CHAPTER 5

ASSESSMENT OF MICRORNA PROFILE & MICRORNA MODULATION BY SILENCING /INHIBITION (LNA)FOR AUGMENTATION OF ISLET DIFFERENTIATION FROM HBMSCS

5.1. Introduction

microRNAs are small non-coding RNAs that play an essential regulator of a key process in cellular development, differentiation, and regeneration. These tiny 21 to 23 nucleotide sequences are situated within a host genome and are transcribed under the regulator of RNA polymerase II. The initial transcripts of miRNA genes, termed pri-miRNAs, are processed by the nuclear microprocessor complex in the nucleus, known as pre-miRNAs. Pre-miRNAs are transported to the cytoplasm through exportin-5 from nucleus and refined by dicer and miRNA-induced silencing complex (RISC), to generate approximately 22 nucleotide mature miRNA (Winter *et al.* 2009), and causes translational repression and/or degradation of the targeted mRNA sequence, as described in the previous chapter-1 (Section:1.11), resulting in the downregulation of the protein encoded by the gene target. Although precise molecular mechanisms underlying the pathogenesis of diabetes remain unclear, current discoveries in understanding post-transcriptional gene regulation by microRNAs (miRNAs) has unlocked a new vicinity of research.

5.1.1. miRNAs and Islet differentiation

Recent reports demonstrating that miRNAs play momentous roles in stem cell proliferation, differentiation, and maintenance. Pancreatic β cell-specific disruption of the miRNA network leads to gradually hampered glucose homeostasis by defecting insulin secretion and eventually progression of diabetes development (Kalis *et al.* 2011). Also, studies conducted in the human fetal pancreas demonstrated that define a class of microRNAs alter the degree of their expression during pancreatic development (Rosero *et al.* 2010). Reports demonstrated that the dynamic expression profile of miRNAs during human pancreas development is unique and necessary for pancreatic β-cell formation and maturation (Lynn *et al.* 2007, Bravo-Egana *et al.* 2008). Also, microRNAs are predominately expressed in mature pancreatic α and β cells of human pancreatic islets (Klein *et al.* 2013). Several highly expressed microRNAs were identified in ILCCs differentiated from hESCs (Chen *et al.* 2011, Wei *et al.* 2013). The pioneer miRNA investigated in pancreatic islet development as well as maturation and further demonstrated to control insulin synthesis and secretion was miR-375. Further, miR-375 has been described to be regulated by pancreatic transcription factors such as PDX1 and NEUROD1, both have a crucial role in pancreatic β-cell development and function. Moreover, there are pieces of evidence that constitutive miR-375 expression in hESCs led to the expression of pancreatic β cell markers as well as the production of insulin in response to

glucose in ILCCs in the absence of any extrinsic factors (Reyhaneh Lahmy 2013). Furthermore, similar groups presented a new approach for the differentiation of human iPS cells into ILCCs via overexpression of miR-375 without any external stimulator (Lahmy *et al.* 2014). Several reports indicate that miR-375 may perform predominately with miR-124a, both of which are remarkably expressed in pancreatic β-cells. Previously, miR-124a was reported as a brain-associated miRNA in mouse and human, the function of which is presumably to promote neuronal-specific lineage (Makeyev *et al.* 2007, Sasidharan *et al.* 2017). Recently, miR-124a regulates the expression of various transcription factors involved in early pancreatic islet differentiation, such as the *SOX17, GATA6, FOXA2, NEUROD1, PDX-1* gene (Figure:5.1). FOXA2 plays a vital role in insulin secretion through the regulation of channel protein such as Kir6.2 and Sur1 in pancreatic islets (Baroukh *et al.* 2007, Liu *et al.* 2011, Jing *et al.* 2014, Sebastiani *et al.* 2015, Liew *et al.* 2019).

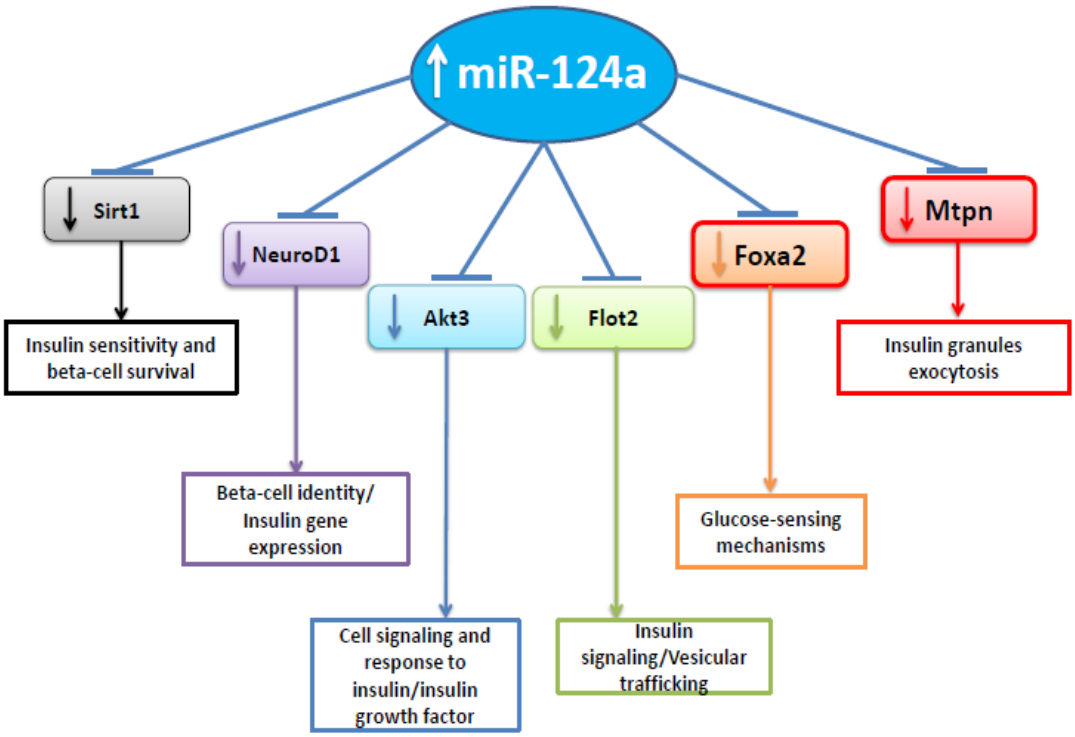


Figure 5. 1: microRNA 124a and its function related to pancreatic islets. miR-124a negative regulates the expression of various transcription factors and genes (related to insulin secretion) in pancreatic β-cells. This figure is adopted from (Sebastiani *et al.* 2015).

A detailed analysis of the comprehensive expression of miR-7 has been done in human pancreatic β-cell development (Correa-Medina *et al.* 2009). Further, miR-342 targets

pancreatic transcription factors such as MAFB and FOXA2, which are involved in pancreatic islet development and function. miR-16, miR-15a, miR-15b, and miR-195, target pancreatic transcription factor NGN3, which has a role in pancreatic β-cell differentiation and regeneration (Joglekar *et al.* 2007). At glance, microRNAs are probable to play an important regulatory role in pancreatic islet differentiation, and they possibly manipulated to enhance pancreatic β-cell function.

To identify potential microRNA gene targets, we analyzed the coordination between the expression profile of 27 microRNAs and their corresponding mRNA targets, predicted by bioinformatic software tools and review of literature in order to understand pancreatic islet differentiation, potential unveiling prospective therapeutic target to treat diabetes using stem cell therapy (Figure:5.2). In addition to their regulation by mRNA and protein, miRNAs can also be controlled by growth factors and bioactive molecules (Tili *et al.* 2010, Maciel-Dominguez *et al.* 2013). Several growth factors, differentiation factors, and bioactive molecules and inducible microRNAs, seem to act cooperatively to support pancreatic islet development, differentiation. Specifically, we focus here on bioactive molecules and their role in the regulation of microRNAs.

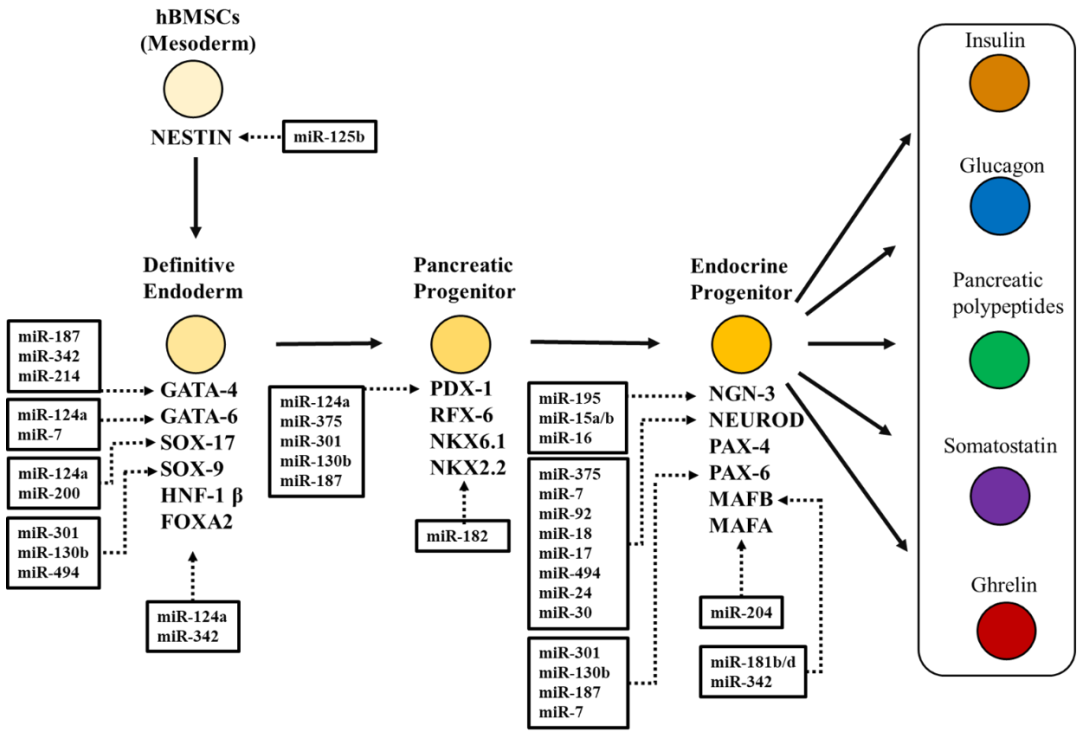


Figure 5. 2 A schematic diagram show possible role of microRNAs in islet differentiation from hBMSCs: overview of microRNAs associated with all-important the pancreatic transcription

factors involving in islet differentiation process. microRNA can act as both enhancer and suppressive during islet differentiation from hBMSCs (Joglekar *et al.* 2009, Rosero *et al.* 2010, Fernandez-Valverde *et al.* 2011, Klein *et al.* 2013, Dumortier *et al.* 2016).

