-related marker in both oxygen tensions, with no significant differences (Figures 30-31).
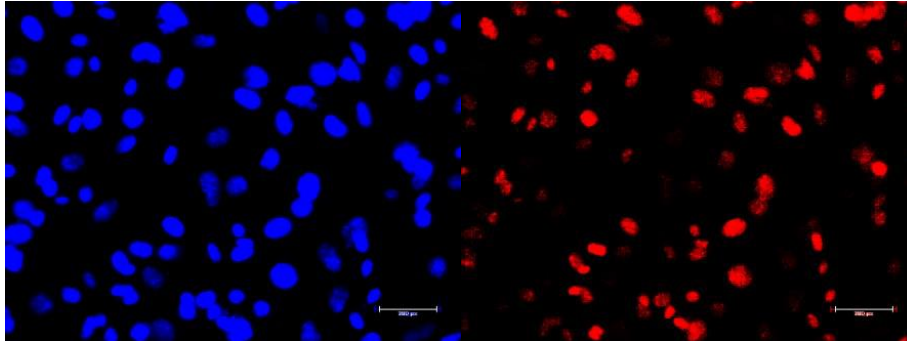
Figure 30: immunostaining analysis for Oct4 in 20% O_2 after 5-Aza CR treatment. Clear signal of Oct4 (red) and DAPI (blue).
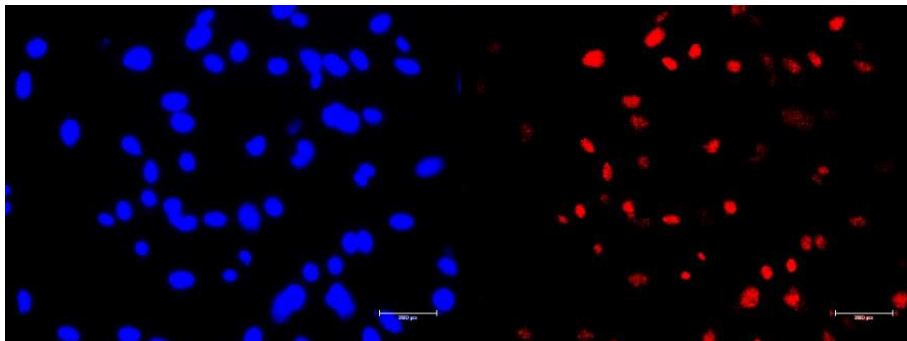
Figure 31: immunostaining analysis for Oct4 in 5% O_2 after 5-Aza CR treatment. Clear signal of Oct4 (red) and DAPI (blue).

Specifically, 5-azacytidine treated fibroblasts displayed an average of 88% ± 2,65 of Oct4 positive cells in high oxygen and 83% ± 3,44 in low oxygen (Histogram 2).
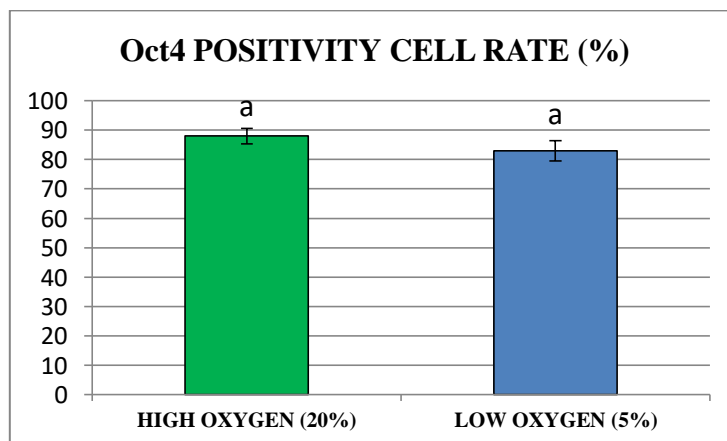
Histogram 2: Oct4 positivity cell rate in murine cells maintained in 20%) and 5% O_2.

4.1.1.2.2 C-Peptide immune-localization

Immunocytochemistry analysis for C-Peptide, carried out at the end of the pancreatic induction (day 10), showed the presence of this pancreatic marker in all experimental groups, except for NOD 5% O_2 that was not able to complete the differentiation process. In particular, no significant differences among groups were detected with cells able to express C-Peptide with an average of 34,28% ± 1,70 (Figure 32).
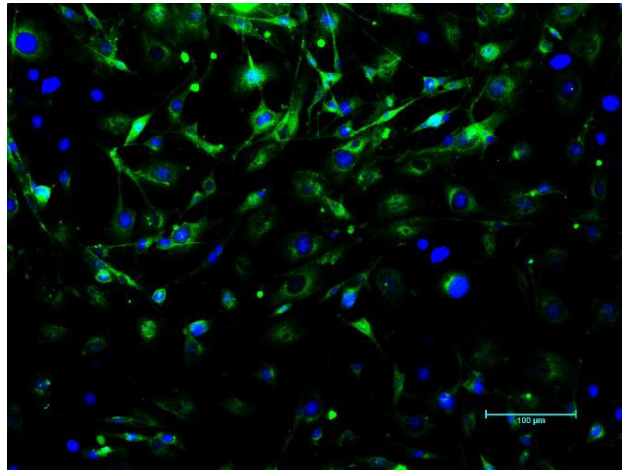
Figure 32: representative image of immunostaining of murine cells at the of the differentiation process. Clear signal of C-Peptide (green) and DAPI (blue).

4.1.1.3 Insulin Release

At the end of the pancreatic differentiation process, an ELISA test specific for Insulin was performed, after cell stimulation in hyperglycemic conditions. The data obtained showed that all experimental groups were able to release insulin. In particular, C57 BL/6J cells differentiated in high oxygen showed a significant lower insulin release (**2,95 ± 0,09 µg INS/µg DNA)**, compared to the other samples (p≤0,05). (Table 12; Histogram 3).

EXP. GROUPS	17,5 mM (µg INS/µg DNA)	20 mM (µg INS/µg DNA)
NOD 20% O$_2$	0,47 ± 0,18	**4,44 ± 0,44 (a)**
C57 20% O$_2$	0,20 ± 0,12	**2,95 ± 0,09 (b)**
C57 5% O$_2$	0,26 ± 0,10	**4,41 ± 0,20 (a)**
CD-1 20% O$_2$	0,27 ± 0,09	**4,98 ± 0,35 (a)**
CD-1 5% O$_2$	0,38 ± 0,04	**5,20 ± 0,13 (a)**

Table 12: **insulin release in different experimental groups (insulin release is expressed as mean value ± SD). Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).**
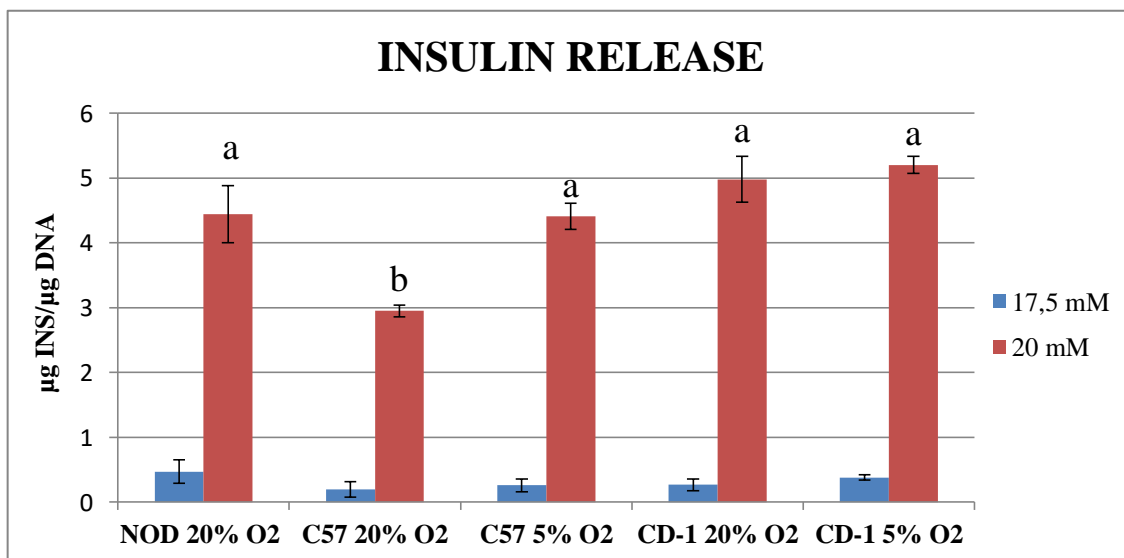
Histogram 3: **insulin release in C57, NOD and CD-1 cells differentiated in different oxygen tensions (insulin release is expressed as mean value ± SD). Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).**

4.1.2 Section 2

These experiments were carried out subjecting NOD cells to 5% oxygen only after their differentiation performed in 20% O_2, since these cells did not survive in low oxygen concentrations during the pancreatic induction process.

4.1.2.1 Insulin Release

ELISA tests were performed in these experimental groups as well, using the same modalities described before.

The results obtained showed that NOD cells remain viable for up to four days in hypoxic conditions. However, their ability to release insulin significantly decreases to concentrations ranging around **1,58 ± 0,27 μg INS/μg DNA** (Table 13; Histogram 4).

EXP. GROUPS	17,5 mM (μg INS/μg DNA)	20 mM (μg INS/μg DNA)
NOD 20% (ctrl)	0,40 ± 0,11	**4,38 ± 0,24 (a)**
NOD 20% + Day 11 20%	0,32 ± 0,08	**4,55 ± 0,33 (a)**
NOD 20% + Day 11 5%	0,14 ± 0,02	**4,39 ± 0,14 (a)**
NOD 20% + Day 11-14 20%	0,3 ± 0,03	**3,38 ± 0,03 (b)**
NOD 20% + Day 11-14 5%	0,13 ± 0,01	**1,58 ± 0,27 (c)**

Table 13: insulin release in different experimental groups (insulin release is expressed as mean value ± SD) Different superscripts indicate statistical differences among the samples(SPSS software, p≤0,05).
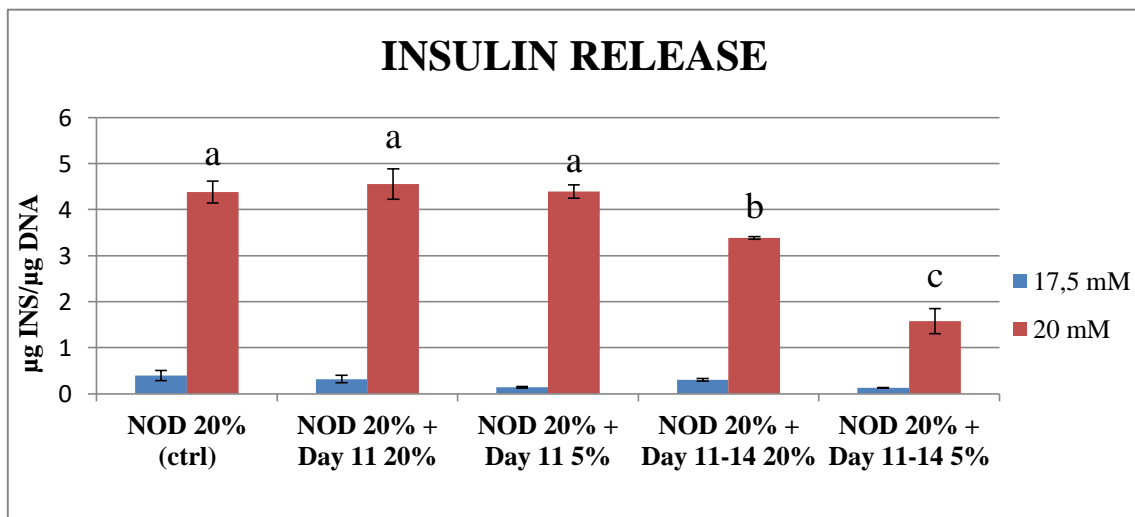
Histogram 4: insulin release in NOD cells maintained in different oxygen tensions (Insulin release is expressed as mean value ± SD). Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05)

4.2 Establishment of the best glucose concentrations leading to cells that respond to glucose variations in a physiological way

4.2.1 Section 1

4.2.1.1 Cell morphology and viability

Human dermal fibroblasts were isolated in high glucose concentrations (25 mM), plated on plastic and differentiated in the standard 17,5 mM glucose as well as in 5,5 and 8,5 mM of glucose.

At the end of the differentiation process, cells cultured in low glucose concentratrions (both 5,5 and 8,5 mM) showed an unusual arrangement (distinct parallel pattern), characterized by a thin and elongated structure (Figure 33), compared to those maintained in high glucose (spheric cell clusters, pancreatic islet structure; Figure 34).
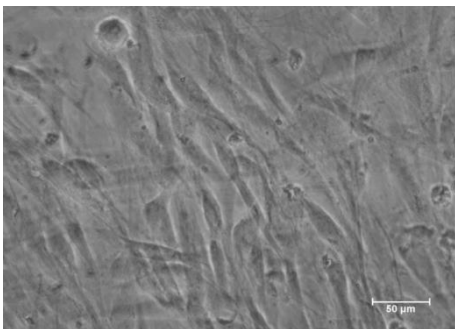
Figure 33: representative image of cells differentiated in low glucose concentrations (5,5 mM), day 36.

Figure 34: representative image of cells differentiated in high glucose concentrations (17,5 mM), day 36.

4.2.1.2 Immunocytochemical analysis

Immunostaining analysis performed on cells differentiated in high glucose concentrations (CTRL) showed the expression of C-Peptide (Figure 35) and Insulin, with an average of 38,15% ± 3,51 and 37,55% ± 4,13 of positive cells respectively. On the other hand, cells cultured in low glucose (Samples 1-6) displayed a negative expression of Insulin and C-Peptide.
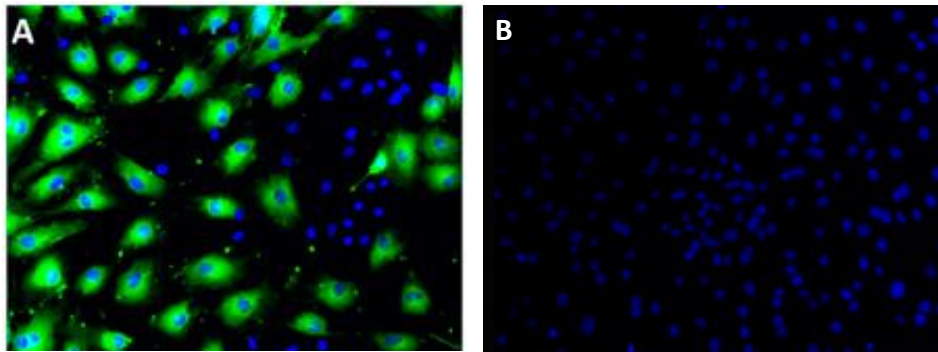
Figure 35: immunostaining of CTRL (day 36): C-Peptide (green) and DAPI (blue) (A). Immunostaining of Low Glucose samples (day 36): C-Peptide is not present, DAPI in blue (B).

4.2.1.3 Insulin Release

At the end of the pancreatic differentiation process, an ELISA test specific for insulin was performed, after cell stimulation in hyperglycemic conditions. The data obtained showed that cells differentiated in high glucose (CTRL) were able to release an average of **6,55 ± 0,22 µg INS/µg DNA.** On the other hand, cells cultured in low glucose (Sample 1-6) were not able to produce insulin in response to the hyperglycemic stimulation (Histogram 5).
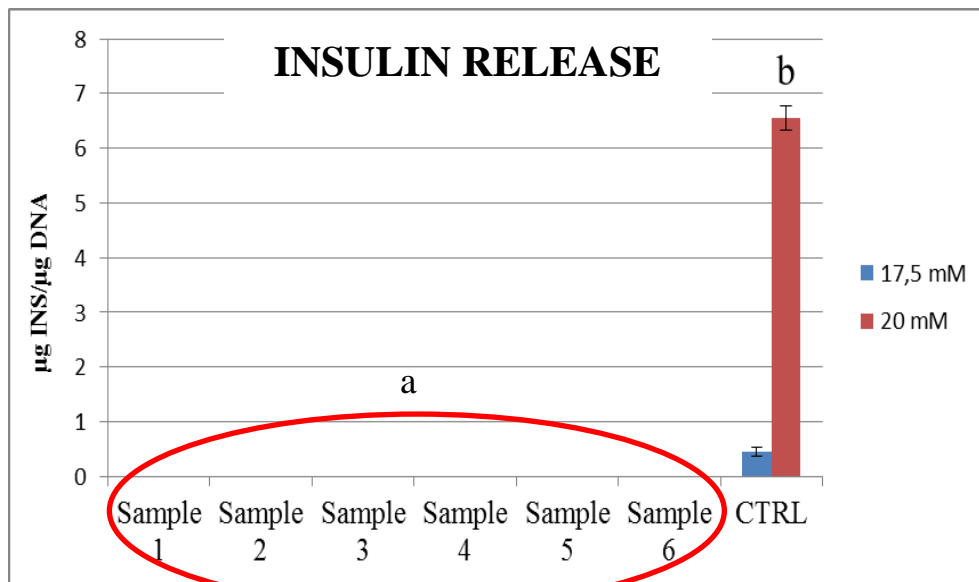
Histogram 5: insulin release in human cells differentiated in low glucose conditions (samples 1-6) and high glucose (CTRL). (Insulin release is expressed as mean value ± SD).
Different superscripts indicate statistical differences among the samples(SPSS software, $p \leq 0,05$).

4.2.2 Section 2

4.2.2.1 Gene expression at different time points

Since the results obtained from the previous experiment showed that cells in low glucose were not able to differentiate, it was designed a second experiment in which human cells (isolated in standard 25 mM glucose) were subjected to standard and low glucose concentrations untill day 14 of the process. Along this period, the expression of 5 important genes was monitored, in order to characterize them during the early pancreatic differentiation.

The results obtained displayed an alteration of the expression of these genes in low glucose samples (Histogram 6).
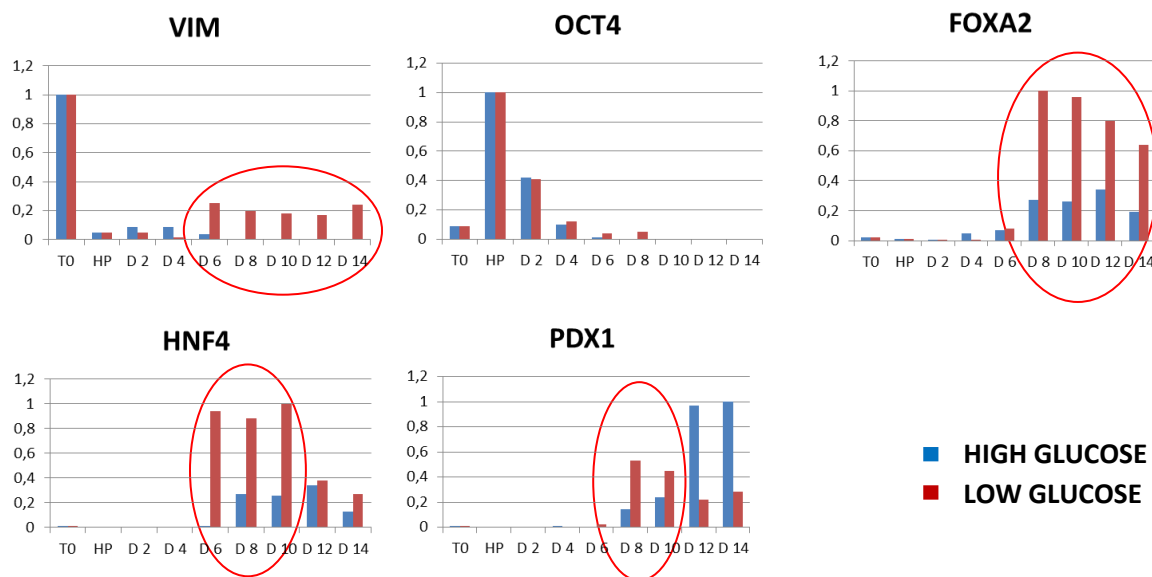
Histogram 6: gene expression at different time points in high glucose (blue) and low glucose samples (red) (gene expression levels are reported with the highest expression set to 1 and all others relative to this).

