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# MicroRNA expression profiling reveals tissue-specific and disease-associated signatures in diabetes mellitus

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# 1- INTRODUCTION

## 1.1- MicroRNAs

### 1.1.1- MicroRNA gene structure

Since their first discovery, in 1993, by Victor Ambros research group, microRNAs (or miRNAs) partially revealed what for years remained in shadow: the role of "junk" DNA in eukaryotes genome. In fact what for years has been called "junk", now it is not anymore*(Lee RC et al. 1993)*. MicroRNAs represent a class of small non-coding RNAs with peculiar functions in control of gene expression at post-transcriptional level *(Zamore PD et al., 2005).* The microRNAs mature form is a single stranded RNA, 19-22 nucleotides long, whose maturation steps take place in part in the nucleus and in part in the cytoplasm.

MicroRNAs are transcribed from their own genes scattered in all chromosomes in humans, except for Y chromosome *(Rodriguez et al., 2004).*To date, the microRNAs database (miRBase) (www.miRbase.org), in which annotated sequence of miRNAs genes are reported and constantly updated, shows that 1424 miRNAs genes are scattered throughout the human genome; of note, this number is continuously growing up both in man and in other species. Since the initial miRBase publication, the number of annotated sequences has shown an astonishing growth, from 218 entries in 2002 to over 16000 entries in 2011 (Fig.1).

Most miRNA genes are located in intergenic regions (intergenic miRNAs) almost >1kb away from annotated/predicted genes, although some miRNAs were found in intronic regions, within protein coding-genes or in non-coding genes (intronic miRNAs) (Fig.2) *(Kim VN et al., 2006).* Intergenic miRNAs are transcribed as autonomous units with their own promoter/regulatory region, from both RNA Polymerase II or III; about a 50% of intergenic miRNA are found in close proximity to other miRNAs, forming extended clusters which are transcribed as single polycistronic unit *(Lee Y et al., 2002*). Intronic miRNAs, residing within protein coding genes or non-coding genes, seem to be transcriptionally related to the expression of their host gene

and processed in consequence of the spliceosome formation *(Lagos et al., 2001; Mourelatos et al., 2002; Lau et al., 2001).*

Transcription of intergenic miRNAs, controlled by their own promoter, generates several Kb-long pri-miRNAs with CAP structures and poly(A) tails, which allow its subsequent processing reactions. On the other hand, the transcription of intronic miRNAs underlies the control of their host mRNAs using the same promoter, and involves protein complexes of mRNAs splicing machinery (Lee, Y. *et al*., 2004).

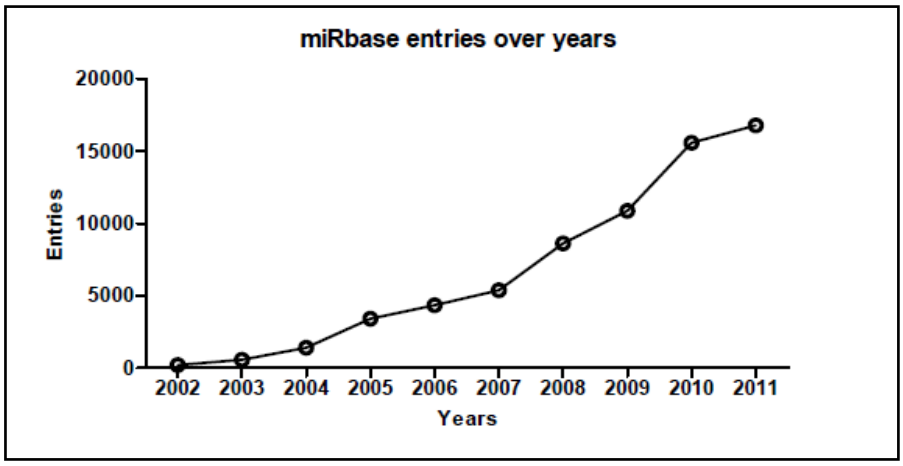


**Figure 1**.Number of miRNAs annotated sequences in miRNA database miRBASE, from 2002 to 2011.

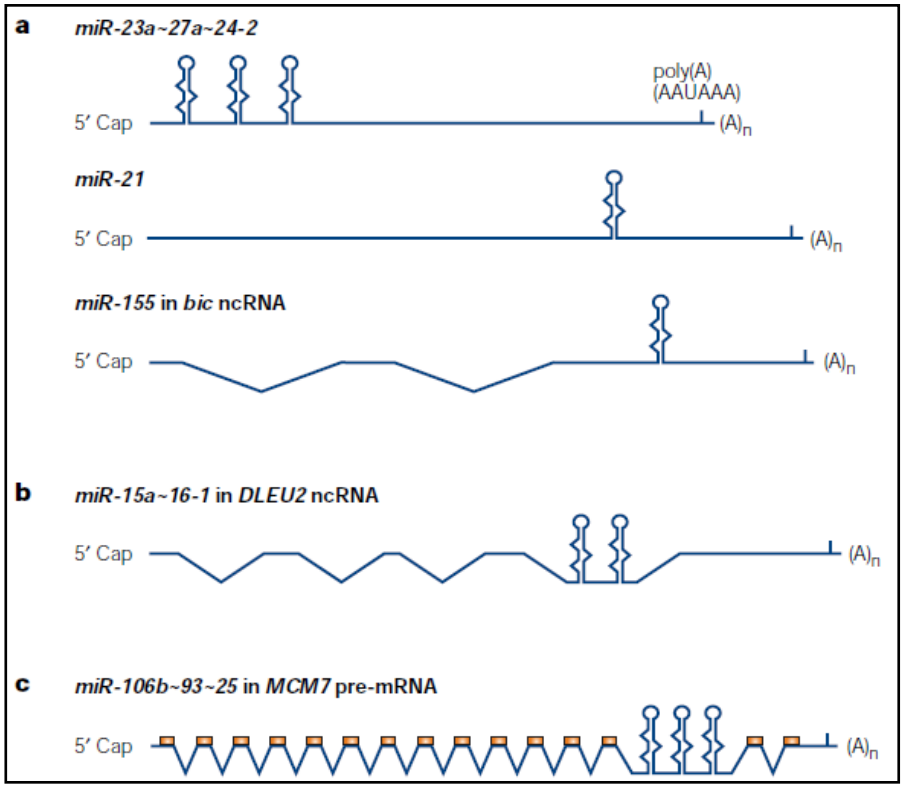


**Figure 2**.MiRNA genes within genome. A. Example of intergenic miRNAs. B) Example of intronic miRNAs in non-coding transcripts. C. Example of intronic miRNAs in protein-coding transcript.

### 1.1.2- MicroRNAs biogenesis and maturation

Transcription of miRNA gene is mediated by RNA polymerase II or III, which produce a primary transcript, called pri-miRNA. This transcript contains the 7-methylguanosine cap and a poly(A) tail. Pol II-dependent transcription enables temporal and positional control over miRNA expression so that a specific set of miRNAs can be expressed during several different cellular stages and under specific conditions and in specific cell types. Pri-miRNAs are long primary transcripts that contain a local stem-loop structure, which is essential for following processing reactions.

This stem-loop structure is cleaved in the nucleus by the RNase III Drosha to release the precursor of miRNA (pre-miRNA). Drosha is a large protein of 160 kDa, which is conserved in animals, that requires a cofactor, the DiGeorge syndrome critical region gene 8 (DGCR8) protein in humans (also known as Pasha in Drosophila and C.elegans) *(Lee, Y. et al., 2003)*. Drosha and DGCR8 form a large 650 kDa complex in humans and a 500 kDa complex in Drosophila, which is known as the microprocessor complex. Because DGCR8 (or Pasha) contains two double-stranded RNA-binding domains (dsRBDs), it is believed to assist Drosha in substrate recognition, although the precise biochemical role remains to be understood *(Han, J. et al.,2004)*. Because the next processing enzyme is confined to the cytoplasm, the Drosha product pre-miRNA, needs to be exported to the cytoplasm. Export of pre-miRNA is mediated by one of the Ran-dependent nuclear transport receptors, exportin-5 (Exp5) *(Lund, E. et al., 2004)*. Once in the cytoplasm, pre-miRNAs are processed into 22-nt miRNA duplexes by the cytoplasmic RNase III Dicer, a highly conserved protein of 200 kDa. The cleavage products [22-nt miRNA/miRNA star (miRNA/miRNA*) duplexes]are thought to be quickly unwound by helicase (Argonaute protein), and a single mature strand, preferentially the most thermodynamically stable, can be asymmetrically incorporated into the RNA-induced silencing complex (RISC) where they can then act by translational repression (by a cleavage-incompetent RISC) or mRNA degradation (by a cleavage-competent, Slicer-containing RISC). The counterpart of the mature miRNA from the duplex that is generally regarded as a passenger strand called miRNA* (miRNA "star"), whose regulatory capacity has not been systematically examined in vertebrates, is usually degraded.

In particular, mature miRNAs are incorporated into the effector complexe, which isknown as, 'miRISC' (miRNA-containing RNA-induced silencing complex) by associating with Argonaute proteins. The RISC complex composition is still not completely clear, but what is known is that it is composed by Dicer and a dsRNA-binding protein named TRBP (Transactivating

Response RNA Binding Protein) that recruits argonaute proteins like hAGO2 (human argonaute protein 2). The Argonaute protein family was first identified in plants, and members are defined by the presence of PAZ (Piwi-Argonaute-Zwille) and PIWI domains (Peters L. *et al*., 2007). It can be divided into the Ago subfamily and the Piwi subfamily. Ago proteins are ubiquitously expressed in many organisms. In mammals, for example there are eight Argonaute genes: human Ago1, Ago3 and Ago4 genes are clustered on chromosome 1, whereas the Ago2 gene is located on chromosome 8. The human PIWI subfamily comprises HIW1, HIW2, HIW3 and HILI that are encoded by genes on chromosomes 12, 11, 22 and 8, respectively. Studies on isolated PAZ domains from different organisms revealed that this domain contains a specific binding pocket that anchors the characteristic two nucleotide 3'-overhang that results from digestion of RNAs by RNase III (*Lingel et al., 2003*); instead PIWI domains show extensive homology to RNase H. Indeed, biochemical *in vitro* studies have shown that some are endonucleases, and these are often referred to as 'slicers'. In humans, only Ago2 has slicer activity *(Peters L. et al., 2007).* After processing operated by Dicer, only a strand of the dsRNA produced is selected as leading strand for post-transcriptional gene silencing. Ago2 plays a fundamental role in leading strand selection: thermodynamic stability of the dsRNAs ends establishes which strand is selected. The strand with relatively unstable pairing at 5' end is generally chosen and thus remains as component of the silencing complex (Fig.3) *(Okamura K, et al. 2004).*

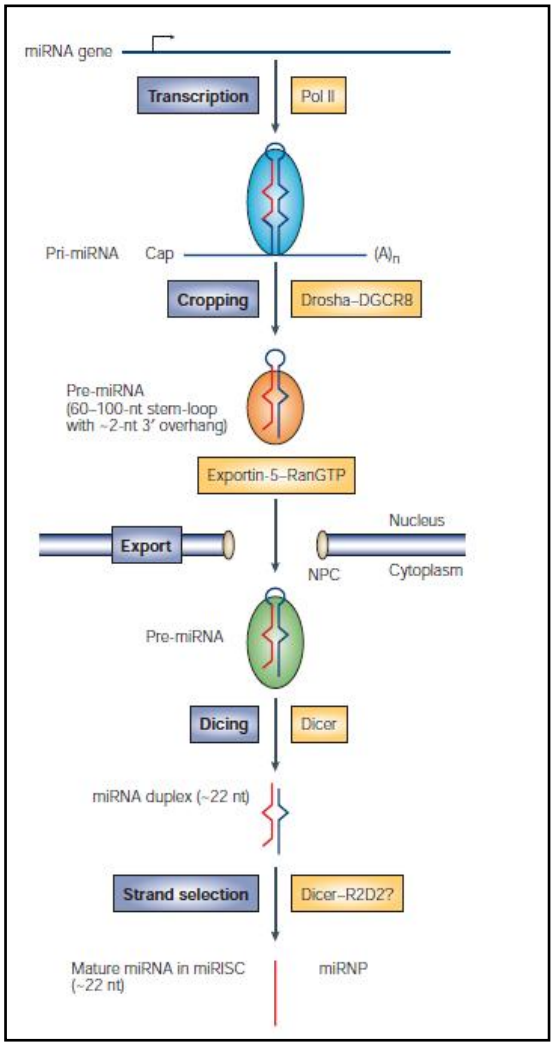


**Figure3.** Mechanism of miRNAs maturation: from transcription to miRNA strand selection

### 1.1.3- Post-transcriptional regulation mechanism

MiRNAs negatively regulate gene expression at post-transcriptional level by mediating translational repression or degradation of the mRNA targets. The main actor of these two different mechanisms is the protein complex RISC. Translational repression is thought to occur when the leading strand, through an imperfect pairing to the target mRNA, directs RISC to bind the 3' untranslated regions (3'UTRs) of the targets. This imprecise matching causes a bulge in the duplex formed between the miRNA and its mRNA and results in blocking the target mRNA (usually the 3'UTR) from being translated and in protecting the target mRNA from destruction by endonucleases (Fig.4b). This complexes are then transported into particular cytoplasmic loci

called *p-bodies(processing bodies)* which are deprived of the translational machinery but retain proteins involved in mRNA degradation. *P-bodies* are sites in the cytoplasm in which, in addition to the mRNA component, essential components of the mRNA degradation pathway (Argonaute proteins and miRNAs) are localized, together with additional proteins, including the mRNA decapping enzymes (Dcp1/Dcp2), the 5'-3' exonuclease, Xrn1, Dhh1p, Pat1p and, in mammalian cells, GW18 that are responsible of the transcript *de-capping* through which the mRNAs capture and degradation occur *(Sen GL, et al., 2005)*.This mechanism is used by miRNAs for the endogenous regulation of different gene products. In contrast, mRNA degradation or "*slicing*", is thought to occur by siRNAs which form perfect/near perfect interactions with their target mRNAs, which results in the cleavage of the mRNA known as RNA interference (Fig.4a). The perfect complementation presumably triggers the action of Slicer, which in mammals is putatively thought to be the Argonaute2 (Ago2) endonuclease acting either alone or together with other Argonaute or unknown proteins within the RISC complex. Slicer mode of action is by cleaving the target mRNA between the 10th and the 11th nucleotide from the 5'end of the miRNA. Seven nucleotides at 2-7 positions (relative to the 5' end of miRNA), known as seed sequences, are crucial in binding to the target mRNA. These series of events lead to the silencing of target genes (Shet U.et al., 2003).

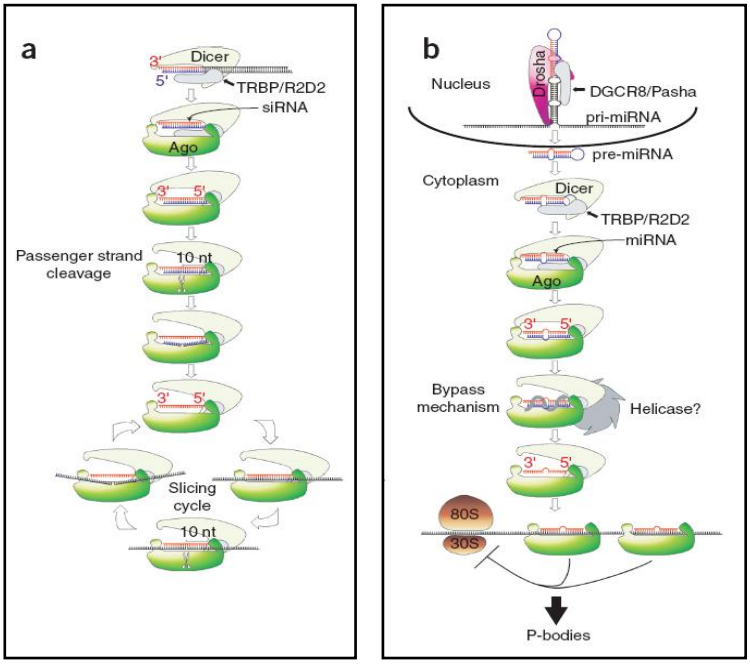


**Figure 4**. A. Mechanism of mRNAs degradation upon perfect pair matching between miRNA and mRNA target. B. Translational inhibition by imperfectly matching between miRNA and mRNA.

### 1.1.4- MicroRNAs and disease

MiRNAs are present in many organisms as well viruses, flies, worms, mammals, and plants, where they regulate fundamental cellular processes such as cell differentiation, cell proliferation, and apoptosis (*Miska EA et al., 2005*). Moreover, it has been shown that some miRNAs display developmental and tissue specificity, demonstrating that their expression can be regulated both temporally and spatially. This observation suggests that miRNAs specific expression may execute a fundamental role in morphological development and organogenesis as well as many other functions mainly dependent on the role of their gene targets (*Miska E. A. et al., 2003).*

In the human genome, miRNAs are involved in the regulation of approximately 30% of all of the protein-coding genes, evidencing their pivotal role in the regulation of cell functions *(Jonh B. et al., 2007).*

Most of the evidences of the involvement of microRNAs in diseases come from cancer research. It has been demonstrated that about 50% of human miRNA genes are located in cancer associated regions or at fragile sites of chromosomes. Many genomic profiling studies indicated a deregulation of miRNAs expression in various tumors, and some miRNAs are reported as partially responsible for altered cell replication in cancer (e.g. miR-17-92 or miR-155 in B-cell Lymphomas). Recent studies on the tumor-suppressing or tumor-promoting activities by miRNAs, support the hypothesis that miRNA could exploit roles as oncogenes or as oncosuppressor genes; this is the case of miR-15 and miR-16 which have a role as suppressor of BCL-2 proapoptotic gene that is frequently overexpressed in B-cell chronic lymphocytic leukemia (B-CLL) *(Calin G.A., et al., 2008).*

Although miRNAs have been found as important modulators of tumor cell growth, they have been also involved in many other diseases. Indeed, they have been characterized as implicated in the regulation of several functions of the immune system and some of their alterations were directly linked to immunological disorders. Autoimmune diseases represent a group of immunological disorders in which altered miRNA expression has been found to play important roles. For example, miR-146a, miR-155 and miR-16 have been found altered in T-cells isolated from rheumatoid arthritis patients and miR-326 has been found to be downregulated in peripheral blood mononuclear cells (PBMCs) of patients with autoimmune multiple sclerosis (*Stanczyk J. et al. 2008; Du C. et al. 2009*).

MiRNAs have been also well involved in a series of metabolic disorders and their role in several other diseases is still growing up.

## 1.2- Diabetes Mellitus

Diabetes mellitus (DM) comprises a group of metabolic disorders characterized by a disturbance in glucose homeostasis, resulting in increased blood glucose levels, due to low insulin production/secretion by pancreatic beta cells or to a low responsiveness by insulin target tissues (skeletal muscle, adipose tissues, liver etc.).

Diabetes mellitus has now reached epidemic proportions in several areas of the world, even if it is more common in the more developed countries where it affects at least 5% of the population. In year 2000, according to the World Health Organization and United Nations, at least 171 million people worldwide suffered from diabetes. Its incidence is increasing rapidly, and global projections suggest that by 2030, this number will almost double *(Wild et al., 2004*). With an increasing incidence worldwide, Diabetes Mellitus will be a leading cause of morbidity and mortality. Several distinct types of diabetes mellitus exist and are caused by a complex interaction of genetics and environmental factors. Diabetes mellitus is classified, depending on the etiology, in several groups, of which the two main forms are type 1 (previously known as insulin dependent or juvenile onset) and type 2 diabetes (non-insulin dependent or adult onset).

### 1.2.1- Type 1 Diabetes Mellitus (T1DM)

Type 1 diabetes mellitus (T1DM) results from the selective autoimmune destruction of pancreatic insulin producing beta-cells and it is therefore characterized by absolute insulin deficiency. In this form of diabetes, in fact, there is the development of a T-lymphocyte (both $CD4^+$ and $CD8^+$) and B-lymphocyte (with disease-specific autoantibodies production) response against autoantigens of the pancreatic beta-cell. Moreover, the islets of Langerans are gradually involved in an inflammatory process, called insulitis, characterized by an infiltrate of T lymphocytes and macrophages that slowly causes beta-cell destruction, thus leading to a decreased insulin production (*Narendran P et al., 2005*).

Although many factors are reported to contribute to T1DM onset, it is now clear that a cooperation between genetic background and environment, plays a fundamental role in the establishment of the disease(*Larsen and Alper, 2004*). It has been demonstrated that genetic predisposi-

tion is necessary but not sufficient to T1DM onset while environmental factors, such as specific viral infections, are reported to be crucial for the establishment and progression of the autoimmune reaction against beta-cell antigens *(Dahlquist G et al., 2001).*

Data obtained using animal models, like NOD (Non-Obese Diabetic)mice and BB (BioBreeding) rats, which spontaneously develop an equivalent form of human type 1 diabetes, helped to identify several self-antigens capable to stimulate an autoimmune response.