5.1.2. microRNAs and Bioactive molecules

plant-derived bioactive molecules like resveratrol, curcumin, quercetin, and other animal derived agents such as vitamin A, vitamin D have been found to positively regulate the expression of miRNAs, stimulating cell differentiation, proliferation and apoptosis (Gavrilas *et al.* 2016).

Most of the research has been done with bioactive molecules in the context of microRNA related to inflammation-associated diseases. Several reports showed that bioactive molecules can regulate microRNAs expression such as, curcumin modulate microRNA expression profile in human pancreatic carcinoma cell line (Sun *et al.* 2008). Similarly, antroquinonol, the bioactive compound derivative of *Antrodia camphorata* has been reported to alter the expression level of miRNAs in lung cancer cells (Kumar *et al.* 2011). Further, Tunca *et. al.*,2012 demonstrated that *Olea europaea* plant extract have anticancer activity in human glioblastoma cell line by modulating the microRNA expression (Tunca *et al.* 2012). Similar studies revealed that iso-flavonoid glyceollins compound, extract from soy lead to significant alterations of the miRNA expression profile in breast cancer (Rhodes *et al.* 2012). Vitamin A derivatives (retinoic acid) also alter the expression of several microRNAs under both conditions *in vitro* and *in vivo* stem cell differentiation (Perri *et al.* 2017). In embryonic stem cells, treatment with retinoic acid significantly downregulates miR-200, which significantly increases the expression of Oct4 and Nanog, which are key pluripotent markers and relevant to the organ development (Zhang *et al.* 2015). Further, selenium also regulates microRNAs, which play an important role in inflammation and oxidative stress (Alehagen *et al.* 2017). Thus, the above pieces of evidence suggest that natural bioactive compounds play a very crucial role in altering the expression profile of microRNAs.

5.1.3. MicroRNA Manipulation strategies: Inhibition (LNA):

The miRNAs have an exceptional peculiarity which is very interesting for developing a novel drug, as they are tiny (small), with mostly conserved sequences among species (Friedman *et al.* 2009). Apart from growth factors and bioactive molecules, microRNA expression can be manipulated either by overexpression or inhibition (Czech 2006). Recently, microRNA

inhibitors, a very potent oligonucleotide-based antisense technology is being developed against target miRNAs.

As we described in review and literature chapter number :3(section:1.11.3-B), Locked nucleic acid (LNA) is a synthetic miRNA with very high affinity and specificity towards, particularly targeted miRNA. LNA is a chemically modified powerful RNA analogue generated by using 2'-O,4'-C methylene bridge (Figure: 5.3). Further, LNA also acquire a strong compatibility against correspondent miRNA along with elevate T_m of 2 to 8°C per LNA monomer (Petersen and Wengel 2003).

Additionally, the researcher demonstrated the mechanism of miRNA silencing approach using seed targeting modified with phosphorothioate oligonucleotides, 8-mer tiny LNA, which is fully modified with phosphorothioate oligonucleotides and complementary to the miRNA seeding sequences (Figure: 5.4). miRNA inhibition by anti-miRs (LNA) requires optimization of the oligonucleotides for increased binding affinity, improved nuclease resistance, and *in vitro & in vivo* delivery (Obad *et al.* 2011).

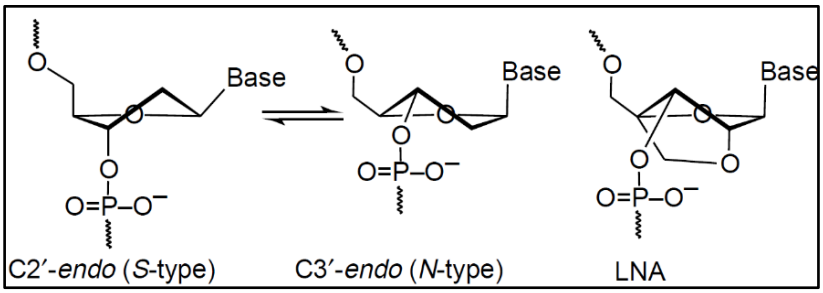


Figure 5. 3 : Representative image depicted that the molecular formation of locked nucleic acid (LNA), which represents the locked C3'-endo and C2'-endo sugar conformation. This figure is adopted from (Petersen and Wengel 2003).

With improved understanding of the mechanisms whereby miRNAs repress essential gene expression and the development of more specific and stable mimics and inhibitors of miRNAs, this could lead to better approaches to address the "functionality of differentiated pancreatic islets" in diabetes.

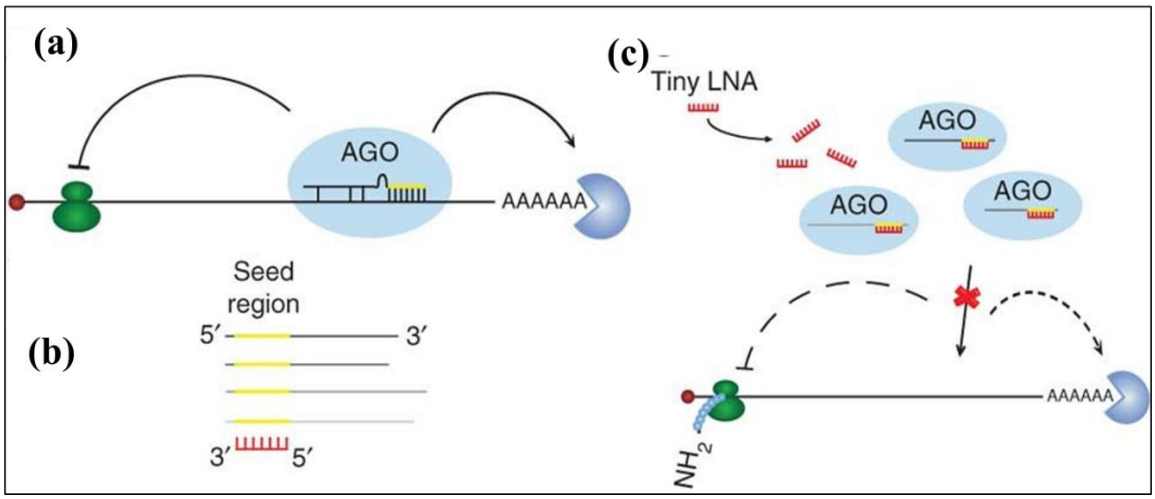


Figure 5. 4 Representative overviews of microRNA inhibition molecular mechanism of short LNAs (a) microRNAs bind to 3' UTR target sites on mRNA and lead to either translational constrain or degradation of mRNA (b) This short LNAs is constructed in such way that it can complementary to seed region. (c) The LNA is shown high binding functional inhibition of miRNA, which to restriction of target mRNA .This figure is adopted from (Obad *et al.* 2011).

This strategy helped to optimize the potential for *in vitro* differentiation of hBMSCs into ILCCs for use as preclinical research and future clinical trial. There is increasing attention to developing a novel pharmacological drug for diabetes treatment based on targeting microRNAs during pancreatic islet differentiation.

Based on the previous work done in chapters (chapter numbers 3 and 4), we noticed that obtaining ILCCs from hBMSCs is achievable. Preliminary studies in the field of islet biology, highlight the role of microRNAs in pancreas islet development and regeneration; however, none have addressed the possible role of microRNAs during pancreatic islet differentiation from hBMSCs. Hence, we addressed the following key questions ;(1) Does microRNAs play a crucial aspect in islet differentiation from hBMSCs? (2) Can we identify highly expressed candidate microRNAs during islet differentiation? (3) Whether the delivery of microRNA inhibitor (LNA) to cell clusters via unassisted transfection agent (Gymnosis) would be successful? (4) Whether inhibiting specific microRNA leads to a beneficial effect and fasten the islet differentiation process from hBMSCs?

Based on key questions and review of literature, we hypothesize that during the differentiation of hBMSCs into ILCCs; there might be specific microRNAs that mediate the pancreatic lineage transition, thus affecting the stage of differentiation of hBMSCs into ILCCs. Identification of

candidate microRNAs will help us to take a step further in optimizing (Inhibiting microRNAs) the quality of islets obtained.

To prove our hypothesis, our approach was to decipher microRNAs profile during islet differentiation which will further provide a platform for manipulating miRNA expression or function by LNA technology. To fulfil this, we have following sub-objectives:

(a) microRNAs expression profile during Islet differentiation

(b) Identified candidate microRNAs expression during islet differentiation

(c) microRNA power inhibitor [Power Lock Nucleic Acid (LNA)] LNA-has-miR-124a study.

Thus, for achieving our objectives, we used experimental design/plan of work as shown in (figure :5.5,5.6.5.7).