In particular, in low glucose samples Vimentin transcription decrease after 5-aza treatment but its expression was visible again starting from day 6, in contrast to the control where Vimentin expression disappeared along the process. This particular Vimentin transcription pattern in low glucose samples indicate that a cell type reversion occurs during the early differentiation, with a substantial part of cells returning to express this typical fibroblast marker instead of carrying on towards the pancreatic lineage.

In contrast, Oct4 expression trend was similar in both glucose concentrations with a peak after 5-aza treatment, followed by a sharp decrease along the process. FOXA2, HNF4 and PDX1 expressions were altered in low glucose samples. These samples displayed higher levels of these early pancreatic genes, the expression of which appears to be prematurely turned on. So, low glucose affect both the correct timing and levels of early pancreatic genes expression. In particular, a glucose-sensitive window was identified around day 6-10 of *in vitro* differentiation, corresponding to the embryonic primitive gut tube/posterior foregut stage *in vivo*.

4.2.3 Section 3

Since a glucose sensitive window was identified around day 6-10 of the pancreatic induction process, it was performed a third experiment subjecting cells (isolated in standard 25 mM glucose) to low glucose concentrations during the first two pancreatic differentiation steps only (Activin A medium and Activin A + Retinoic medium; day 1-9; Table 9).

4.2.3.1 Morphology and viability

Cells from <u>GROUP A</u>, consisting in cells differentiated in low glucose (8,5 mM) until day 9 (corresponding to the Activin A and Retinoic Acid steps) and, then, in high glucose till the end of the process, did not complete their differentiation and began to detach from the support on day 23 (Figure 36).

In contrast, cells from <u>GROUP B,</u> consisting in cells differentiated in standard high glucose concentrations (Table 9), successfully proceeded along the pancreatic differentiation and displayed the expected aggregates (Figure 37).
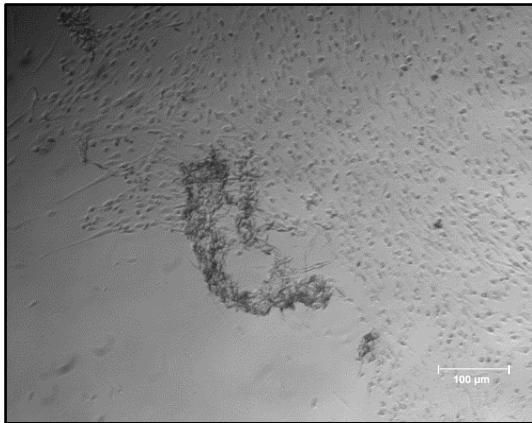
Figure 36: representative image of cells from GROUP A, day 23.
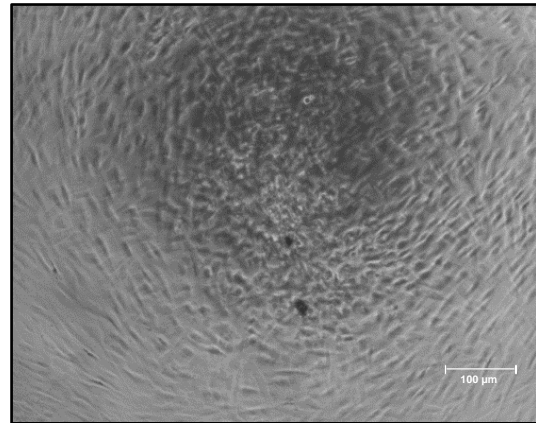
Figure 37: representative image of cells from GROUP B, day 23.

4.2.3.2 Gene expression at different time points

Gene expression results showed that cells from GROUP A displayed a gene expression trend comparable to that one illustrated in Section 2, with both the correct timing and levels of early pancreatic genes expression affected (Histogram 7).

Furthermore, although these cells were able to release Insulin, they did not complete the differentiation process and began to detach from the culture dish at day 23.
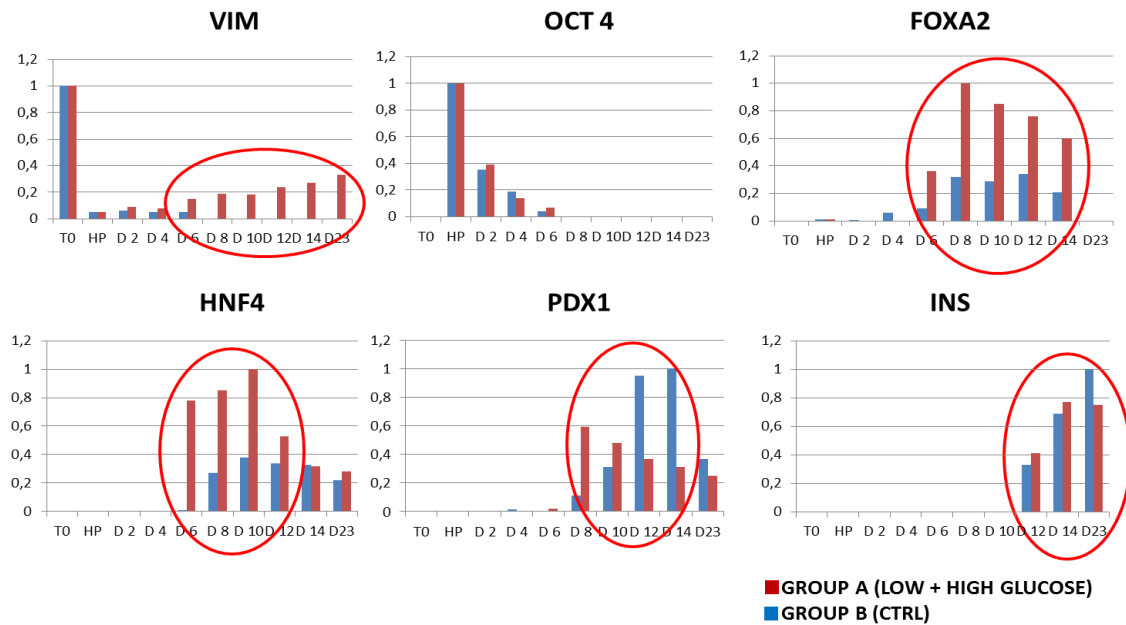
Histogram 7: gene expression at different time points in Group A (red) and Group B (blue)
(gene expression levels are reported with the highest expression set to 1 and all others relative to this).

The results obtained in these first three sections indicate that low glucose environment interferes with the normal differentiation process, altering the expression of some early pancreatic genes, resulting in cells not able to complete their conversion towards the pancreatic lineage.

4.2.4 Section 4

4.2.4.1 Mouse model

4.2.4.1.1 Cell morphology

Murine dermal fibroblasts were isolated both in high (25 mM) and low glucose concentrations (5,5 mM), plated on plastic and differentiated both in the standard 17,5 mM and in 5,5 mM of glucose. At the end of differentiation, cells from all experimental groups lost the typical elongated shape of untreated fibroblasts and acquired an epithelioid morphology (Figure 38), with no significant differences.
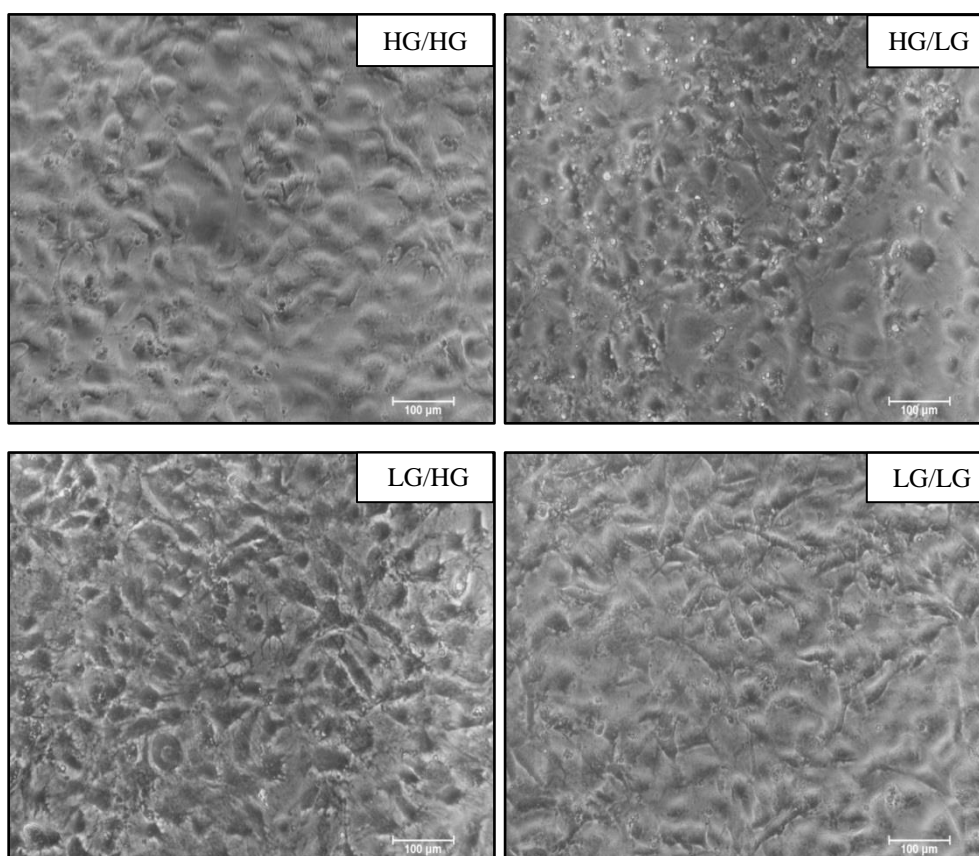
Figure 38: representative image of murine fibroblasts isolated and differentiated in all conditions (end of differentiation, day 10).

4.2.4.1.2 Immunocytochemistry

Cells displayed positivity for Insulin in all experimental groups, with different immune-positive percentage. Efficiency towards β cell differentiation, measured by counting cells that expressed Insulin, was 40 ± 4,4% in HG/HG, 25 ± 3,5% in HG/LG, 83 ± 3,4% in LG/LG and 72 ± 4,1% in LG/HG (Figure 39).
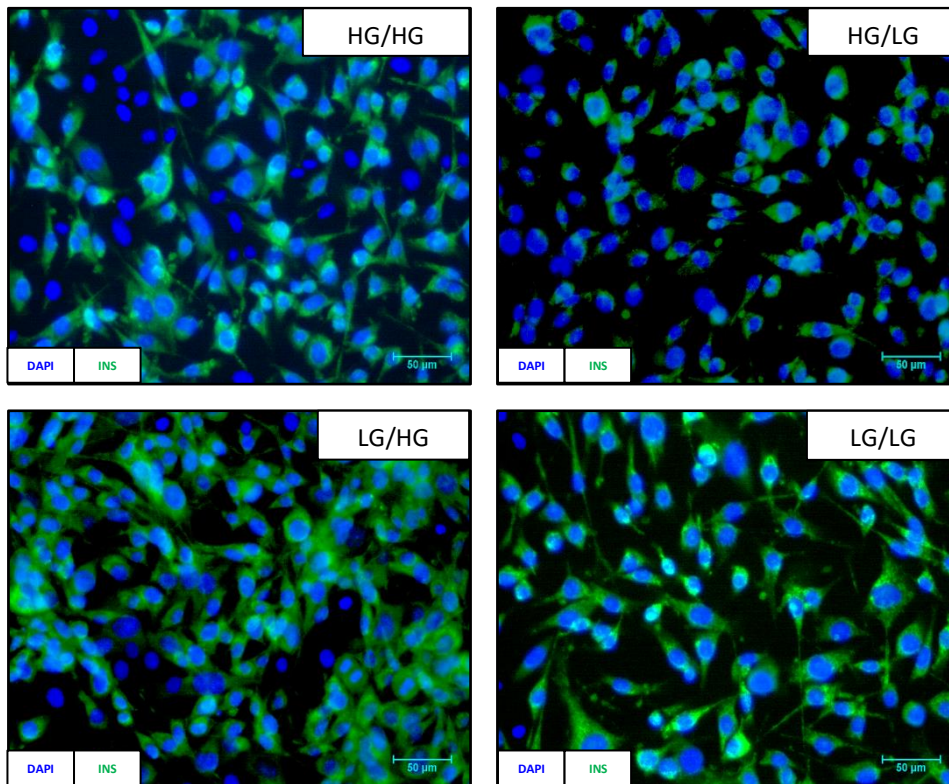
Figure 39: immunolocalization of Insulin in all mouse experimental groups (end of differentiation, day 10)

4.2.4.1.3 Insulin Gene expression

Cells isolated and differentiated in all conditions were able to express Insulin at the end of the differentiation process (Histogram 8). However, significantly higher Insulin levels were expressed in cells isolated and differentiated in low glucose (LG/LG; 1 ± 0,001 fold), compared to those isolated in low glucose and differentiated in high glucose concentrations (LG/HG; 0,84 ± 0,07 fold). Furthermore, cells isolated in high glucose and differentiated in both glucose concentrations showed a significant lower Insulin expression (HG/HG 0,51 ± 0,08 fold; HG/LG 0,28 ± 0,05 fold).
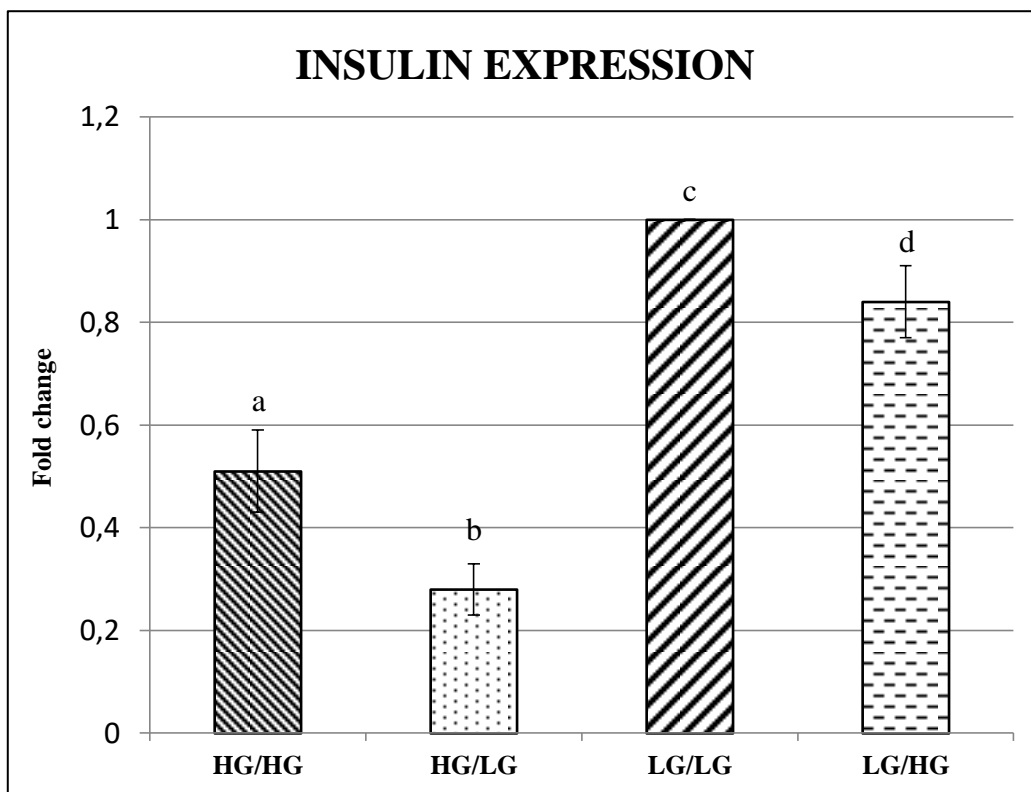
Histogram 8: **insulin gene expression in all different experimental groups.**
(gene expression levels are reported with the highest expression set to 1 and all others relative to this).
Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).

4.2.4.1.4 Insulin release

In this experiment, cells were stimulated for 1 hour with the physiological 8,5 mM (mouse blood glucose concentration after meal) as well as with the standard 20 mM D-glucose. All samples were able to respond to 1 hour exposure to 8,5 mM and 20 mM glucose both and actively release Insulin in cell supernatants conditions (Table 14; Histogram 9). Furthermore, basal insulin release was measured collecting media used during the differentiation (5,5 mM in HG/LG and LG/LG; 17,5 mM in HG/HG and LG/HG).

EXP. GROUPS	5,5 mM (basal) (µg INS/µg DNA)	8,5 mM (µg INS/µg DNA)	17,5 mM (basal) (µg INS/µg DNA)	20 mM (µg INS/µg DNA)
HG/HG	-	ND	$0,28 \pm 0,07$ (a)	$5,88 \pm 0,46$ (b)
HG/LG	$0,38 \pm 0,10$ (a)	$2,81 \pm 0,35$ (c)	-	$4,11 \pm 0,43$ (d)
LG/LG	$0,42 \pm 0,08$ (a)	$7,46 \pm 0,38$ (e)	-	$10,41 \pm 0,63$ (f)
LG/HG	-	ND	$0,33 \pm 0,11$ (a)	$9,37 \pm 0,45$ (f)

Table 14: insulin release in different experimental groups (insulin release is expressed as mean value ± SD) Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05). (ND = not detected)
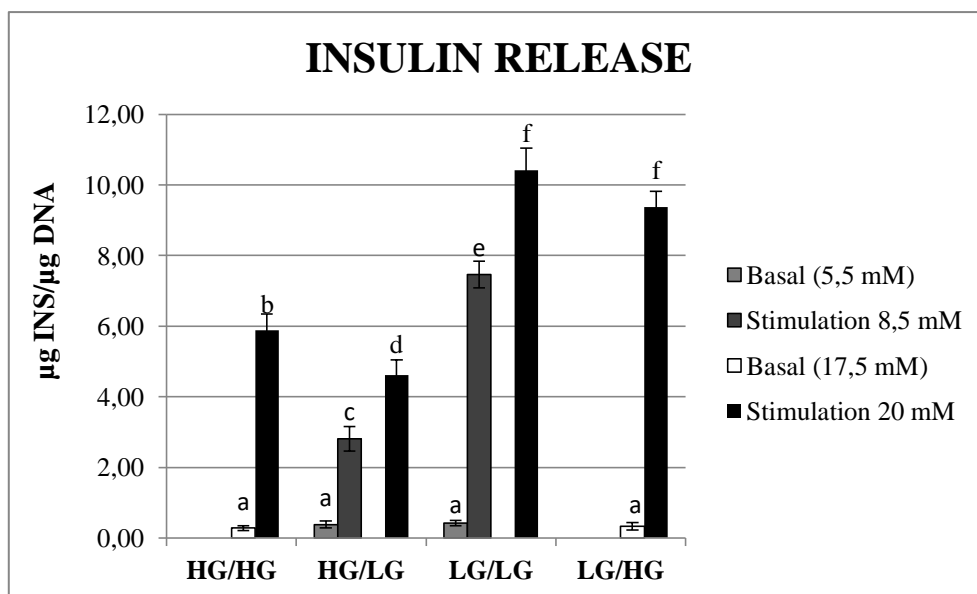
Histogram 9: insulin release in all different samples (Insulin release is expressed as mean value ± SD) Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).