The autoimmune process starts in the preclinical phase, as evidenced by the presence of autoantibodies in the serum against disease-specific autoantigens. The four major autoantigens so far identified are insulin, GAD65 (glutamic acid decarboxylase, 65kDa form),IA2 (Insulin autoantigen 2, an intracellular phosphatase) and ZnT8 (islet zinc transporter 8).

T1DMseems to be associated with a number of susceptibility alleles. The major susceptibility genes are located in the HLA region on chromosome 6 that contains genes encoding for class II MHC molecules, which present antigens to helper T cells and are involved in initiating the immune response.

### 1.2.2- Type 2 Diabetes Mellitus (T2DM)

Type 2 diabetes mellitus (T2DM) represents the most diffused endocrine-metabolic disorder worldwide, accounting for 90% of the total cases of diabetes mellitus. It is characterized by three pathophysiological abnormalities: 1) peripheral insulin resistance, that is the inability of the target tissues (mainly skeletal muscle, liver and adipose tissues) to respond to blood concentrations of insulin; 2) impaired insulin secretion, that is the incapacity of the beta-cells to compensate this resistance in the phases before the overt hyperglycemia and, finally, 3) excessive hepatic glucose production (*Bell GI. et al., 2001*). No single etiologic factor has been defined as the cause of T2DM, although important risk factors include age, ethnicity, family history together with obesity. The behavior of the beta-cell-mediated insulin secretion during the course of the natural history of T2DM is variable: in fact the insulin levels can be increased, normal or decreased, but, relatively to the circulating glucose concentrations, they are not sufficient to guarantee normoglycemia.

In the early stages of the disorder, glucose tolerance remains normal, despite insulin resistance, because the pancreatic beta-cells compensate by increasing insulin output (synthesis- secretion / beta-cell mass outgrowth).As insulin resistance and compensatory hyperinsulinemia progress, the pancreatic islets in certain individuals are unable to sustain the hyperinsulinemic state and

Impaired Glucose Tolerance (IGT), characterized by elevations in postprandial glucose, then develops. A further decline in insulin secretion and an increase in hepatic glucose production lead to overt diabetes with fasting hyperglycemia. Furthermore, the natural history of type 2 diabetes entails progressive deterioration in beta-cell function associated with loss of beta-cell mass due to apoptosis.

The physiological mechanisms that regulate the beta-cell mass are on the one side beta-cell neogenesis (differentiation from precursor cells), beta-cell proliferation, and beta-cell hypertrophy (increased cell size) that increases beta-cell mass, and, on the other side, beta-cell death, primarily through apoptosis, and beta-cell atrophy (decreased cell size) that decreases beta-cell mass *(Akermann AM et al., 2007).*In individuals not affected by T2DM, the balance between these two different phenomena, maintains the beta-cell number constant throughout lifetime. In people affected by T2DM, instead, beta-cell loss is greater than the cellular growth, so that over time, there is an absolute reduction of beta-cell mass. It is still unknown why beta-cells are lost in individuals with T2DM, although a number of hypotheses have been raised. For example, elevated glucose and free fatty acids (e.g. gluco-lipotoxicity) or oxidative stress as a consequence of gluco-lipotoxicity may induce beta-cell apoptosis.

## 1.3- MicroRNAs and Diabetes

### 1.3.1- MicroRNAs in the regulation of beta-cell function

The first indication of an involvement of miRNAs in the regulation of beta-cell function, derived from a work by Stoffel and colleagues who identified two novel miRNAs specifically expressed in human pancreatic islets: miR-375 and miR-376 *(Poy M. et al., 2004)* . One of these, miR-375, has been reported to have important roles in beta cell physiology *(Avnit-Sagi T. et al., 2009)*. Indeed, it has been demonstrated that miR-375 negatively regulates glucose-stimulate insulin secretion, mainly controlling the expression of Myotrophin (Mtpn). Mtpn is a protein with a role in remodeling F-Actin filaments, indirectly controlling vesicle binding to plasma membrane. The reduction of Mtpn levels by RNA interference mimicked the overexpression of miR-375 on insulin secretion *(Poy M. et al., 2004)*.

MiR-375 has been also demonstrated to have another important target clearly involved in the regulation of PI3 kinase signaling pathway: 3'phosphoinositide-dependent protein kinase-1 (PDK1). The reduction of PDK1 levels by miR-375 overexpression led to a decrease in the stimulatory action of glucose on insulin gene expression and DNA synthesis (*El Ouaamari A et al., 2008)*.

Recently, specific miR375 knockout mice have been generated by Poy N. and colleagues. They observed a strong hyperglycemia mainly derived by increased number of alpha-cells and consequent high glucagon plasma levels. In contrast, a decreased beta-cell mass has been reported, mainly due to the low proliferation rate of existing beta-cells; moreover, beta cells from miR-375 KO ob/ob mice are reported to be unable to compensate for high demand of peripheral insulin by target tissues, resulting in a severe diabetic condition *(Poy MN. et al., 2009)*.

Several other miRNAs are able to influence the function of adult beta-cells. For instance, miR-9 has been demonstrated to be necessary for optimal insulin release in response to glucose. When overexpressed in beta-cells, miR-9 leads to impairment of glucose-induced insulin secretion caused by the inhibition of the transcription factor Onecut-2 (OC-2). OC-2 has been demonstrated to be a transcriptional repressor of Granuphilin/Slp4, a Rab-GTPase associated with secretory granules, with negative effects on insulin secretion. An increase in miR-9 levels has been demonstrated to decrease the levels of OC-2, which in turn allows the transcription of Slp-4 and finally a decreased insulin secretion *(Plaisance V. et al., 2006)*.

Another microRNA able to modulate the expression of several components of the insulin secretory machinery is miR-124a. As for miR-9, miR-124a is preferentially expressed in neuronal cells and in pancreatic islets. It has been demonstrated that the overexpression of this miRNA in beta-cell line MIN6 leads to the negative modulation of several components of exocytotic machinery: SNAP25, Rab3a, Synapsin-1a, Rab27a and Noc-2. Rab27a is a direct target of miR-124a, while the downregulation of other components is the result of a not-yet elucidated miR-124a indirect effect. The alteration of these components elicited an increase in basal insulin secretion while damaging the secretion upon glucose stimulus, when the secretory machinery is under stress condition *(Lovis P. et al., 2008).*

In addition, miR-124a directly targets another important component of the beta-cell function: Foxa2 *(Baroukh N. et al., 2007).* The role of Foxa2 in beta-cells is now well established. It cooperates with other transcription factors to maintain beta-cell phenotype, in part eliciting the transcription of Pancreatic duodenal homeobox-1 (Pdx-1). It also regulates the expression of genes of the glucose sensor machinery, such as KiR6.2 or Sur-1 (subunits of the ATP-sensitive K+ channel) *(Wang H. et al., 2002).*

Baroukh and colleagues also demonstrated that overexpression of miR-124a in insulin producing cell lines led to higher intracellular $Ca^{2+}$ levels at basal condition and to a defective $Ca^{2+}$cytosolic release in response to glucose. However, they did not observe any change in insulin release.

More recently, another study by Hennessy and colleagues defined a set of 10 microRNAs which were downregulated in glucose non responsive MIN6 cells compared to glucose responsive ones. They focused mainly on miR-410, miR-130a and miR-200a demonstrating that functional inhibition of these miRNA led to a decreased capability of MIN6 to secrete insulin upon glucose stimulation. Conversely, miR-410 overexpression was associated to an improved glucose-stimulated insulin secretion. However, these Authors did not reveal the mechanisms involved in these modulations; consequently, further studies are necessary to have a more complete picture of the system.

### 1.3.2- MicroRNAs and pancreas development

Development of pancreas is a complex process involving several transcription factors and signaling pathways. The pancreatic tissue derives as dorsal and ventral outpouching of the anterior

midgut region of embryo and a series of specific transcription factors delineates it from the development of other tissues such as liver, gallbladder and duodenum.

Although it has been demonstrated that during development of these organs, the overlap of several transcription factors is quite significant, microRNA expression profile clearly differs, thus suggesting the importance of microRNAs in specifying cell fate and tissue development.

To understand the contribution of microRNAs in pancreas development several research groups have evaluated their role during pancreas development in zebrafish, mouse and man. One of the pioneering studies on the role of microRNAs during pancreas development, was performed on zebrafish. Kloosterman and colleagues used morpholinos (2'-O- methyl oligonucleotides, targeting genes or miRNAs sequences) technology to temporally knockdown microRNAs during zebrafish embryo development. They silenced 13 miRNAs, all conserved between zebrafish and mammals and observed relevant defects only knocking down miR-375. Indeed, the lack of miR-375 during zebrafish pancreas development caused defects in the islets morphology, maybe compromising also their function. Moreover, this effect was also confirmed using different morpholinos, targeting other sequences in the miR-375 precursor, showing the specificity of the observed effects *(Kloosterman P. et al., 2007)*.

The same year, the group directed by Michael German performed a wide miRNAs cloning analysis on pancreata from e14.5 mouse embryos. They identified 107 known mouse miRNAs highly expressed in the developing pancreas. The authors found that miR-375 was overrepresented in the cloning experiment and they specifically analyzed it using in situ hybridization. In-situ analyses showed colocalization of miR-375 in pancreatic ductal epithelium (the main source of endocrine and exocrine progenitor cells) of developing pancreas (e14.5) with Pdx-1, showing the importance of miR-375 during normal development *(Lynn FC et al., 2007)*.

To determine the importance of miRNAs during pancreas development, they also ablated the RNase domain of Dicer1 using a Pdx-1/Cre mediated deletion starting at e9.5. The pancreas-specific Dicer1-KO mice died early after birth (P3) and showed a strong reduction in ventral and dorsal pancreas while the remaining tissue was compromised, with a dramatic disorganized epithelium *(Lynn FC et al., 2007)*.

A more recent work described the contribution of microRNAs during human pancreas development. Joglekar and colleagues analyzed the expression of the 4 islet-specific miRNAs (miR-7, miR-9, miR-375, miR-376) during human fetal pancreatic islet development at different points of gestation. They observed high expression levels of all four miRNAs during development. Specifically, they demonstrated a gradually increased expression of miR-7 and miR-375 during development/differentiation corresponding to the increase observed in insulin transcript;

the two other miRNAs, miR-9 and miR-376, although reported to be expressed at higher levels, did not show such a regulation. The behavior observed for miR-7 and miR-375, that gradually increased during pregnancy, may be addressed to an important function of these miRNAs, which could regulate cell fates within developing islets. For miR-375, Joglekar and colleagues also performed a single cell analysis in order to identify putative expression differences between insulin positive and insulin-negative cells from third trimester of pregnancy. They found that pri-miR-375 was expressed in almost all islets cells, but mature miRNA showed a scattered expression pattern maybe due to a different post-transcriptional processing of pri-miR-375, which may indeed be a regulatory step in islet development *(Joglekar MV. et al., 2009)*.

Another miRNA found to be important for pancreatic islet development is miR-124a. As reported above, miR-124a has been demonstrated to be a master regulator of insulin secretion. Baroukh and colleagues found that this miRNA is also well represented during mouse pancreas development, and may therefore regulate the major steps in pancreatic islet generation (*Baroukh.et al,2007*). They analyzed miR-124a expression during different stages of mouse pancreatic development, observing an increased expression of this miRNA at e18.5 compared to e14.5. As mentioned above, miR-124a targets an important regulator of beta-cell identity also well involved in beta-cell differentiation: Foxa2. This miRNA may then play an important role during pancreas development by regulating the differentiation cascade downstream Foxa2 control (Fig.5).

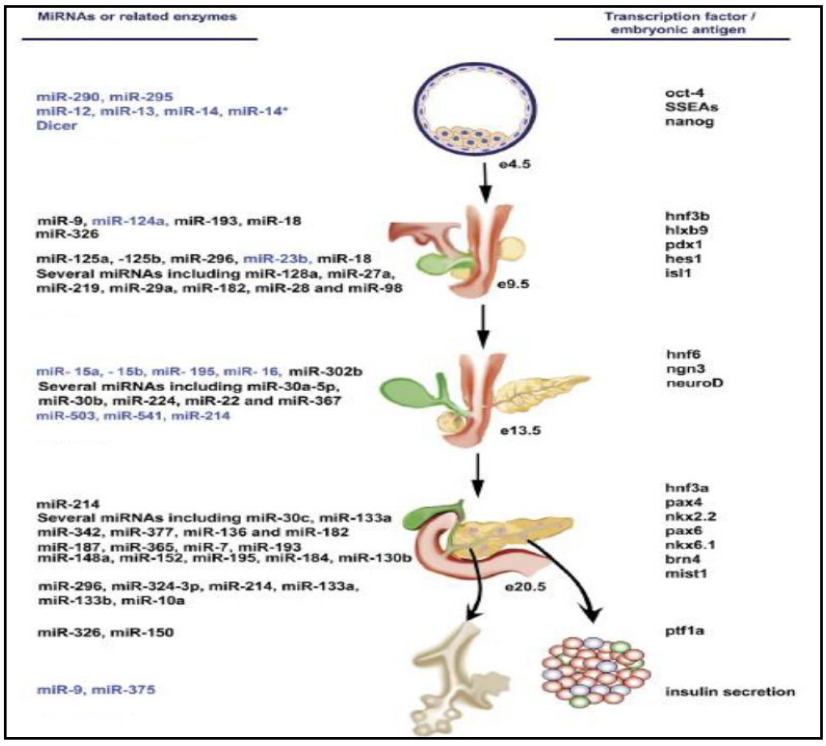


**Figure 5**. Transcription factors and microRNAs involved in mouse pancreas development. MicroRNAs with experimental evidence of involvement in development are reported in blue.

### 1.3.3- MicroRNAs and beta-cell regeneration

Pancreatic injuries derived from several different sources (viral infection, gluco-lipotoxicity etc etc), may compromise the function of secretory cells (both endocrine and exocrine cells).
The study of pancreas regeneration, especially the mechanism of beta-cell regeneration, has been one of the major issues in recent years.
Although the regeneration process is not yet well understood, now we know that different mechanisms are suggested to contribute to generation of new pancreatic tissue (in particular beta-cells) *(Bonner-Weir S. et al., 2010)*:

- Neogenesis from ductal precursor cells
- Replication of pre-existing beta-cells
- Differentiation of an intermediate multipotent cell type

According to several authors, different types of injury may activate different pathways of regeneration, although the exact mechanisms are not yet well understood. Moreover, it has been suggested that islet regeneration in adult organisms recapitulates embryonic developmental pathways. In fact, many studies illustrated that pancreatic transcription factors that are expressed in a temporal fashion during development are re-expressed during regeneration.
As for pancreas development, microRNAs may play a pivotal role also in beta cell regeneration. Joglekar and colleagues provided a comparison between the expression profile of 283 miRNAs during different stages of pancreas development and during regeneration. Firstly, they observed that the majority of transcription factors present in developing pancreas were the same involved in the regenerating one, with the only exception of neurogenenin-3 (Ngn3) *(Joglekar MV et al.,2007*. Ngn3 marks all pro-endocrine cells during pancreas development and is therefore considered an early hierarchical transcription factor necessary to determine the endocrine fate of islet cells. The expression of Ngn3 was absent in mouse pancreas after 4 weeks from 70% of pancreatectomy, even if neo-islets were clearly observed. The miRNA expression profile revealed that 4 miRNAs (miR-15a, miR-15b, miR-16, miR-195) are expressed at least 200fold higher in regenerating pancreas as compared to different embryonic days of mouse pancreas development. Moreover, these miRNAs are predicted to bind Ngn3, possibly explaining the absence of Ngn3 protein during regeneration. Inhibition of these miRNAs in regenerating pancreatic cells using anti-sense specific inhibitors, induces the expression of Ngn3 and its

downstream players Neurod1 and Nkx2.2, while the overexpression during pancreas development showed a reduction in the number of hormone producing cells.
Although few studies regarding microRNAs and regeneration are available, these data represent a good evidence of how microRNAs may play important roles in pancreas regeneration.

### 1.3.3- MicroRNAs in the regulation of autoimmunity

Regulation of the immune system is vital to preventing many pathogenic disorders including autoimmune diseases, and mammals have developed a complex system of molecular mechanisms for immune regulation, in order to maintain self-tolerance while allowing immune responses to foreign pathogens. Recently, it has become evident that microRNAs play an important role in regulating immune responses as well as immune cell development (Fig6) *(Cobb BS et al.,2006; O'Connell RM. et al., 2010; Baltimore D. et al.,2008).*
To this regard, miR-155 has been shown to be a key factor for innate and acquired immune responses. MiR-155 is required for T-cell differentiation and function, germinal centre B-cell responses and responses to bacterial and viral infections .*(Tili E. et al., 2007.* Experiments in mice deficient in miR-155 showed immunodeficiency and defects in the function of B- and T-cells, and of dendritic cells Another study in mice demonstrated an important role for miR-155 in the differentiation of T-helper cells and in the establishment of germinal centers.
MiR-150, a miRNA specifically expressed in mature lymphocytes, can block B-cell development when it is expressed prematurely; nevertheless, it allows normal T-cell activation by targeting transcription factors such as c-Myb *(Zhou B. et al., 2007).* MiR-181a is involved in B- and T-cell differentiation and modulates T-cell sensitivity in response to antigens during T-cell development and maturation by down-regulating the expression of multiple phosphatases in the TCR signaling pathway. In addition, miR-17-92 is essential for adult B cell development and regulates the transition from pro-B cell to pre-B cell. Finally, several studies have demonstrated that miR-223 is implicated in granulopoiesis, in the regulation of granulocyte maturation and function, and in the inflammatory response *(Chen CZ. Et al., 2004).*
It is becoming increasingly clear from cell culture and animal studies that proper miRNA regulation is critical for the prevention of autoimmunity. However, it is not yet well understood whether miRNA dysregulation could play a role in autoimmune disease pathogenesis in man. Recently, several studies have uncovered a possible role for miRNAs in some autoimmune dis-

ease such as rheumatoid arthritis, systemic lupus erythematosus, and autoimmune multiple sclerosis.

A recent work by Hezova and colleagues analyzed miRNA profiling in T-regulatory cells (Treg) inT1D patients compared to controls. Tregs have been reported to be critical regulators of autoimmunity. Unbalancing Treg amount within immune system has been demonstrated to be critical for development of autoimmunity. Hezova and colleagues found that 2 microRNAs, miR-342 and miR-191, were decreased, while miR-510 was upregulated in Treg from T1DM patients. Moreover, they compared Treg cell population with other T-cell populations from blood of normal subjects and detected a specific miRNA signature of Treg cells, demonstrating the importance of microRNAs also in Treg function *(Hezova R. et al., 2010).*

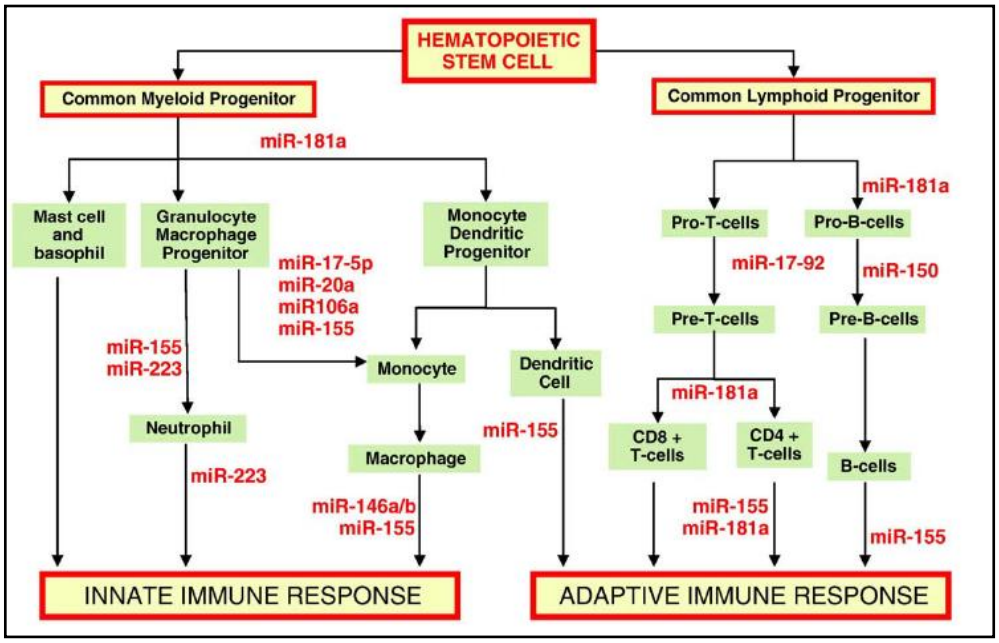


**Figure 6.** MicroRNA roles in development and function of the immune system components

## 2- AIMS

The aim of this work was to gain insights into possible role of microRNA in events associated to the regulation of (auto)immune response in type 1 diabetes and of beta cell mass in function both in type 1 and in type 2 diabetes.