5.2. Experimental design of chapter:5

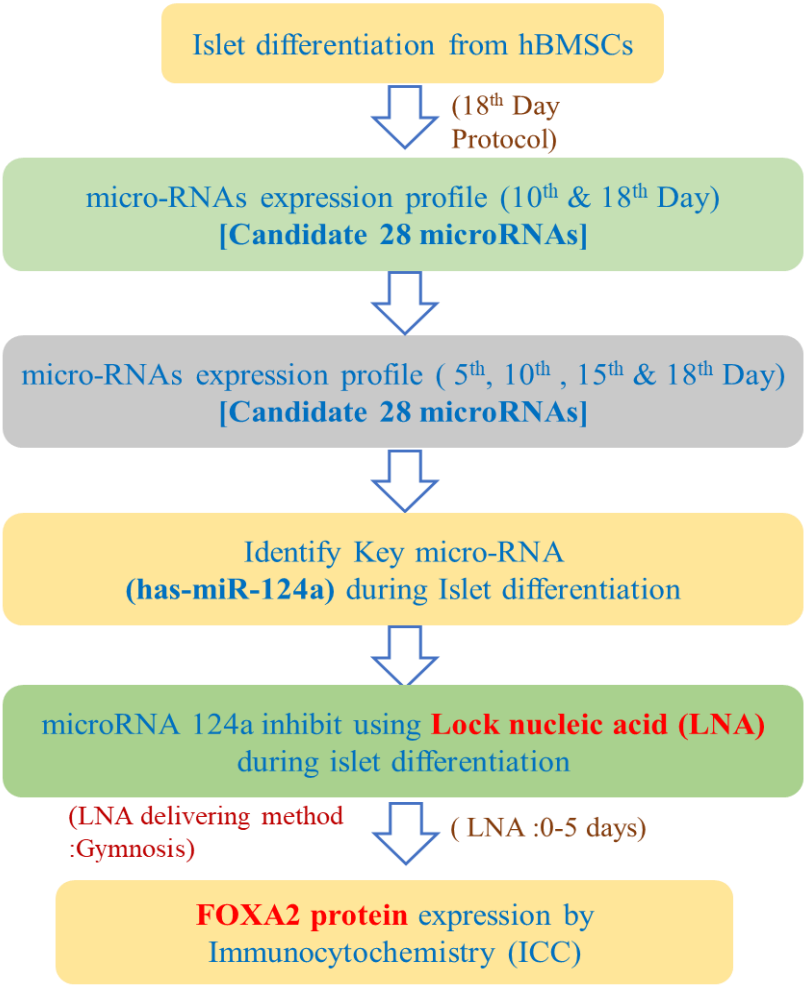
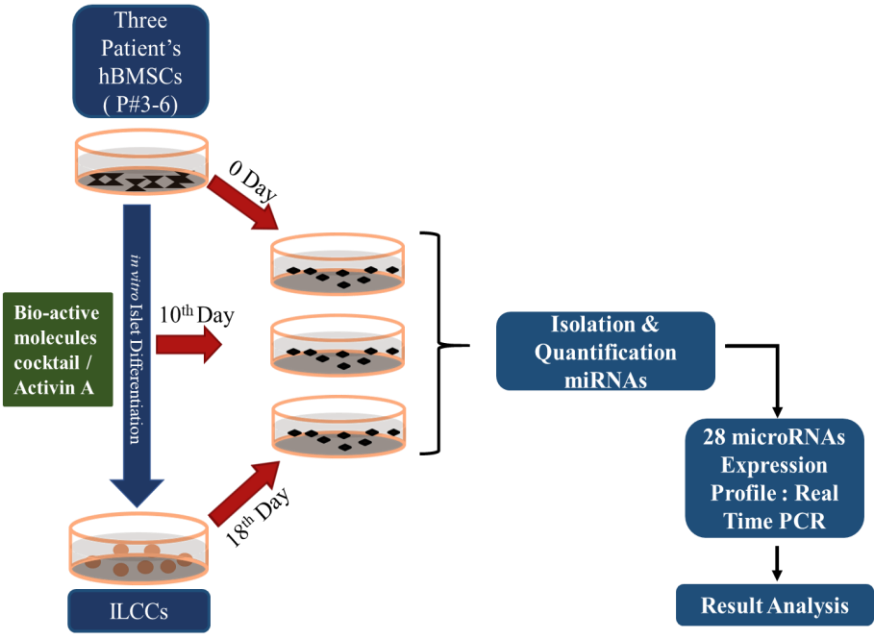


Figure 5. 5 Representative flow chart of experimental design

5.3. Plan of work: microRNAs profile

(i) microRNAs expression profile during Islet differentiation (Day 10th and Day 18th)



(ii) microRNAs expression profile during Islet differentiation (Day 5th, Day 10th Day 15th and Day 18th)

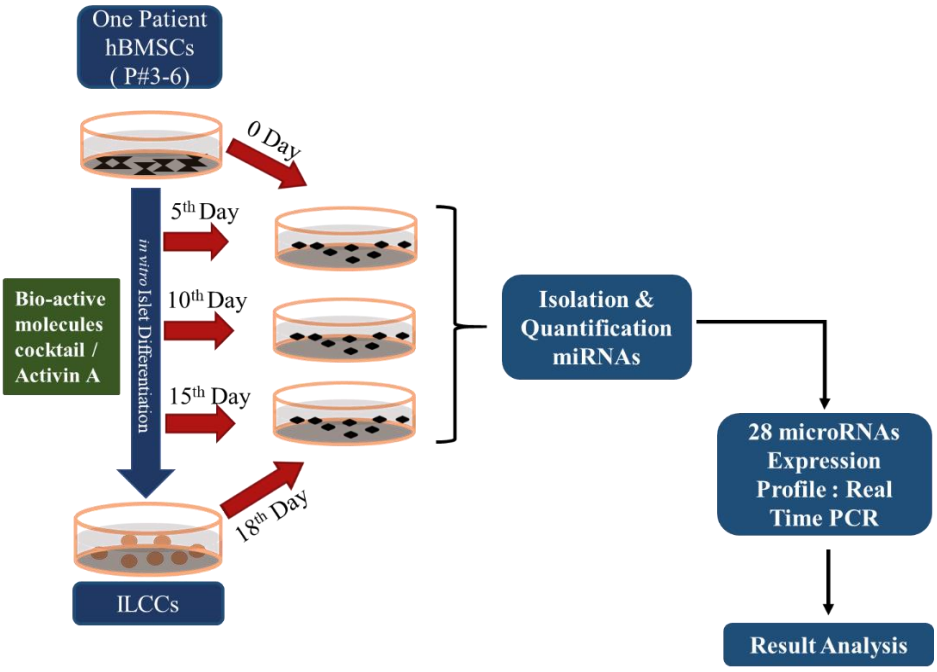


Figure 5. 6 Plan of work for microRNAs expression profile during islet differentiation from hBMSCs using activin A and bioactive molecules cocktail.

(iii) microRNA manipulation: Power inhibitor LNA-has-miR-124a

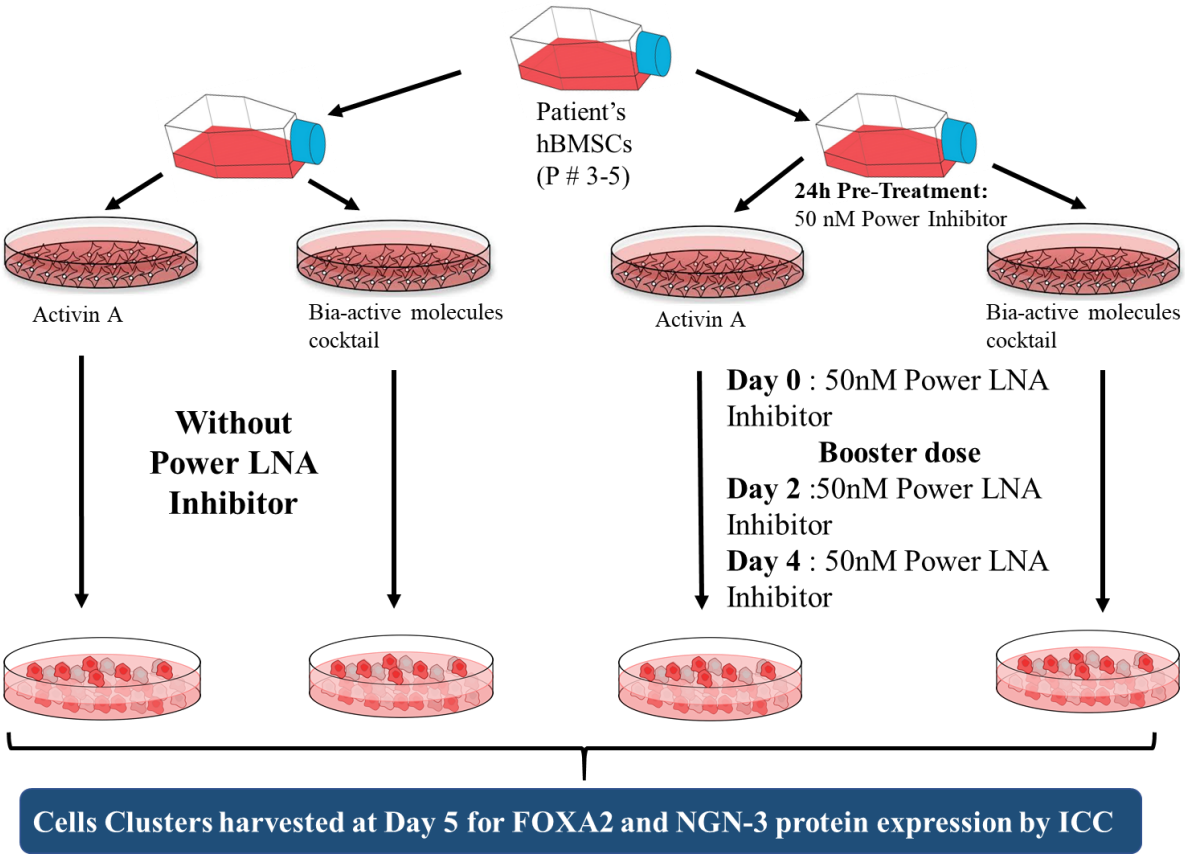


Figure 5. 7 Plan of work for inhibiting microRNAs -124a using power inhibitor LNA.

5.4. Material & Method

5.4.1. Materials:

A. Chemicals and cell culture media:

All chemicals, media used in the present study were procured from Sigma Aldrich, USA, and Thermo Scientific, USA. Molecular biology reagents and cDNA and PCR kits were procured from ABI. All plasticware were purchased from Nunc and corning, USA.