4.2.4.1.4.1 Insulin release after stimulation with 8,5 mM glucose

Fibroblasts isolated in high glucose and differentiated in low glucose conditions (HG/LG) were able to release Insulin even when stimulated with physiological hyperglycemic levels (8,5 mM), producing $2,81 \pm 0,35$ µg INS/µg DNA. On the other hand, HG/HG and LG/HG cells were not able to produce insulin when stimulated with 8,5 mM glucose.

Interestingly, fibroblasts isolated and differentiated in low glucose (LG/LG) ensure a significantly increase of insulin release when stimulated with 8,5 mM of glucose. As a matter of fact, these cells appear to be more responsive to insulin challenge, even when stimulated with 8,5 mM, with a significantly higher release ($7,46 \pm 0,38$ µg INS/µg DNA), compared to the other experimental groups described above.

4.2.4.1.4.2 Insulin release after stimulation with 20 mM glucose

After differentiation, cells from all experimental groups, stimulated with 20 mM of glucose, were able to respond releasing Insulin. Once again, the highest Insulin release values were obtained in cells isolated in low glucose and differentiated in both conditions, respectively equal to $10,41 \pm 0,63$ µg INS/µg DNA in LG/LG and $9,37 \pm 0,45$ µg INS/µg DNA in LG/HG. On the other hand, cells isolated in high glucose were able to produce significantly lower insulin amounts ($5,88 \pm 0,46$ µg INS/µg DNA in HG/HG and $4,11 \pm 0,43$ µg INS/µg DNA in HG/LG).

4.2.4.1.5 Class 1 Glucose Transporters (GLUT 1-4) gene expression

The expression of four glucose transporters (GLUT1, GLUT2, GLUT3, GLUT4) was measured in all experimental groups, including untreated fibroblasts isolated in high (T0 HG) and low glucose (T0 LG), as visible in Histogram 13.

4.2.4.1.5.1 GLUT1 expression

All samples were able to express GLUT1 at the end of the differentiation process, with a significant higher expression in cells isolated in high glucose and differentiated in both glucose conditions (HG/HG 1,03 ± 0,08 fold; HG/LG 0,86 ± 0,11 fold; Histogram 9), compared to the others. On the other hand, cells isolated in low glucose and differentiated in both glucose concentrations showed a significant lower expression of GLUT1, respectively 0,59 ± 0,10 fold in LG/HG and 0,40 ± 0,12 fold in LG/LG (Histogram 10). Finally, the expression of GLUT1 appear to be more evident in untreated fibroblasts isolated in low glucose (T0 LG) than those isolated in high glucose (T0 HG).
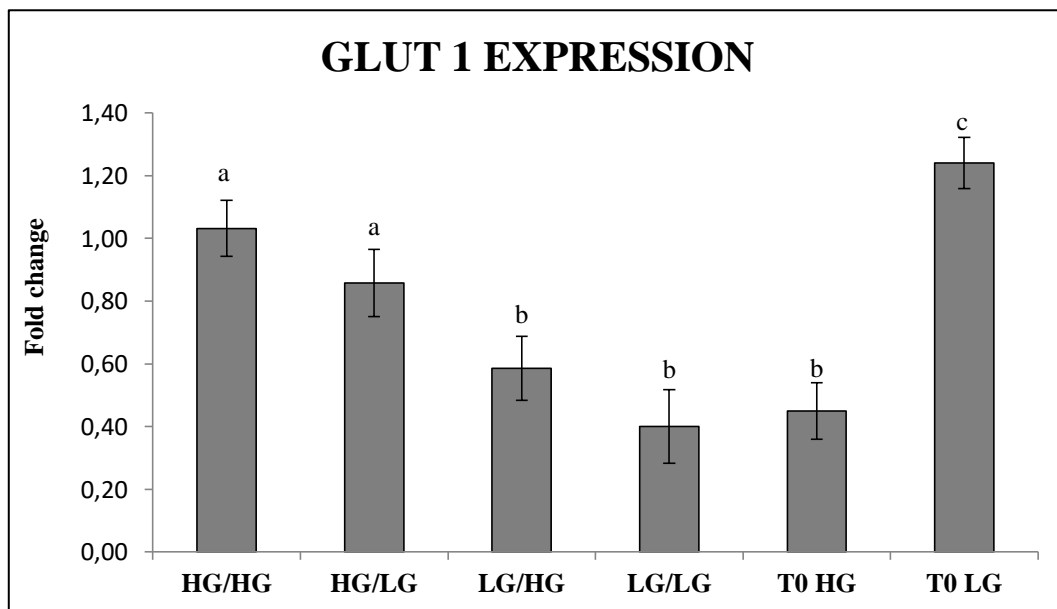
Histogram 10: GLUT1 gene expression in all experimental groups.
Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).

4.2.4.1.5.2 GLUT2 expression

The expression of GLUT2 was observed in all experimental groups, with a significant higher expression in cells isolated in high glucose (1,21 ± 0,07 fold in HG/HG and 0,89 ± 0,05 fold in HG/LG; Histogram 11). On the other hand, cells isolated in low glucose were able to express GLUT2 too, although they displayed a significant lower expression (0,15 ± 0,03 fold in LG/LG and 0,49 ± 0,04 fold in LG/HG; Histogram 10), compared to those isolated in high glucose. Finally, the expression of this gene in T0 HG and T0 LG was not detected.
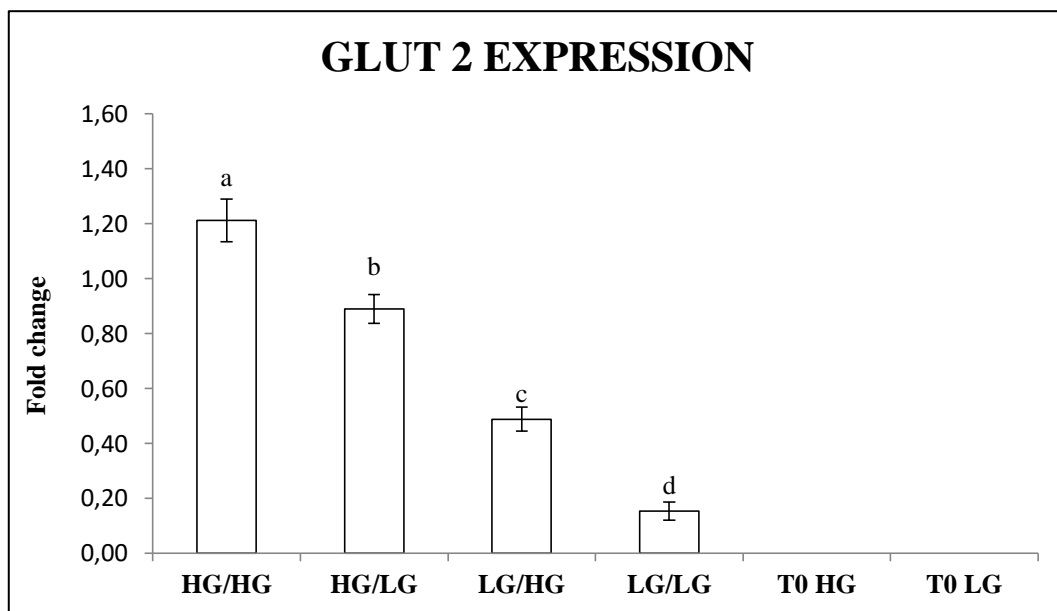
Histogram 11: GLUT2 gene expression in all experimental groups.
Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).

4.2.4.1.5.3 GLUT3 expression

GLUT3 expression was not detected in all experimental groups.

4.2.4.1.5.4 GLUT4 expression

Excluding untreated fibroblasts (T0 LG and T0 HG), the expression of GLUT4 was observed in all experimental groups, with varying yields (Histogram 12). In particular, LG/LG showed a significant overexpression of GLUT4 (12,85 ± 1,49 fold) compared to LG/HG (2,58 ± 0,51 fold), HG/LG (0,28 ± 0,08 fold) and HG/HG (0,04 ± 0,01 fold) (Histogram 12).

These results suggest that cell isolation performed in low glucose promotes the expression of GLUT4 at the end of the pancreatic differentiation carried out both in low and high glucose concentrations.
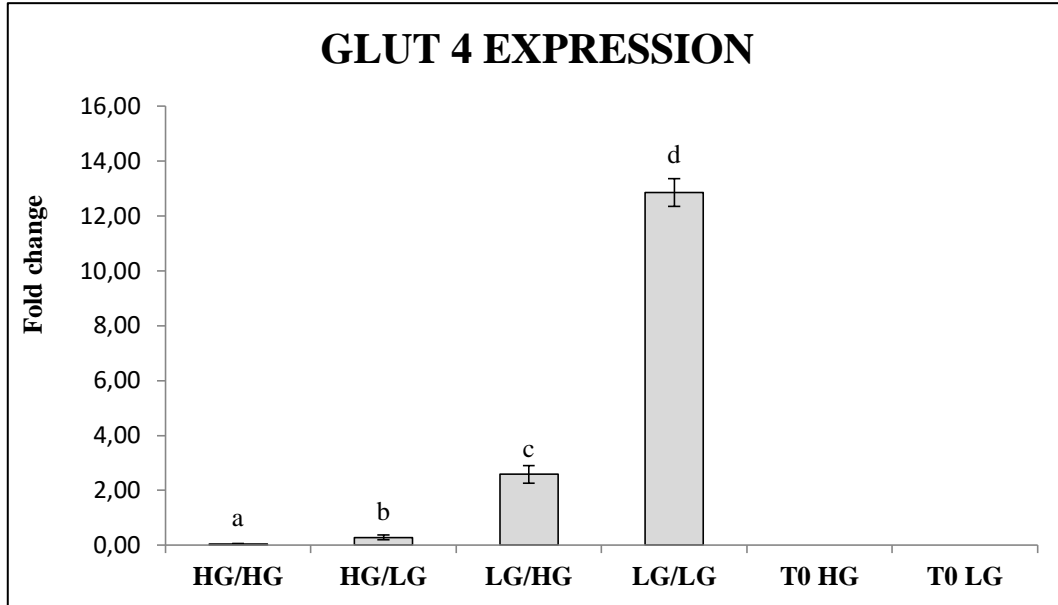
Histogram 12: GLUT4 gene expression in all experimental groups.
Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).
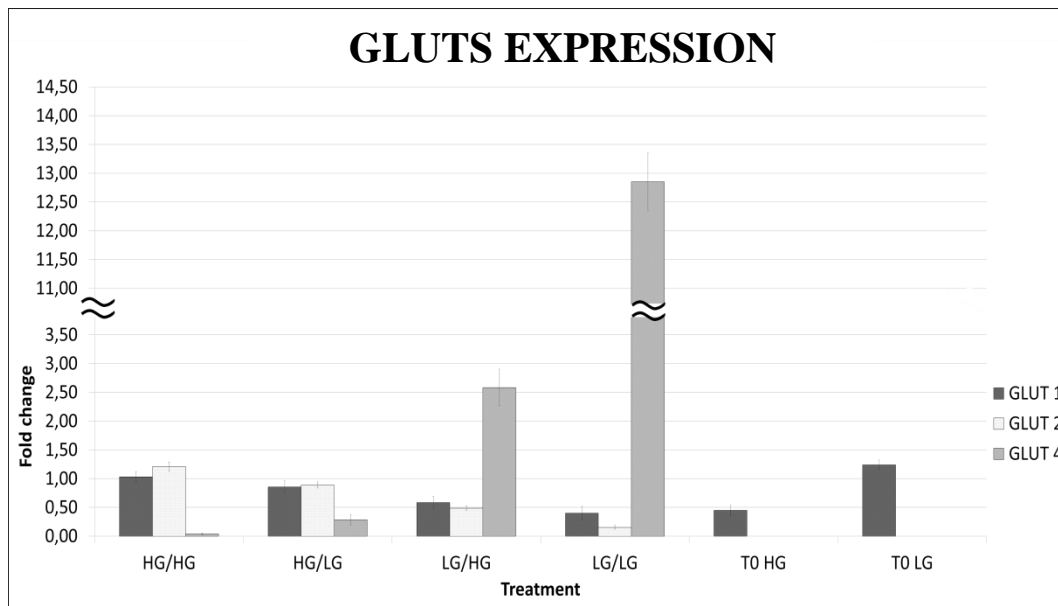
Histogram 13: GLUTS gene expression in all experimental groups.

4.2.4.2 Human model

4.2.4.2.1 Cell morphology

Human dermal fibroblasts were isolated both in high glucose (25 mM) and in low glucose concentrations (5,5 mM), plated on plastic and differentiated in both standard and low glucose levels. Significant cell morphology differences were visible among all experimental groups (Figure 40).
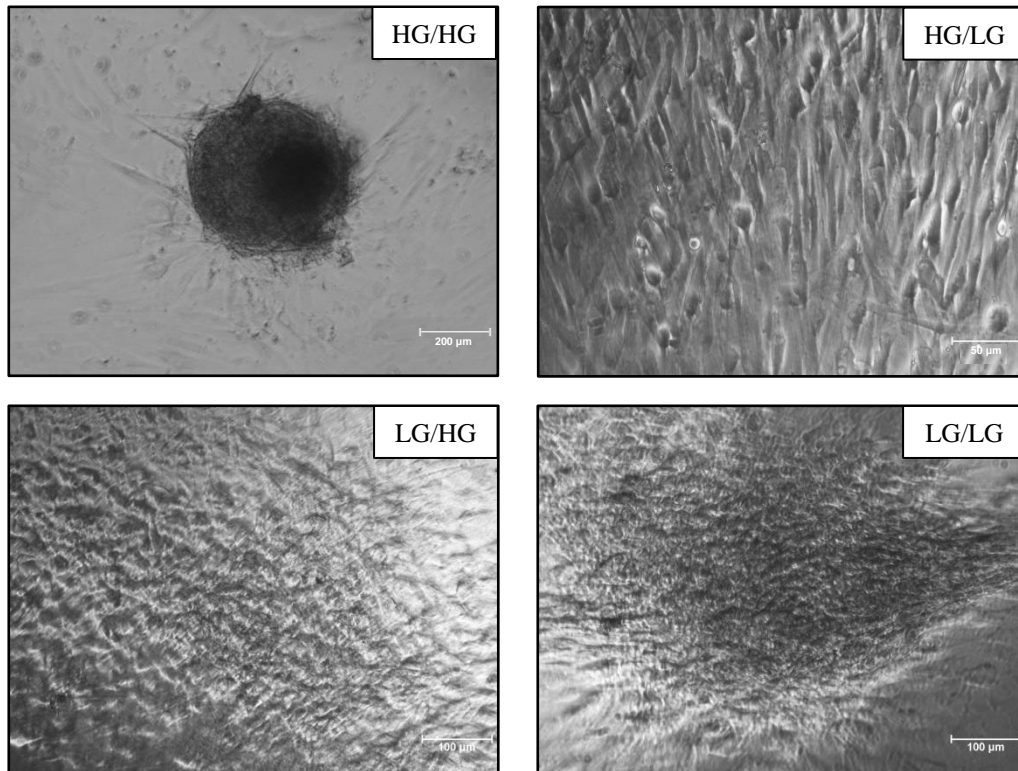
Figure 40: human fibroblasts isolated and differentiated in all glucose conditions, day 36.

In particular, HG/HG cells aggregated in spheric cell clusters (similar to the pancreatic islet structure), as expected from this experimental group. On the other hand, HG/LG cells showed an unusual arrangement, characterized by a thin and elongated structure, comparable with the results obtained in Section 1 (Paragraph 4.2.1.1; Figure 33).

Finally, fibroblasts isolated in low glucose and differentiated in both glucose levels (LG/HG; LG/LG) remain viable and began to organize themselves in cell clusters (as HG/HG sample) but, interesting, they failed to complete their aggregation process, also when maintened in culture for more than standard 36 days (Figure 40).

4.2.4.2.2 Insulin Gene expression

HG/HG, LG/HG and LG/LG experimental groups were able to express Insulin at the end of the differentiation process (Histogram 14). Significantly higher Insulin levels were expressed in cells isolated and differentiated in high glucose (HG/HG; $1 \pm 0,001$ fold), compared to those isolated in low glucose and differentiated in both high and low glucose concentrations (LG/LG $0,66 \pm 0,04$ fold; LG/HG $0,59 \pm 0,08$ fold).
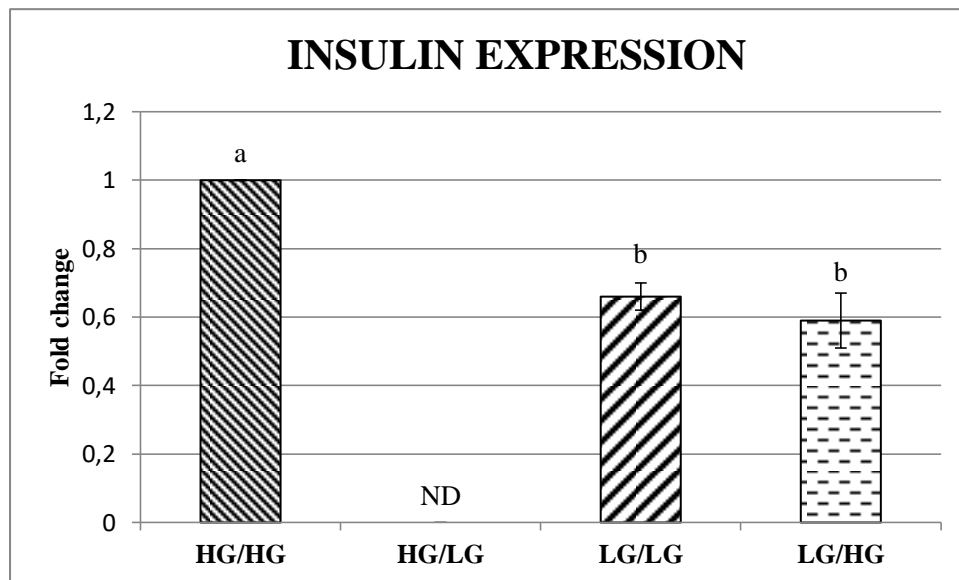
Histogram 14: insulin gene expression in all different experimental groups.
(gene expression levels are reported with the highest expression set to 1 and all others relative to this).
Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).
(ND = not detected)

4.2.4.2.3 Insulin release

At the end of the pancreatic differentiation process, cells were stimulated for 1 hour with 8,5 mM as well as 20 mM D-glucose, with the same modalities described above. Glucose-dependent insulin release was assessed as previously described. HG/HG, LG/HG and LG/LG samples were able to respond to 8,5 and 20 mM glucose challenge both and actively release insulin in cell supernatants (Table 15; Histogram 15).