# 3- MATERIALS AND METHODS

## 3.1- Human islet preparation and culture

For experiments on T2DM, human pancreatic islets were obtained from 4 non-diabetic (aged 58,2±7,1 years) and 4 T2DM multi-organ donors (64,2±11,3years).
For experiments on beta-cell expansion and regeneration, human pancreatic islets were obtained from 6 non-diabetic multi-organ donors (aged 43.2±9.7 years).
Islets were prepared as described previously *(Lupi R. et al., 1999)*. Briefly, purified islets were prepared by intraductal collagenase solution injection and density gradient purification. At theend of the isolation procedure, islets were resuspended in M199 culture medium(supplemented with 10% serum and antibiotics) and cultured at 27°C in a $CO_2$incubator until ready for experimental procedure.
High glucose treatment was performed culturing non-diabetic human islet for 24 h in M199 culture medium (supplemented with 10% FBS and 1X Antibiotic/Antimicotic) containing 22.2 mmol/l of glucose. Islets cultured for 24h in 5.5mmol/glucose (basal condition) were used as control.

## 3.2- T1DM patients recruitment and PBMC isolation

A group of 19 T1DMpatients (Tab.1) were enrolled at the Diabetes Unit of Le Scotte University Hospital (Siena,Italy). These 19 patients (11 females and 8 males, aged 20-75) were divided into three groups: 1) Ab[-] (patients negative for both GADA and IA2-A; n=5); 2) Ab[+] (patients positive for GADA or IA-2A; n=10); 3) Ab[++] (patients positive for both GADA and IA-2A; n=4). The mean disease duration was 9.2 years with a range from 0 to 23 years. PBMCs were obtained from each patient using Lympholite-H extraction gradient and subsequently stored at -80°C until ready for RNA extraction.

3.3- Cell Culture and transfections

MIN6 cell line and pseudoislets

MIN-6 -cells (passages 50-60) were maintained at 37°C and 5% $CO_2$ in DMEM supplemented with 10% fetal bovine serum, 2mmol/l glutamine (Sigma) and 1X Antibiotic/Antimicotic (Sigma). The medium was changed every 4 days and monolayers were passaged and used for experiments when 80% confluent. MIN6 pseudoislets formation has been performed plating $5x10^5$ cells in 100mm Ø non-treated Petri dishes and cultured for 15 days. Medium was changed every 5 days.

Pseudoislets after 15 days of culture were used for transfection experiments using mmu-miR-124aa-1, mmu-miR-124aa-2, mmu-miR-124aa-3 hairpin inhibitor and mmu-miR-124a mimic (Dharmacon). 24 well plates were used for transfection experiments and 200 pseudoislets were plated in each well. After 24h, culture medium was replaced with transfection medium composed by Dharmafect 2 transfection reagent (Dharmacon) together with 50nM mmu-miR-124a-1-2-3 hairpin inhibitors mix or mimic. After 48h incubation, transfection medium was replaced with new fresh one and pseudoislets incubated for other 72h.siGLO Red transfection control reagent (10 nM) (Dharmacon) was used to verify transfection efficiency. Scrambled hairpin inhibitor or mimic were used as transfection negative control. Transfected pseudoislets were then harvested or tested for insulin secretion assay.

HEK-293T cell line

HEK-293T cells were maintained in DMEM, 10% foetal bovine serum, 2mmol/l Glutamine, and 1X Antibiotics/Antimicotic at 37°C and 5% $CO_2$ and used for experiments when confluent.

Beta-TC1 and Alpha-TC1-6 cell lines

Beta-TC1 cells (passages 10-20) were culture in DMEM, 15% Horse serum, 2.5% fetal bovine serum, 2mmol/l Glutamine, and 1X Antibiotics/Antimicotic at 37°C and 5% $CO_2$.

Alpha-TC1 clone6 were maintained in DMEM 10% fetal bovine serum, 2mmol/l Glutamine, and 1X Antibiotics/Antimicotic at 37°C and 5% $CO_2$. Palmitic acid treatment was performed as follows. Briefly, 0.5mM of Palmitic acid preparation was melted in boiling water and then filtered using 0.22uM mesh filters. The right quantity of Palmitic acid was then added to the medium after 24h of cell culture. Treatment was performed for 48h and then the cells were harvested for subsequent analysis.

Human pancreatic islets derived mesenchymal cells (hPIDM)

hPIDM cells were obtained as follows: Approximately 50 islet equivalents, without dissociation, were cultured in 100mm plastic tissue culture dishes (Falcon; Becton Dickinson, San Jose, CA, USA) in growth medium (GM) (modified RPMI 1640 medium (11.1mM glucose) (Sigma Aldrich, St. Louis, Mo, USA) supplemented with 10% FBS (Stem Cell Technologies Inc., Vancouver, BC, Canada), 2mmol/L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 250 ng/ml amphotericin B, (Sigma)) and maintained at 37°C in 5% $CO_2$ and 95% humidified air. After 15 days of culture, adherent islets were detached with 0.25% trypsin-2mM EDTA (Sigma) and seeded at a density of 12000 cells/$cm^2$ for two passages and subsequently at a density of 4000 cells/$cm^2$. Culture medium was replaced every 3 days *(Gallo R.et al., 2007*).

To induce a pancreatic endocrine differentiation, $6x10^5$ cells were plated in six-well plastic dishes with differentiation medium consisting in RPMI 1640 supplemented with insulin (10 mg/ml), transferrin (5.5 mg/ml), sodium selenite (6.7 ng/ml) and 1% BSA (all from Sigma). Cells maintained in GM were used as controls. Culture medium was changed every 3 days.

For time course expression analysis hPIDM cells were harvested at 3, 7, 11, 14, 18, and 21 days of pancreatic differentiation program.

3.4- Dual Luciferase reporter activity assay

MiR-124a targeting assay was performed subcloning FoxA2-3'UTR and Mtpn-3'UTR into pRL-TK vector plasmid and cotransfected with firefly luciferase pGL3 vector and hsa-miR-124a mimic (Dharmacon) at a concentration of 50nM. Luciferase activity was assayed after 24h with the Dual-Luciferase Assay (Promega, Madison, WI).Transfections were performed in HEK-293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with manufacturer's instructions.

3.5- Laser Capture microdissection (LCM)

Murine pancreatic tissues were extracted from wild type C57Bl-6 mice and immediately frozen using a rapid protocol (15'') in order to preserve nucleic acids integrity. Briefly, an isopentane chamber surrounded with dry ice and ethanol was prepared at least 40' before pancreas extrac-

tion. Mouse pancreas were OCT embedded, frozen, incubated in dry ice for 1h and finally stored at -80°C until ready for cryostat sectioning.

Immediately prior to LCM, 5-8 um frozen murine pancreatic sections were dehydrated and stained in 70% ethanol for 30 seconds, DEPC treated $H_2O$ for 10 seconds, Hematoxilyn Mayer's for 30 seconds, DEPC treated $H_2O$ for 10 seconds, 70% ethanol for 30 seconds, 95% ethanol for 30 seconds, ethanol 100% for 2 minutes and xylene for 5 minutes.

The sections were air dried for 5 minutes and LCM was performed using Arcturus XT Laser Capture Microdissection system (Arcturus engineering, Mountain view, CA). LCM was performed by melting thermoplastic films mounted on transparent LCM caps (Arcturus) on selected central core of murine islets containing beta cells or on selected periphery containing non-beta cells.

For the smallest spot size, the system was set to the following parameters: 90mW for the IR laser power, 12 msec pulse duration, and 10um spot size. The thermoplastic film containing the microdissected cells was mounted on ExtracSure Sample Extraction device and incubated with 10 ul of Extraction buffer (Picopure RNA extraction kit) for 30 minutes at 42°C. Each microdissection was performed in 30-45 minutes, during which no more than 4 sections were processed. 20 sections were used to obtain enough RNA for the RT-Real Time PCR.

### 3.6- Insulin secretion assay

Insulin secretion in response to glucose was assessed as previously described *(Marselli L. et al., 2001).* Briefly, following a 45 min pre-incubation period at 3.3 mmol/l glucose, 20 pseudoislets were plated into 6-wells plastic dishes and kept at 37°C for 45 minutes in Krebs-Ringer bicarbonate solution (KRB), 0.5% albumin, pH 7.4, containing 3.3 mmol/l glucose. At the end of this period, medium was completely removed and replaced with KRB containing either 3.3 mmol/l or 16.7 mmol/l glucose. After additional 45 min incubation, the medium was removed. Samples (500 µl) from the different media were stored at -20°C prior measurement of insulin concentration by IRMA

## 3.7- RNA extraction

RNA extraction from human pancreatic islets, from cell line samples and from PBMCs has been performed using the phenol-chloroform method. Cell pellets have been lysed adding 1ml of TRIZOL (Invitrogen), a solution containing phenol and guanidine isothiocyanate and homogenized with a syringe. Homogenized cells have then been centrifuged at 12000 rpm, 4°C, for 10' to eliminate the non-solubilized debris;200 µl chloroform has been added to the supernatant and, after a centrifugation at 12000 rpm for 15' at 4°C, the upper watery phase containing RNA has been recovered. RNA has been precipitated adding 500 µl cold isopropanol and centrifuged at 12000rpm at4°C, for 10'. The RNA containing pellet, after a wash with 1 ml 70% cold ethanol followed by a centrifugation at 12000 rpm for 5' at 4°C, has been resuspended in a volume ranging between 20 and 40 µl nuclease Free $H_2O$ on the basis of the pellet size.
RNA concentration has been evaluated measuring absorbance at 260nm using Nanovue spectrophotometer system (GE Healthcare). All samples had a ratio of absorbance 260/280 nm between 1.8 and 2.3
Total RNA extracted from each LCM sample was performed using PicoPure RNA isolation kit (Arcturus). RNA extraction quality was evaluated with RNA Pico Chips (Agilent Technologies)using Agilent 2100 Bioanalyzer with a RIN cutoff of >5 for Picopure extracted samples and >7 for phenol-chloroform extracted samples.

## 3.8- RT-Real Time PCR for gene expression assay

The reverse transcriptase reaction has been performed using up to 1 µg RNA, which has been retro-transcribed adding 1 µl dNTPs (100mM), 0.2 µl Random primers (50mM) and 0.8 µl Nuclease free $H_2O$. After 5' incubation at 65°C the following reagents have been added: 4 µl Buffer RT 5X, 1 µl Rnase OUT (40U/µl) (Invitrogen), 2 µl Diothiothreitol (DTT) (0.1µM). After incubation at RT for 10', 1 µl the Multiscribe$^{TM}$ RT enzyme (50U/µl) (Applied Biosystems) has been added and the following cycles have been performed:42°C x 50', 70°C x 15'.
Quantitative analysis of mRNA expression of genes of interest has been performed by Real Time PCR, using Taqman$^{TM}$ gene expression assays (Applied Biosystem).
For each reaction 25ng of corresponding cDNA has been used and added to a mix composed by: 12.5ul of Taqman Universal PCR Master Mix, 1.25ul Taqman gene expression assay and Nuclease Free $H_2O$ at 25ul final volume. We have used the following cycles of amplification:

50.0° for 2',95.0°for 10', 95.0° for 15''60.0°for 1'(these last two steps were repeated for 40cycles).

3.9- RT Real Time PCR for microRNAs single assay expression and array profiling

RT-stem loop Real-Time PCR was performed to evaluate miRNAs relative expression. Assays for miRNA profile analysis and for the internal controls were carried out according to Applied Biosystem protocols (Foster City, CA). Briefly, RT reactions containing 10ng RNA, specific stem-loop primers for each miRNA, 1X buffer, dNTPs reverse transcriptase and RNAse inhibitor were incubated in a DOPPIO™ Thermalcycler (VWR) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. Then, Real Time PCR for miRNA expression levels was performed using miRNA specific TaqMan MGB probe and TaqMan universal master mix in an Applied Biosystem 7900HT PCR system in 96 well plates, in duplicate. MiRNAs expression levels were normalized to the internal controls smallRNAs RNU6, RNU48, RNU44, RNU6B. The comparative threshold cycle method was used to calculate the relative miRNA expression among sample groups.

For control or T2DM pancreatic islet samples, the expression profiling on 150 microRNAs was performed using Taqman 96 wells microRNA assays custom designed (Applied Biosystem) following the manufacturer instructions.
For hPIDM cells, microRNAs expression profiling has been evaluated using Taqman microRNAs fluidic cards array A and B. This technology allowed us to perform a complete screening on a total of 754 microRNAs splitted into panel A card (which contains assays for most generally expressed and interesting microRNAs), and into panel B card (which contains assays for low expressed and "star" microRNAs).
Before loading Taqman array cards, specific cDNA has been prepared using a stem loop primers mix, including a total of 384 microRNAs primers (panelA or panelB primers mix), following the same reaction as for single assay microRNA RT.
Taqman microRNA microfluidic cards were then loaded using: 1X Taqman Universal Master mix II (Applied Biosystem, Foster, CA)and 500ng cDNA from each sample.

### 3.10- MicroRNAs target genes computational prediction

Gene targets of selected microRNAs were predicted using online prediction algorithms Targetscan 5.1(www.targetscan.org), PicTar (http//pictar.mdc-berlin.de) and MiRanda(http://www.microrna.org);only genes belonging to pancreatic beta-cell pathways were taken into consideration.

### 3.11- Statistical analysis

Statistical analysis was performed using both Student T test and Mann Whitney U test, depending on the experimental procedures and the sample size.
For Taqman microRNAs profiling analysis, the majority of analyses were performed using Real time Statminer software (Integromics).

# 4-RESULTS

## 4.1- MicroRNAs and T1DM

### 4.1.1- Hsa-miR-326

A recent study by Du et al. reported an increased expression of miR-326 in peripheral blood mononuclear cells (PBMCs) from patient with autoimmune multiple sclerosis. The upregulation of miR-326 was most evident in patients with a relapsing state of the disease respect to the remitting one, evidencing how high levels of this microRNA were associated with a state of ongoing autoimmunity. The authors also found that the alteration of miR-326 expression was specific to the Th-17 T-cell subset, which was demonstrated to be involved in pathogenesis of autoimmune diseases *(Du C. et al. 2009).*

Given the similarity of molecular autoimmune onset among different diseases, we analyzed the miR-326 expression in PBMCs from T1DM patients with or without ongoing islet autoimmunity. To distinguish between patients with or without islet autoimmunity, we subdivided patients into islet autoantibody positive and negative, taking into consideration the two main autoantibody characterizing the clinical progression of T1DM: Glutamate Acid Decarboxylase antibody (GADA) and Islet-Antigen 2 antibody (IA-2A) (Table1).

| GROUP | SAMPLE NAME | SEX | AGE (YEARS) | DISEASE DURATION(YEARS) | GADA | IA-2A |
|---|---|---|---|---|---|---|
| *Ab[-]* | 1 | M | 41 | 18,0 | - | - |
| | 2 | M | 29 | 2,8 | - | - |
| | 3 | F | 20 | 14,8 | - | - |
| | 4 | F | 36 | 9,4 | - | - |
| | 5 | F | 38 | 22,3 | - | - |
| *Ab[+]* | 6 | M | 34 | 0,0 | + | - |
| | 7 | M | 75 | 0,1 | + | - |
| | 8 | M | 38 | 15,3 | - | + |
| | 9 | F | 32 | 10,4 | - | + |
| | 10 | F | 23 | 4,7 | + | - |
| | 11 | F | 50 | 22,7 | + | - |
| | 12 | F | 51 | 23,0 | + | - |
| | 13 | F | 39 | 10,3 | + | - |
| | 14 | F | 58 | 16,2 | + | - |
| | 15 | F | 65 | 4,3 | + | - |
| *Ab[++]* | 16 | M | 20 | 0,0 | + | + |
| | 17 | M | 21 | 0,0 | + | + |
| | 18 | M | 22 | 0,0 | + | + |
| | 19 | F | 43 | 8,8 | + | + |

**Table 1.**

4.1.2- miR-326 expression in PBMCs from T1DM patients

By using quantitative Real Time PCR, we analyzed miR-326 expression in the three groups described in Table1. Firstly, we compared Ab[-] patients versus total Ab[+] patients, regardless of the single or double positivity to GADA or IA-2A. We found miR-326 significantly increased ($p<0.05$) in PBLs from Ab[+] patients (2.68±0.35 fold increase), vs Ab[-] patients (Fig.7a). Next we compared Ab[-] patients to Ab[+] and Ab[++] patients, separately. We found a significant increase ($p<0.05$) in miR-326 expression both in Ab[+] and Ab[++] (2.93±0.46 and 2,05±0.38 fold increase, respectively), but we did not found any difference in Ab[+] versus Ab[++] patients (Fig.7b).

We did not find any correlation between disease duration and miR-326 expression levels in PBLs from T1D patients.

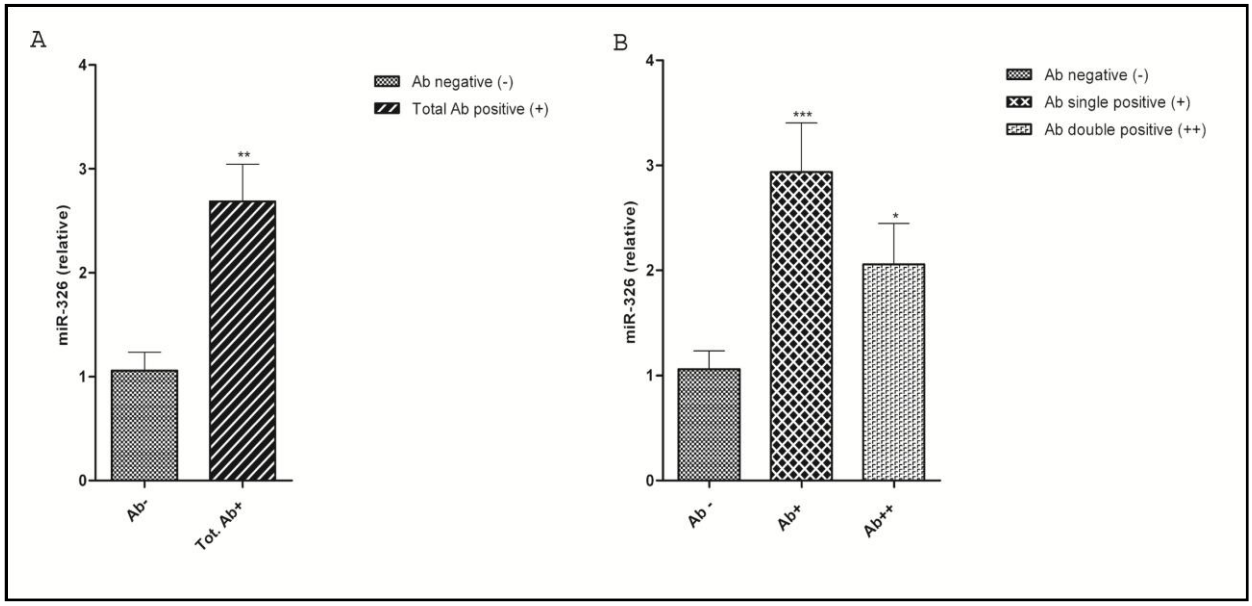


**Figure 7**. A: Real time qPCR analysis of hsa miR-326 in PBLs from type 1 diabetic patients. Autoantibodies negative (n=5) vs total autoantibodies positive patients (n=14) are reported.

B: Real time qPCR analysis of hsa miR-326 in PBLs from type 1 diabetic patients. Autoantibodies negative (n=5) vs total autoantibodies single positive patients (n=10) and vs double positive patients (n=4) are reported.

Data are presented as mean ± s.e.m. Statistical analysis was performed using Mann Whitney U test (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ vs autoantibody-negative subjects)

4.1.3- MiR-326 target genes

In the light of the above results, we looked for potential predicted mRNA target genes of miR-326, which could be consequently modulated as a consequence of such increased expression. By the online prediction algorithm Targetscan 5.1 we found 2 genes of interest: 1) the already known Erythroblastosis virus E26 oncogene homolog 1 (Ets-1) (Fig.8a); 2) Vitamin D receptor (VDR) (Fig8.b). According to Targetscan 5.1 algorithm we found at least 4 target sequences for miR-326 into the 3'UTR of VDR, indicating this gene as a predicted bona fide miR-326 target gene.