B. microRNAs kits:

All microRNAs related kits were purchased from Qiagen/Exiqon, USA.

5.4.2. Methods:

A. hBMSCs culture and *in vitro* islet differentiation protocol (18th Day protocol)

hBMSCs from three non-diabetic (Normal) human subject isolated, characterized and differentiated into ILCCs using BMC and activin A as described earlier in the chapter: 3 (section: 3.3.2-H)

B. microRNAs isolation

microRNAs's isolation was performed using the miRNeasy mini kit (catalog number: 217004) as represented in figure:5.8. The specific details of the isolation protocol are described in the miRNAeasy Mini Kit manual (Qiagen). We measured the total RNA (Including microRNAs) concentration using a carry 60 UV-Visible spectrometer (Agilent Technology Inc) at lambda 260 and 280 nm.

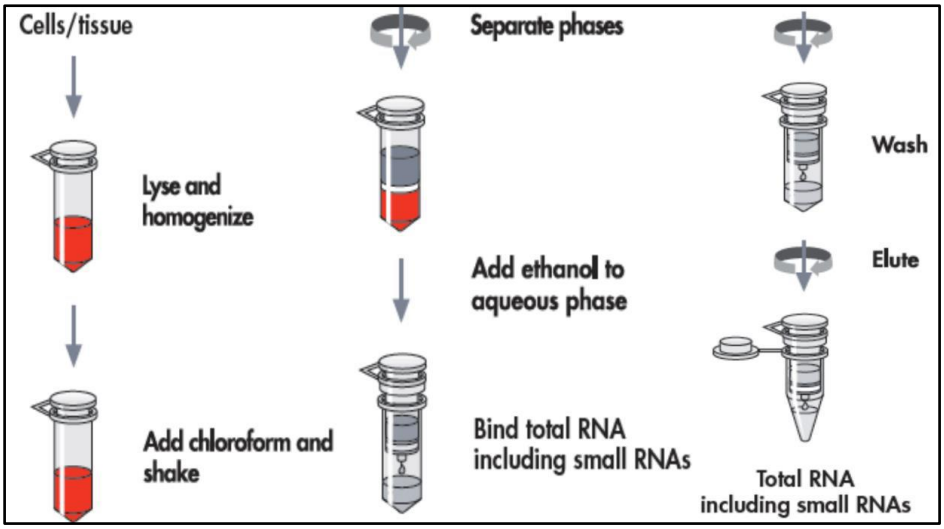


Figure 5. 8 The schematic diagram shows a step-wise protocol for isolating total RNA along with miRNA by utilizing miRNeasy MINI Kit (Qiagen, Germany).

C. microRNAs profile:

2 µg of isolated total RNA was reverse transcribed into respective cDNA utilizing miScript II RT Kit (Qiagen, catalog number:218160). The exact details of the cDNA synthesis protocol are illustrated in the miScript II RT kit manual (Qiagen). The synthesized cDNA is diluted as per manufacturer's guideline and then subjected to qPCR using miScript miRNA PCR Arrays (uses FAST SYBR Green Chemistry). miScript miRNA PCR Arrays system contains all requirements to detect and quantification of miRNAs such as forward primer to specific mature

miRNA and miScript PCR reagents. microRNAs profiling was performed with Custom miScript miRNA PCR Arrays (Qiagen: Catalog number: 331231) for human microRNAs, which allows a quantitative assessment of 32 microRNAs using quant studio 12k flask real-time PCR system (Applied Biosystem, USA) as a described in the figure: 5.9. The precise details of PCR arrays are illustrated in the miScript miRNA PCR arrays kit manual (Qiagen). We customized this array in 32×3=96 format for covering all 32 microRNAs including experimental positive control and negative control. The forward primers used were validated to be microRNA specific, embedded in a tailor-made microRNA PCR Array and very sensitive Fast SYBR Green Chemistry is utilized. The details of the custom microRNAs array map are described in Appendix (E).

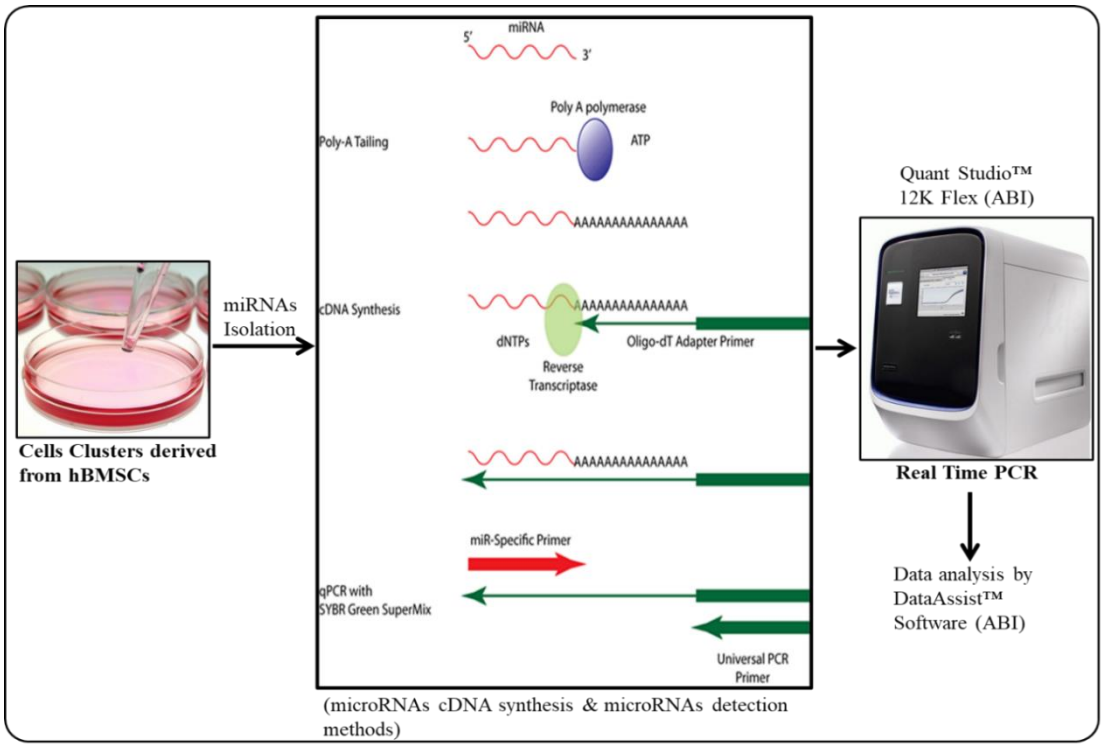


Figure 5. 9 Schematic diagram on microRNAs cDNA synthesis and expression profile strategies based on Poly-A Talling and SYBR green dye methods (qPCR).

Data assist software (ABI, USA) was used to analyze the quantification cycle (Ct) value, Ct more than 35 cycles were considered as undermined values, which is defined as a number of cycles at which SYBR green fluorescence signal is significantly higher than the set threshold; expression of every microRNA was represented from the threshold cycle (Ct), and relative quantification level were analyzed utilizing the RQ = $2^{-\Delta\Delta Ct}$ method after normalization with endogenous control RNU6 (Small nucleolar RNA), which is expressed evenly in almost all

samples. To inspect samples that may be contaminated with DNase /RNase nucleases, we took benefit of the multiple quality controls parameters such as positive PCR control (PPC), miRNA reverse transcription control (miRTC), and c.elegans-miR-39, which were provided in the Custom miScript miRNA PCR array kit (Qiagen, Germany).

D. microRNA Power Inhibitor LNA:

The candidate miRNA selected from the above study is targeted for inhibition by microRNA inhibitor (LNA^{TM} -Exiqon) which will be delivered unassisted in the culture without any transfection agents (Gymnosis). Details of Power Inhibitor LNA (miRCURY LNA^{TM} miRNA power inhibitor)**:** This power inhibitor is extremely specific to mature microRNA 124a & it strongly binds with only mature microRNA 124a. Hence, it's called LNA-has-miR-124a (Catalog number: 339131 YI04102198-DDA). The oligonucleotide inhibitor sequence 5'G*C*A*T*T*C*A*C*C*G*C*G*T*G*C*C*T*T*A3' (HPLC purified, Exiqon) were used for inhibiting has-miR-124a expression during islet differentiation. The miR-124a inhibitor obtained from Exiqon (Qiagen, USA), has fully Phosphorothioate (PS)-modified backbones (bonds indicated by "*" in inhibitor sequence), which enhanced stability in cell culture *in vitro* study as well as pharmacokinetic and pharmacodynamic properties *in vivo*. We followed manufacture's instruction (miRCURY LNA Inhibitor Handbook) for Power Inhibitor dilution, experimental dose, *in vitro* delivery methods. Untreated cells/cell clusters were cultured parallel to the LNA anti-miR-124a transfected cells/ cell clusters. The efficiency of the transfection was investigated by confocal fluorescent microscopy.

E. Immunocytochemistry by laser-scanner confocal microscopy:

Cell clusters were collected on day 5 in activin A treated and BMC with LNA treated or non-treated both conditions. Then, the aforementioned cell clusters are subjected to immunocytochemistry (ICC) experiments to identify the effect of LNA on FOXA2 and NGN-3 protein expression. We have performed ICC in these cell clusters as described earlier in the chapter: 4 (section number: 4.4.4).