EXP. GROUPS	5,5 mM (μg INS/μg DNA)	8,5 mM (μg INS/μg DNA)	17,5 mM (μg INS/μg DNA)	20 mM (μg INS/μg DNA)
HG/HG	-	ND	0,20 ± 0,04 (a)	6,77 ± 0,16 (c)
HG/LG	ND	ND	-	ND
LG/LG	0,18 ± 0,05 (a)	2,75 ± 0,18 (b)	-	4,14 ± 0,23 (d)
LG/HG	-	ND	0,16 ± 0,06 (a)	3,89 ± 0,27 (d)

Table 15: insulin release in different experimental groups (insulin release is expressed as mean value ± SD) Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05). (ND = not detected)
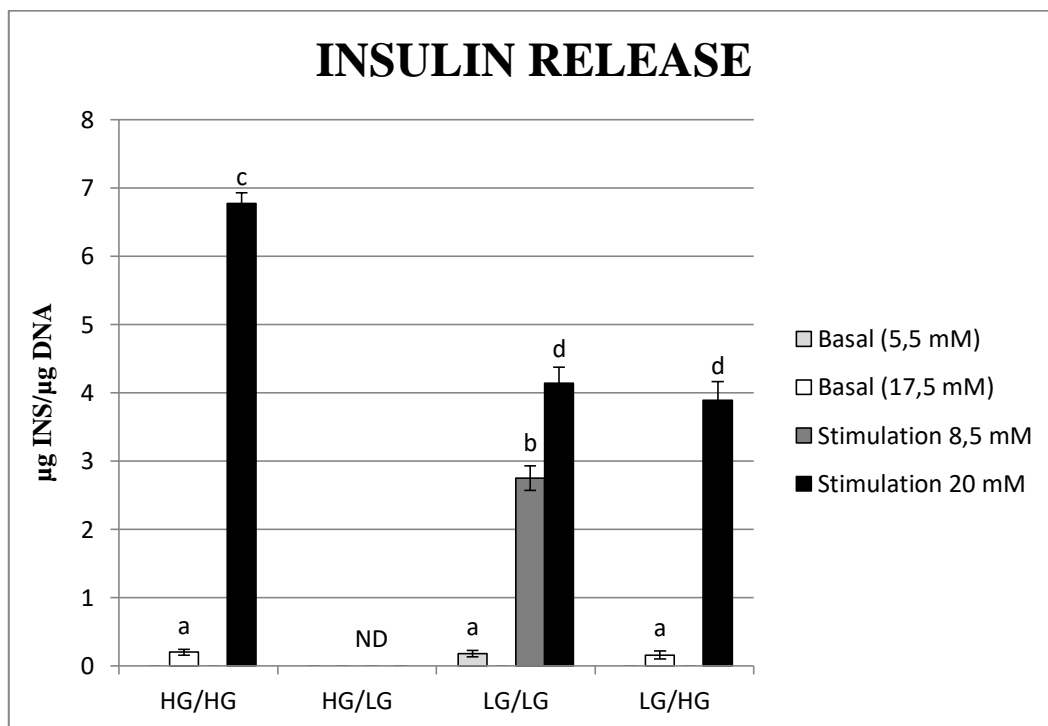
Histogram 15: insulin release in all different samples (Insulin release is expressed as mean value ± SD) Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).

4.2.4.2.3.1 Insulin release after stimulation with 8,5 mM glucose

Fibroblasts isolated in high glucose conditions (HG/HG; HG/LG) were not able to produce insulin when stimulated with 8,5 mM glucose. On the other hand, cells isolated and differentiated in low glucose (LG/LG) were able to produce an average amount of Insulin equal to $2,75 \pm 0,18$ µg INS/µg DNA. Conversely, LG/HG cells were not able to release insulin when stimulated with 8,5 mM of glucose.

4.2.4.2.3.2 Insulin release after stimulation with 20 mM glucose

At the end of pancreatic differentiation process, HG/HG, LG/LG and LG/HG cells were able produce Insulin, after stimulation with 20 mM of glucose.

In human model, a significant higher insulin release was obtained in cells isolated and differentiated in high glucose (HG/HG), as opposed to what happened in the mouse model. More precisely, HG/HG differentiated cells were able to produce an average of $6,77 \pm 0,16$ µg INS/µg DNA while LG/LG and LG/HG $4,14 \pm 0,23$ and $3,89 \pm 0,27$ µg INS/µg DNA respectively.

4.3 Use of 3-D systems and evaluation of mechanosensing related responses

4.3.1 Cell morphology

4.3.1.1 PTFE culture system

Cells differentiated in PTFE showed no morphological differences between samples maintained in both oxygen tensions. In particular, cells aggregated in spherical structures and maintained this organization along the differentiation process (Figure 41).
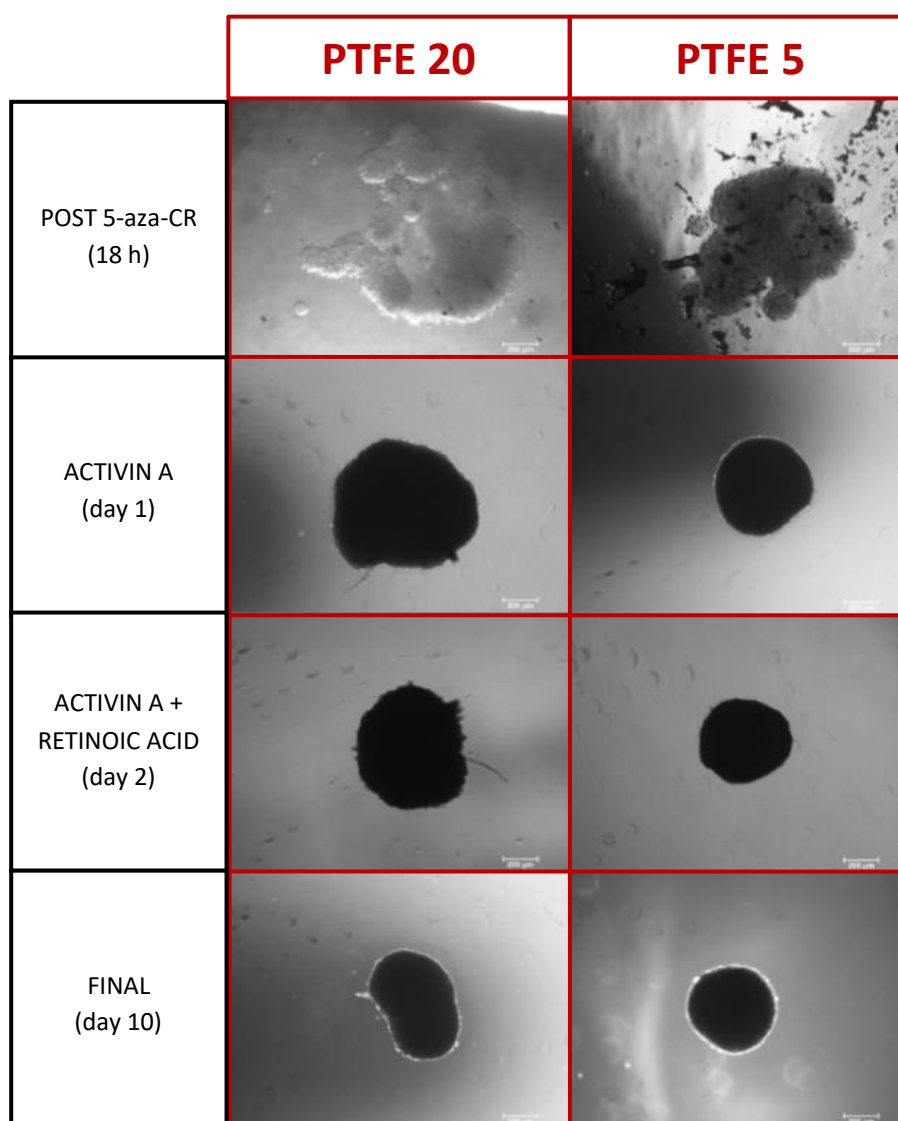
Figure 41: cell morphology of mouse fibroblasts differentiated in PTFE in 20% and 5% oxygen.

4.3.1.2 PAA gels culture system

Cells differentiated on 1 kPa soft gel in high oxygen (1 kPa 20), maintained the monolayer morphology for the first days of the process and, then, they organize themselves into several spherical clusters. On the other hand, cells differentiated on 4 kPa and on 1 kPa gel in low oxygen (1 kPa 5; 4 kPa 20; 4 kPa 5) maintained the monolayer morphology along the entire process (Figure 42).
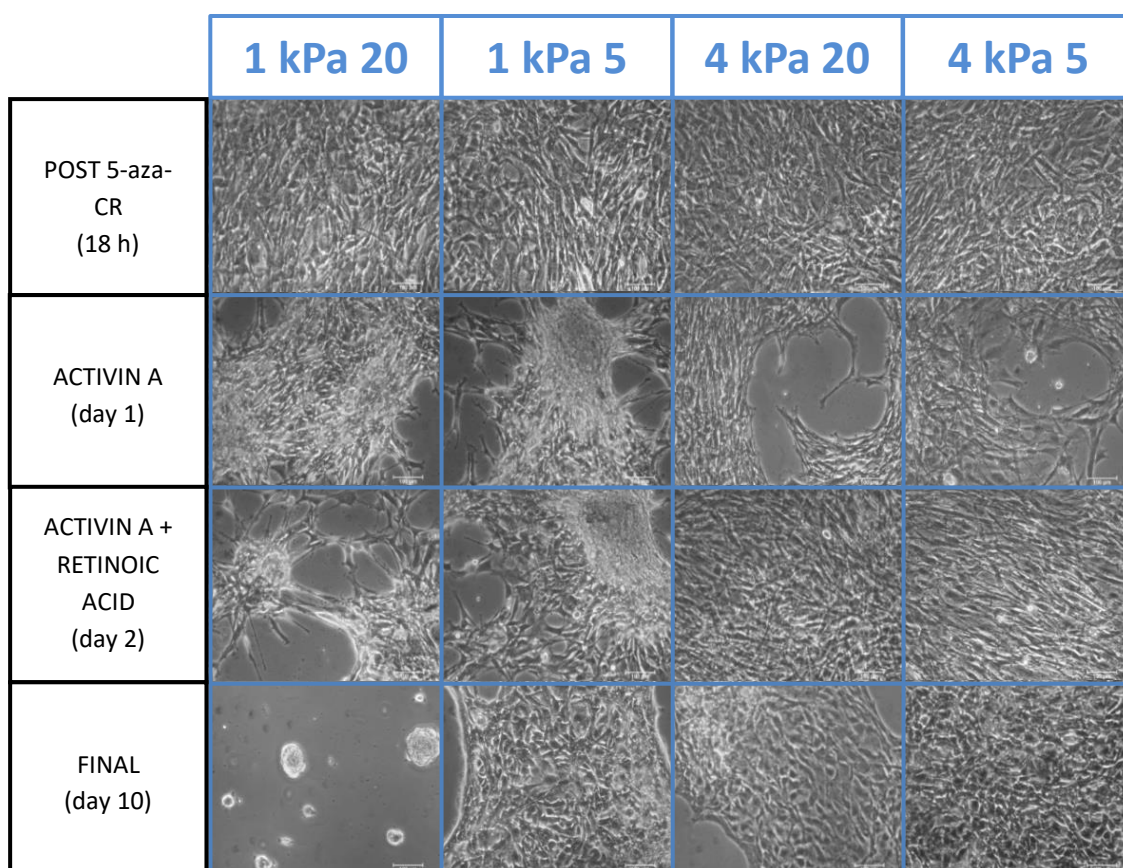
Figure 42: cell morphology of mouse fibroblasts differentiated
on PAA gels (1kPa; 4kPa) in 20% and 5% oxygen.

4.3.1.3 Standard plastic culture system (CTRL)

Cells differentiated on plastic dishes (as control) showed the typical morphology of mouse fibroblasts converted into insulin-producing cells. In particular, after 5-aza-CR treatment, cells changes their morphology, with the typical elongated morphology of untreated fibroblasts being replaced by a round or oval shape and with cell size becoming considerably smaller. Along the process cell populations acquired an epithelioid morphology in both high and low oxygen concentrations (Figure 43).

No significant morphological differences between samples maintained in different oxygen tensions were observed.
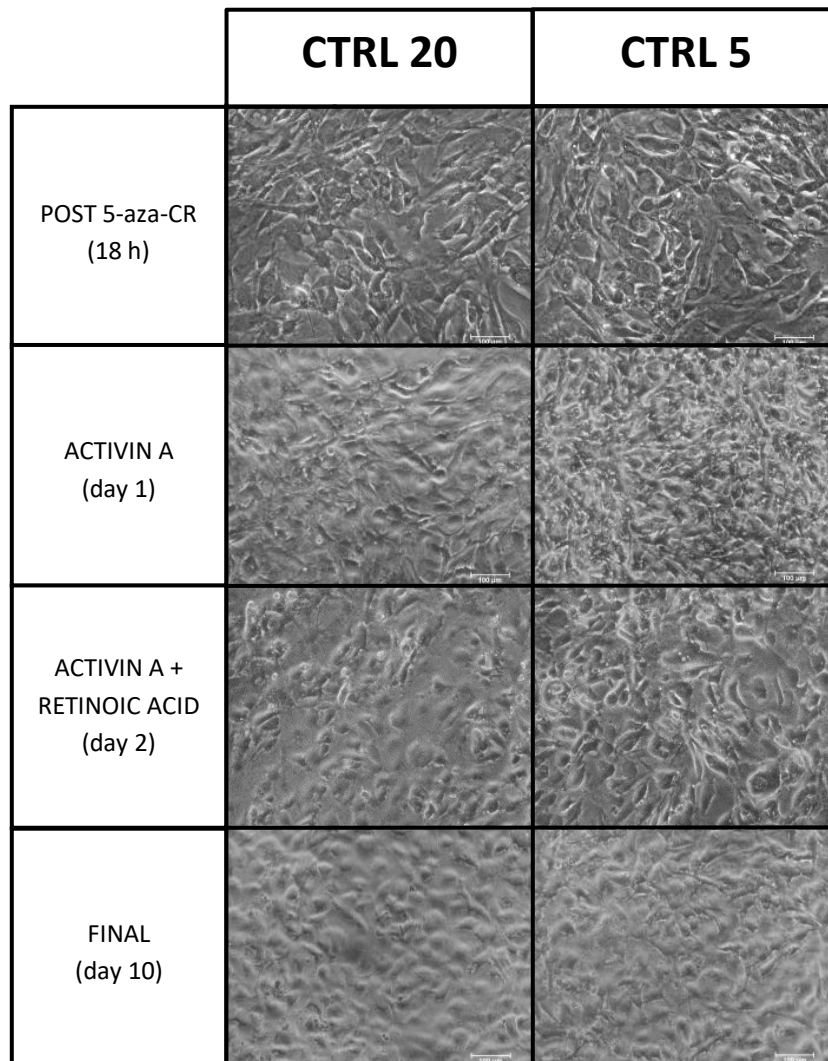
Figure 43: cell morphology of mouse fibroblasts differentiated on standard plastic dishes in 20% and 5% oxygen.

4.3.2 Insulin release

At the end of the pancreatic differentiation process, an ELISA test specific for Insulin was performed, after cell stimulation with hyperglycemic conditions. The data obtained showed that all samples were able to respond to glucose challenge and actively release Insulin in cell supernatants (Table 16; Histogram 16). In particular, cell differentiated in PTFE in low oxygen (PTFE 5) displayed a significant higher Insulin than those in PTFE in high oxygen (5,71 ± 0,15 and 2,58 ± 0,21 µg INS/µg DNA respectively). Similarly, cells converted on 1 kPa PAA gels increase their ability to release Insulin, especially in low oxygen (6,25 ± 0,21 µg INS/µg DNA). In contrast, cells differentiated on 4 kPa PAA gels display a significant lower amount of Insulin in both oxygen tensions, compared to the other experimental groups, controls included.

EXP. GROUPS	17,5 mM (µg INS/µg DNA)	20 mM (µg INS/µg DNA)
PTFE 20	0,44 ± 0,18	2,58 ± 0,21 (a)
PTFE 5	0,26 ± 0,06	5,71 ± 0,15 (b)
1 kPa 20	0,21 ± 0,04	5,62 ± 0,13 (b)
1 kPa 5	0,28 ± 0,09	6,25 ± 0,21 (c)
4 kPa 20	0,13 ± 0,05	1,85 ± 0,09 (d)
4 kPa 5	0,15 ± 0,03	1,63 ± 0,18 (d)
CTRL 20	0,19 ± 0,08	3,47 ± 0,12 (e)
CTRL 5	0,30 ± 0,11	4,86 ± 0,19 (f)

Table 16: **Insulin release in all experimental groups (Insulin release is expressed as mean value ± SD). Different superscripts indicate statistical differences among the samples(SPSS software, p≤0,05).**
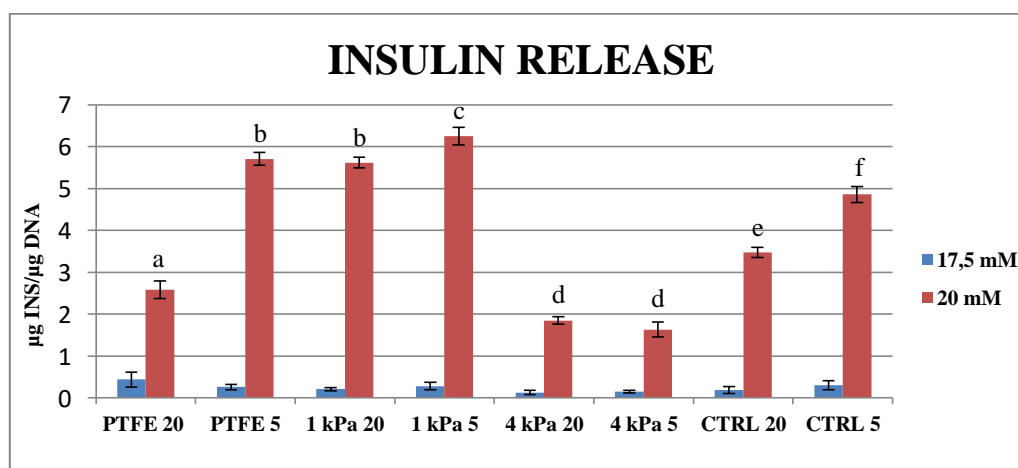
Histogram 16: **Insulin release in all experimental groups (Insulin release is expressed as mean value ± SD). Different superscripts indicate statistical differences among the samples (SPSS software, p≤0,05).**

4.3.3 Gene expression

4.3.3.1 Ki67 expression

The expression of Ki67 (gene that encode for a nuclear protein associated to the cellular proliferation) showed a balanced relation between cell death and proliferation in all experimental groups, compared to untreated fibroblasts (T0). This data showed us that cells did not increase their number maintaining a constant cell density during the differentiation (Histogram 17).
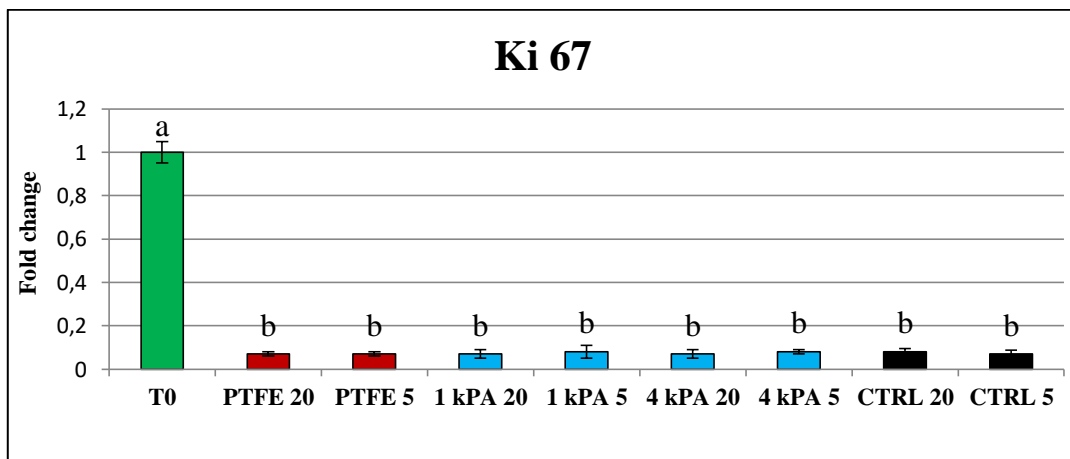
Histogram 17: Ki67 gene expression in all experimental groups.