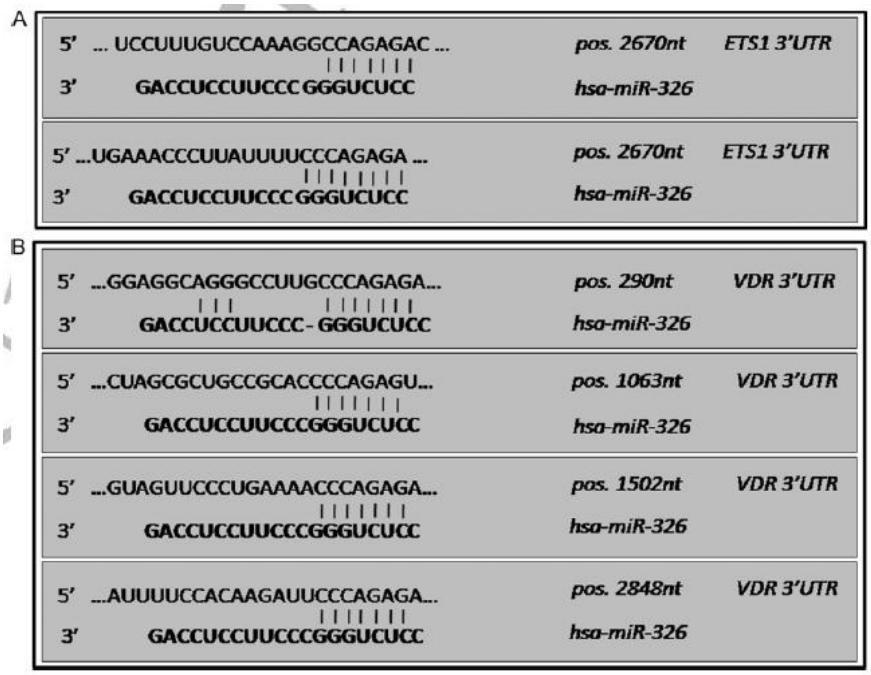


**Figure 8**.Targetscan 5.1 mRNA target prediction for hsa-miR-326. (A) *Ets-1* 3_-untranslated region shows two target sequences for hsa-miR-326. (B) *Vitamin D receptor* 3'-untranslated region shows four target sequences for hsa-miR-326. Positions of the first nucleotide of the seed sequences within 3'-untranslated region are reported.

## 4.2- MicroRNAs and T2D

### 4.2.1- MicroRNAs expression profiling in T2DM human pancreatic islets

MiRNAs have been demonstrated to control beta cell function mainly acting by modifying insulin secretory machinery genes expression. Extensive studies using T2D animal models reported alteration of miRNAs expression on both pancreatic islets and insulin target tissues. We analyzed the expression profile of 150 microRNAs on four normal and four T2D human pancreatic islets preparation, using RT Real Time PCR and Taqman 96-well plates (custom designed).

We identified 142 microRNAs well expressed in the samples, using a Ct cutoff of 35 (Fig.9). Of these 142 miRNAs, 14 were differentially expressed between non-diabetic and T2D samples ($p<0.05$). Specifically, 6 miRNAs resulted upregulated and 8 miRNAs downregulated in T2D islets. Among these 14 microRNAs, we identified 6 of them with interesting target genes in terms of beta-cell physiology: 3 of them were upregulated (miR-124a, miR-337,miR-138)(Fig.10a) and 3 downregulated (miR-184, miR-187, miR-31)(Fig10b).

As a negative control for profiling analysis, we included C.Elegans miRNA cel-lin-4, which, as expected, was found not to be expressed in the samples analyzed; hsa-miR-16 was used as positive miRNA control.

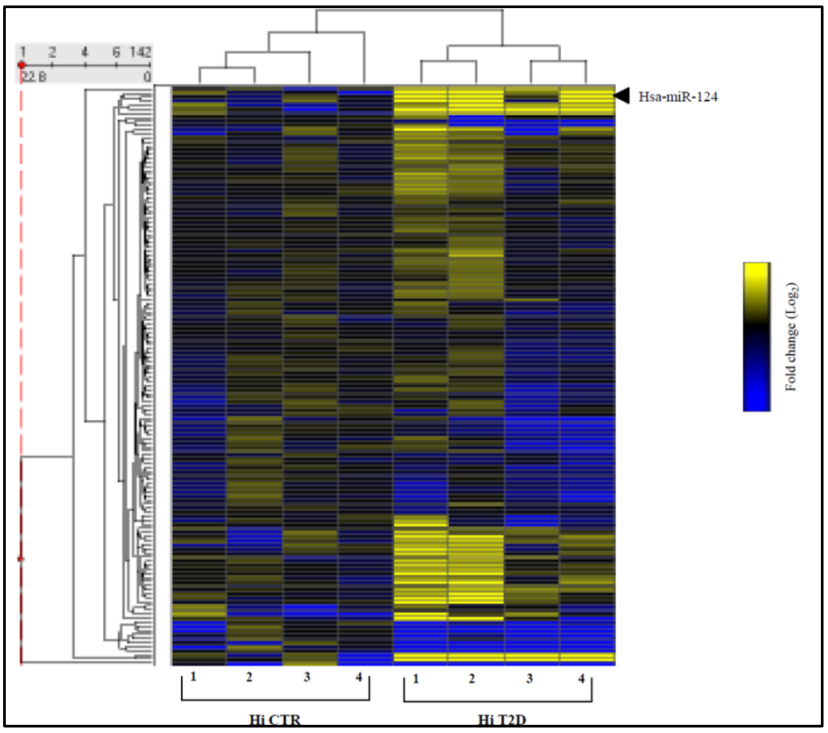


**Figure 9.**Hierarchical clustering heatmap analysis of microRNAs profiling on 4 normal and 4 T2DM human pancreatic islets

*

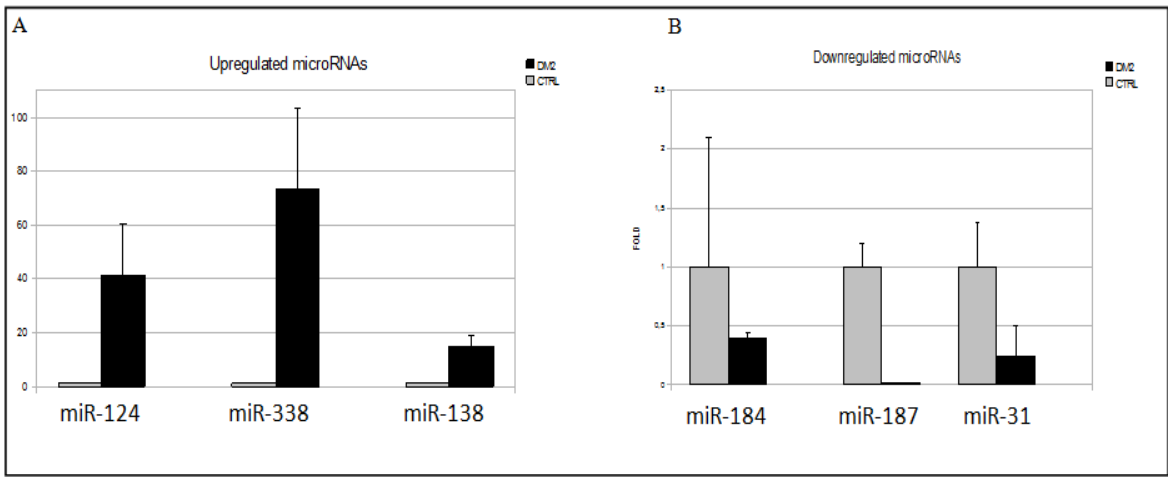


**Figure 10.**MicroRNAs RT Real time PCR on control/T2D Human islets. A. Upregulated microRNAs in T2DM pancreatic islets, with a putative role in beta cell physiology. B. Downregulated microRNAs in T2DM with a putative role in beta-cells. Values are reported as mean of relative expression (n=3). Statistical analysis was performed using Mann Whitney U test (*=p<0.05, **=p<0.01, ***=p<0.001)

4.2.2- MiR-124a

- MiR-124a is upregulated in T2DM

Among upregulated microRNAs, we interestingly identified miR-124a. Three of four T2D pancreatic islets donors showed a strong increase in miR-124a expression levels (Fig.11a). We statistically found a miR-124a significant increase (31.2± 8.2 fold increase; $p<0.01$) in T2D pancreatic islets compared to islets from age-matched control donors (Fig.11b).

Of relevance, we found that the expression levels of miR-375, considered as the most important endocrine pancreatic miRNA, did not show any alteration between normal and T2D islets.

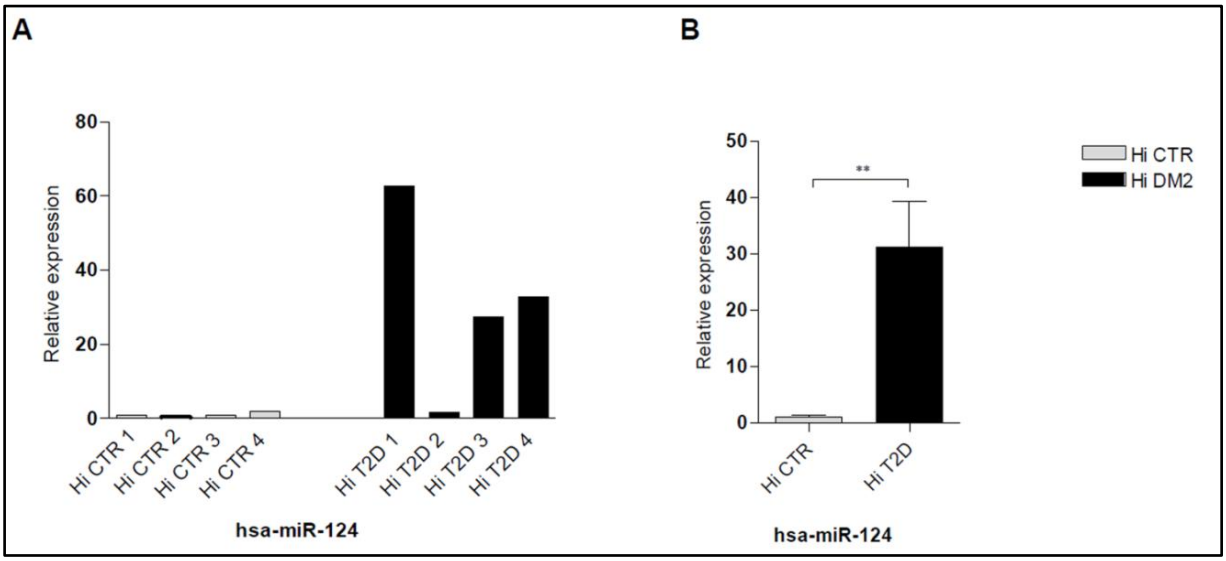


**Figure 11**.A. RT-Real time PCR profiling of microRNAs (miR-124a) on 8 human islet samples (4 normal/4T2DM). B. Relative expression of miR-124a in CTR and T2DM Hi. Mean±s.e.m values are reported. Statistical analysis was performed using Mann Whitney U test (**=$p<0.01$).

- miR-124a is not modulated by high glucose

In order to exclude the possibility that miR-124a could be modulated by high glucose concentration, which can occur in T2D condition *in vivo*, we treated normal human islets for 24h with elevated glucose levels (22mM) to simulate a hyperglycemic status, comparing high glucose treated islets with basal glucose concentration (5.5mM) treatment. We therefore measuring miR-124a expression levels through Rt-Real Time PCR on three human islets preparation derived from three different age-matched donors, and we found that its expression did not significantly change upon high glucose treatment (Fig.12).

Our findings are in accordance with the recent work of R.G Fred et al. which found that human islets miR-124a levels were unaffected by high glucose treatment.

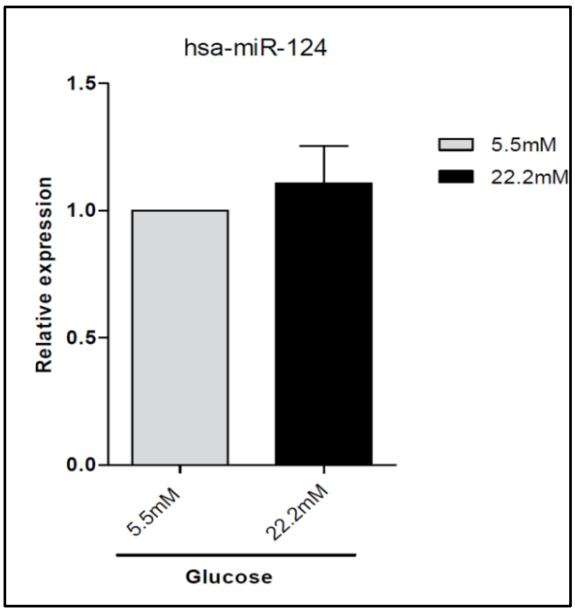


**Figure 12.**RT Real Time PCR of miR-124a in Human islets treated with basal or high glucose. No significant differences were reported.

- MiR-124a is a biological modulator of beta-cell genes

Using a combination of online prediction algorithms (Targetscan5.1, miRanda and Pictar), we looked for miR-124a target genes, mainly focusing on those belonging to pivotal pathways that control beta-cell physiology and function. Therefore, we selected participating to: glucose sensing apparatus, insulin exocytotic machinery, beta cell mass control and beta-cell identity pathways.

We identified following genes with one or more miR-124a target sequences in their 3'UTR: Flot2 (Flotillin-2), NeuroD1(Neurogenic differentiation 1), Akt3 (v-akt murine thymoma viral oncogene homolog 3), Sirt-1(Sirtuin-1)and experimentally validated target Mtpn (Myotrophin) (Table2) *(Wojcik M. et al.,2009; Taoka M.et al., 2003; Accili D. et al.,2004; Wang HF. Et al.,2002)*. For the other experimentally verified miR-124a target gene, Foxa-2 (Forkhead box A2), we did not found any predicted miR-124a target sequence using any of the three algorithms, but given the importance and the data already available about miR-124a and Foxa-2, we chose to include it in our analyses.

For all target genes selected, we verified a 100% conservancy between human and mouse.

To investigate the influence of miR-124a on transcript levels of selected target genes, we inhibited its function in Min-6 derived pseudoislets. Although monolayer Min-6 cells reveals the ability of glucose-stimulated insulin secretion (GSIS) similar to isolated pancreatic islets, Min-6 derived pseudoislets have been demonstrated to be more responsive to glucose changing respect to monolayer, making them more suitable to test the effect of miR-124a deprivation on beta cell function. We transfected pseudoislets cultured for 15 days with 50mM mmu-miR-124a inhibitor, changing the transfection medium after 48h and harvesting them after 72h post second transfection. By RT-Real time PCR we evaluated the mRNA expression of selected target genes. Following miR-124a inhibition, target genes should hypothetically show an upregulation of their expression in consequence of a lack of repression. Indeed we found a significant upregulation of all six selected target genes transcript levels following miRNA inhibition (Fig.13). The two most important selected target genes, Foxa2 and Mtpn, showed a ~2,0 fold increase ($p<0.05$), indicating a role of miR-124a in controlling these genes which made part of pivotal beta-cell pathways.

| Entrez ID | Gene name | Gene symbol | Function |
|---|---|---|---|
| 2319 | Flotillin-2 | FLOT2 | Flotillin forms a ternary complex with CAP and Cbl, directing the localization of the CAP-Cbl complex to a lipid raft subdomain of the plasma membrane, regulating glucose uptake |
| 4760 | Neurogenic differentiation 1 | NEUROD1 | NeuroD1 forms an heterodimer with beta2/E47 transcriptional factor, inducing insulin gene transcription |
| 10000 | v-akt murine thymoma viral oncogene homolog3 | AKT3 | AKT kinases are known to be regulators of cell signaling in response to insulin and growth factors. They are involved in a wide variety of biological processes including cell proliferation, differentiation, apoptosis, tumorigenesis, as well as glycogen synthesis and glucose uptake |
| 23411 | NAD-dependent deacetylase sirtuin-1 | SIRT1 | Sirtuins may function as intracellular regulatory proteins with mono-ADP-ribosyltransferase activity. Alteration of ATP/ADP ration could influence insulin secretion |
| 136319 | Myotrophin | MTPN | Myotrophin is involved in remodeling of F-Actin filaments, regulating the boundary of secretion vesicles to cell membrane |
| 3170 | Forkhead box A2 | FOXA2 | It is involved in development of endocrine pancreas. Transcriptional regulator of many specific beta-cell factors |

**Table 2.** miR-124a putative and experimentally validated target genes (underlined). Gene ID as well as their function in beta-cells are reported.

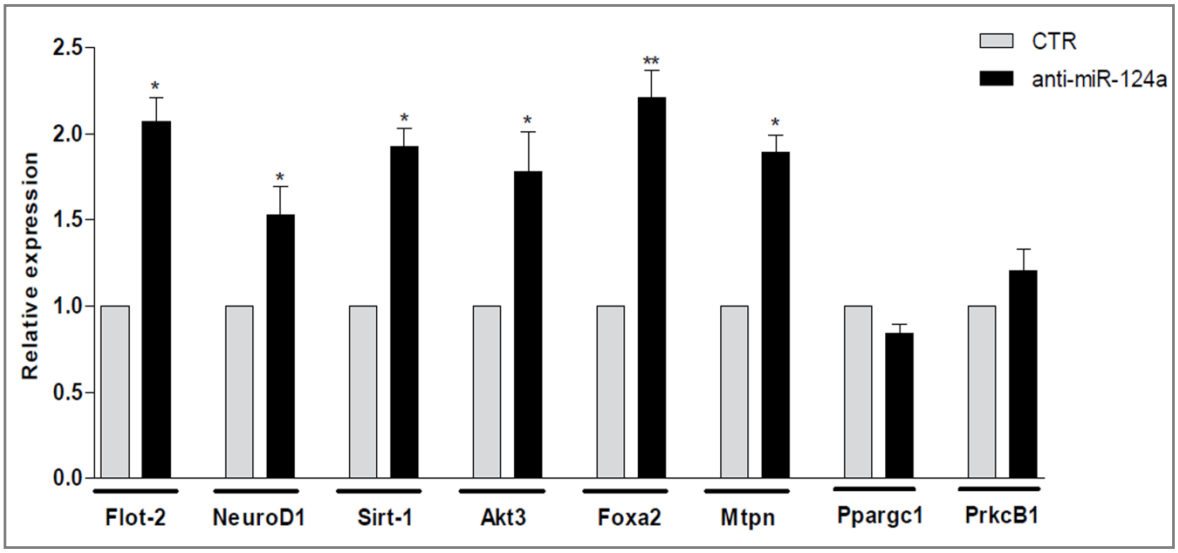


**Figure 13**. RT-Real Time PCR of miR-124a target genes upon miR-124a inhibition**.** Two genes related to beta cell metabolism and function (Ppargc1, PrkcB1), but not predicted to be miR-124a target genes were reported as not modulated. Mean±s.e.m. values are reported. Statistical analysis was performed using student *t* test (*=p<0.05, **=p<0.01).

- MiR-124a binds to the 3'UTR of Foxa2 and Mtpn

In order to confirm the targeting of miR-124a on human 3'UTR of Foxa2 and Mtpn we performed a dual luciferase assay experiment. This assay allows us to detect the actual targeting of miR-124a on 3'UTR of the selected genes.

We observed that the level of luciferase activity was lower in HEK-293T cell overexpressing miR-124a, both for FoxA2 and Mtpn and, moreover, in the case of target sequence mutation, this decrease was completely restored, demonstrating the activity of miR-124a on that sequences (Fig.14).