5.5. RESULTS:

5.5.1. microRNAs expression profile (*In vitro* differentiation of hBMSCs into ILCCs)

After an extensive review of the role of microRNAs in pancreas developments, we came up with 27 candidate microRNAs (Rosero *et al.* 2010, Fernandez-Valverde *et al.* 2011, Fernández-Hernando *et al.* 2013, Özcan 2014, Filios and Shalev 2015). We selected 27 candidate microRNAs whose targets have been identified and whose role in islet differentiation has been studied in other systems like embryonic stem cells to pancreatic islet differentiation. In our present study, bone marrow (BM) samples were acquired from three non-diabetic human subjects. We characterized hBMSCs (Passage # 2-6) using flow cytometry and differentiated into Islet like cell clusters (ILCCs) using activin A and bioactive molecules cocktail (BMC) as discussed in chapter number : 3. To identify microRNAs that are involved in pancreatic islet differentiation from hBMSCs, a microRNAs profile array using a custom miScript miRNA PCR Arrays (Qiagen) has been performed. These cells clusters were harvested on day 10^{th} and day 18^{th}, along with undifferentiated hBMSCs as day 0 control for further analysis. From the heat map, we could observe that in comparison to the endogenous control RNU6, the expression of candidate miRNAs was varying during the differentiation protocol, between control undifferentiated hBMSCs and islet differentiation sample at day 10^{th} and 18^{th} in both (BMC) and activin A (Positive differentiation control) groups (Figure:5.10). At first, a heatmap was utilized to demonstrate the dynamic expressed miRNAs (rows) and differentiated stage of hBMSCs(columns) through color intensity. Based on the heatmap of our microRNA's expression data, activin A and BMC group revealed dynamic microRNAs expression profile on day 10^{th} and day 18^{th} in all three patients, sharing some similarities in their microRNA's expression profile (Figure:5.10). Interestingly, numerous microRNAs reported previously in studies of mouse/human pancreatic development/regeneration and pancreatic islet function were also found expressed during islet differentiation from all three-human subject of hBMSCs in both BMC and activin A groups. We found that few downregulated miRNAs belonged to the human bone marrow-derived mesenchymal stem cells -regulating the family of microRNAs (i.e., miR-335, miR-494, etc.), which is essential for hBMSCs maintenance and renewal, while in contrast, the upregulation of a set of microRNAs (i.e. miR-375, miR-146a, miR-124a, miR-195, miR-15, miR-16) occurs during islet differentiation from hBMSCs.

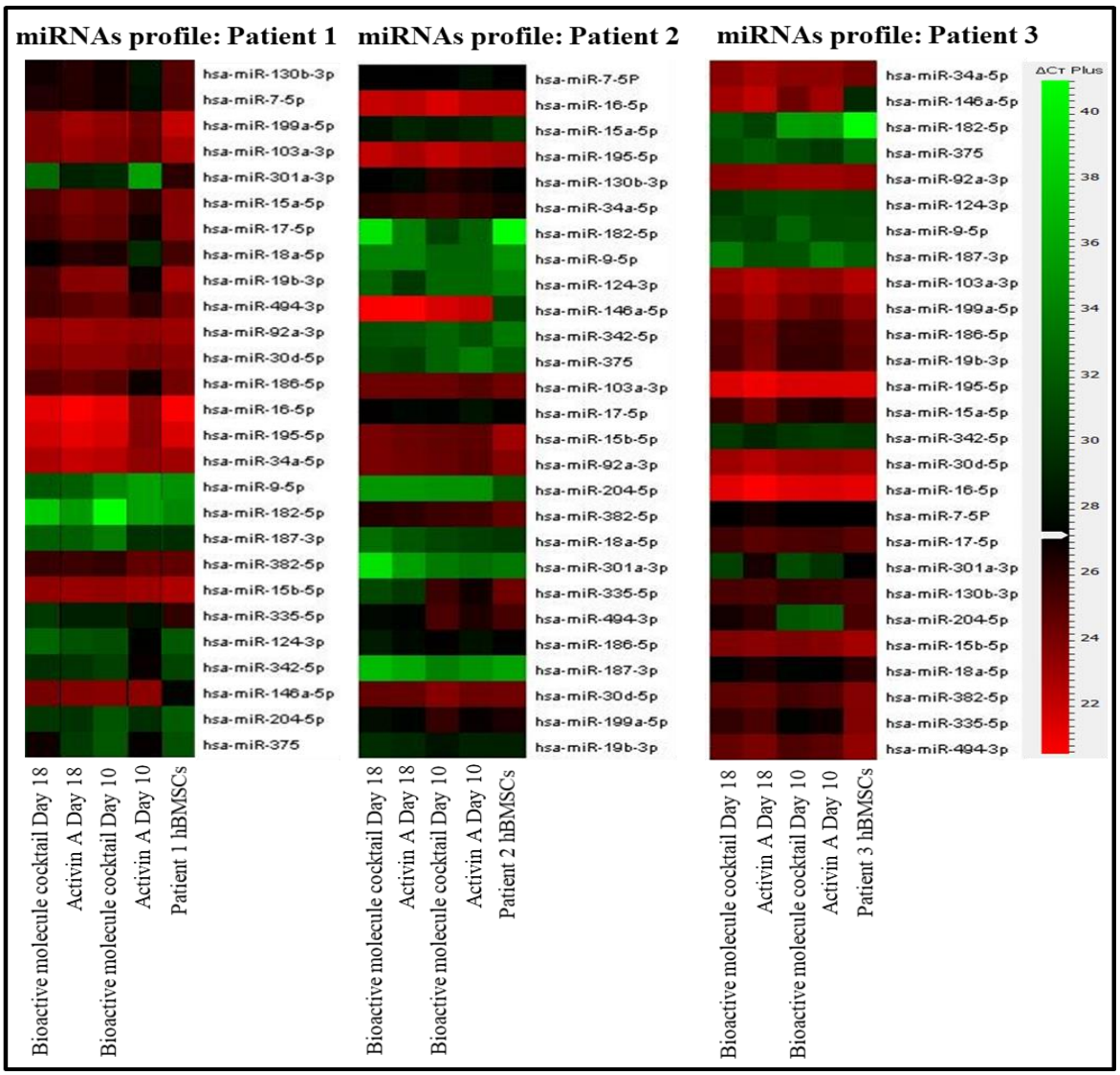


Figure 5. 10 Heat map depicting the expression of 28 candidate miRNAs in Islet like cell clusters obtained at 10th Day and 18th Day of differentiation process from BMSCs from three different Non-diabetic patients. Undifferentiated hBMSCs as the undifferentiated control; Endogenous control=RNU6; BMC group as test group & activin A as a positive control group for islet differentiation. MicroRNAs expression values are reported as ΔCt values in scale colors (from red –highest expression- to green- lowest expression). Heat map (Global View) generated by Data assist software -ABI, in which distance measure by Pearson's correlation and average linkage as the clustering method (N=3).

Further, the heatmap result showed that BMC and activin A group shared a similarity in their microRNAs expression profiles with hBMSCs-derived islet-like cells clusters. Thus, from our results, we can achieve with utmost assurance that hBMSCs are differentiating into islet-specific lineages. However, the results also indicate the need for further investigation of

important candidate microRNA highly specific in islet differentiation from hBMSCs. Hence, we further scrutinized microRNAs (11 microRNAs) which are known to be directly involved in the regulation of very crucial transcription factors such as FOXA2, PDX1, NGN-3, etc playing role in the early stage of the islet differentiation process, and pancreatic islet functionality. These microRNAs were also involved in catering the oxidative stress as well as regulation of other essential transcription factors.

As presented in figure:5.11, eleven microRNAs that had been identified to change during islet differentiation, such as miR-375, miR-124a, miR-146a, miR-195,miR-7, miR-34a,miR-182, miR-187,miR-204,miR-342, and miR-382 were detected in BMC and activin A both groups. Out of elven microRNAs, seven microRNAs have shown up-regulation in BMC & activin A group, and two microRNAs have shown down-regulation in the BMC group on 10th Day of islet differentiation (normalized with undifferentiated hBMSCs).

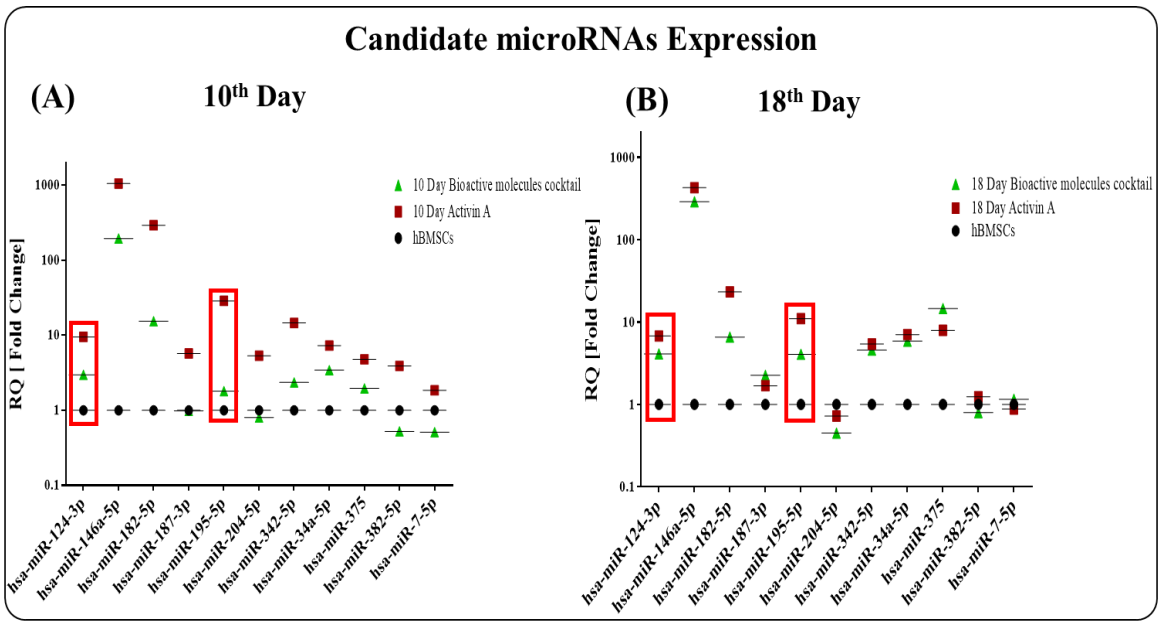


Figure 5. 11: Candidate microRNA expression at 10th& 18th Day of islet like cell clusters of three different patients based on three patient's microRNA expression profile (previous heat map). [A] Graph represents 11 highly up-regulated microRNAs on 10th day of islet differentiation [B]Graph represent 11 highly up-regulated microRNAs on 18th day of islet-like cell clusters. Undifferentiated hBMSCs as the undifferentiated control; Clusters differentiated using activin A as the positive experimental control; Endogenous control=RNU6 (N=3).