4.3.3.2 Insulin expression

The results obtained showed that 1 kPa gel and PTFE system induced significant higher Insulin expression than plastic and 4 kPa gel, especially in low oxygen (Histogram 18). Furthermore, comparing the efficiency of the two systems tested, 1 kPa PAA gel ensured a higher Insulin transcription than PTFE.
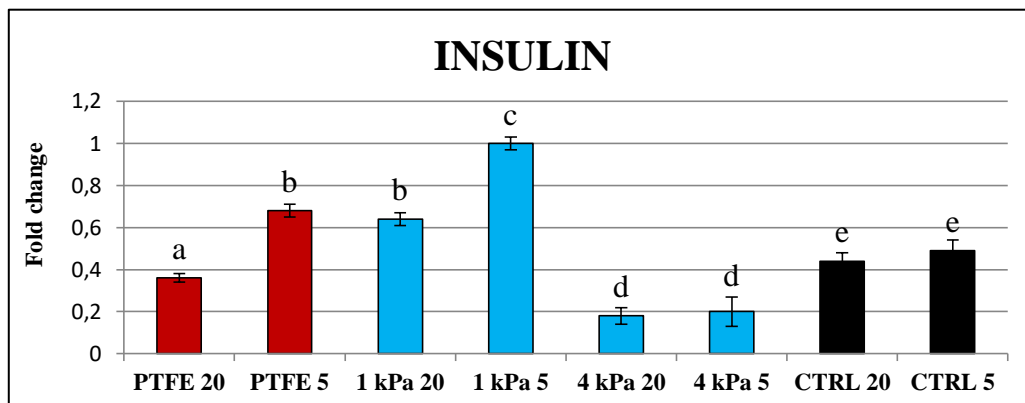
Histogram 18: Insulin gene expression in all experimental groups.

4.3.4 Immunocytochemistry

Immunocytochemistry analysis performed on the PAA gel samples confirmed the presence of three main pancreatic hormones (Insulin; Glucagon; Somatostatin), especially in 1 kPa soft gel maintained in both oxygen tensions (Figures 44-45).
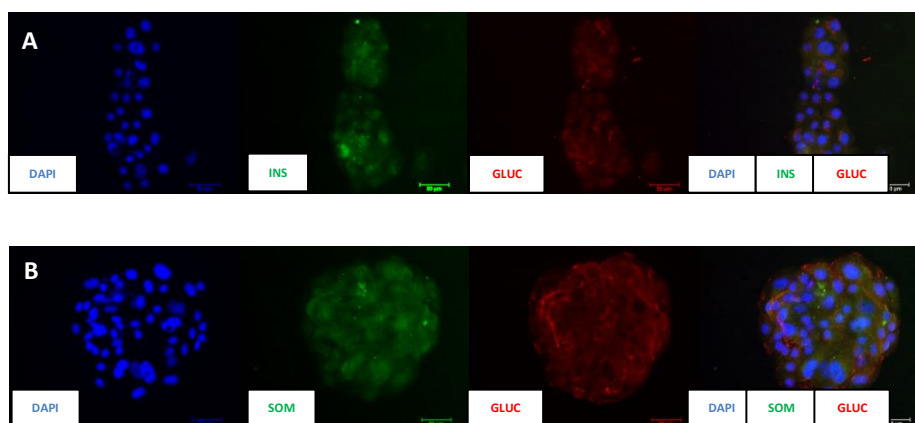
Figure 44: Immunostaining of cells differentiated on 1 kPa soft gel in 20% oxygen, day 10.
Clear signal of Insulin (green), Glucagon (red) and DAPI (blue) (A).
Immune-localization of Somatostatin (green), Glucagon (red) and DAPI (blue) (B).
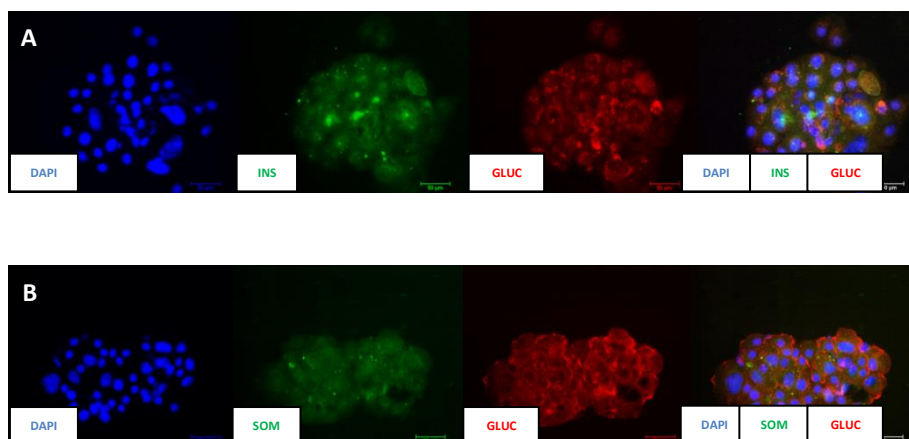
Figure 45: Immunostaining of cells differentiated on 1 kPa soft gel in 5% oxygen, day 10.
Clear signal of Insulin (green), Glucagon (red) and DAPI (blue) (A).
Immune-localization of Somatostatin (green), Glucagon (red) and DAPI (blue) (B)

The results obtained in the 4 kPa gel samples showed a significant lower immune-localization of these pancreatic markers compared to those visible in 1 kPa soft gel (Figures 46-47).
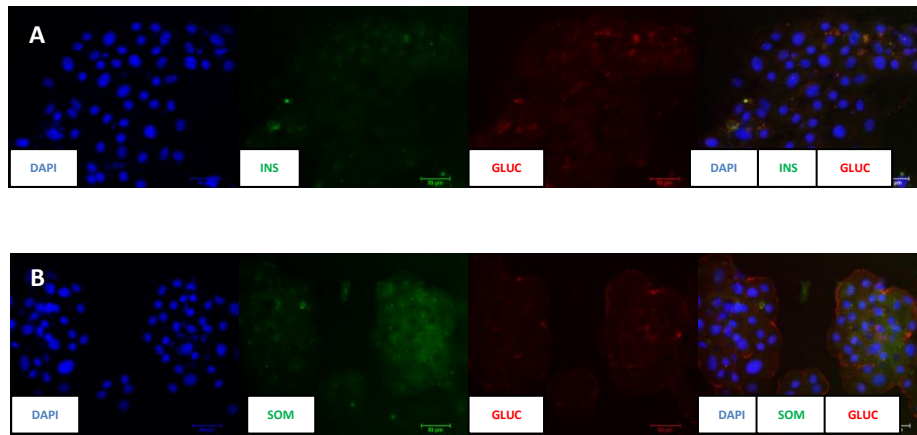
Figure 46: **Immunostaining of cells differentiated on 4 kPa soft gel in 20% oxygen, day 10.**
Signal of Insulin (green), Glucagon (red) and DAPI (blue) (A).
Immune-localization of Somatostatin (green), Glucagon (red) and DAPI (blue) (B).
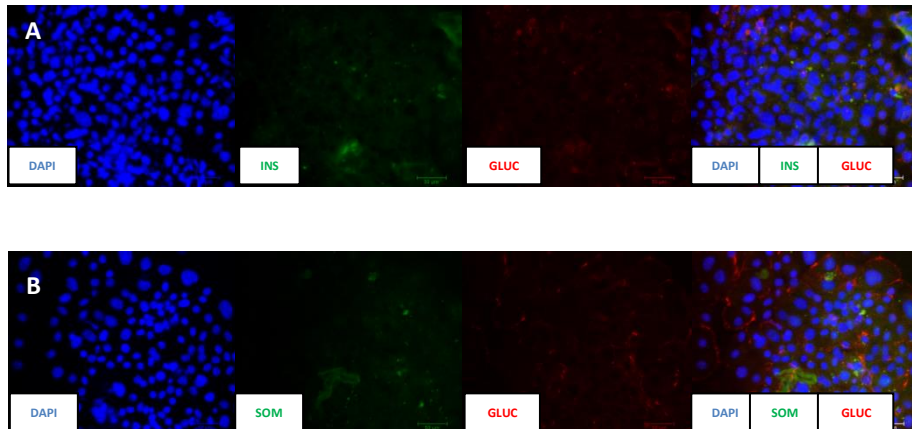
Figure 47: **Immunostaining of cells differentiated on 4 kPa soft gel in 5% oxygen, day 10.**
Signal of Insulin (green), Glucagon (red) and DAPI (blue) (A).
Immune-localization of Somatostatin (green), Glucagon (red) and DAPI (blue) (B).

4.3.5 Image analysis

Fluorescence intensity analysis for Insulin and Glucagon showed compartmentalization of the signal and increased mono-hormonality of the cells (Figure 48; Histogram 19). The increasing presence of cells able to produce only one pancreatic hormone indicates a high cell maturity and differentiation level and this represents one of the most eligible goal for the cell therapy.
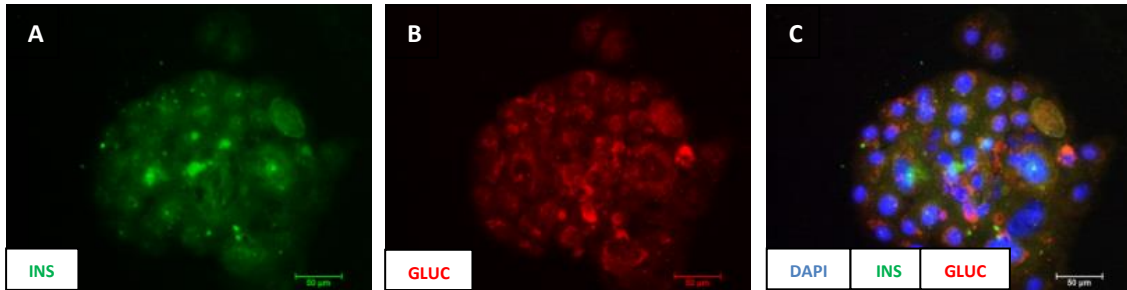
Figure 48: immunostaining of cells differentiated on 1 kPa soft gel in 5% oxygen, day 10. Immune-localization of Insulin (A), Glucagon (B) and merged picture of Dapi, Insulin and Glucagon (C).
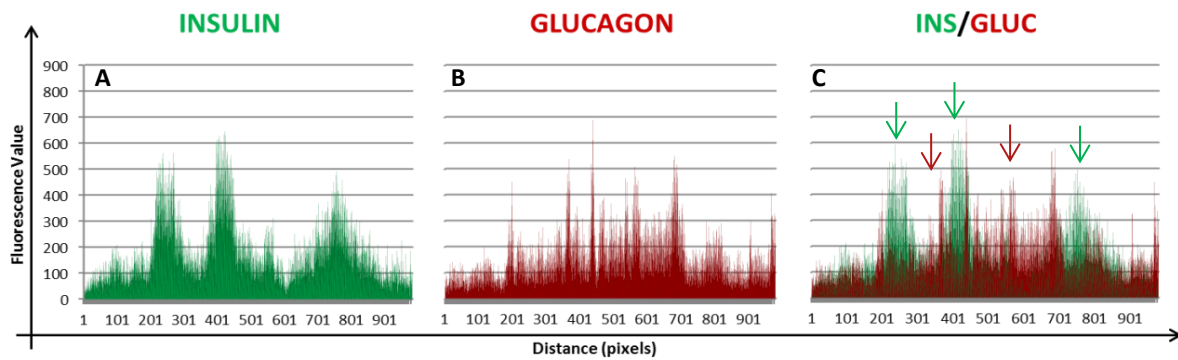
Histogram 19: fluorescence intensity analysis for Insulin (A), Glucagon (B) and Insulin/Glucagon (C).

Similarly, fluorescence intensity analysis for Somatostatin and Glucagon showed an analogue compartmentalization of the signal and increased mono-hormonality of the cells (Figure 49; Histogram 20), reinforcing the concept previously described in Insulin-Glucagon image analysis.
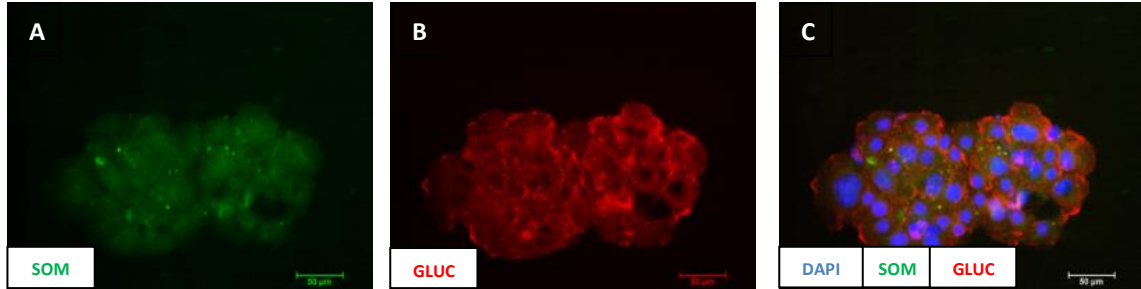
Figure 49: **immunostaining of cells differentiated on 1 kPa soft gel in 5% oxygen, day 10. Immune-localization of Somatostatin (A), Glucagon (B) and merged picture of Dapi, Somatostatin and Glucagon (C).**
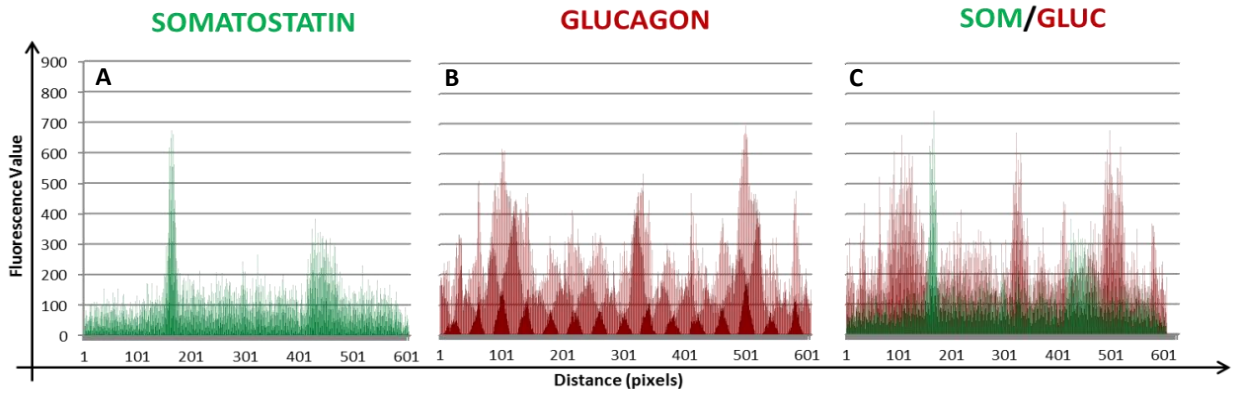
Histogram 20: **fluorescence intensity analysis for Somatostatin (A), Glucagon (B) and Somatostatin/Glucagon (C).**

4.4 The role of the pluripotency related gene Oct4 in the molecular mechanisms driving epigenetic conversion and phenotype switch