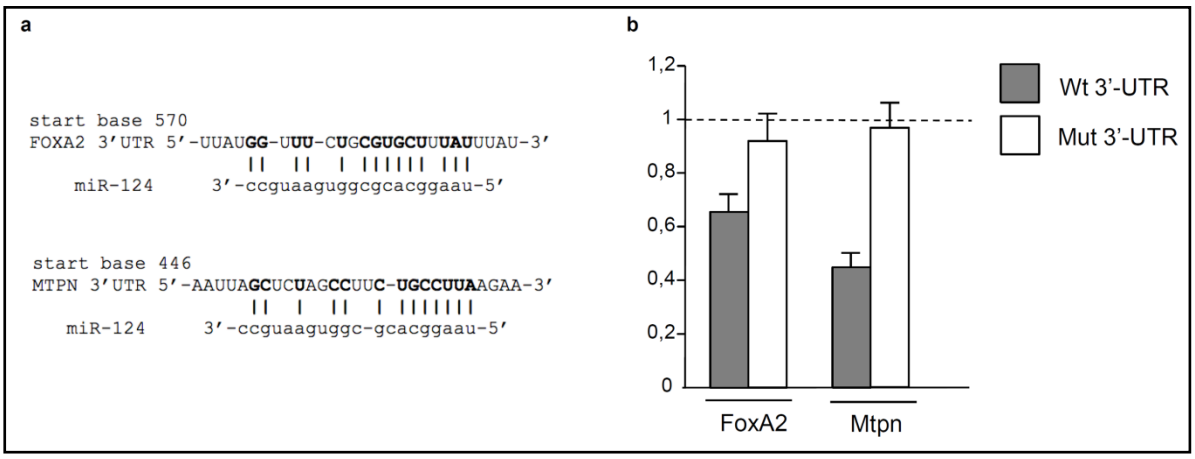


**Figure 14**. A. Representation of miR-124a and FoxA2 or Mtpn transcript interaction. B. Levels of luciferase activity in HEK293 cells overexpressing miR-124aa and transfected with the wild-type 3'-UTR vector (wt) or its mutant derivative lacking the miRNA-binding sites (mut). Data are indicated as ratios with respect non targeting RNA (dashed line).

- MiR-124a overexpression deregulates glucose stimulated insulin secretion

In order to verify the effects of high miR-124a expression on insulin secretion, we overexpressed it in Min6 cells derived pseudoislets to simulate the biological situation we have found in human islets.

We transfected 15-days old Min6 pseudoislets with mmu-miR-124a mimic or scrambled as negative control and, after 48h+72h transfection, as previously did for miR-124a inhibitors transfection, we measured insulin secretion under basal or stimulated glucose concentration (3.3mM and 16.7mM).

We observed that under basal condition (3.3mM glucose), overexpression of miR-124a did not alter insulin secretion respect to control; after the glucose stimulus (16.7mM), the insulin secretion in consequence of high levels of miR-124a diminished of 45.4% (54.6±3.2% of the control) (Fig.15), demonstrating that miR-124a overexpression alters insulin secretion only when the exocytotic machinery is fully working up.

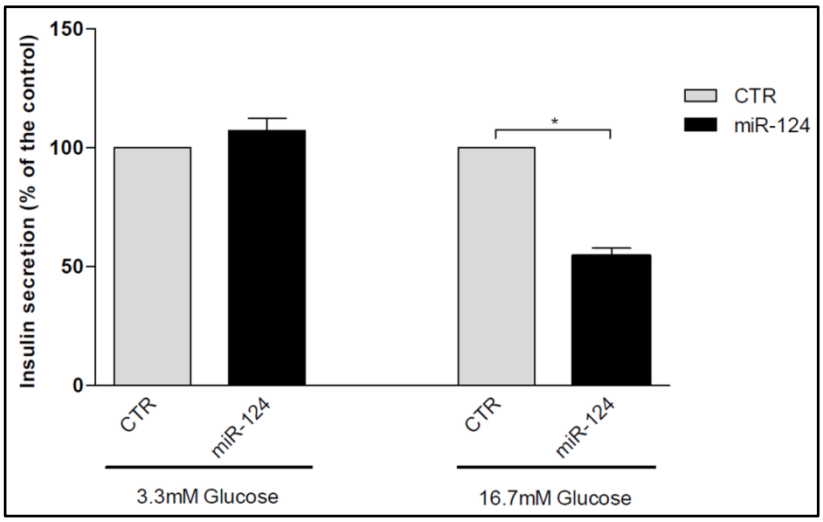


**Figure 15.**Insulin secretion assay on Min6 pseudoislets under basal (3.3mM) or stimulating (16.7mM) glucose concentrations upon miR-124a overexpression. Data at basal or stimulating glucose concentrations were analyzed respect to scrambled CTR.

4.2.3- MiR-184

- MiR-184 is downregulated in T2DM islets

Among downregulated microRNAs we firstly focused on miR-184.

Despite the relative high variability among patients, we detected a significant down-regulation of miR-184 in 4 T2DM pancreatic islets respect to 4 normal islets (Fig.16a). Specifically, we observed approximately 10 fold decrease in miR-184 expression in diabetic islets respect to normal one (Fig16b).

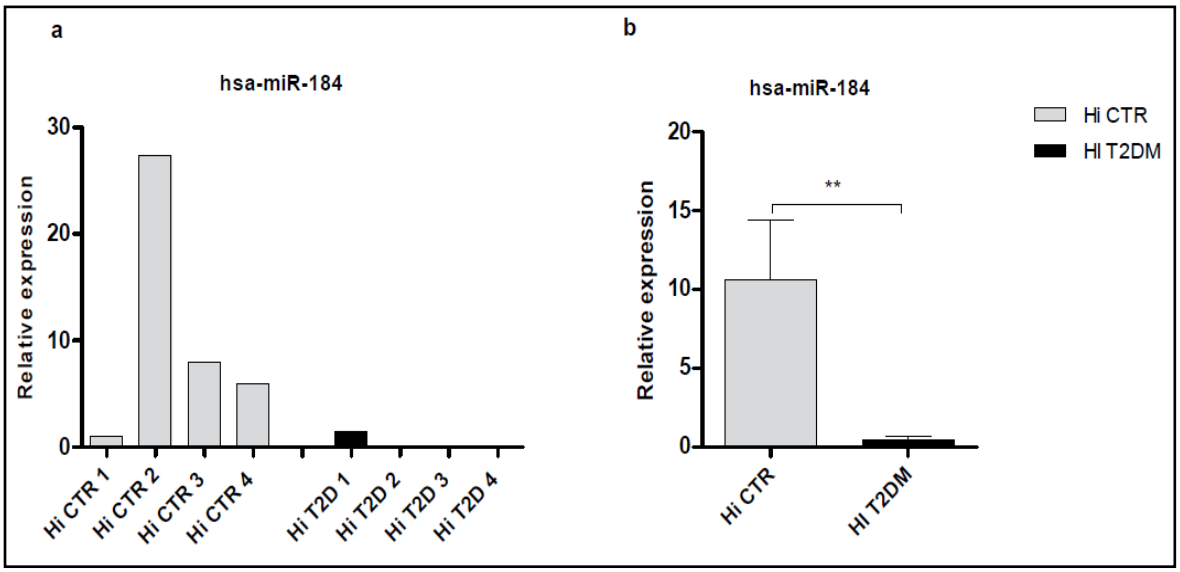


**Figure 16.** A. RT-Real time PCR profiling of microRNAs (miR-184) on 8 human islet samples (4 normal/4T2DM). B. Relative expression of miR-184 in CTR and T2DM Hi. Mean±s.e.m values are reported. Statistical analysis was performed using Mann Whitney U test (**=p<0.01)

- MiR-184 is differentially expressed between alpha and beta-cells

In order to verify the possibility that miRNAs found to be modulated (both upregulated and downregulated) in T2DM could be differentially expressed between the two major cell populations within islet (alpha and beta-cells), we evaluated their expression in the mouse alpha and beta cell lines, Alpha-TC1-6 and Beta-TC-1(Fig.17). These cell lines derive respectively from mouse glucagonome and insulinome, but they are widely used to verify molecular processes in order to simulate the islet environment.

Through RT-Real time PCR we observed that miR-184 is highly expressed in Beta-TC1, but not in alpha TC1-6.Moreover, we verified this specific signature between alpha and beta cell lines using cells at different culture passages in order to exclude any possible variation depending on passage number (Fig.18). We did not find any differential expression between alpha-TC1 and Beta-TC1 for the above mentioned miR-124a.

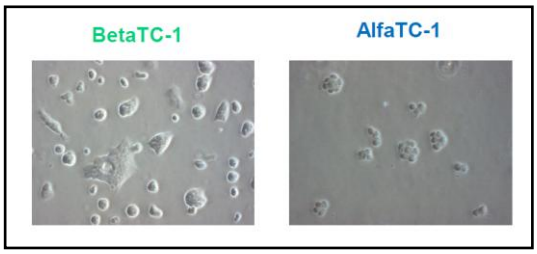


**Figure 17**. Right. Phase contrast microscopy of betaTC-1 cells. Left. Phase contrast microscopy of alphaTC-1 cell line.

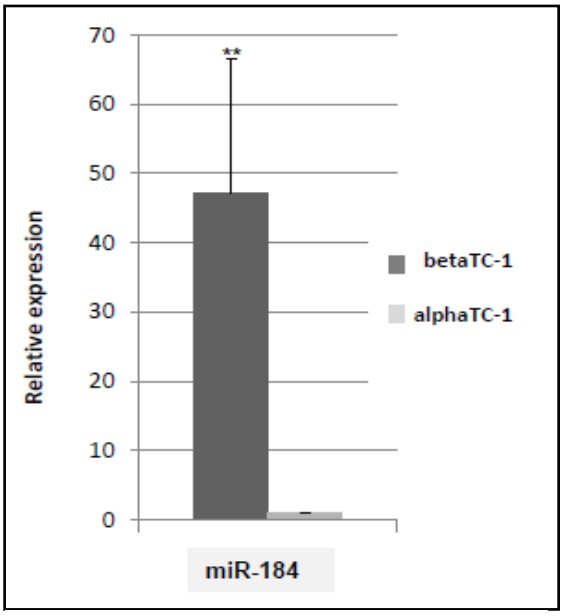


**Figure 18.** RT Real Time PCR of miR-184 in beta and alpha TC-1 cell line. . Mean± S.D. values are reported. Statistical analysis was performed using Mann Whitney U test (**=p<0.01)

In order to exclude the possibility that this specific differential expression was due to specific characteristics of these transformed cell lines, we evaluated the expression of miR-184 in laser captured samples of beta-cell and alpha-cell enriched tissue samples obtained directly from mouse pancreatic sections by LCM.

The Laser Capture Microdissection is a novel technique that allows the specific capture of single cells or portion of tissue from frozen sections for subsequent analysis of gene and protein expression. The particular mouse islet architecture (with a beta-cell core surrounded by a mantle enriched in alpha and delta-cells)(Fig. 19), allowed us to specifically capture tissues enriched in beta (islet core) or in alpha-cells (islet periphery) (Fig.20) *(Brissova, M. et al., 2005).*

The tissue captured were then properly processed in order to extract the RNA and consequently to evaluate the expression of selected microRNAs and genes.

Firstly, we evaluated the differential expression of insulin and glucagon between islet core and islet periphery in order to measure the quality of captured samples. We observed that insulin gene expression was up to 2.5 fold higher in islet core than in periphery; conversely glucagon gene expression was up to 6 fold higher in islet periphery than in islet core. These data demonstrate the specific enrichment in beta and alpha cells in the two samples.

Once verified the quality of our samples, we measured the miR-184 specific expression. In accordance with the findings on mouse islets cell line, we detected a higher expression (up to 6 fold), in enriched beta-cell respect to enriched alpha cell sample (Fig.21).

The specific expression of miR-184 in beta but not in alpha cell could be conclusive of two important observations: 1) miR-184 exerts specific functions within beta-cells. 2) the downregulation of miR-184 observed in T2DM human pancreatic islets may completely derive from beta-cell component.

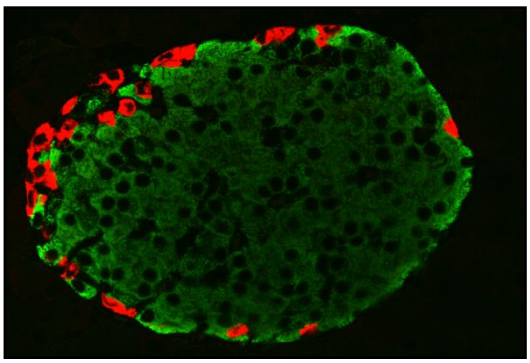

**Figure 19.**Immunofluorescence analysis of Insulin (green) and Glucagon (red) positive cells in mouse pancreatic islets. The particular architecture is shown by the peripheral position of the glucagon positive cells (alpha cells).

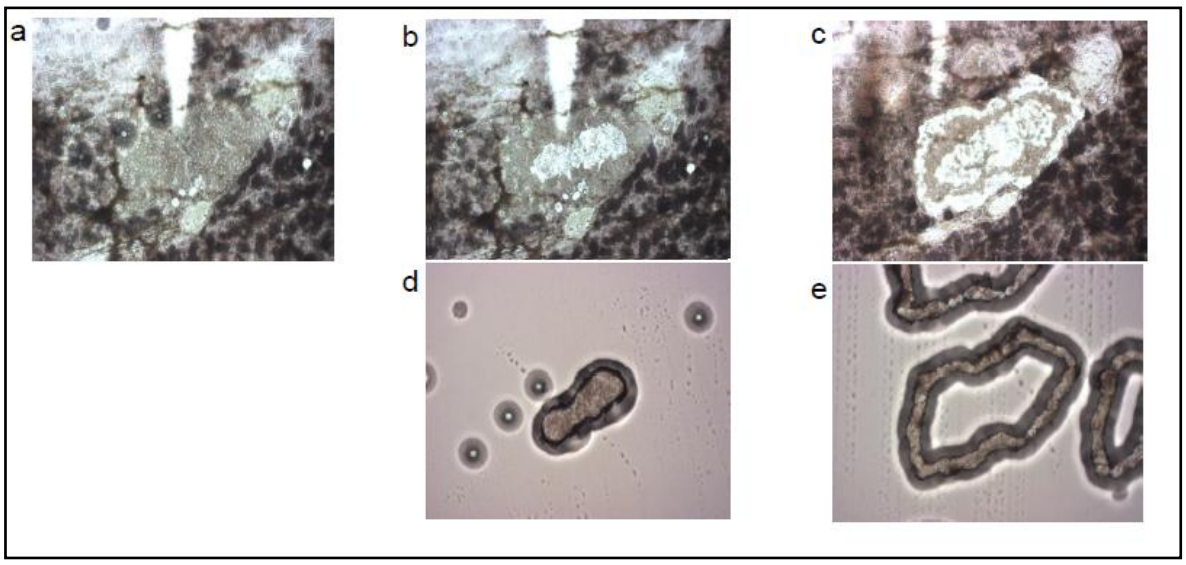


**Figure 20.** Laser Capture Microdissection of mouse pancreatic islets core (beta cells enriched) and periphery (alpha cells enriched). A. Intact islets before capture. B,D. Islet core after capture. C,E. Islet periphery after capture.

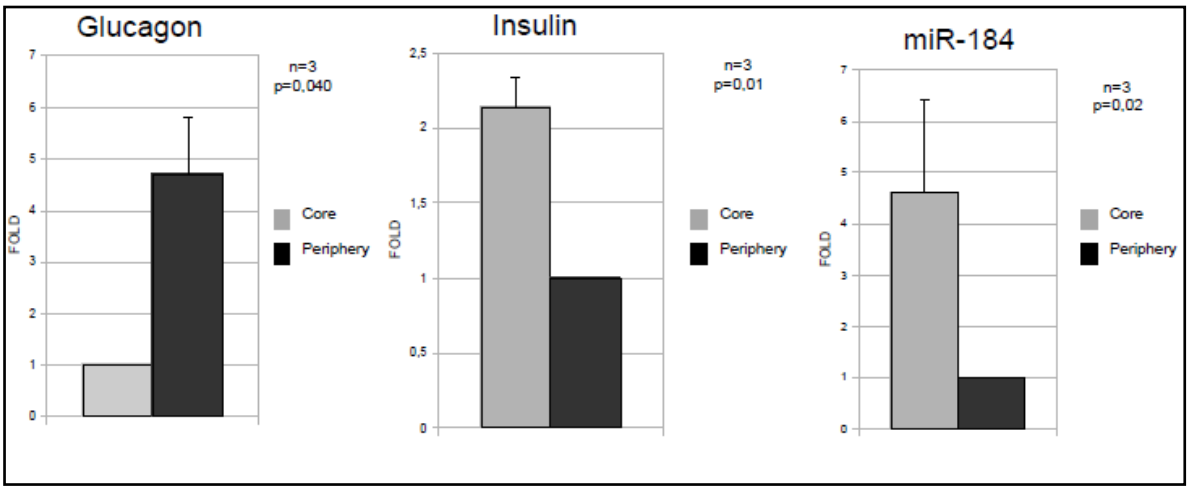


**Figure 21**. RT-Real Time PCR analysis of Glucagon, Insulin and miR-184 on mouse pancreatic islet core and periphery. Resulte are reported as mean ±S.D. Student T test was performed to evaluate the significance of data.

- miR-184 modulates insulin signaling

Using Targetscan algorithm and Pictar we looked for miR-184 target genes with a specific function in beta-cell physiology. We interestingly detected two genes both implicated in the modulation of insulin signaling: Ship2 and Prkc-beta.

Insulin signaling has been demonstrated to be of upmost important in insulin target tissues (skeletal muscle, liver, adipose) as well as in beta-cell itself. Insulin signaling pathway controls cell metabolism, gene expression growth and differentiation *(Muller D. et al.,2006)*.

The binding of insulin on its own receptor activates the receptor kinase to phosphorylate specific residues within its subunits. This autophosphorylation further stimulates the receptor kinase to phosphorylate other cellular proteins such as insulin receptor substrate (IRS) or SHC protein, transmitting the signal. IRS family proteins are the first intracellular effector of insulin signaling. Activation of IRS-1 by receptor kinase activates other cellular signaling protein including the SH2 domain of the phosphatidylinositol (PI) 3-kinase. PI 3-kinase is involved in vesicle trafficking as well as in some metabolic effects of insulin *(Gual P. et al., 2005)*.

SHIP2 protein or INPPL1 is a Phosphatidylinositol (PtdIns) phosphatase that specifically hydrolyzes the 5-phosphate of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) to produce PtdIns(3,4)P2, thereby negatively regulating the PI3K (phosphoinositide 3-kinase) pathways. Indeed it plays a central role in regulation of PI3K-dependent insulin signaling. It has been demonstrated that its overexpression attenuates PI 3-kinase downstream signaling and therefore AKT activation, therefore negatively regulating insulin signaling *(Dyson JM et al., 2005)*.

PRKCB is part of a family of enzymes that are involved in controlling the function of other proteins through phosphorylation of hydroxyl group of serine and threonin aminoacid residues. PRKC beta has important effects on insulin signaling by modulating serine phosphorylation of IRS-1,leading to IRS-1 inactivation and by inducing phosphorylation (activation) of Src Homology phosphatase 2 (SHP2) that is known to dephosphorylation of IRS-1 (inactivation) *(Ishizuka T et al.,2004).*

It is possible to speculate that downregulation of miR-184 may lead to an upregulation of its target genes Ship2 and Prkc-beta, thus negatively regulating insulin signaling in beta-cell itself, possibly contributing to its dysfunction (Fig.22).

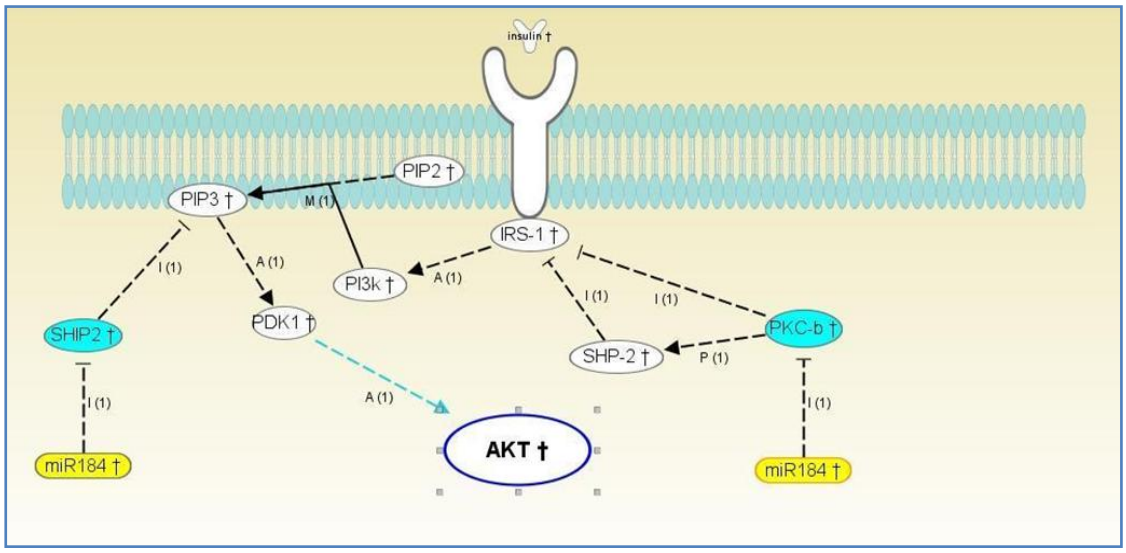


**Figure 20.**.Putative mechanism of action of miR-184. Downregulation of miR-184 may result in upregulation of Ship2 and PKC-beta leading to inactivation of insulin signaling in beta-cells and to their dysfunction.