Our results showed referential expression of miR-375, miR-182, etc. in 18th Day ILCCs which indicates a potential role in islet differentiation and maintenance of islet functionality. miR-

146a is reported as an indicator of oxidative stress. We found the highest expression of miR-146a as compared to other microRNA expressions in both groups with remarkably low expression in the BMC group on 10^{th} and 18^{th} day of islet differentiation (Figure:5.11). miR-34, which is supposedly known to play a role in stress response was unaltered in either group as compared to hBMSCs. miR-195 is been known to target NGN-3 levels (master transcription regulator for the endocrine pancreas). In our study, the miR-195 expression profile showed approx. 30-fold and 10-fold increase at day 10 and day 18 respectively, in activin A group, whereas only 3-fold and a 6-fold increase in BMC group on 10 and day 18 respectively, indicating higher NGN-3 protein expression (Figure:5.11). Similarly, miR-124a, which targets FOXA2 transcription factors (Early endoderm marker) showed approximately 10-fold and 9-fold increase in activin A. while only a 3-fold and 8-fold increase in the BMC group, on day 10 and day 18 respectively. Moreover, miR-7 and MiR-375 are well-known for their role in the development of pancreatic endocrine lineage, ironically, we detected only basal level of miR-7 expression and higher expression of miR-375 on 18^{th} day as compared to 10^{th} day during islet differentiation in both BMC and activin A groups (Figure:5.11).

5.5.2. Dynamic changes in microRNAs expression Profile during multistage islet differentiation (5^{th},10^{th},15^{th} and 18^{th} day).

After studying the microRNAs profile at 10^{th} day and 18^{th} day in three non-diabetic patients, we further investigated same microRNAs expression (Selected 28 microRNAs) profile at an early stage of islet differentiation i.e. 5^{th} day and middle phase of islet differentiation i.e.15^{th} day along with 10^{th} and 18^{th} day in one sample of non-diabetic, hBMSCs during islet differentiation in both BMC and activin A group (Figure:5.12).

Based on the heat map (hierarchical clustering) of our microRNAs expression data, our results revealed a distinct color pattern with a diverse expression profile, most dynamically changing during differentiation in both in BMC and activin A group. There was reproducibility in the microRNA profile in the different stages of different samples from the same group suggesting good consistency.

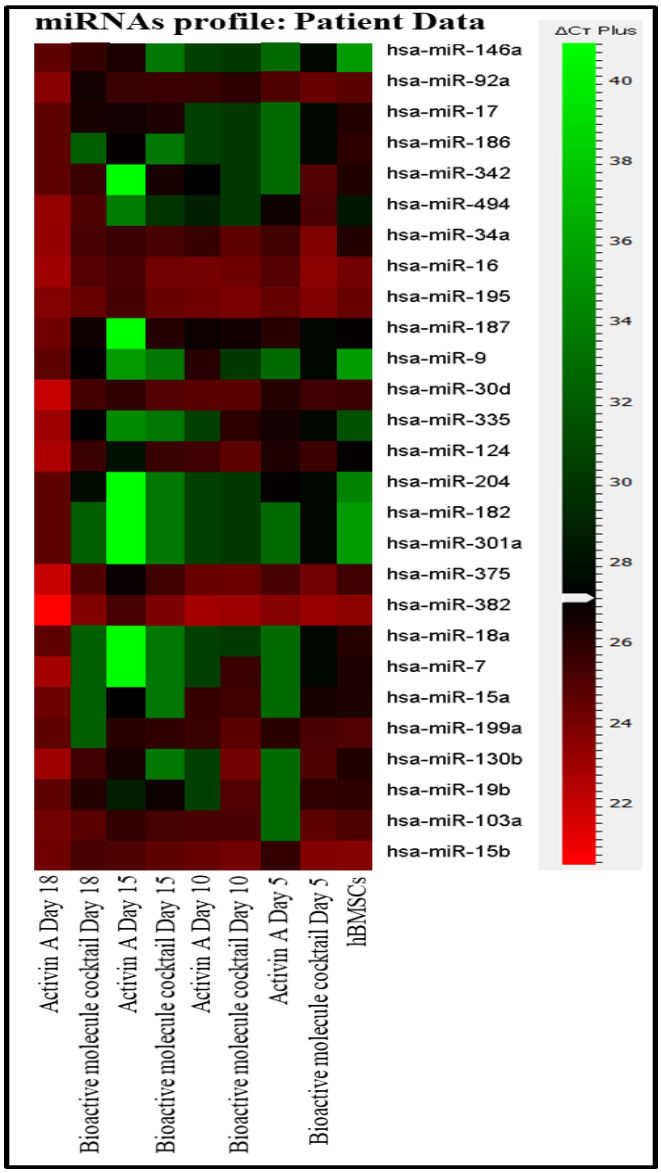


Figure 5. 12 Heat-map depicting the expression of 28 candidate miRNAs in Islet like cell clusters obtained at 5th Day, 10th Day,15th Day and 18th Day of differentiation process from BMSCs from one non-diabetic patient. Undifferentiated hBMSCs as the undifferentiated control; Clusters differentiated using activin A as the positive experimental control; Endogenous control=RNU6; MicroRNAs expression values are reported as ΔCt values in scale colors (from red –highest expression- to green- lowest expression). Heat map (Global View) generated by data assist software -ABI, in which distance measure by Pearson's correlation and average linkage as a clustering method.

Further, we noted the upregulation of miR-124a and miR-195 on 5th and 15th day of differentiation in both BMC and activin A group. Since miR-124a and miR-195 are known to hinder islet differentiation by downregulating its target FOXA2 and NGN-3 protein expression

respectively, we further focused only on miRNA124a expression because it regulates FOXA2 transcription factor, a direct upstream regulator of NGN-3.

5.5.3. microRNA 124a & its target FOXA2 gene and protein expression

To understand the molecular mechanisms underlying miR-124a mediated regulation of islet differentiation, we searched for potential targets of miR-124a using the previously published reports (Figure:5.1). The target genes of miR-124a contain several mRNAs, some of which play prominent roles during the pancreatic islet development, including FOXA2, and SNAP25, Rab3A, etc. Amongst this FOXA2 was selected for further analysis as in our earlier chapter (Chapter: 4), the FOXA2 gene and protein expression were found to be most dynamically changed during sequential differentiation into ILCCs from hBMSCs.

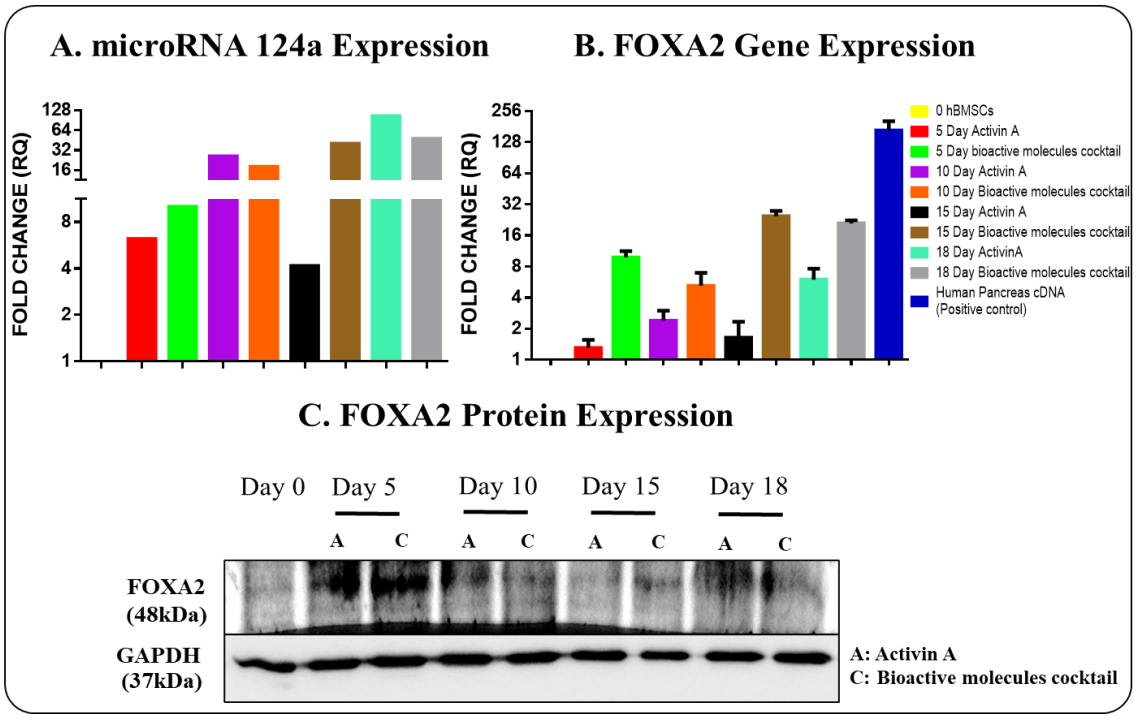


Figure 5. 13: microRNA, gene expression, and protein expression during the islet differentiation stage: Day 5, Day 10, Day 15, and Day 18. A: hsa-miR-124a expression profile during the process of islet differentiation - RNU 6 as microRNA endogenous control. B: FOXA2 gene expression profile β-actin as endogenous control; Human pancreas cDNA as a positive control [Data adopted from the previous chapter : 4] C: FOXA2 protein expression by western blot technique- (hBMSCs at P #3-6 as Day 0, undifferentiated cells control; GAPDH as endogenous control) [Data adopted from the previous chapter:4]

We compared microRNA 124a and its target FOXA2 gene (qPCR)and protein expression (western blot) in one platform (Figure:5.13). Notably, the dynamic expression of miR-124a showed specific "W" shaped patterns during the differentiation (Figure:5.13) in both BMC and activin A groups, which are coincided with decreased FOXA2 protein expression while FOXA2 gene expression was increased during differentiation. Thus, miR-124a and their target gene FOXA2 are probably to play crucial regulatory roles in differentiation of pancreatic islet, and they may be exploited (Inhibit) to enhance the proportion of pancreatic β-cells and insulin synthesis in the differentiated ILCCs. Next, we inhibited key microRNA (miR-124a) using antisense/ knockdown techniques for the betterment of the islet differentiation process.