4.4.1 Section 1

4.4.1.1 In vitro functional assay of the Oct4 CRISPR/Cas9 vectors

To investigate whether our Oct4 CRISPR/Cas9 vectors were functional active, a dual fluorescent reporter system (C-Check), based on CRISPR/Cas9 induced-DNA double strand breaks in an episomal vector (Zhou et al., 2016), was used. In particular, two C-Check vectors (for human and murine Oct4 respectively) were generated, carrying synthetic Oct4 CRISPR/Cas9 target sites. The generated C-Check vectors were then transfected into HEK293T cells and results obtained led us to choose **T2** and **T3** CRISPR/Cas9 sgRNAs in human model (Figures 50-51) and **T2** and **T4** CRISPR/Cas9 sgRNAs in murine model (Figures 52-53).
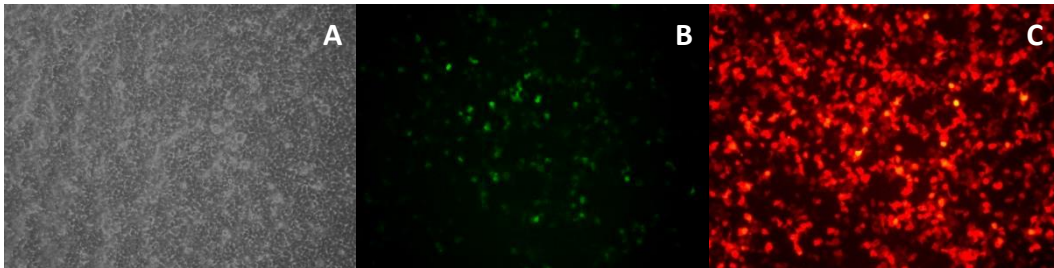
Figure 50: Oct4 C-Check vector with human T2 CRISPR/Cas9 sgRNAs into HEK293T cells (A): expression of Green Fluorescent Protein (B) and mCherry Fluorescent Protein (C).
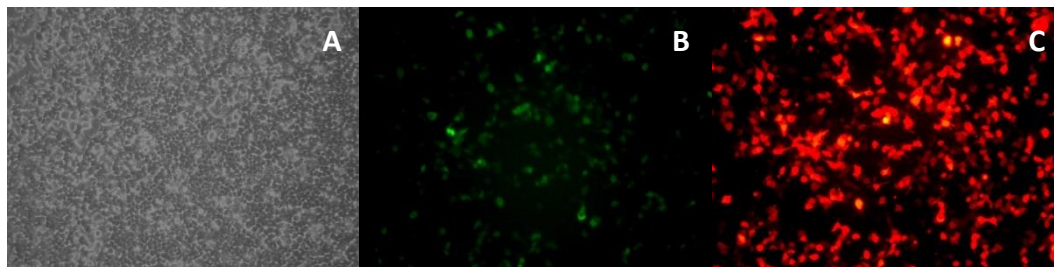
Figure 51: Oct4 C-Check vector with human T3 CRISPR/Cas9 sgRNAs into HEK293T cells (A): expression of Enhanced Green Fluorescent Protein (B) and mCherry Fluorescent Protein (C).
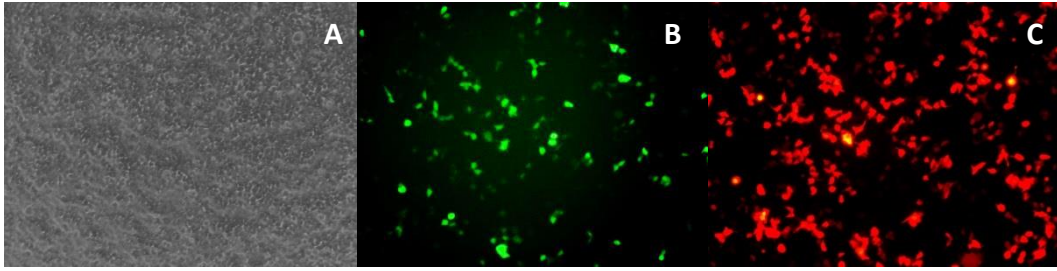
Figure 52: Oct4 C-Check vector with mouse T2 CRISPR/Cas9 sgRNAs into HEK293T cells (A): expression of Enhanced Green Fluorescent Protein (B) and mCherry Fluorescent Protein (C).
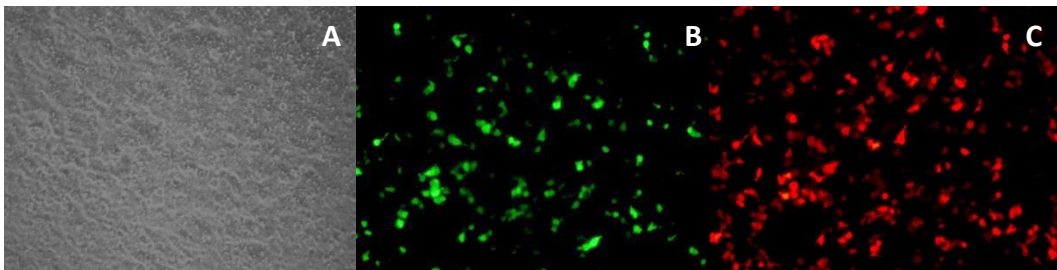
Figure 53: Oct4 C-Check vector with mouse T4 CRISPR/Cas9 sgRNAs into HEK293T cells (A): expression of Enhanced Green Fluorescent Protein (B) and mCherry Fluorescent Protein (C).

4.4.1.2 Oct4 KO fibroblasts generation

Once the most promising human and murine sgRNAs couples have been identified (T2-T3 in human and T2-T4 in murine model), fibroblasts were plated and, when these cells achieved about 70% confluence, *in vitro* validated CRISPR/Cas9 vectors were transfected into the cells, in order to generate Oct4 KO fibroblasts.

Then, 24 hours after, cell transfection effectiveness was tested through fluorescent analysis for GFP protein (included into vectors), marker of vector integration efficiency (Figures 54-55).
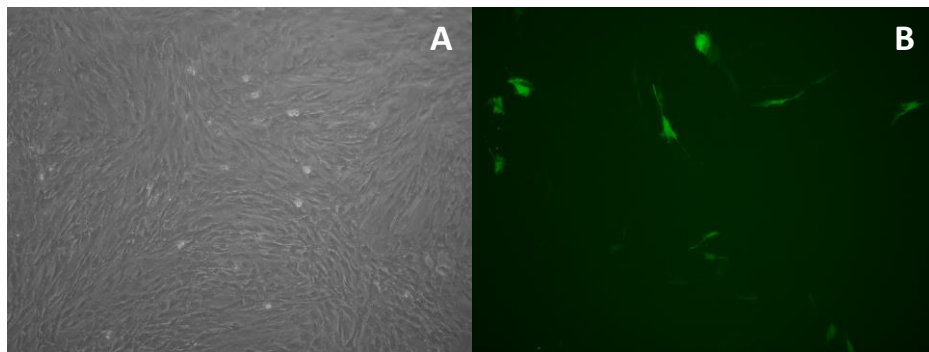
Figure 54: representative image of transfected mouse fibroblasts:
bright-field (A) and GFP positive cells (B).
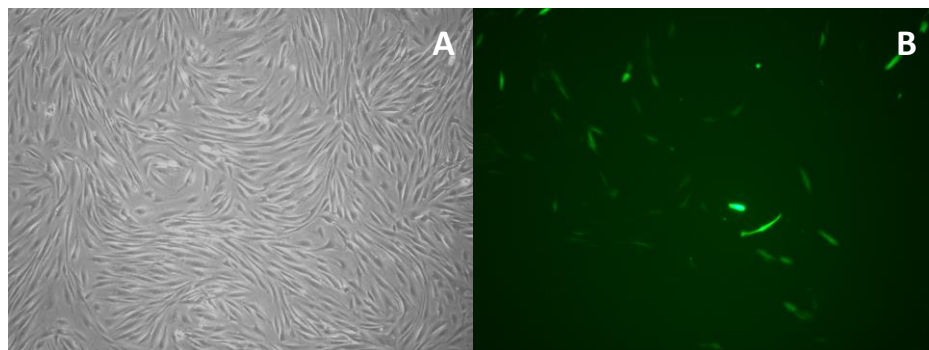
Figure 55: representative image of transfected human fibroblasts:
bright-field (A) and GFP positive cells (B).

The results obtained showed a GFP-positive cell rate equal to 15% in human model and 10% in mouse transfected fibroblasts (Figures 54-55), indicating that transfection efficiency was not very high.

Moreover, both human and mouse transfected cells showed a normal growth and their morphology was not different from untreated fibroblasts, with their typical elongated and spindle structure (Figures 56-57).
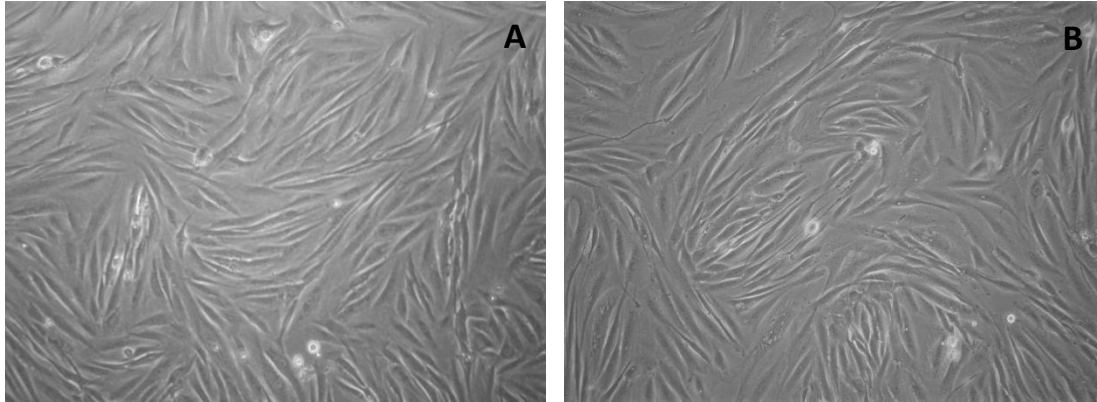
Figure 56: untreated human fibroblasts (A) and transfected human fibroblasts (B).
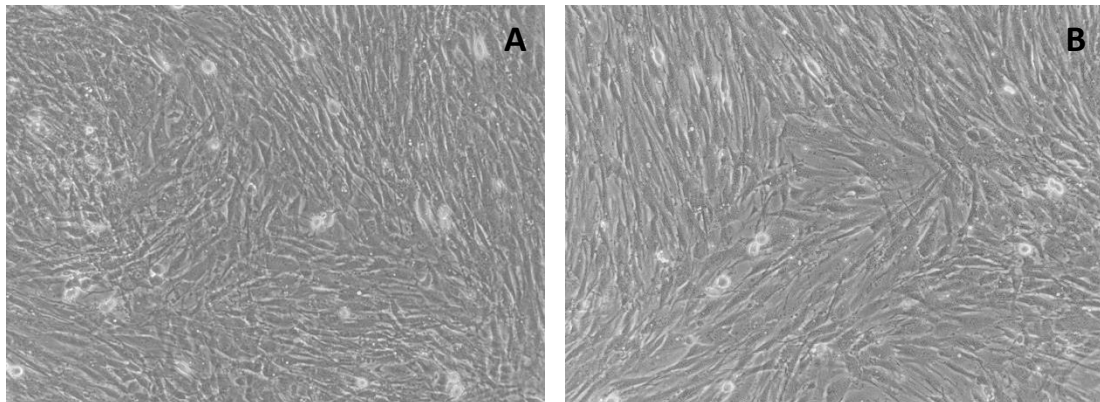
Figure 57: untreated mouse fibroblasts (A) and transfected mouse fibroblasts (B).

Then, Puromycin selection has been applied in order to generate pure Oct4 KO clones and to obtain pure KO cell lines. In particular, at the end of the selection process, eleven clones were obtained in the mouse altogether but, in contrast, selection carried out in human fibroblasts gave unsatisfactory results and cells suffered significantly during the puromycin selection.

Furthermore, FACS-based sorting has been applied also but, unfortunately, unsatisfactory results were obtained in both human and mouse samples.

4.4.1.3 Screening PCR after Puromycin selection (Mouse model)

At the end of the selection process, DNA extraction and amplification have been performed to genotype correct Oct4 KO clones. Firstly, the amplification of the whole Oct4 genomic area was performed and, subsequently, a further screening PCR was carried out, amplifying the T3–downstream genomic region, with the modalities described above (Section 3.4.1.4). Eleven clones were obtained in the mouse altogether, with varying yields. PCR screening demonstrated 2 clones (KO1; KO3), in particular, displaying a high KO cell rate, although a heterogeneous population also composed by WT cells still persist, as visible in Figures 58-59 (correct KO clones circled in red).

Figure 58: amplification of whole Oct4 genomic region in mouse treated cells.
Correct Oct4 KO clones are circled in red (~ 400 bp).

Figure 59: amplification of T3–downstream genomic region in mouse treated cells.
Correct Oct4 KO clones are circled in red.

4.4.1.4 Immunocytochemistry

The most promising KO clones (KO 1; KO 3) and WT mouse cell lines were subjected to the 5-azacytidine treatment, as previously described.

Immunocytochemistry analysis for Oct4 performed after 5-azacytidine treatment, showed the presence of this pluripotent-related marker in both mouse KO and WT samples, as visible in figures 60 and 61 respectively.
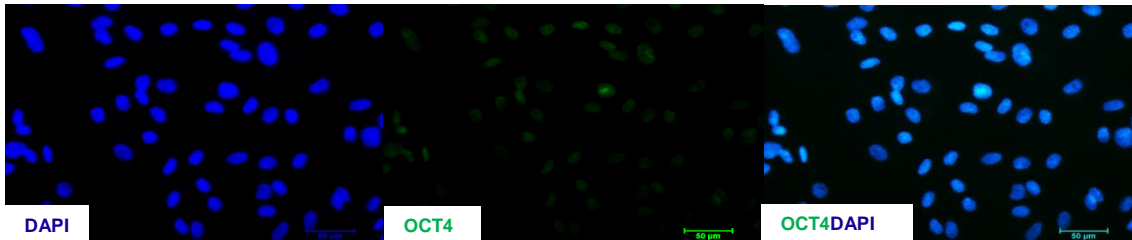
Fig.60: representative image of immunocytochemical localization of Oct4 in KO mouse fibroblasts (KO 1 clone).
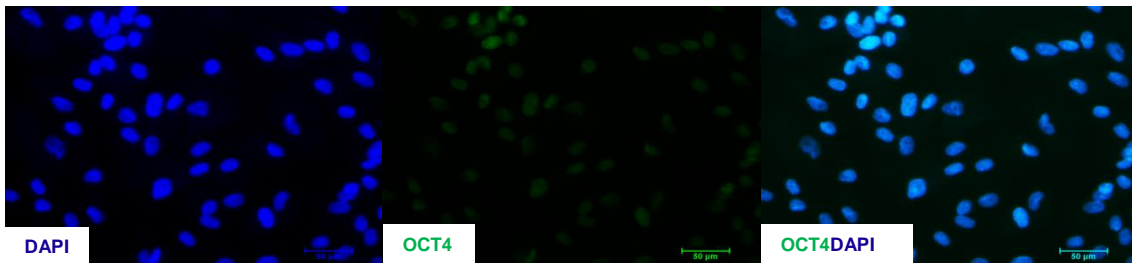
Fig.61: Representative image of immunocytochemical localization of Oct4 in WT mouse fibroblasts.

In particular, a rate of 86% ± 4,11 of cells expressing Oct4 was observed in WT mouse cell line (Figure 60). On the other hand, it was observed an Oct4 expression decrease in KO cells, with 65% ± 5,35 of cells expressing Oct4 (Figure 61), confirming the presence of a consistent KO cell population.

4.4.1.5 Screening PCR at the end of pancreatic induction process (Mouse model)

Both KO (KO 1; KO 3) and WT mouse cell lines were subjected to the pancreatic induction protocol, as previously described. Then, at the end of the differentiation process, DNA extraction and amplification have been performed to verify the maintenance of the Oct4 genomic region deletion in KO clones. The genotyping of WT lines was performed too, as a control.
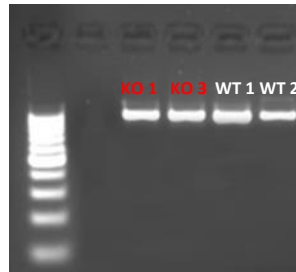
Figure 62: amplification of whole Oct4 genomic region in KO and WT mouse samples.
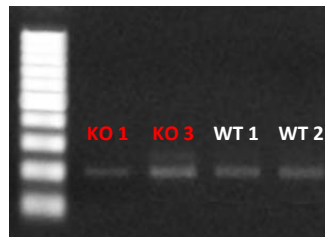
Figure 63: amplification of T3–downstream mouse Oct4 genomic region
in KO and WT mouse samples.

The results obtained displayed the persistence of Oct4 genomic sequence positivity in KO clones, showing a genomic profile comparable to WT cells. In particular, DNA amplification of both whole Oct4 and T3-downstream sequences displayed that a small number of non-KO cells probably escaped the selection and actively proliferated, outnumbering the KO ones (Figures 62-63).

4.4.2 Section 2

4.4.2.1 Oct4 KO fibroblasts generation

In this second experiment, human and mouse fibroblasts were plated both on plastic and on 1 kPa PAA gels. When these cells achieved about 70% confluence, cells were transfected with the same modalities previously described but, in this case, all four *in vitro* validated CRISPR/Cas9 vectors (T1-T2-T3-T4) were used.

No significant morphological differences were observed between cells plated on plastic and on 1 kPa PAA gel, as visible in figures 64 and 65.
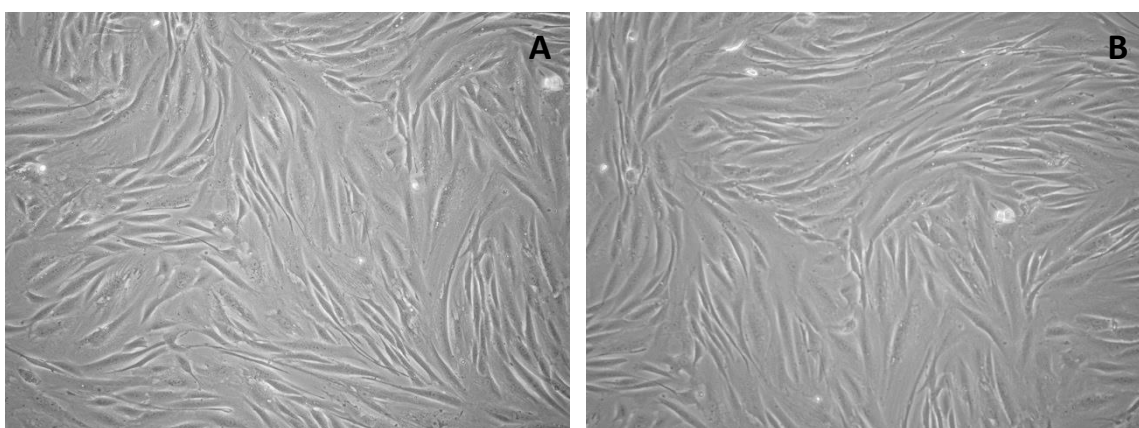
Figure 64: human fibroblasts plated and transfected on plastic (A) and on 1 kPa PAA gel (B).
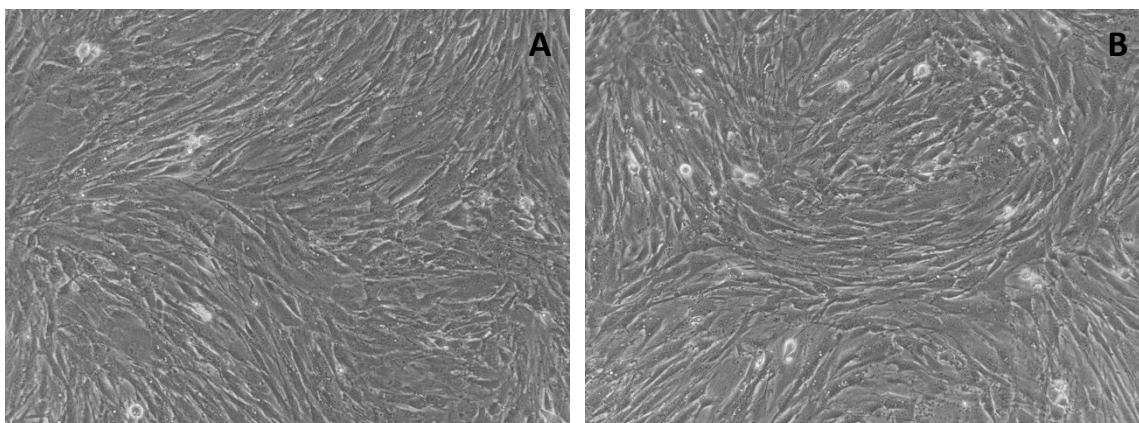
Figure 65: mouse fibroblasts plated and transfected on plastic (A) and on 1 kPa PAA gel (B).