4.2.3- MiR-187

- MiR-187 is downregulated in T2DM

Another downregulated miRNA in T2DM human pancreatic islets is miR-187. We detected a significant downregulation of miR-187 in all four T2DM patients vs control subjects, without relative variability among patients (Fig.23a, Fig.23b).

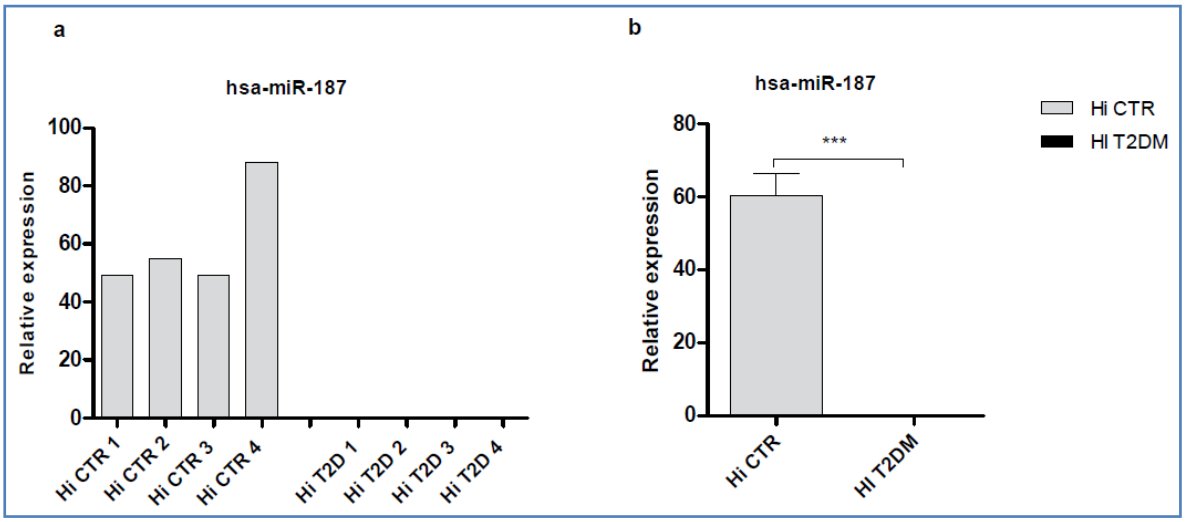


**Figure 21.**RTReal time PCR profiling of microRNAs (miR-187) on 8 human islet samples (4 normal/4T2DM). B. Relative expression of miR-187 in CTR and T2DM Hi. Mean±s.e.m values are reported. Statistical analysis was performed using Mann Whitney U test (***=p<0.001).

- miR-187 target genes with a role in alpha cell function

By using Miranda and Targetscan algorithm we computationally predicted miR-187 target gene with a possible role in beta-cell or islet functions. We interestingly detected two genes with an important role in physiology of alpha cell but no one with a known pivotal role in beta-cell: Glucagon and Pax6 (Fig.24).

Glucagon is the major hormone produced by the alpha cell, while Pax6 has been reported to be as one of the last transcription factor that occurs during alpha cell fate specification.

Recently, the role of alpha-cell in the pathogenesis of T2DM has been reviewed and some authors reported convincing data about the central role of the alpha cell in T2DM. It is possible to speculate that miR-187 downregulation observed during T2DM, may lead to dysfunction of alpha-cells maybe acting through its alpha-cell specific target genes.

Glucagon mouse 3'UTR


Pax6 mouse 3'UTR


Pax6 Human 3'UTR


**Figure 22**.miR-187 target genes prediction by using miRanda algorithm and confirmed with targetscan.

- The FFA, palmitic acid modulates the expression of miR-187 in alpha-TC1 cell line

We tested the possibility that miR-187 in alpha cell could be modulated by lipotoxicity, which usually occurs during T2DM. It has been demonstrated that lipotoxicity acts also on alpha cell components of the islets mainly leading to cell dysfunction than causing cell death as usually happen for beta-cell *(Piro S. et al.,2010)*..

We treated the mouse alpha cell line, alpha TC-1, with one of the mayor components of the FFA: the palmitic acid. The alphaTC-1 cells were cultured for 24h and palmitic acid was then added to the medium for 48h. We evaluated the expression of miR-187 using RT-Real Time PCR and detected a mayor downregulation of miR-187 of 50% respect to control ($p<0.05$) (Fig.25).

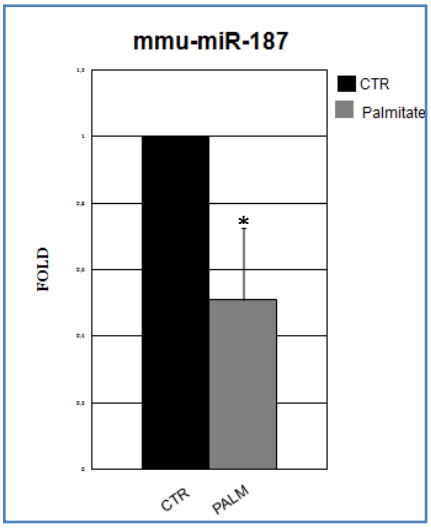


**Figure 25**. RT-Real Time PCR of miR-187 upon AlphaTC-1 Palmitate treatment. Mean ±S.D. values are reported. Student T test was performed (*=$p<0.05$)

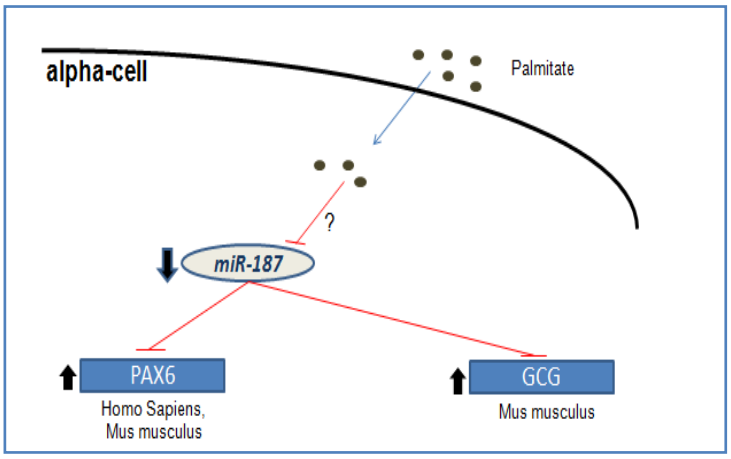


**Figure 26.** Putative mechanism of action of palmitate on miR-187 expression and its predicted targets on alpha cells.

## 4.3- MicroRNAs in Human Pancreatic Islets derived Mesenchymal (hPIDM) cells expansion and differentiation

### 4.3.1- hPIDM expansion from human pancreatic islets and pancreatic differentiation

In order to obtain proliferating hPIDM cells, human pancreatic islet-enriched fractions were cultivated in 100mm culture dishes, using RPMI 1640 medium supplemented with 10% FBS (GM) at 1 islet/cm$^2$.After 2 days all islets attached to the surface of the dishes and during the following 2 weeks they changed their original architecture and acquired a morphology characterized by a central cluster of cells surrounded by a migrating population of flattened epithelial cells. After 14 days cells were trypsinized and transferred to new culture dishes. The cells adhered to the new dishes by a large majority and proliferated. When they reached confluence were subcultured again. After the three passages previously performed, hPIDM cells were subcultured 4000 cells/cm$^2$ every 8 days to adequately expand them. The cells doubled their quantity every 6 days and were cultured for up to 20 passages (Fig. 27).

During expansion the islet cell population acquired a mesenchymal-like phenotype (hPIDM cells). According to us this process resembles the epithelial mesenchymal transition usually occurring during embryo development and during tissue regeneration upon injuries. Indeed it has been demonstrated that human beta-cells are able to undergo the EMT process and to proliferate (*Thiery JP et al.,2009).* This concept has been demonstrated using genetic lineage tracing (lentivirus based) in order to follow the fate of beta-cells during islets cell expansions *(Russ HA et al., 2008).* Subsequently, these hPIDM cells, can be induced to differentiate into hormone-expressing islet-like clusters. In order to evaluate the ability of hPIDM cells to differentiate into a pancreatic endocrine phenotype, cells were cultured for 21 days in the specific pancreatic endocrine differentiation medium previously described (SFM). After the first week, cells formed adherent clusters, but subsequently they detached from the culture plates and acquired an islet-like morphology (Fig.28)

We performed RT- Real Time PCR gene expression profile of mesenchymal or endocrine pancreatic genes on native human islets, proliferating hPIDM cells and differentiated hPIDM cell (Fig. 29).

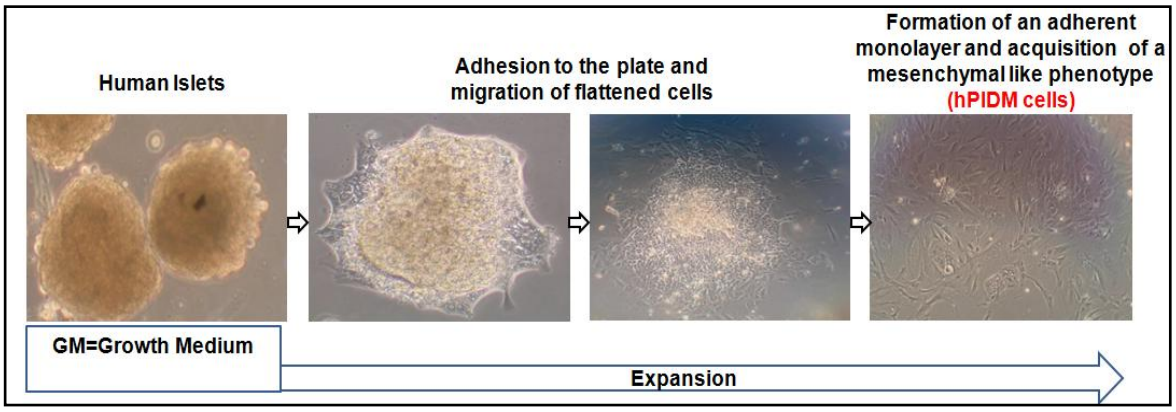


**Figure 27.** Stages of hPIDM generation/expansion from human islets.

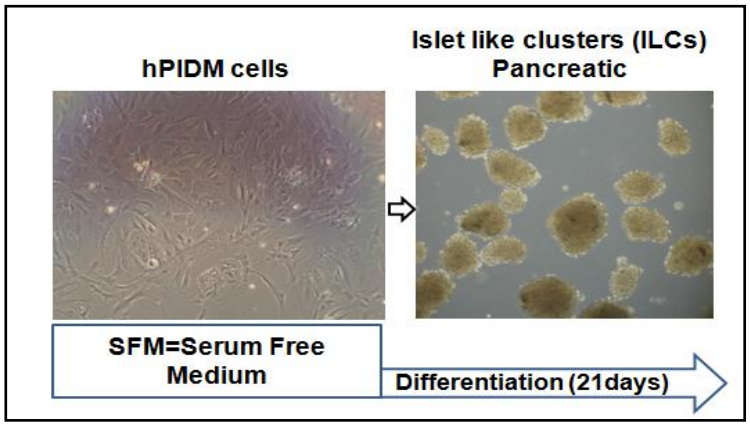


**Figure 28.**hPIDM cells differentiation towards endocrine pancreatic phenotype upon 21days of culture.

In the graphs above, we selected 5 of 92 analyzed genes mainly involved in endocrine phenotype (Fig.30a) and 5of 92 in mesenchymal one (Fig 30b). We observed that during the expansion phase (Hi → hPIDM GM), islet cells lost their typical pancreatic expression markers (Insulin, Glucagon, Somatostatin, Isl-1, Neurod1) while reacquiring their expression during the endocrine pancreatic differentiation process (Fig.30a).

Mesenchymal specific genes (SMA, Vimentin, Snail1/2, Twist1) were, indeed, expressed during generation/expansion phases of hPIDM cell, while were downregulated (not for all genes) during differentiation step (Fig.30b).

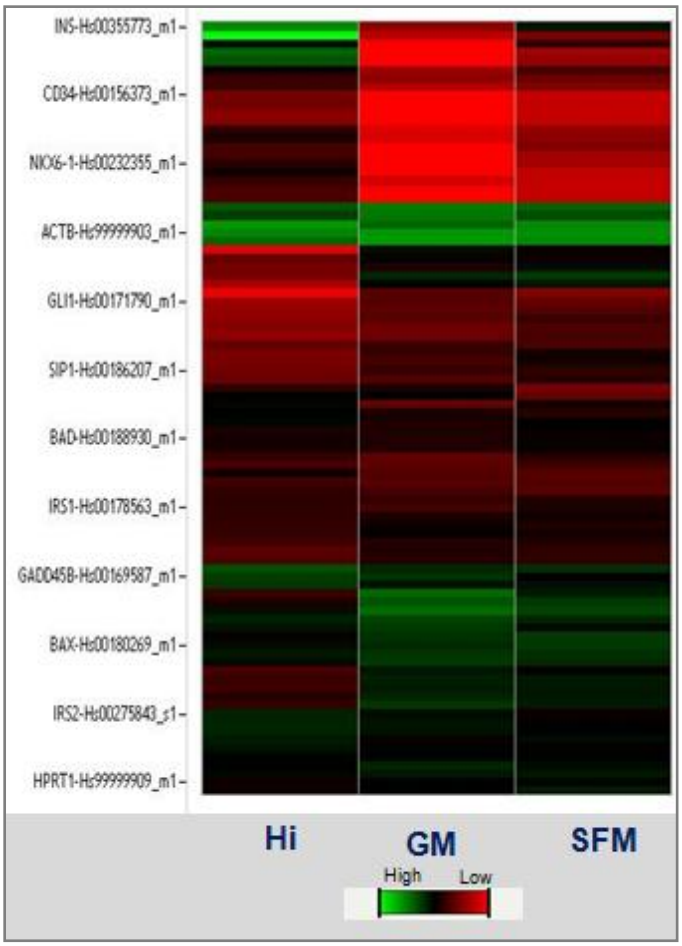


**Figure 29.** Hierarchical clustering heatmap analysis on 92 genes using Statminer software.

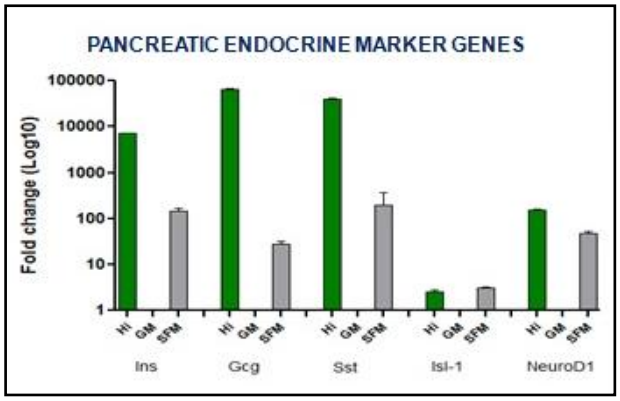


**Figure 30a.**

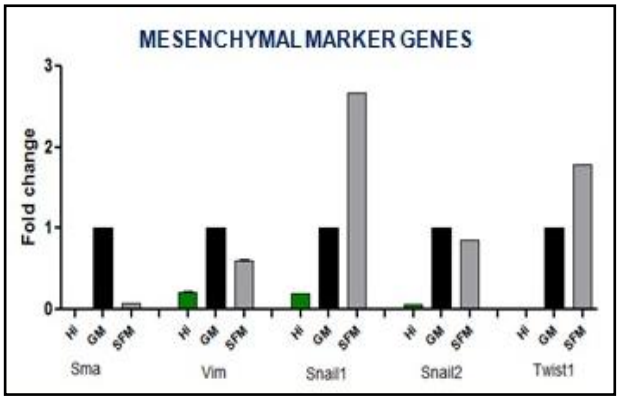


**Figure 30b.**

4.3.2- MicroRNAs profiling during expansion and differentiation of hPIDM cells

Through RT-Real Time PCR (Taqman microRNA Assay) we analyzed the expression profile of 754 unique microRNAs in human native islets (HI) derived from 3 healthy multiorgan donors, in hPIDM cells (GM) derived from other 3 healthy multiorgan donors and in islet-like cells derived from the differentiation of the same hPIDM cells (SFM). The results obtained

from the analysis of the expression of the 754 microRNAs, were analyzed with Real Time StatMiner® Software (Fig.31). Each of the three samples (splitted into 2 panels: A and B) reported in figure 30 is represented by a column, while the rows stand for each microRNA analyzed. The scale colour from green to red represents the normalized levels of microRNAs using smallRNAs U6, RNU48 and RNU44 as endogenous controls. Of the 754 microRNAs analyzed, 277 were found expressed in native human islets, 254 in hPIDM cells and 228 in differentiated hPIDM cells. Moreover, the expression of some of the 754 microRNAs analyzed, resulted to be modulated during the epithelial-mesenchymal transition into hPIDM cells (8 microRNAs upregulated and 136 downregulated) and during their endocrine pancreatic differentiation (16 microRNAs upregulated and 6 downregulated). We firstly focused on those microRNAs, which showed two particular modulation patterns during expansion and re-differentiation: microRNAs downregulated during expansion and upregulated during differentiation (Modulation pattern A- Fig.32a) and microRNAs upregulated during expansion and downregulated upon differentiation (Modulation pattern B- Fig.32b)

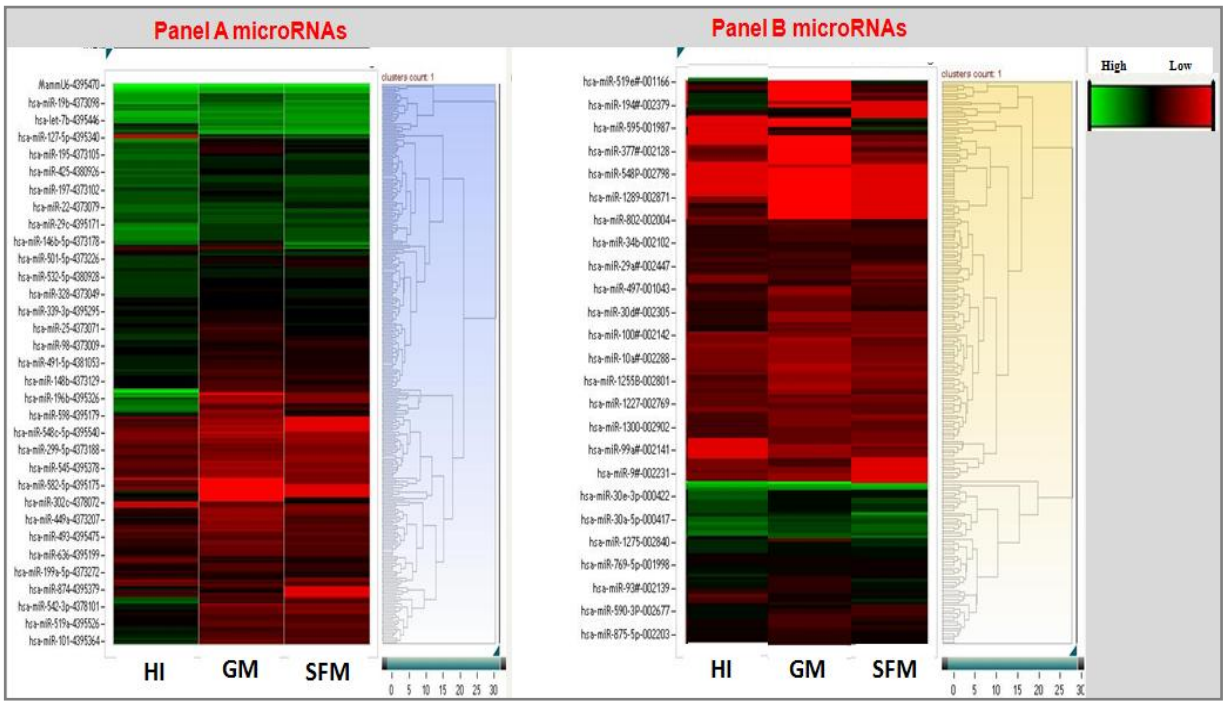


**Figure 31.** Hierarchical clustering heatmap analysis using Statminer software on 754 microRNAs splitted into panel A(containing the most expressed and well known miRNAs) and panel B (less expressed and minor forms of microRNAs( e.g. miRNAs star)).