5.5.4. microRNA power inhibitor (LNA-hsa-miR-124a) study

To confirm whether microRNA 124 inhibition has a beneficial effect on islet differentiation, we performed a power inhibitor LNA study during islet differentiation in both BMC and activin A groups. We employed power LNA, which has one major advantage of gymnotic delivery, which efficiently delivers microRNAs inhibitor to suspension 3D cell clusters that are otherwise very difficult to transfect.

The anti-miRNA inhibitors (124a) are sequence-specific oligonucleotides that specifically target and knockdown miRNA-124a molecules. Based on previously published reports, we standardized and finally determined 50nM as an optimal concentration of miR-124a power inhibitor for both group BMC and activin A. Thus, within our multi-step differentiation protocol (ILCCs from hBMSCs), cell clusters were incubated (Gymnotically) with a power LNA inhibitor, and then ICC was performed to determine important pancreatic transcription factors FOXA2 on day 5.

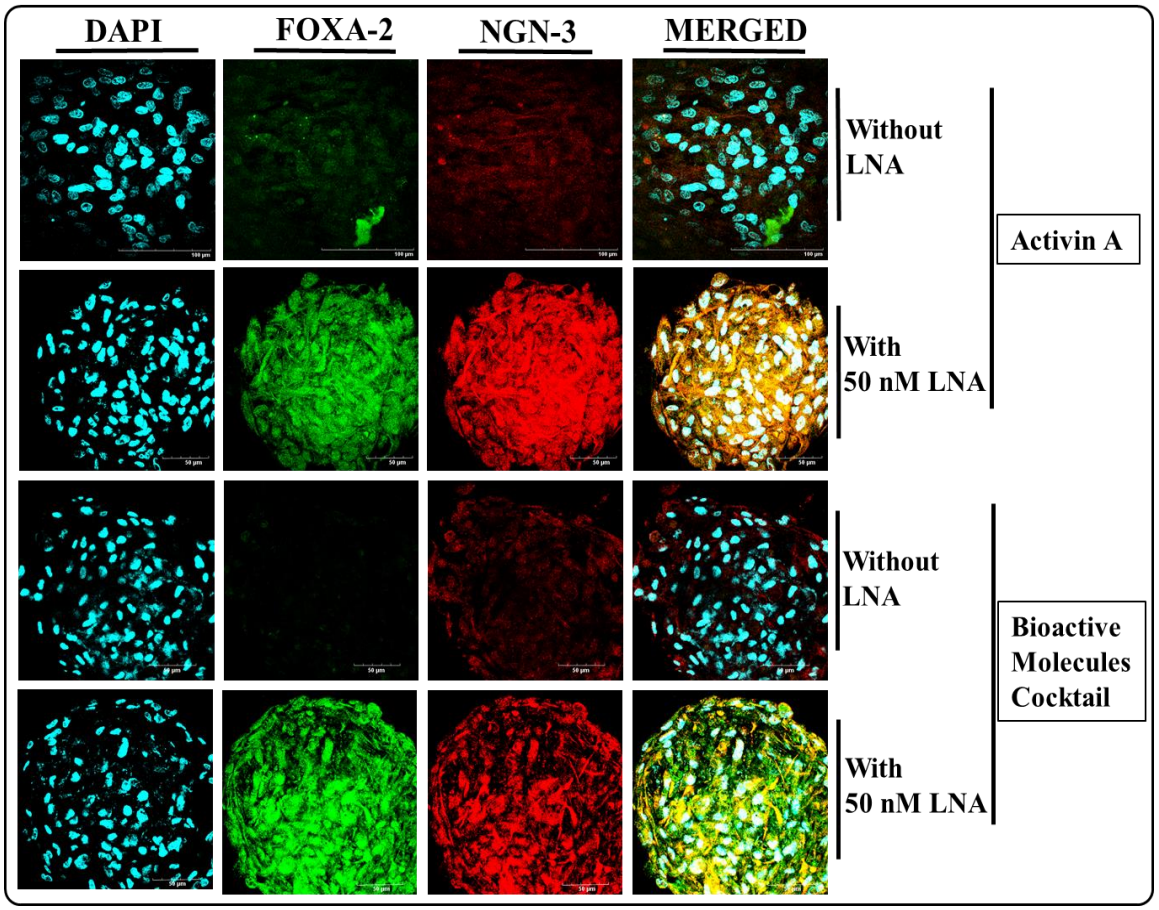


Figure 5. 14 Immunofluorescent staining (using confocal microscopy) of FOXA-2 and NGN-3, definitive endoderm and pancreatic endocrine progenitor marker at 5th Day of islet differentiation treated with and without power LNA-anti-miR124a (50nM concentration -Gymnosis method) in both BMC and activin A groups. FOXA2 and NGN-3 protein extremely strongly expressed in power LNA-anti-miR-124a treated samples as compare to non-treated samples in both BMC and Activin A group. DAPI was used as nuclear counterstain (blue colour); FOXA2 (FITC), NGN-3 (AF555). Red and Green represent AF555 and FITC conjugates, respectively [Magnification 60 X; scale bar: 50 µM] (n=3).

To gain maximum inhibition of targeted microRNA, pre-treatment (-24 hrs.) and booster treatments were applied in three-time points, days 0, day 2, and day 4 with power LNA-has-miR-124a in both BMC and activin A group. Our results demonstrate that 50 nM conc. significantly linked with the inhibition of miRNA124a. Immunocytochemistry of protein FOXA2 demonstrated that the control group (without LNA^{TM} inhibitor) had a basal level of expression while its upregulation was evident in cell clusters treated with 50nM (LNA-anti-miR124a) in both BMC and activin A treatment group. Thus, The LNA anti-miR-124a-treated group maintained a relatively high FOXA2 protein expression which further, caused the high

expression of NGN-3 (an important downstream transcription factor of FOXA2), a major upstream regulator of insulin, in LNA treated group as compared to the control (Figure 5.14), indicating that it triggers pancreatic β-cells differentiation process at an early phase. Additionally, we also noticed that microRNA124a inhibition (at 50nM concentration) did not affect any change in the number of cell clusters nor morphology of cell clusters in both groups. Treatment with power inhibitor LNA showed no effect on FOXA2 and NGN-3 transcription factor expression in only the hBMSCs group (Figure:5.15). Additionally, the secondary antibody isotype control study showed no expression of FOXA2 and NGN-3 in hBMSCs (Figure:5.15). Overall, these novel findings suggest that the LNAanti-miR-124a treatment can accelerate the islet differentiation process from hBMSCs in both BMC and activin A group.

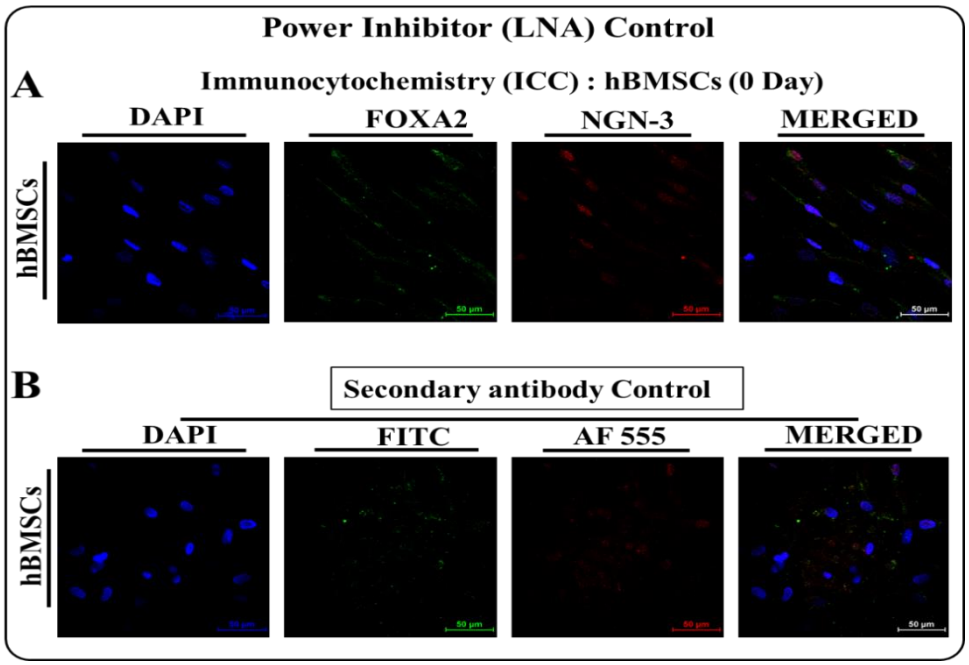


Figure 5. 15 Immunofluorescent staining (using Confocal microscopy) of FOXA2 and NGN-3 protein expression. (A) Undifferentiated hBMSCs show minor protein expression of FOXA2 and NGN-3 at 5th Day of islet differentiation. (B)undifferentiated hBMSCs show negligible secondary antibody control of FITC, AF 555 expression.

5.6. Discussion

Numerous factors modulate the expression of proteins, one being regulation by small non-coding RNAs -microRNAs. Many recent studies have shown that several microRNAs are up-regulated and down-regulated during stem cell differentiation. As of now most of the reports addressing the microRNA role in pancreatic development/islet differentiation were conducted using mouse embryonic model cell system. However, with the increasing accessibility of

human mesenchymal stem cells, there is an opportunity to study the role of microRNAs in islet differentiation/pancreas development. We took the benefit of the hBMSCs for investigating the role of microRNAs in pancreatic islet differentiation. We selected 27 important microRNAs whose targets have been recognized and whose role in islet differentiation has been investigated using other stem cell systems like hESCs (Wei *et al.* 2013, Chen *et al.* 2014). Henceforth, we isolated, characterized and differentiated hBMSCs, from 3 non-diabetic human subjects, into ILCCs using BMC and activin A. Candidate microRNAs expression profile using customized microRNAs qPCR array revealed alter the expression of microRNAs like miR-375, miR-146a, miR-195, miR-34a, miR-124a, miR-7, etc.