Then, 24 hours after trasfection, fluorescent analysis for GFP protein were performed to quantify GFP-positive cell percentage and, as a consequence, to test the transfection effectiveness (Figures 66-67).
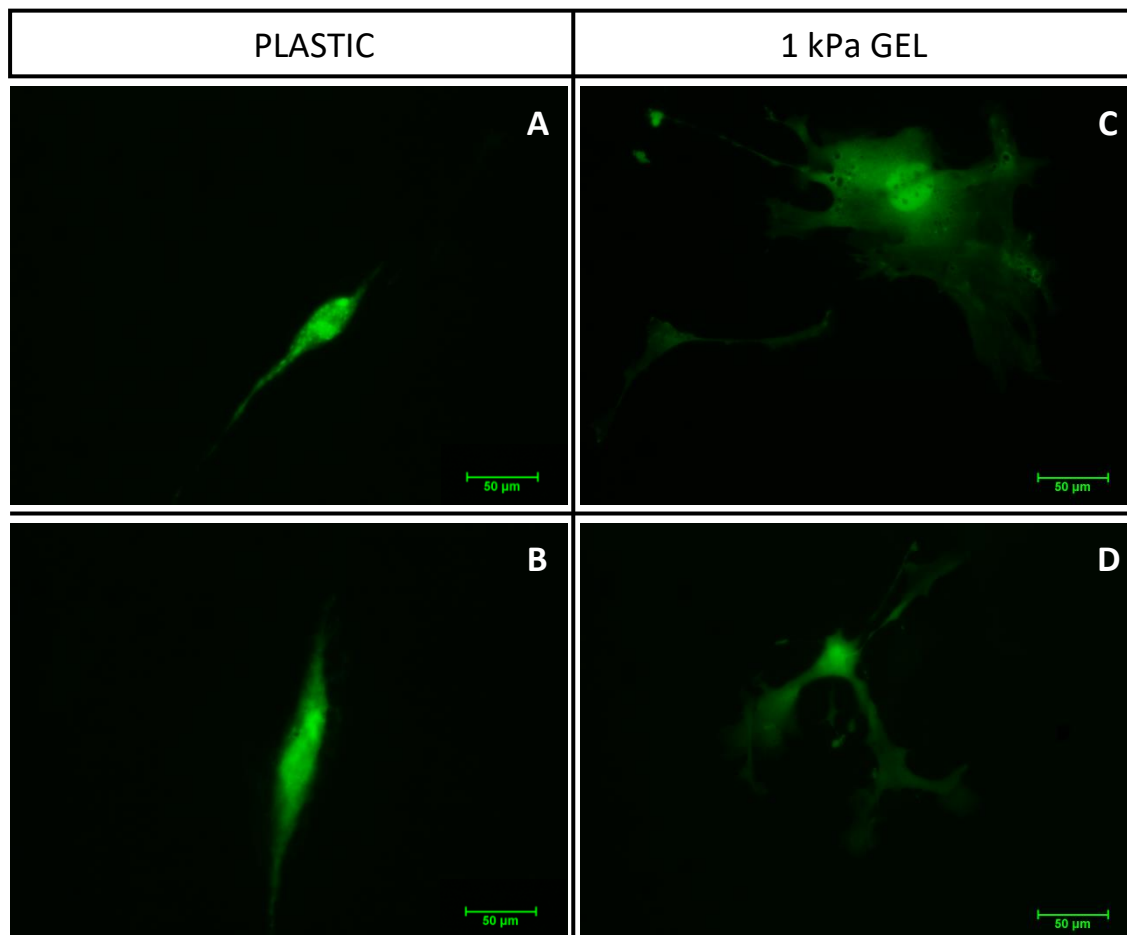
Figure 66: GFP expression in human transfected fibroblasts plated on plastic (A, B) and on 1 kPa PAA gel (C,D).
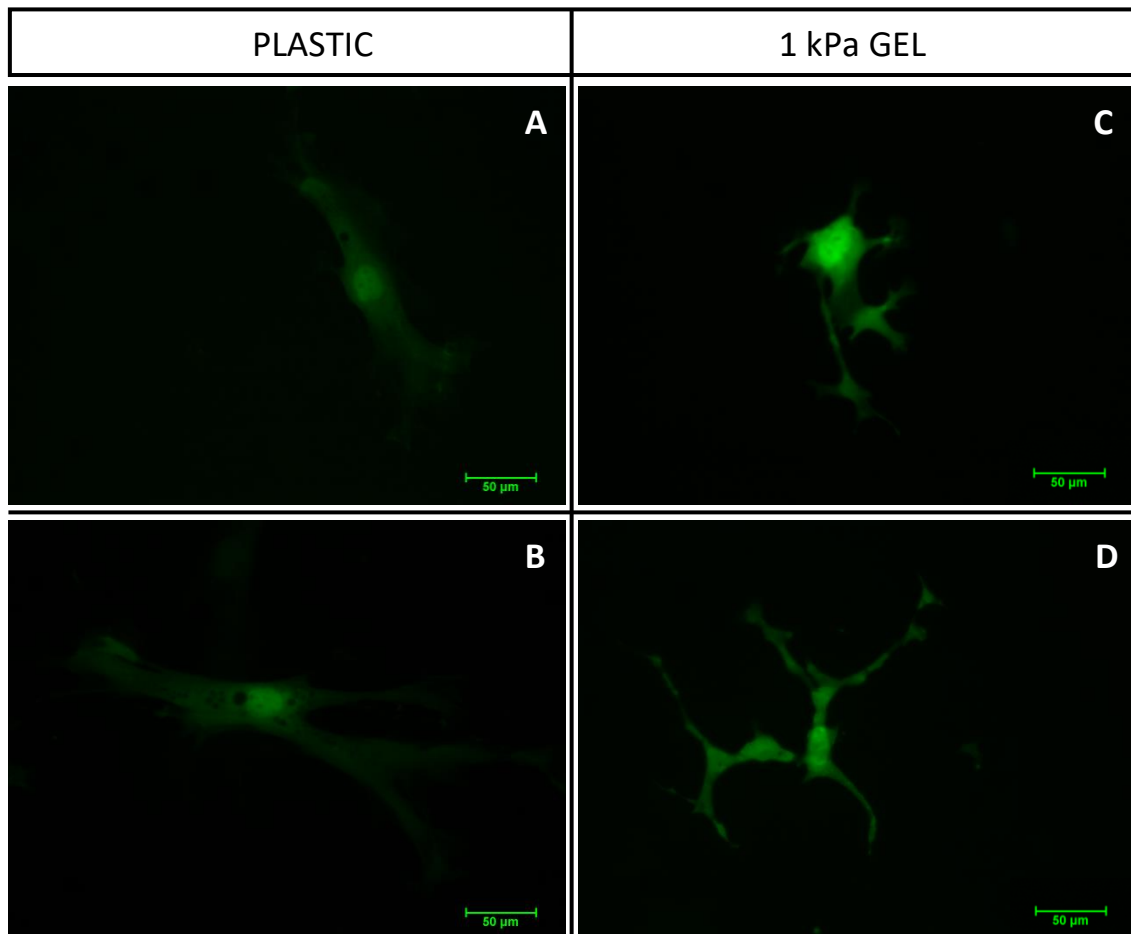
Figure 67: GFP expression in mouse transfected fibroblasts plated on plastic (A, B) and on 1 kPa PAA gel (C, D).

Fluorescence analysis displayed a very few number of fibroblasts expressing the GFP signal, with a positive cell rate equal to 2% in human and 3% in mouse transfected fibroblasts (Figures 66-67), indicating that transfection efficiency was too low in all samples.

However, an interesting aspect observed is related to the particular and unconventional morphology acquired by both mouse and human transfected fibroblasts maintained on 1 kPa gels. As a matter of fact, the few GFP-positive cells present in culture after treatment displayed a peculiar and "dendritic-like morphology" (Figures 66C, 66D, 67C and 67D). On the other hand, transfected fibroblasts cultured on plastic maintained their morphology, as visible in pictures 66A, 66B, 67A and 67B.

Then, Puromycin selection has been applied in all transfected samples but, unfortunately, it was impossible to establish a pure KO cell line because of the insufficient number of transfected cells remained viable after selection. Thus, these cells were not able to proliferate and increase their number and, for this reason, it was impossible to apply the pancreatic differentiation process.

CHAPTER 5:
DISCUSSION

5. DISCUSSION

5.1 Assessment of the optimal oxygen tension to be used in culture

It is generally accepted that mammalian development occurs at very low oxygen levels prior to the onset of blood circulation (Simon & Keith, 2008). Therefore, it is reasonable to expect that pancreatic development take place at similar low oxygen concentrations, at least until the advent of blood flow in the organ (Colen et al., 1999).

The results obtained in this thesis showed that oxygen plays a role in the epigenetic conversion and pancreatic induction process. This is particularly evident in cells derived from NOD mice, which are physiologically predisposed to the onset of diabetes, and whose cells differentiated in 20% oxygen only and were unable to adapt a pancreatic phenotype in low oxygen conditions, that resulted highly unfavorable for their viability. As a matter of fact, NOD derived cells suffered and died by day 3 in low oxygen (Figure 24, Histogram 1). On the other hand, if moved to 5% of oxygen, after differentiating in normal oxygen conditions, they remained viable for up to four days even in hypoxic conditions, although with a decreased ability to release Insulin (Histogram 4).

Despite many reports describe a major influence of oxygen on pancreatic islet survival and function (Carlsson et al., 2003; Carlsson et al., 2002; Chase et al., 1979; Kazzaz et al., 1999; Ko et al., 2008; Papas et al., 1996), the first systematic study on oxygen participation in the development of the pancreas was available only recently (Fraker et al., 2007). This may be due to the inability of standard culture methods to deliver oxygen in a physiological way, which has complicated the appropriate design of *in vitro* studies. This limitation was finally overcome with the development of novel culture vessels (perfluorocarbon-based culture devices - PFC/Si) designed to maintain relatively constant oxygen levels throughout cellular aggregates (Fraker et al., 2007). In particular, Fraker et al. performed their experiments on mouse pancreatic buds demonstrating that enhanced oxygenation (either 21% or 35% O_2) promotes cell differentiation *in vitro* (Fraker et al., 2007). Besides, this study shows that high oxygen enhances pancreatic bud growth, without oxygen-related stress, promoting endocrine over exocrine differentiation. In addition, the gene expression profile of pancreatic buds cultured in this way was largely indistinguishable from that observed during normal native

development at corresponding time points, suggesting that physiological oxygenation is critical for appropriate pancreatic development (Fraker et al., 2007). Furthermore, a recent study carried out on human Induced Pluripotent Stem Cells (hiPSCs) and on mouse Embryonic Stem Cells (mESCs) (Hakim et al., 2014) demonstrates that a very high oxygen tension (60% O_2) facilitates and promotes the differentiation of these cells into Insulin-producing cells and into endocrine progenitors with a clear expression increase of several early pancreatic markers (SOX17, FOXA2, PDX1, Ngn3). In line with this, Shah et al. showed that enhanced oxygen delivery after the inflow of blood would seem a likely permissive factor for initiating differentiation, with endocrine areas that ended to be more highly oxygenated (Shah et al., 2011). Similarly, subjecting pregnant rats *in vivo* to a hypoxic environment, by gradually decreasing the O2 level from 21% to 8%, indicated that pancreatic cell differentiation depends on cell oxygenation, with hypoxia decreasing β-cell development (Heinis et al., 2010). This is not limited to rodents, since Heinis et al. showed that mouse and human fetal pancreases displayed very few β cells at 3% O_2. Altogether, these data demonstrate that hypoxia decreases β-cell differentiation in a conserved manner between rats, mice and humans (Heinis et al., 2012) and highlight the importance of high oxygen conditions during the pancreatic differentiation process. These results are fully in agreement with those obtained in our NOD model, where cells need high oxygen levels to complete their differentiation and, to this regard, we may hypothesize that this may be due to the high metabolic consumption required by late stages of pancreatic differentiation. Then, further studies are required in order to elucidate this aspect and, to this regard, a distinct possibility could be related to the evaluation of the involvement of HIF signaling pathway, which has been described to have a key role during β-cell differentiation (Heinis et al., 2012; Fraker et al., 2009; Diez et al., 2007; Czech, 2006; Gunton et al., 2005; Levisetti & Polonsky, 2005; Pugh & Ratcliffe, 2003).

Interestingly, these results are likely to be specific for pancreatic differentiation since other differentiation processes require hypoxia condition in order to increase cell proliferation, viability and functional efficiency. A clear example is represented by the Neural Crest Stem Cells (Morrison et al., 2000), Hematopoietic Cells (Adelman et al., 1999; Cipolleschi et al., 1993; Parmar et al., 2007), Bone Marrow Mesenchymal Stem Cells in rats (Lennon et al., 2001) and ESCs cultures (Ezashi et al., 2005; Harvey et al., 2004). There is no clear explanation for this point and further experiments are

mandatory in order to clarify the relation between oxygen specific concentration and distinct differentiation pathway. In addition, the data obtained in the present project, show a completely different trend when C57 BL/6J and CD-1 mice cells were used. In both populations, differentiation to Insulin secreting cells succeeded regardless to oxygen tensions. In detail, C57 BL/6J cells maintained in low oxygen conditions ensured a significantly higher Insulin release than those cultured in 20% oxygen tension (Histogram 3). CD-1 cells were able to release higher but not significantly different Insulin amount in low oxygen, compared to those differentiated in high oxygen (Histogram 3). Therefore, oxygen did not appear to be a critical point for the success of the differentiation process in both cells types. These results disagree with those described above, however are in line with a recent study aimed to the *in vitro* differentiation of umbilical cord blood mesenchymal stem cells (UCB-MSCs) into Insulin producing cells, where hypoxia was shown to effectively direct MSCs differentiation into early β-cell progenitors and endocrine cells (Sun et al., 2015). Similarly, adipose derived MSCs (AMSCs) differentiated in 3% oxygen displayed an increased number of cells expressing pancreatic transcription factors (Yoo et al., 2014). Altogether, these data suggest that genetic background and cell type may have a profound effect in the responses to local oxygen concentrations. Cell type-specific behavior can be appreciated along differentiation processes and, more in particular, during the *in vitro* pancreatic induction process, in the presence of different oxygen tensions.

5.2 Establishment of the best glucose concentrations leading to cells that respond to glucose variations in a physiological way

In healthy physiological condition, blood glucose is tightly maintained from 5.5 mM for fasting to 7.0-8.5 mM after eating (Ceriello & Colagiuri, 2008). However, most of the protocols used *in vitro* (Shi et al., 2005; D'Amour et al., 2006; Zhang et al., 2009; Rezania et al., 2012; Pennarossa et al., 2013; Pagliuca et al., 2014;) differentiate cells in high glucose concentration (17.5 mM), which is a very un-physiological condition but results in good efficiency. It must be considered, however, that high glucose has been shown to be deleterious for Stem Cells functions and a cause of abnormalities during embryogenesis, due to an increase of oxidative stress and a reduction in inositol levels, one of the principal second messenger of Insulin action (Wentzel et al., 2001). Based on these considerations, we attempted to test the effect of physiological glucose concentration on endocrine pancreatic cell differentiation, converting human and mouse fibroblasts both in 5.5 mM (fasting blood glucose level) and in 8.5 mM (blood glucose level after meal). The results obtained showed that glucose plays a major and specific role in pancreatic endocrine cell development. Cells maintained in low glucose levels were not able to differentiate into insulin producing ones and displayed an unusual thin and elongated morphology, arranging in a distinct parallel pattern and peculiar structure (Figure 33). Moreover, immunocytochemical analysis showed a positive expression of Insulin and C-Peptide in cells differentiated in standard high glucose levels but not in those cultured in hypoglycemic conditions (Figure 35), that were unable to respond to 20 mM glucose challenge (Histogram 5). This finding is in agreement with a study showing that glucose is necessary for embryonic pancreatic endocrine cell differentiation and development, as well as crucial for both α and β cell specification (Guillemain et al., 2007). In addition, previous studies demonstrated that high glucose promotes β-cell replication *in vitro* and *in vivo* at the 20 to 30-mmol/l concentration (Bonner-Weir et al., 1989). Those observations have been supported by Zalzman et al. that demonstrated that high glucose (25 mmol/l) culture of immortalized PDX1-expressing human fetal hepatocytes promoted the production, storage and release of Insulin in a regulated manner (Zalzman et al., 2003). Expression pattern of early pancreatic markers also showed an alteration in low glucose samples (Histogram 6). In

125

particular, hypoglycemic conditions appeared to increase the levels of expression of crucial early pancreatic genes, such as FOXA2, HNF4 and PDX1, that were stimulated by low glucose levels and prematurely turned on. When analysis was performed at later days, FOXA2 and HNF4 marker continued to be overexpressed in low glucose cells whereas, PDX1 was transcribed at higher level in high glucose concentration. Although it is difficult to explain these results, it must be noted that during embryonic life, the final number of β-cells will depend on the proliferation of early PDX1-positive pancreatic progenitor cells (Bernard et al., 1999; Bhushan et al., 2001) and it is possible low glucose may cause a wrong timing in the regulation of transcription. In support to this observation, Cao et al. demonstrated that high glucose is necessary for complete maturation of PDX1-VP16-Expressing Hepatic Cells into functional Insulin-Producing Cells (IPCs) (Cao et al., 2004). Interestingly enough, even limiting the use of low glucose concentration to the early step of differentiation (day 1-9), cells were unable to complete the process, began to detach from the growth support around day 23 (Figure 36) and died. This is in agreement with a study demonstrating that culture in low-glucose concentrations induced apoptosis in pancreatic β-cells (Van De Casteele et al., 2003).

One key point, in our understanding is represented by the conditions adopted during the isolation and establishment of primary cultures. According to our findings, when cells - both of human and murine origin - were directly isolated in low and physiological glucose concentrations they successfully differentiated in these conditions as well as in high glucose. The initial environment appeared to set cell future behavior and response to glucose levels, with cells being able to adapt to similar or higher concentrations (LG/LG; LG/HG; HG/HG), but unable to cope with lower glucose ones (HG/LG). Altogether this suggests that pancreatic differentiation efficiency was mainly affected by the starting derivation conditions, regardless to the glucose conditions used during the endocrine pancreatic induction process.

Cell ability to respond to glucose stimulation, requires the presence of specific receptors, namely the Class I Glucose Transporters family (GLUT 1-4), that are intrinsic membrane proteins which differ in tissue-specific expression and response to metabolic and hormonal regulation (James D.E, 1994; Muekler, 1994). Consistent with the results described above, the widely expressed isoform GLUT1 was detected in all experimental groups (Histogram 10) indicating that converted cells procure their basal glucose

requirement through a canonic pathway (Muekler, 1994). These data are in agreement with previous works, indicating that GLUT1 is ubiquitously expressed in adult mammalian cells and tissues (Cura & Carruthers, 2013; Mueckler, 1994; James D.E, 1994; Gould & Holmant, 1993; Hudson et al., 1992; Piper, R.C. et al., 1992; Asano, T., et al, 1992; Bell & Lin, 1990). GLUT2 expression was detected in all samples as well, with a significantly increased level in samples isolated in high glucose (HG/HG and HG/LG; Histogram 11). This gene represents the major glucose transporter isoform expressed in pancreatic β-cells, hepatocytes, kidney and absorptive epithelial cells of the intestinal mucosa (Cura & Carruthers, 2013; Mueckler, 1994; Tal et al., 1992; Thorens, 1992; Thorens et al., 1988; Fukumoto et al., 1988). Its presence in converted fibroblasts confirms that cells underwent an epigenetic switch and adopted a pancreatic phenotype, and is in line with previous works that demonstrated GLUT2 gene expression in ESCs differentiated toward the pancreatic lineage (Pilar Vaca et al., 2006; T León-quinto et al., 2004). In contrast to these results, the expression of GLUT3 was not detected in any of the samples. However this is not surprising since the transporter is distinctive of brain, placenta, spermatozoa and testis membranes, where it is strongly expressed (Haber et al., 1993).