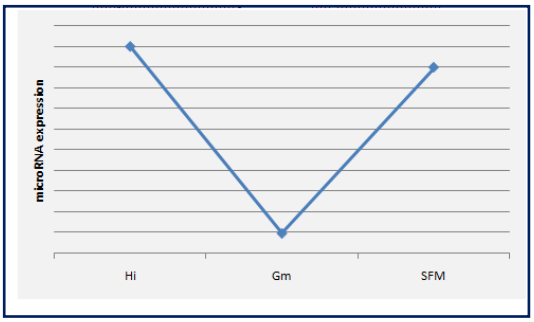


**Figure 32a. Modulation pattern A** detected for some of the miRNAs analyzed among Hi, hPIDM GM and hPIDM SFM.

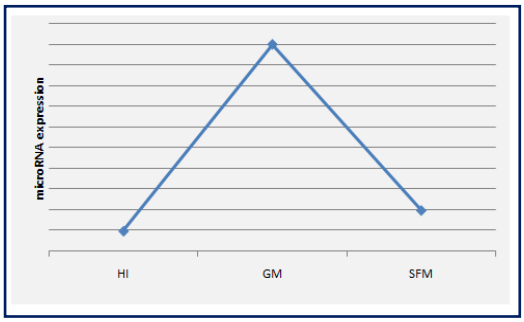


**Figure 32b. Modulation pattern B** detected for some of the miRNAs analyzed among Hi, hPIDM GM and hPIDM SFM.

### 4.3.3- MicroRNAs belonging to modulation pattern A

We interestingly found that some of the modulated microRNAs showed a peculiar modulation patterning. Indeed these microRNAs were strongly downregulated during hPIDM expansion and newly expressed upon pancreatic endocrine differentiation. 15 microRNAs (miR-146-5p, miR-16, miR-185, miR-324-3p, miR-340, miR-345, miR-374a, miR-375, miR-598, miR-93, miR-200 family (miR-200a, miR-200b, miR-200c, miR-429, miR-141) showed this kind of modulation pattern. Of these 15 microRNAs potentially involved in beta-cell differentiation, we focused our attention on miR-375 and miR-200 family microRNAs, because of their importance in beta-cell physiology and epithelial phenotype maintenance respectively.

- miR-375

Expression profile analysis on HI, GM and SFM hPIDM cells, revealed that miR-375 is highly expressed in pancreatic human islets, downregulated duringexpansion of hPIDM cells (GM) and up-regulated upon re-differentiation (SFM). These data were confirmed using single assay Real Time PCR and normalized with U6 and RNU48 (Fig.33).

This miR-375 expression pattern suggests a potential role in the definition of beta-cell identity and function and in human beta-cell differentiation program.

To determine whether miR-375 expression starts to be upregulated during 21 days of pancreatic differentiation program, we analyzed its expression in a time course expression experiment. We also performed insulin expression time course experiment in parallel with miR-375. We found that both miR-375 and insulin were mainly upregulated during first 7-11 days of pancreatic differentiation program (Fig.34). Moreover, these changing of both miR-375 and insulin expression were in line with main morphological changes of hPIDM cells during pancreatic differentiation program.

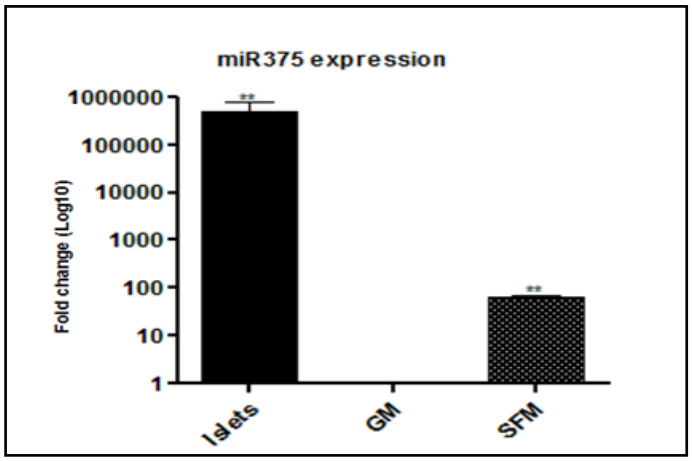


**Figure 33.** miR-375 expression profile confirmed in single assay on HI, GM hPIDM, SFM hPIDM cells. Mean ±s.e.m. values are reported. Mann Whitney U test was performed (**=p<0.01)

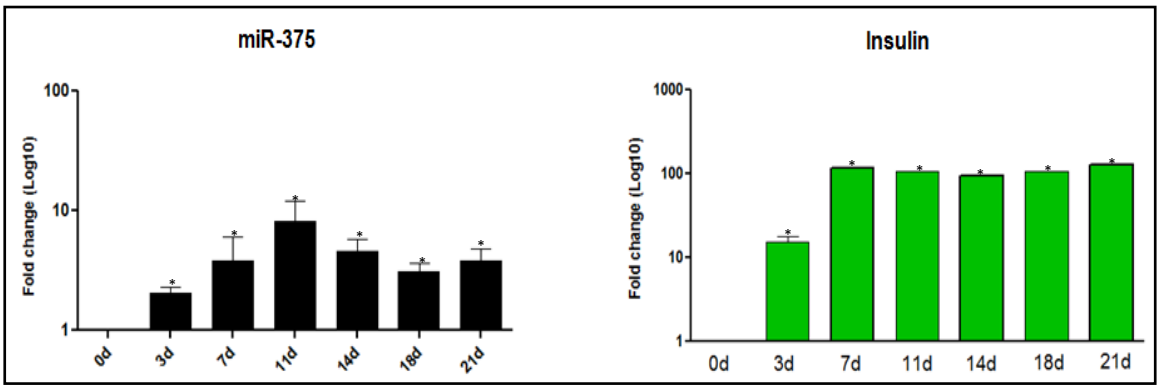


**Figure 34.** miR-375 (right) and insulin mRNA (left) time course expression during 21 days of endocrine pancreatic differentiation program. The main cell morphological changes are in line with main expression changes during differentiation steps. Mean ± s.e.m. values are reported. Mann Whitney U test was performed (*=p<0.05)

- miR-200 family

MicroRNAs belonging to miR-200 family (miR-200a, miR-200b, miR-200c, miR-429, miR-141) are mapped in two separate clusters: miR-200a, miR-200b and miR-429 genes localize in chromosome 1, while miR-141 and miR-200c genes in chromosome 12 in human.

MiR-200 family has been recently linked to the induction of epithelial differentiation *(Gregory PA et al., 2008).* In particular, all five members of the microRNA-200 family have been shown to be markedly downregulated in cells that undergone epithelial-to-mesenchymal-transition (EMT) in response to transforming growth factor (TGF-β) *(Xu J. et al., 2009).* Furthermore, recent studies demonstrated that over-expression of miR-200 family microRNAs (individually or in combination) represses EMT. Computational analysis and experimentally validated data, revealed that two important regulators of cell epithelial phenotype, zinc-finger E-box binding homeobox 1 (Zeb1) andSMAD-interacting protein 1 (Zeb2 or SIP1), represent the main target genes of miR-200 family microRNAs. These genes are transcriptional regulators that mainly control gene expression through repression. They belong to the Zeb family and are characterized by two zinc-finger clusters at each end, whose simultaneous binding to bipartite E-boxes mediates the interaction with regulatory DNA sequences. Their central region contains a Smad (proteins activated after TGF-β receptor complex stimulation)-interaction domain, a homeodomain and a CTPB binding domain. TGF-β signaling induces the expression of ZEB proteins during EMT through an indirect mechanism and, upon activation, these transcription factors interact with Smad3 and directly repress the expression of epithelial marker genes (ie. claudins, ZO, E-cadherin, Plakophilin, crumbs3) and concomitantly activate mesenchymal gene expression (ie. N-cadherin, vitronectin, fibronectin). One of the most interesting target genes of Zeb1 and Zeb2 is E-cadherin *(Korpal M. et al., 2008).* The latter is a transmembrane adhesion receptor which characterizes a type of epithelial junctions, the adherens junctions, organized as a belt and located adjacent to the tight junctions in the basolateral surface compartments of epithelial cells, connecting to cytoskeletal microfilaments. The cytoplasmic domains of E-cadherin bind tightly to β-catenin, a cytoplasmic protein that interacts with α-catenin, which in turn anchors to the actin cytoskeleton, either directly or indirectly via actin-binding proteins. Loss of E-cadherin is considered as an essential, but not sufficient, event of EMT that initiates a series of signaling events and major cytoskeletal reorganization. During EMT, the adherens junction complexes disassemble and the actin cytoskeleton reorganizes from an epithelial cortical alignment associated with cell-cell junctions into actin stress fibers that are anchored to focal adhesion complexes *(Mongroo PS. et al., 2010*).

Through RT-Real Time PCR we interestingly found that miR-200 family microRNAs are highly expressed in native human islets, downregulated during hPIDM cells expansion and newly upregulated upon re-differentiation program (Fig.35). These data are in accordance with literature data, which assigned a specific epithelial role to miR-200 family microRNAs and confirmed their role during EMT of islet cells.

Then we looked at miR-200 microRNAs target genes, Zeb1 and Zeb2, and subsequently at E-cadherin expression. In our analysis we found that Zeb1 and Zeb2 expression increased during expansion of hPIDM cells in accordance with the observation that their microRNAs repressors (miR-200 family) decreased, and that E-cadherin was downregulated in hPIDM cells, as expected from the upregulation of Zeb1 and Zeb2. However the expression of both Zeb1 and Zeb2 and E-cadherin continued to advance in the same way, instead of oppositely changing, when hPIDM cells were induced to re-differentiate into pancreatic endocrine phenotype (Fig. 36).

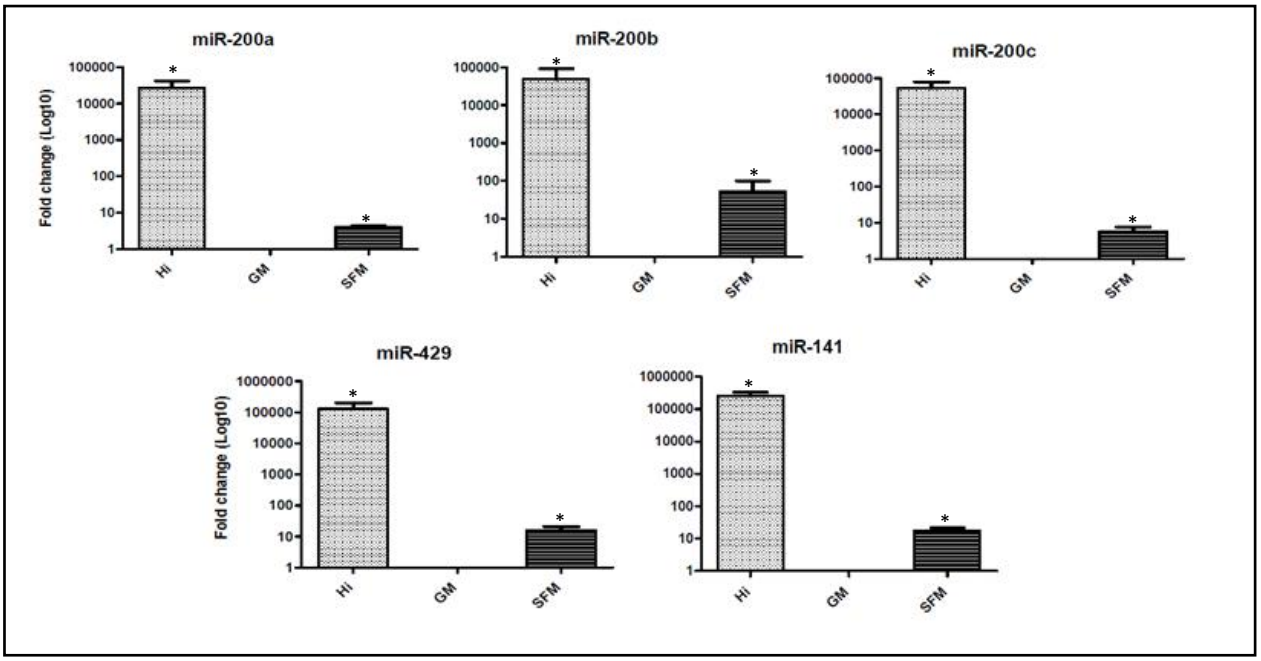


**Figure 35.** miR-200 family microRNAs expression profile confirmed in single assay RT Real Time PCR. Mean ± s.e.m. values are reported. Mann Whitney U test was performed (*=p<0.05).

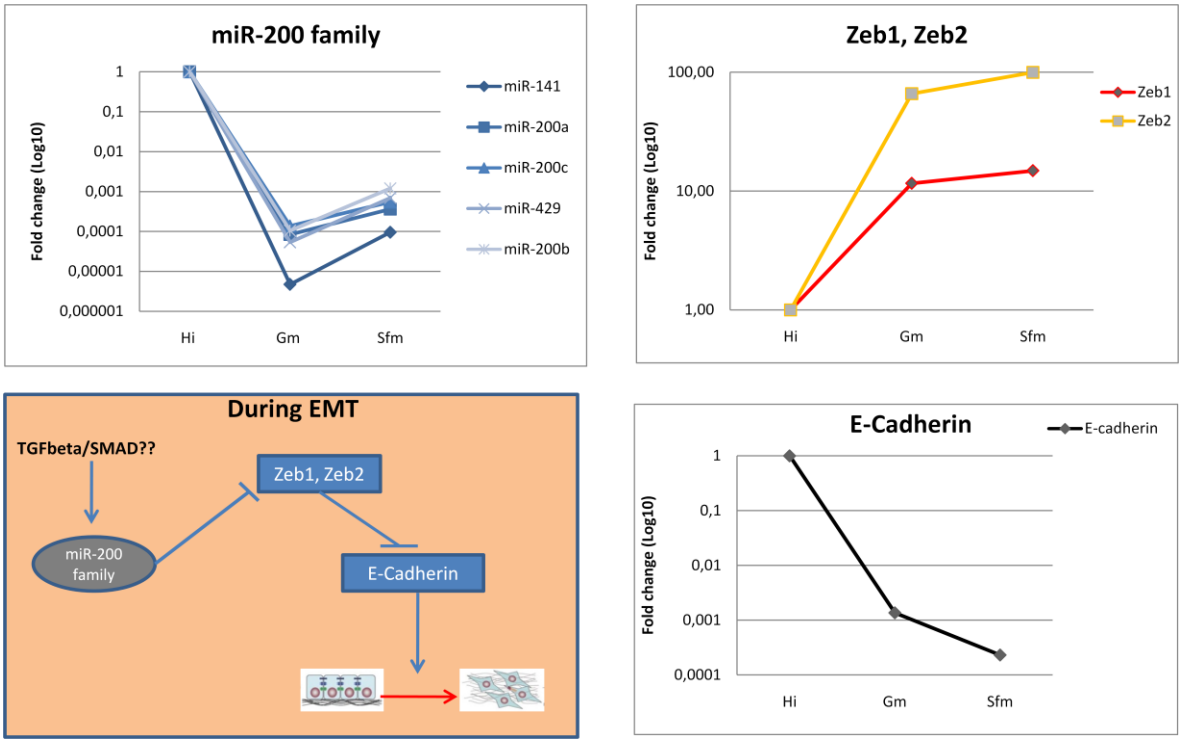


**Figure 36**. RT- Real Time PCR expression profile of miR-200 family targets (Zeb1 and Zeb2) and the transcriptional target of Zeb1 /2, E-Cadherin, on Hi, GM hPIDM cells, and SFM hPIDM cells.

### 4.3.4- MicroRNAs belonging to modulation pattern B

In addition to the observation that the expression of 15 microRNAs of the 754 initially analyzed resulted to be downregulated when the human islets undergo EMT and upregulated during re-differentiation, it was also observed that 5 microRNAs are upregulated during hPIDM cell expansion and downregulated upon re-differentiation program. These microRNAs belong to the miR-302-367 cluster.

- miR-302-367 cluster

MiRNA-302-367 cluster is codified in the human chromosome 4 and consists of nine different miRNAs co-transcribed in a polycistronic manner: miR-302a, miR-302a*, miR-302b, miR-302b*, miR-302c, miR-302c*, miR-302d, miR-367 and miR-367*. Very recently was identified and characterized the putative promoter of the miR-302-367 gene that constitutes of a strongly conserved region of approximately 500 bp immediately upstream of the transcriptional start that contains a conventional TATA box *(Barroso-del Jesus et al., 2009)*.The miR-302-367

promoter activity depends on the ontogeny and hierarchical cellular stage. From an ontogeny standpoint of view, the core promoter specifically drives the transcription of a reporter gene in ESCs but not in somatic cells (either stem cells or differentiated cells). This indicates that the miR-302-367 promoter activity is restricted to an embryonic stage of development being turned off later in development. From a hierarchical point of view, the miR-302-367 promoter is active on ESCs but its activity decays upon differentiation of ESCs (*Barroso-delJesus et al., 2009).* To summarize this promoter transcriptional activity confers to miR-302-367 cluster a ESC-specific expression. In fact this miRNA cluster is known to be specifically expressed in human embryonic stem cells (hESCs) and in induced pluripotent stem cells (iPSCs), but not in differentiated cells. Recent studies demonstrated that ectopic expression of miR-302-367 cluster induces pluripotency, allowing the exit from G1 phase. Actually, cyclin D1 and Cdk4 have been recently found to bepost-transcriptionally regulated by miR-302 in hESCs *(Greer Card DA. et al.,2008).*

As aforementioned, increasing evidence supports the role of miR-302-367 in a variety of biological processes essential in ESC biology such as cell cycle, apoptosis, differentiation and maintenance of "stemness". The miR-302-367 rather than being at the top of the stem cell hierarchy, is likely to function as a crucial intermediate regulator since it is a downstream transcriptional target of Oct3/4, Sox2 and Nanog, that are essential transcription factors for ESCs identity and iPSCs induction.

We found that the main members of miR-302-367 cluster (miR-302a, miR-302b, miR-302c, miR-302d, miR-367) were absent in native human islet but well expressed in hPIDM cell upon expansion. Their expression were then downregulated when hPIDM cells were re-differentiated towards endocrine pancreatic phenotype (Fig.37). These data were confirmed in single assay RT-Real Time PCR on 6 different human islet donors.

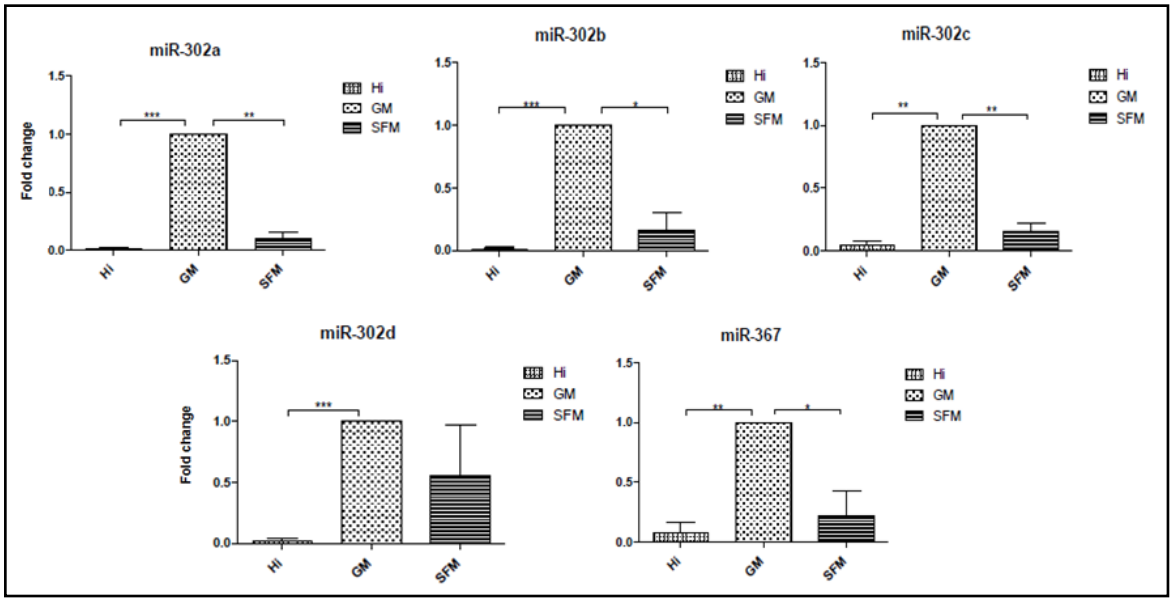


**Figure 37.**RT Real Time PCR expression profile of miR-302-367 miRNA members (miR-302a, miR-302b, miR-302c,miR-302d, miR-367). Mean ± s.e.m. values are reported. Mann Whitney U test was performed (*=p<0.05,**=p<0.01, ***=p<0.001)

Moreover, we performed a time course expression analysis of all members of miR-302-367 cluster during the 21 days of differentiation program. We detected a major downregulation during the first seven days of differentiation program comprises all the members of miR-302-367 cluster (Fig. 38). As for miR-375 time course expression analysis, this expression profile is in line with the mayor morphological changes observed during differentiation. Moreover miR-302-367 time course expression is parallel but opposed to that of miR-375, evidencing the opposite signature of these two miRNAs during the hPIDM cells expansion and differentiation.

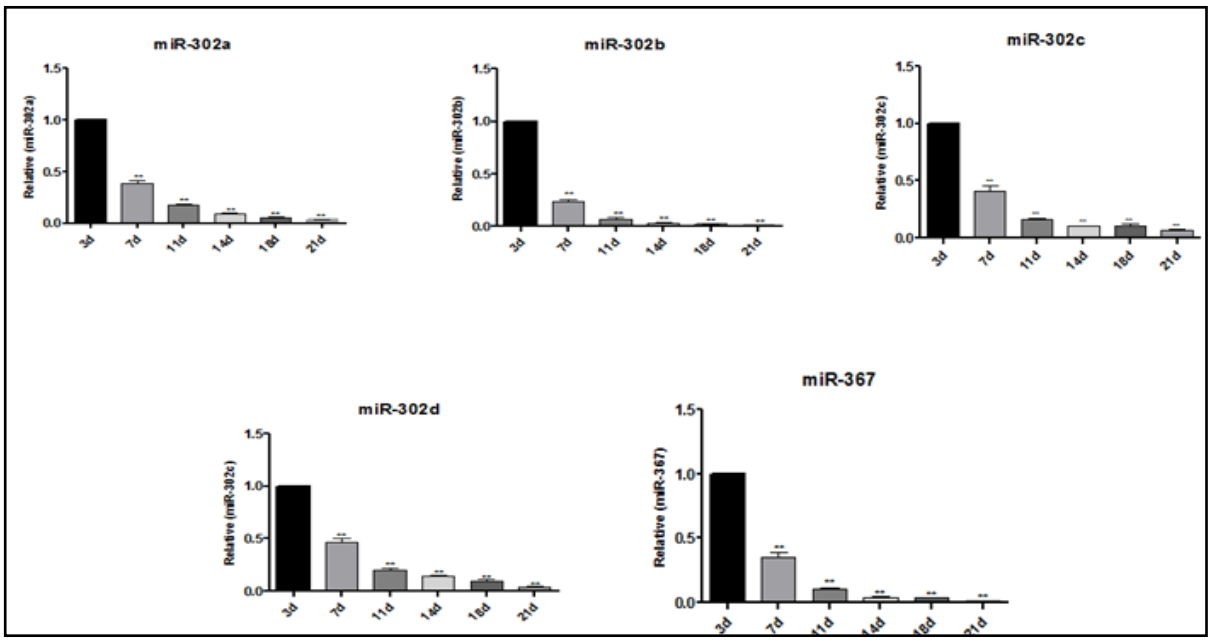


**Figure 38.**Time course expression analysis of miR-302-367 cluster.

# 5- DISCUSSION

## 5.1- MicroRNAs and T1DM

T-cell autoimmune attack of pancreatic beta cells represents the major issue during T1D pathogenesis. Specific T-cell clones against several autoantigens, proliferate and then were recruited from periphery to pancreatic islets, forming typical lymphocytic infiltrate. Moreover, the presence of humoral autoimmunity, measured through detection of GAD and IA-2 autoantibodies in patients' blood serum, is an important evidence of T-cell autoimmune reaction against autoantigens. The breakdown of beta cell-specific self-tolerance by T-cell involves a number of dysregulated events intrinsic and extrinsic to T-cells. Enhanced T-cell sensitivity as well as resistance to activation-induced cell death or impaired signaling pathway could lead to establishment of autoimmune reaction.

Recently, microRNAs were reported to be essential for normal immune T-cell function. Indeed, some specific miRNAs were found altered in several autoimmune pathologies and were directly or indirectly linked to T-cell function or development *(O'Connell RM. 2010)*.

We have showed that miR-326, a miRNA previously found altered in Th17-cell subset during autoimmune multiple sclerosis, was increased in PBMCs from T1D patients with positivity for at least one of the two autoandibodies measured (GADA or IA-2A), respect to patients negative for both ones.

Our data suggest that miR-326 could be directly or indirectly correlated to autoimmune reaction in place. We did not show any association of miR-326 with lymphocytic subpopulation normally present in PBLs, but we can hypothesize a specificity of miR-326 alteration in one of the multiple lymphocyte subsets rather than a general phenomena.

MiR-326 precursor gene is located within the chromosome 11 in the intron 1 of the beta-arrestin gene (Arrb1). Although some intronic microRNA have their own promoter and consequently possible independent expression, it has been shown that their expression could be consequent to the expression of their host genes *(Lutter D et al., 2010)*. It is possible to hypothesize that expression of miR-326 is positively correlated to the expression of beta arrestin gene. It has been demonstrated that beta arrestin1 gene regulates T cell survival through enhanced expression of the proto-oncogene Bcl2. In a recent work Pei and colleagues found that expression

levels of beta arrestin1 were increased in CD4+ T cells from mice with experimental autoimmune encephalomyelitis; moreover they additionally report that this gene could modulate the severity of streptozotocin-induced T1D in mice *(Shi Y. et al.,2010)*. Other autoimmune pathologies have been demonstrated to be characterized by increased levels of Arrb1, making the possibility of dependent expression of miR-326 to its host gene very realistic also in T1D *(Hu Z. et al., 2011)*. Therefore, it is possible to speculate that increased levels of miR-326 in PBLs from T1D patients with autoimmune reaction in place, may be the consequence of Arrb1 upregulated transcription.

Generally, microRNAs are believed to function by targeting multiple functionally related genes, inhibiting their expression. In the immune system miR-181a was strictly associated with modulation of the sensitivity of T-cell receptor (TCR) to peptide antigens and its inhibition lead to impairment of positive and negative selection by increasing negative regulators, whereas miR-150 controls T-cell normal activation by targeting transcription factors as cMyb *(Li Li QJ.et al.,2008; Xiao C. et al.,2007)*.

We found that a possible predicted target gene of miR-326, not experimentally validated, is VDR. VDR 3'UTR posses at least 4 possible miR-326 target sites according to Targetscan 5.1 prediction. It is possible to speculate that VDR could be one of the miR-326 target genes and that the upregulation of miR-326 may lead to inhibition of VDR expression. VDR is expressed in most cells of the immune system, including activated T-cells and it is now clear that Vitamin $D_3$ signaling pathway play an important role in immune modulation *(Von Hessen MR. et al., 2011)*. In animal models of T1D, the administration of high doses of Vitamin $D_3$ was shown to prevent the disease mainly through immune regulation while generation of VDR null mice led to impairment and disorders of the immune function *(Mathieu C. et al., 2005)*.

We hypothesize that an upregulation in miR-326 expression could lead to decreased presence of VDR in immune cells, impairing their response during T1D autoimmunity. Experimental confirmation of VDR as target of miR-326 will be necessary to validate our hypotheses.

Improving our knowledge on microRNA miR-326 network during normal and diseased immune system could help to develop new therapeutic strategies for autoimmune diabetes and to elucidate its role in Vitamin $D_3$signaling in immune cells.

## 5.2- MicroRNAs and T2DM

MicroRNAs represents a new frontier of gene expression regulation. Recently, many authors have focused their attention on the role of microRNAs in many disorders, including type 2 diabetes. However, due to the scarcity of human pancreatic islets from T2D patient donors, many of the efforts in this field have been concentrated on T2D animal models and on rodent pancreatic cell line (from both beta and alpha cells). The involvement of microRNAs in beta cell function and development has been clearly demonstrated, especially in Dicer1-KO or specific miRNA-KO animal models. Here we have reported that microRNAs expression profiling of 4 T2DM human pancreatic islet samples compared to 4 normal ones, resulted in several specific microRNAs alteration.

Interestingly, among altered microRNAs, we have found miR-124a. Many authors clearly characterized the function of miR-124a in beta-cell with particular attention to the role of this miRNA in insulin secretion. Indeed, it has been demonstrated that miR-124a targets several genes made part of exocytotic machinery and therefore could be essential for beta-cell function. In 3 of 4 (75%) ofT2D pancreatic islet samples we have observed an important upregulation of this miRNA respect to normal subjects. Although the observed miR-124a alteration resulted statistically significant including all of 4 T2D samples vs 4 normal, it is clear that a confirmation on a more numerous samples set is of upmost importance. However, given the importance of miR-124a in beta-cell physiology, its strong alteration during T2D could be one of the possible mechanisms, which cooperate to induce the beta-cell dysfunction. Since miRNAs upregulation usually leads to enhanced inhibition of their target genes, we looked for miR-124a target genes mainly involved in specific beta-cell function. We found that all of six selected target genes were upregulated upon miR-124a silencing. It is possible to speculate that upregulation of miR-124a, as we observed in human islets, could therefore leads to negative modulation of its target genes, strongly influencing beta-cell function at several different levels; among selected target genes, we confirmed the real targeting of miR-124a on Mtpn and FoxA2 through luciferase assay. Both genes strongly regulate insulin exocytotic or glucose-sensing machinery and, consequently, their possible alteration may interrupt the correct mechanism of exocytosis. Myotrophin is involved in the distal stages of insulin secretion controlling the F-actin filaments formation and vesicles docking to plasma membrane. FoxA2 is known to control the expression of genes mainly involved in glucose-sensing apparatus, such as Pdx1 gene, but also in insulin release through transcriptional control of Kir6.2 (inward rectifier potassium channel member 6.2) and Sur1 (sulfony-

lurea receptor 1), both subunits of ATP-dependent $K^+$channel. A consequence of miR-124a increased levels could be the diminished transcriptional activity of Mtpn and FoxA2 with a possible impairment of beta-cell glucose-sensing mechanism and insulin secretion. An evidence of insulin secretion impairment following miR-124a increased expression is well represented in Min6 derived pseudoislets. Using miR-124a mimicking, simulating the T2D human islets microRNAs molecular environment, we observed an altered insulin secretion upon exposition to high glucose stimuli, which requires highly functional exocytotic machinery to obtain an adequate response. Insulin secretion has been found to be normal when Min6-pseudoislets were cultured in basal glucose condition upon high miR-124a expression levels. These data partially explain that when beta-cells underlie stress conditions (e.g. high glucose), the molecular machineries controlling glucose sensing and insulin secretion apparatus are not enough functional to allow normal responses as seen in rest conditions. Although Mtpn and FoxA2 represent two important regulators of beta cell function, posttranscriptionally controlled by miR-124a, even other genes may underlie this control. In fact, miR-124a has also direct or indirect control on the other 4 predicted genes (Flot2, Akt3, Sirt1, NeuroD1). We observed a significant downregulation of these 4 predicted miR-124a target genes upon its upregulation in Min6-pseudoislets, but until direct targeting in their 3'UTR by miR-124a will be proven, such a regulation could be exerted also through an indirect mechanism. However, the effects of miR-124a upregulation, direct or indirect, may hit also other beta-cell pathways, possibly contributing to T2D dysfunction.

The other miRNAs we have focused on is the miR-184. We observed a downregulation of miR-184 in T2DM human pancreatic islets respect to normal ones. Interestingly we demonstrated that this miRNA is preferentially expressed in beta-cells. We observed this differential expression using betaTC-1 and alphaTC-1 as well as laser capture microdissection of core and periphery of mouse pancreatic islets (respectively enriched of beta and alpha cells) Although miR-184 differential expression has been proven on mouse cell line and mouse tissue, it is possible to speculate that, given the genetic conservancy of miR-184, this differential expression could be also well represented in human samples. However, until evidences, we cannnot be sure about the conservancy of the molecular mechanisms between mouse and human.

A strong evidence of the importance and specificity of miR-184 in beta-cells is given by the important role of its target genes, Ship2 and Prkc-Beta. They are both involved in the regulation of insulin signaling, particularly acting as negative modulators of insulin signal transmission. Given the recently acquired importance of autocrine insulin signaling on beta-cell, its regulation could be of primary importance within their dysfunction in T2DM. It is possible to hypothesize that downregulation of miR-184 in human beta-cell during T2DM condition may lead to upregulation

of its target genes (Ship2 and Prkc-beta). The upregulation of these two genes provokes an attenuation of insulin signaling which may represent part of the dysfunction of beta-cell during diabetic condition.

Next we focused on miR-187. We observed a strong downregulation of this miRNA in all of 4 T2DM human pancreatic islets respect to normal ones. Interestingly, when we looked at miR-187 target genes with a possible role in islets function, we only found genes specifically involved in alpha cell components of the islets. Indeed, among miR-187 target genes we found Glucagon and Pax6. Glucagon is the mayor hormone produced by alpha cells while Pax6 is involved in their differentiation and identity. Although we observed the alteration of miR-187 expression in entire islets samples, it is possible to speculate that its effector function may be more important in alpha than in beta-cells. Moreover, we also investigated the possible causes participating in the alteration of miR-187 expression. We tested the effects of FFA palmitic acid on alphaTC-1 cell line measuring the expression of miR-187. We observed that upon 48h of palmitate treatment, the alphaTC-1 cells decreased their miR-187 expression, maybe resembling what happens during T2DM.

Although we have found that palmitate has an important effect on miR-187 downregulation, we do not know the exact molecular mechanism acting during this phase and therefore it will require more analysis to understand the interaction between miRNAs expression and islets environment during T2DM.

### 5.3- MicroRNAs and hPIDM cells expansion and differentiation

Existing treatments for type 1 and type 2 diabetes primarily focus on replacing insulin and improving beta-cells function, but there are growing evidences supporting the hypothesis that diabetes and overt hyperglycaemia are mainly due to the inadequate mass of functional pancreatic beta-cells. Indeed, increasing beta-cell mass could be a potential strategy to improve the possibility of diabetes therapy or even a cure. To reach this goal, islet transplantation has been used to treat some patients affected by diabetes, but since the availability of islets from pancreas donors is limited, it is impossible to intervene using this method on a large scale. Starting from these assumptions, the idea of looking for alternatives sources of beta-cells is becoming increasingly important. These beta-cells should be able to secrete insulin, but also to properly release it after the stimulus induced by glucose. Several attempts have been made to identify stem/progenitors cells within pancreatic tissue as potential source for transplantable insulin-producing tissue, but unfortunately the origin of new beta-cells in adult pancreas is not yet known. High expectations have been recently raised by increasing experimental evidence that cells with a fibroblast-like morphology can be obtained from cultures of human pancreatic islet cells. On the basis of these results, it was initially suggested that these mesenchymal-type cells originate from beta-cells after having undergone a reversible epithelial-to-mesenchymal transition. This transition would allow the expansion of the cells in the presence of serum, while, following serum removal, the cells could reacquire an endocrine phenotype, which synthesizes and releases insulin *(Gershengorn MC et al., 2004; Russ HA et al., 2009).* Therefore, understanding the processes of beta-cell differentiation and proliferation might allow *in vitro* cultivation of beta-cells in considerable amounts to be transplanted into patients with diabetes, obtaining an important replacement therapy. In addition to this, identifying the molecular regulators of beta-cell mass development, maintenance, and expansion might provide new therapeutic targets for diabetes. Some recently identified microRNAs have been found to play a role in beta-cell functionand have been later studied in developing pancreas. Here we have reported that, during human pancreatic islets beta-cell expansion (acquisition of a mesenchymal like phenotype), and during subsequent re-differentiation, there is an important microRNAs modulation. The expression analysis of 754 microRNAs, demonstrated that 144 of them are modulated during human pancreatic islet de-differentiation (EMT) into hPIDM cells (8 upregulated and 136 downregulated) and 22 during endocrine pancreatic differentiation (16 upregulated and 6 downregulated), suggesting a potential role of such microRNAs in beta-cell regeneration. We

also found that some microRNAs belong to two particular patterns of modulation: 15 microRNAs, belonging to pattern of modulation A, resulted downregulated during EMT and upregulated upon re-differentiation program (miR-146-5p, miR-16, miR-185, miR-324-3p, miR-340, miR-345, miR-374a, miR-375, miR-598, miR-93, miR-200 family (miR-200a, miR-200b, miR-200c, miR-429,miR-141)), 5 microRNAs belonging to modulation pattern B resulted upregulated during EMT and downregulated during re-differentiation (miR-302-367 cluster).

Of the microRNAs belonging to modulation pattern A, miR-375 and miR-200 family were further analyzed. MiR-375 is the most abundant and islet-specific microRNA and it has been found highly expressed in human native islets, decreased in hPIDM cells and increased in islet-like cell clusters (ILCs). Furthermore, we found that the increased expression of miR-375 during re-differentiation is similar to the insulin mRNA expression levels, suggesting a direct connection between them and a potential role of miR-375 as a good target in hPIDM cell differentiation towards beta-cell phenotype. Also miR-200 family members which are demonstrated to actively participate in EMT, were found highly expressed in human islets, absent in hPIDM cells and re-expressed in ILCs. To better investigate the role of this microRNA family, we focused our attention on its target genes expression. We analyzed the expression profile of Zeb1, Zeb2 and E-cadherin in human islets, hPIDM cells and re-differentiated cells (SFM) and we noticed that when miR-200 family members decrease during islet de-differentiation, Zeb1 and Zeb2 increase (as it was expected to be, because they are target of miR-200) and E-cadherin, which is inhibited by Zeb1 and Zeb2 expression, decrease contributing to the loss of epithelial phenotype. However, when miR-200 family members increase during hPIDM re-differentiation, Zeb1 and Zeb2 are not repressed and continue to increase and E-cadherin continue to decrease. These suggest that there are other molecular factors in EMT and beta-cell regeneration that work together or in contrast with miR-200 family. However it must be taken into consideration that the islet-like population derived from hPIDM cells re-differentiation is a mixed group of beta- and non-beta-cells, thus it is highly likely that a lot of molecular mechanisms are not the same and reflexive of the beta-cell development, like they might be in an homogeneous population.

On the contrary, microRNAs of the modulation pattern B were found to be upregulated during EMT and downregulated upon re-differentiation .All these microRNAs belong to the miR-302 cluster (miR-302a, miR-302b, miR-302c, miR-302d, miR-367), which is specifically expressed in human embryonic stem cells (ESCs) and in induced pluripotent stem cells (iPSCs) but not in differentiated cells. In fact this set of miRNAs is required for rapid cell proliferation and cell-

cycle progression. For example, it has been demonstrated that miR302a inhibits the expression of an important G1 regulator, cyclin D1, in hESCs.

We observed that miR-302-367 cluster members are highly expressed in hPIDM multipotent cells, while they are absent in human pancreatic islets and in differentiated hPIDM cell. For these observations, it might be important to determine the role of the human miR-302 cluster in EMT involved in the phenomena of beta-cell differentiation. To do that it may be significant to investigate the behavior of transcription factors required for pluripotency during early embryogenesis and for the maintenance of ESCs identity, such as Oct4, Sox2 and Nanog that bind a conserved promoter region of miR-302 cluster and induce miR-302 microRNAs expression. Moreover analyzing whether the transcriptional activation of miR-302 during EMT is related to the translational repression of its putative targets might provide potential markers for beta-cell regeneration *in vitro*. For example, the identification of the proper regulation of Oct4/Sox2/miR-302-cyclin D1 gene networks would be critical for the success of regenerative strategies that involve pluripotent cells. Moreover, assumed that the Nodal inhibitors lefty1 and lefty2 are post-transcriptionally targeted by miR-302 in hESCs and that miR-302 members become upstream regulators of the TGF-β/Nodal pathway, functioning via Smad2/3 signaling, it could be interesting to analyze the expression profile of lefty1 in islets, hPIDM cells and after the loss of the mesenchymal phenotype. This investigation may provide a valid path to follow in order to maintain a balance between mesenchymal multipotent phenotype and beta-cell specification.

We have demonstrated that beta-cell de-differentiation and re-differentiation produces differential microRNAs expression profile and that one of this microRNAs, miR-375, is actively involved in beta-cell function regulation. Moreover, the regulatory mechanisms that induce mesenchymal or endocrine phenotype are not so simple because they are crossed one each other and regulated by different genes. However the obstacles that can be found during the identification of a specific and reproducible pattern of modulation for all microRNAs and their associated target genes and transcription factors, are due to the fact that after re-differentiation program, a mixed population is generated. The islet-like clusters, in fact, are composed only barely by beta-cells.

In conclusion, we can affirm that hPIDM cells might represent a good substrate to develop an alternative source of insulin-producing cells and that an accurate examination of the microRNAs behavior during beta-cells differentiation could improve the identification of new therapeutic targets to produce beta-cells in vitro for transplantation.

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