Finally, the data obtained in relation to the expression of GLUT4 appear very intriguing. This gene was significantly up-regulated in LG/LG and LG/HG groups, while a negligible expression was observed in HG/HG; HG/LG samples (Histogram 12). GLUT4 is expressed specifically in muscles and fat tissues and is classically referred to as the "Insulin-responsive" transporter (Kraegen et al., 2010; Slot et al., 1991). In our understanding, it is therefore not surprising that the highest GLUT4 expression levels were detected in those groups that showed the highest Insulin release (LG/LG and LG/HG) and suggest the possibility that the high concentration of available insulin may result in the induction of this hormone responsive transporter. Even more interesting is the observation that up-regulation of GLUT4 was detected in the groups where lower GLUT2 expression was measured. (LG/LG and LG/HG), Although, further evidence is required, we may hypothesize the occurrence of a switch between these two glucose transporters in cells isolated in low glucose.

5.3 Use of 3-D systems and evaluation of mechanosensing related responses

In vivo, cells are surrounded by a 3-D organization, mainly composed by extra cellular matrix and neighboring cells (Geckil et al., 2010) but, traditionally, tissue culture has been dominated by growing cells as monolayers. While these 2-D systems are well documented and have enabled approaches to understanding individual cellular phenomena, they lack the ability to reproduce the morphology, 3-D architecture and some biochemical features of cells in the original tissue. In order to create an *in vitro* environment that attempt to replicate a "closer-to-*in vivo*" behavior, cells may be cultured on matrices and scaffolds (Ravi, Kaviya, & Paramesh, 2016). Several studies demonstrated the positive effect of more physiological culture surfaces. For instance, the use of substrates approximating to the elastic moduli of brain (0.1 kPa), pancreas (1.2 kPa), cartilage (3 kPa), muscle (8 to 17 kPa) and bone tissue (40 kPa), could directly drive stem cells to differentiate into neurogenic, pancreatic, chondrogenic, myogenic and osteogenic lineages, respectively (Engler, Sen, Sweeney, & Discher, 2006; Narayanan et al., 2014; Wang, Lai, Han, Tong, & Yang, 2014). The results obtained in the present work indicate that matrix elasticity may have a profound influence on the epigenetic conversion and endocrine pancreatic differentiation process. In particular, significantly higher Insulin transcription and release were visible in cells differentiated on 1 kPa gel and in PTFE systems (Histograms 16, 18). These data are consistent with recent studies that demonstrated the ability of stiffness substrate to influence cell fate and differentiation, and suggested that soft gel systems may enhance cell differentiation towards the endodermal lineage (Candiello et al., 2013; Jaramillo et al., 2015; Richardson et al., 2014), providing a biologically active environment for the cells to proliferate, differentiate and secrete cell specific components (Schellenberg et al., 2014). The significant and positive impact of the use of a correct stiffness and an adequate 3D environment, is also demonstrated by the results obtained with immune-cytochemical analysis. The latter showed a compartmentalization of the signals for Insulin, Glucagon and Somatostatin, demonstrating an increase in mono-hormonality of the cells that were differentiated on 1 kPa PAA gel (Figures 48-49; Histograms 19-20). Interestingly, hormone compartmentalized localization is considered to be suggestive of a mature phenotype and to be distinctive of terminally differentiated cells (Bocian-Sobkowska et al., 1999; Riopel et al., 2014). Similarly, it has been observed that

primitive endocrine cells, typical of early fetal stages, co-express Insulin and Glucagon, while they mature into a mono-hormonal phenotype later in development (Piper et al., 2004; Polak et al., 2000). Our results demonstrate that soft substrates significantly boost the acquisition of a mono-hormonal phenotype, supporting the possibility that epigenetic conversion lead to the derivation of matutre pancreatic, differentiated cells. This observation is consistent with recent studies that demonstrated hydrogel encapsulation system ability to enhance cell differentiation towards the endodermal (Candiello et al., 2013; Jaramillo et al., 2015; Richardson et al., 2014) and the pancreatic lineage (Davis et al., 2012; Jin et al., 2013; Mason et al., 2009; Niknamasl et al., 2014).

Altogether, these data suggest that the use of an appropriate substrate has a general effect on the differentiation of epigenetically erased fibroblasts and indicate a positive impact both on conversion efficiency and on the promotion of a mono hormonal population.

5.4 The role of the pluripotency related gene Oct4 in the molecular mechanisms driving epigenetic conversion and phenotype switch

Epigenetic conversion is a very powerful and promising technique for regenerative medicine. A thorough understanding of the mechanism driving the applied process, is however indispensable when potential applications in clinical management of disease are hypothesized. In order to understand the role of the pluripotency marker Oct4 in the experiment discussed in the present section, we used CRIPR-Cas 9 to delete its genomic region and investigate the result of its KO. In particular, the idea behind this, was to determine whether pluripotency was required for the epigenetic conversion process. The results obtained, although encouraging, were not completely satisfactory. In particular, we obtained a rather low transfection efficiency, especially in the human model. On the other hand, mouse KO cell lines were obtained however, after 5-aza CR treatment, no significant reduction in Oct4 expressing cell percentage was observed. (Figures 60-61). A possible explanation may be that a small number of non-KO cells escaped the selection and actively proliferated, outnumbering the KO ones. This is supported by the observation that, at the end of the experiments, DNA analysis indicated the persistence of Oct4 genomic sequence positivity (Figures 62-63), showing a comparable genomic profile in KO and WT cells.

Presently, there is a paucity of reports related to pluripotency gene deletions obtained through CRISPR/Cas9 approach and we find it difficult to understand the data we generated. Indeed, only very recently Fogarty el al. focused on the use of editing to investigate the function of the pluripotency transcription factor Oct4. However this was applied to human and mouse embryos and did not involve somatic cells (Fogarty et al., 2017), making it difficult to translate their results to our models.

Even the use of a 3D culture system that would improve cell behavior and responses led to a very few number of KO fibroblasts (Figures 66-67), indicating a limited and inadequate transfection efficiency. Based on these results, it was impossible to establish a pure KO cell line and apply the pancreatic differentiation process. An interesting aspect observed is related to the particular and unconventional morphology acquired by both mouse and human transfected fibroblasts maintained on soft gels, that displayed a "dendritic-like morphology" (Figures 66C, 66D, 67C and 67D). This observation is

fully in agreement with several works, affirming that fibroblasts plated on a 3-D collagen matrix formed dendritic extensions (Grinnell et al., 2003; Jiang and Grinnell, 2005; Rhee et al., 2007; Rhee, 2009). In particular, Grinnell et al. observed a new type of "normal" fibroblast morphology and cells projected and retracted a dendritic network of extensions, developeing the appearance of neuronal cells. These morphological changes in fibroblasts were not observed in our previous experiments dedicated to elucidate the role of mechanosensing in epigenetic conversion (see section 3), where mouse cells, plated and differentiated on PAA gels, displayed a classical morphology, typical of fibroblasts and with no indications of neural related changes (Paragraph 4.3.1.2; Figure 42). A possible interpretation about these two different cell behaviors may be related with the confluence used in the experiments. As a matter of fact, the confluence obtained after Puromycin selection is significantly lower than that typical of protocols focused on the use of matrices (Figures 42; 66-67).

In conclusion, although still preliminary, the results obtained support the possibility to establish CRISPR/Cas9 mediated Oct4 gene knock out models. It is undeniable that further experiments are mandatory to optimize genome editing efficiency and, eventually, determine whether and how Oct4 deletion may interfere with the acquisition of 5-Aza-CR induced high plasticity. Furthermore, it will be necessary to understand whether the expression of Oct4 during the brief "high plasticity stage" induced by 5-Aza CR is required for the success of the differentiation process. Data obtained in parallel in our laboratory strongly point to the key role played by the TET proteins and advocate the involvement of specific families of histones, suggesting an even more complex scenario coupling pluripotency, epigenetic erasing and mechano-sensing responses.

CHAPTER 6:
CONCLUSIONS

6. CONCLUSIONS

The data reported in the present thesis reveal some of the mechanisms driving the epigenetic conversion of mammalian fibroblasts into Insulin-secreting cells. These results, beside their impact in the understanding of cell fate commitment and differentiation, may find useful application to increase yield and improve conditions to be applied during cell phenotype switch. In particular, we demonstrated that using culture environments that closely resemble the physiological milieau we may favorably influence the process and improve scaling up. Firstly, our data provides evidence that oxygen plays a role. However, a distinct response is detected in relation to the strain used in the experiments, suggesting that genetic background has a profound effect on the role of oxygen during the *in vitro* differentiation process. This reflects a different susceptibility to the diabetic disease of the strains used in the experiments and it needs to be further expanded with researches applied to the human. Even more intriguing are the data generated with the experiments where we used different concentrations of glucose. These indicated that efficiency was not strictly dependent on the concentration used during differentiation, but, rather, by the conditions applied at cell isolation from the original tissue and early culture. A key point is also represented by the use of 3-D culture systems that match the stiffness typical of the original tissue. In particular, the results achieved suggest that the use of a soft substrate (mimicking pancreatic Young's modulus) was able to increase differentiation and favored the acquisition of a mature pancreatic phenotype, distinctive of terminally differentiated cells. Last but not least, the parallel and pioneer experiments, focused on genome editing of fibroblasts through the CRISPR/Cas9 technique, although still preliminary, appear very promising. They will be further developed in order to clarify the role of the pluripotency master gene Oct4 in epigenetic erasing and the control of somatic cell plasticity.

Altogether, the evidences here presented demonstrate that the use of appropriate and physiological conditions greatly promote cell differentiation and boost efficiency. The thorough understanding of the mechanisms involved are mandatory both for fundamental research as well as for regenerative medicine applications.

CHAPTER 7:
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7. REFERENCES

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Zhou, Y., Liu, Y., Hussmann, D., Br??gger, P., Al-Saaidi, R. A., Tan, S., … Luo, Y. (2016). Enhanced genome editing in mammalian cells with a modified dual-fluorescent surrogate system. *Cellular and Molecular Life Sciences*, *73*(13), 2543–2563. https://doi.org/10.1007/s00018-015-2128-3

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CHAPTER 8:

PhD CANDIDATE ACTIVITIES

8. PhD CANDIDATE ACTIVITIES

Courses:

- 24.11.14 - 09.02.15 Medical Statistic 1
- 26.01.15 - 18.05.15 English 1
- 04.02.15 - 11.02.15 Databases
- 04.03.15 - 25.03.15 Communication 1
- 14.09.15 - 21.09.15 Imaging
- 13.10.15 - 16.10.15 Bioethics
- 29.01.16 - 15.04.16 English 2
- 01.02.16 - 04.04.16 Medical Statistic 2
- 02.03.16 - 23.03.16 Communication 2
- 22.11.16 - 15.12.16 Medical Statistic 3
- 25.01.17 - 22.02.17 Communication 3

Scientific meetings:

- 07.11.2014 "7th Annual Meeting of the Diabetes and Cardiovascular Diseases", Milan (Italy);
- 23.01.2015 "17° Giornata di studio sulle Cellule Staminali". Unistem. Milan (Italy);
- 24.03.2015 "Human Induced Pluripotent Stem Cells" Workshop, Ospedale San Raffaele, Milan (Italy);
- 15 - 17.07.2015 Veterinary and Animal Science days 2015, Milan (Italy);
- 13-16.12.2015 " SALAAM Conference 2015", "SALAAM - Sharing Advances on Large Animal Models" COST Action BM1308 Scientific Meeting, Poznan (Poland);
- 25.05.2016 "The use of data meta-analysis to understand the granulosa-cumulus-oocyte complex in bovine", Milan (Italy);
- 08-10.06.2016 Veterinary and Animal Science days 2016, Milan (Italy);

- 22-23.09.2016 "CRISPR genome editing: From high-throughput screening to disease models", Copenhagen (Denmark);
- 26-29.09.2016 "EpiConcept Conference 2016", "Epiconcept" COST Action FA1201 Scientific Meeting, Giardini Naxos (Italy);
- 27-31.03.2017 Training School SALAAM (Cost Action BM1308): "Principles and procedures of tissue sampling and biobanking including legal and ethical aspects"; LMU München (Germany);
- 12-14.09.2017 "In vitro 3-D total cell guidance and fitness", "CellFit" COST Action CA16119 Scientific Meeting, Albena (Bulgaria);
- 27/09-02/10/17, "2nd International Conference on Stem Cells", Rhodes (Greece).

Oral presentations:

- Veterinary and Animal Science Days 2017, 06-08 June 2017 Milan (Italy);
- Veterinary and Animal Science Days 2016, 08-10 June 2016 Milan (Italy);
- Veterinary and Animal Science Days 2015, 15-17 July 2015 Milan (Italy).

Visit to external laboratories:

06.06.2015 - 06.08.2015 Short Term Scientific Mission (STSM) at the Department of Biomedicine, Aarhus, Denmark. Project title: "GENOME EDITING IN HUMAN AND MURINE SKIN FIBROBLASTS THROUGH CRISPR/Cas9 TECHNIQUE".

Publications:

Scientific posters:

- Zenobi A., Zhou Y., Liu Y. , Luo Y., Brevini T.A.L., Gandolfi F. "Establishment of a CRISPR-CAS9 mediated Oct4 gene knock out model to investigate pluripotency control in epigenetically converted fibroblasts", 2015, Meeting of Cost Action BM1308 Sharing Advances on Large Animal Models – SALAAM, Poznan (PL);

- Zenobi A., Luo Y., Brevini T.A.L., Gandolfi F. "Use of CRISPR/Cas9 to edit OCT4 gene and investigate high plasticity of epigenetically erased fibroblasts", 2016, EpiConcept Conference 2016, Giardini Naxos (IT);

Abstracts:

- Tiziana A.L. Brevini, Elena F.M. Manzoni, Alessandro Zenobi and Fulvio Gandolfi "Epigenetic controls of cell plasticity and commitment", 2nd International Conference on Stem Cells, 2017, Rhodes (Greece);

- Manzoni E.F.M, Pennarossa G., Zenobi A., Ledda S., Gandolfi F. and Brevini T.A.L. "Use of a micro-bioreactor to promote 3-dimensional cell rearrangement and induce, maintain, and stabilize high plasticity in epigenetically erased fibroblasts", 2017, Reproduction, Fertility and Development 29(1) 207-207, https://doi.org/10.1071/RDv29n1Ab196;

- Zenobi A., Gandolfi F., Brevini T.A.L. "Matrix stiffness and oxygen tension modulate epigenetic conversion of mouse dermal fibroblasts into insulin producing cells". 2017, International Journal of Health, Animal Science and Food Safety;

- Zenobi A., Luo Y., Brevini T.A.L., Gandolfi F. "Use of CRISPR/Cas9 to edit OCT4 gene and investigate high plasticity of epigenetically erased fibroblasts", 2016, EpiConcept Conference 2016, Giardini Naxos (Italy);

- Brevini T.A.L., Pennarossa G., Manzoni E.F.M., Zenobi A., Gandolfi F. "Combining 3D culture systems to epigenetic tools", 2016, EPICHEMBIO action meeting "Creating new collaborative research proposals", Groningen (Netherlands);

- Zenobi A., Gandolfi F., and Brevini T.A.L. "High glucose concentrations are required for endocrine pancreatic differentiation of mammalian adult fibroblasts". 2016, International Journal of Health, Animal Science and Food Safety;

- Waszkiewicz E., Zenobi A., Pennarossa G., Gandolfi F., Brevini T.A.L., Franczak A. "Expression of octamer-binding transcription factor 3/4 (oct 3/4) in the myometrium of the pig (sus scrofa domestica)", 2016, The 4TH Winter Workshop of the Society for Biology of Reproduction "Central and Local Regulations of Reproductive Processes", Zakopane (Poland);

- Zenobi A., Zhou Y., Liu Y. , Luo Y., Brevini T.A.L., Gandolfi F. "Establishment of a CRISPR-CAS9 mediated Oct4 gene knock out model to investigate pluripotency control in epigenetically converted fibroblasts", 2015, Meeting of Cost Action BM1308 Sharing Advances on Large Animal Models – SALAAM, Poznan (Poland);

- Zenobi A., Gandolfi F., Brevini T.A.L. "Role of oxygen tension and genetic background during the epigenetic conversion of mouse fibroblasts into insulin secreting cells". 2015, International Journal of Health, Animal Science and Food Safety.

Papers:

- Ghiringhelli M., Zenobi, A., Brizzola S., Gandolfi F., Bontempo V., Rossi S., Brevini T.A.L., Acocella F. "Simple and quick method to obtain a decellularized, functional liver bioscaffold", 2017, Methods Mol. Biol.; https://doi.org/10.1007/7651_2017_97;

- Brevini T.A.L. , Pennarossa G., Manzoni E.F.M., Zenobi A., Gandolfi F. "Mountain high and valley deep: Epigenetic controls of pluripotency and cell fate", 2017, Animal Reproduction 14(1):61-68; https://doi.org/10.21451/1984-3143-AR899;

- Brevini T.A.L., Pennarossa G., Manzoni E.F.M., Gandolfi C.E., Zenobi A., Gandolfi F. "The quest for an effective and safe personalized cell therapy using epigenetic tools", 2016, Clinical Epigenetics 8(1); https://doi.org/10.1186/s13148-016-0283-5;

- Brevini T.A.L., Pennarossa G., Acocella F., Brizzola S., Zenobi A., Gandolfi F. "Epigenetic conversion of adult dog skin fibroblasts into insulin-secreting cells", 2016, Veterinary Journal 211: 52-56; https://doi.org/10.1016/j.tvjl.2016.02.014;

- Pennarossa G., Zenobi A., Gandolfi C.E., Manzoni E.F.M., Gandolfi F., Brevini T.A.L. "Erase and rewind:: epigenetic conversion of cell fate" 2016, Stem Cell Rev 12(2): 163-170; https://doi.org/10.1007/s12015-015-9637-1;

- Brevini T.A.L., Pennarossa G., Maffei S., Zenobi A., and Gandolfi F. "Epigenetic conversion as a safe and simple method to obtain insulin-secreting cells from adult skin fibroblasts", 2015, JoVe (109); https://doi.org/10.3791/53880.

Other activities:

- Tutor for the course of "Embriologia Molecolare: dalla cellula all'apparato" (Molecular Embryology), Prof. Tiziana AL Brevini, Veterinary Biotechnology, University of Milan, 2015/2016;

- Tutor for the project "CUSMIBIO - Una settimana da Bio", University of Milan, 2015, 2016, 2017;

- Tutor for the course of "Imaging analysis of cells and tissues", Prof. Tiziana AL Brevini, Science in Veterinary Biotechnologies, University of Milan, 2015/2016;

- Tutor for the course of "Principi e tecniche di terapia e rigenerazione cellulare UD; Medicina rigenerativa: principi biologici e procedure di laboratorio" (Regenerative Medicine: principles and techniques), Prof. Fulvio Gandolfi, Science in Veterinary Biotechnologies, University of Milan, 2016/2017.

CHAPTER 9:

AKNOWLEDGMENTS

9. ACKNOWLEDGMENTS

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