Early microRNAs profiling of pancreatic β cell differentiation from hESCs disclosed that miR-375, miR-146a, miR-7, and miR-34a (Wei *et al.* 2013), are the most predominately present microRNAs in embryonic stem cell differentiation process. Further, a study on microRNA expression signature showed that several pancreatic specific miRNAs such as miR-375, miR-182, miR-187, miR-124a, miR-342, miR-382, etc., were expressed most abundantly in course of human pancreatic development (Rosero *et al.* 2010). Amongst these, the most important and broadly studied miRNA in pancreas development is microRNA-375 which is, associated with proliferation, differentiation, and regeneration in developing human pancreas (Joglekar *et al.* 2007, Joglekar *et al.* 2009, Lahmy *et al.* 2014). Further, microRNA 375 has been known to target proteins aiding in insulin secretion and considered as an indicator of human islet differentiation (Joglekar *et al.* 2009, Wei *et al.* 2013). Generally, de-differentiation of pancreatic β-cell for *in vitro* expansion has been found to be related with a decrease in miR-375 expression (Nathan *et al.* 2015, Dumortier *et al.* 2016). In our study we observed a gradual increase in miR-375 expression during islet differentiation, indicating that these cells did not participate in the de-differentiation process. A recent study by yuha goa et.al., 2019 showed breakthrough research related to activin A and miR-375. In this study, they found that activin A activates the TGF-β/ smad pathway and formation of smad complex, which directly interacts with promoter sequence of NGN-3 and the pancreas-specific miR-375, to enhance pancreatic islet differentiation from stem cells (Gao *et al.* 2019). Previously, our lab also reported role TGF β and smad pathway in islet differentiation with increased NGN3 expression by treatment of swertisin, one of the key components of BMC (Dadheech *et al.* 2015). Our present results showed increased miR-375 much more in the BMC group on day 18th of islet differentiation as

compared to activin A group, suggesting that the use of bioactive molecules is one of the superior approaches in pancreatic islet differentiation from stem cells.

A report showed that miR-375 may act synergistically with miR-124a, which are amply expressed in pancreatic islets and may regulate the mechanism of insulin secretion myotrophin (Krek *et al.* 2005, Gauthier and Wollheim 2006). Initially, miR-124a was defined as neuronal-specific miRNAs in animals, the function of which is apparently to determine and maintain cell-specific characteristics (Sempere *et al.* 2004). Further, Nadine Baroukh *et. al.,* 2007 explored more role of miR-124a and found that it directly targets FOXA2 transcription factor and K_{ATP} channel components, Kir6.2 and Sur-1 and indirectly regulate PDX-1 and NEUROD1 transcription factors via FOXA2 (Baroukh *et al.* 2007). Based on, our results we could understand the highest miR-124a expression at the end of islet differentiated clusters with high FOXA2 gene expression and undetectable FOXA2 protein. miR-342 also targets FOXA2 and was found to increase on day 10 and day 18 of islet differentiation in both BMC and activin A group (Plaisance *et al.* 2014, Filios and Shalev 2015).

To date, only a few studies have been published *concerning miR-182 expression in islet* biology. It has been previously shown, that miR-182 upregulates insulin promoter activity and insulin transcript levels (Melkman-Zehavi *et al.* 2011). Tal Melkman-Zehavi *et. al.,* 2011 demonstrated that miR-182 expression promotes insulin transcript by downregulating the expression of insulin transcriptional repressors such as BhLhe22 and Sox6 (Melkman-Zehavi *et al.* 2011). Our results showed a prominent expression of miR-182 during islet differentiation in both BMC and activin. J. M. Locke *et. al.,* 2014 displayed that miR-187 is being targeted by protein HIPK3 (homeodomain-interacting protein kinase-3), a notable controller of insulin secretion in pancreatic β-cells (Locke *et al.* 2014). Our results indicated a basal level of miR-187 expression during islet differentiation. We observed primary level of miR-7 expression which negatively regulates α- and β-cell differentiation via direct inhibition of PAX6, which is essential for endocrine pancreatic differentiation (Kredo-Russo *et al.* 2012), indicating that miR-7 may not be a major regulating factor during islet differentiation from hBMSCs.

Several studies showed that the upregulation of miR-195 promotes progenitor cells toward an endocrine progenitor fate via reduction of target NGN3 protein expression a well-known master regulator of the endocrine pancreas. It was also found to be higher in the regenerating pancreas of 90% partially pancreatectomized mouse model (Gradwohl *et al.* 2000, Joglekar *et al.* 2007, Bolmeson *et al.* 2011). As expected, we found a low level of miR-195 expression in

the BMC group as compared to activin A, suggesting that the BMC group stimulates more NGN-3 protein expression for better islet differentiation. miR-146a and miR-34a demonstrate mutual acts in pancreatic islet function and are associated with the development of diabetes (Lovis *et al.* 2008, Li *et al.* 2009, Roggli *et al.* 2010). Yangmei Xie *et. al.*, 2018 analyzed miR-146a and found it an indicator of inflammation and oxidative stress (Xie *et al.* 2018). Our result demonstrated an apical expression of miR-146a in the initial stage of differentiation which declines along with the differentiation process, suggesting high oxidative stress during the initial stage of islet differentiation due to serum-free media conditions. Similarly, several reports displayed that miR-34 is involved in ROS generation and stress response (Andolina and Di Segni Matteo 2017, Cheleschi *et al.* 2017). further, microRNA-34a has been known to target SIRT1 which protects pancreatic beta cells by reduced apoptosis (Backe *et al.* 2014). Aranha *et. al.,*2011 demonstrated that miR-34a is needed for appropriate neural differentiation (Aranha *et al.* 2011). It is understood that neuron and pancreas have shared lots of transcription factors including NGN-3, PAX-6 for a common mechanism to control development (Lumelsky *et al.* 2001). Thus, our results demonstrating, stable moderate expression of miR-34a during islet differentiation seemed to reasonable. Interestingly, the BMC group demonstrated that miR-146a and miR-34a expression were remarkably reduced on the initial stage of islet differentiation (10^{th} day) as compared to activin A, indicating a low level of oxidative stress possibly due to presence of bioactive molecules in BMC group.

Recently, researchers pay more attention to exploring microRNAs manipulation strategy such as overexpression and inhibition of candidate microRNAs/key microRNAs. It has been reported that the overexpression of miR-375 in embryonic stem cells generates insulin-producing cells *in vitro* without the use of any extrinsic factors (Shaer *et al.* 2014, Lahmy *et al.* 2016).

Further, lopez beas *et. al.,* 2018 demonstrated that overexpression of miR-7 in the later stage of islet differentiation process from hESCs, improved islet functionality by increasing insulin secretion (López-Beas *et al.* 2018). However, very recently, Shawna Downing *et. al.*, 2019 confirmed inhibition of miR-7 directly increases REG-1 protein expression, which was linked with initial stage pancreatic islet differentiation (Downing *et al.* 2019). Hence, such modifications may lead to enhancement of pancreatic islet quality in terms of functionality, yield, and viability. Moreover, our microRNAs expression profile data point out that upregulated microRNAs such as miR-124a and miR-195, which are believed to be negatively

involved in regulating islet differentiation. We detected miR-124a and miR-195 highly upregulated which may result in reduced expression of FOXA2 and NGN-3 respectively.

A new approach to overcome this microRNA elevation mayhem in pathological conditions is microRNA inhibition. Such inhibition studies use anti-miR oligonucleotides complementary to the target microRNA, this process of microRNA silencing is validated by studying expression profile for downstream target protein of subject microRNA. Several groups demonstrated microRNAs delivery study using viral vectors such as lentiviruses and adenoviral vectors and polymer-based delivery such as poly lactide-co-glycolide (PLGA) (Yang 2015, Liang *et al.* 2016, Herrera-Carrillo *et al.* 2017). However, all these have some drawbacks including loss of miRNA efficiency and safety issue (Chen *et al.* 2015, Fu *et al.* 2019). We used power LNA-modified oligonucleotides for successful delivery microRNA inhibitor and enhanced miR-124a inhibition. The power LNA modified miRNA delivery system in one of the most advanced *in vitro* delivery systems without any use of transfection reagents and less cytotoxic for a 3D culture like tumorospheres, pancreatic islets and more appropriate and effortless for the *in vivo* delivery system (Baek *et al.* 2018).

The protein expression of FOXA2 after LNA- has-miR-124a inhibitor treatment showed considerable higher expression as compared to the control group (without LNA^{TM} inhibitor) in both BMC and activin A group. Considering that microRNAs inhibition function by rising the expression level of FOXA2 protein, we would expect an increase in all important pancreatic transcription factors such as NGN-3 protein expression. To our knowledge, very few studies have examined the regulation of islet differentiation by using both miRNAs manipulation strategy i.e. inhibition and overexpression. Arefeh Jafarian *et. al.*,2015 demonstrated a similar study on microRNA inhibition and microRNA overexpression. They showed that over-expression of miR-375 and inhibition miR-9 (LNA) had strong positive synergistic effects on pancreatic islet differentiation using bone marrow-derived mesenchymal stem cell source. Interestingly, they showed for the first time that trans-differentiation was induced by manipulating only the levels of miRNA without the use of pancreatic transcription factors or cocktail media (Jafarian *et al.* 2015).

Overall, the indirect and direct effects observed in the present study point to the potentials of microRNAs in accelerating pancreatic islet differentiation by inhibiting only one microRNA such as miRNA-124a during islet differentiation from hBMSCs. Although, inhibiting one

microRNA i.e. miRNA-124a may not be adequate to induce pancreatic islet differentiation from hBMSCs, but it has the potential to expedite the process of pancreatic islet differentiation through early enhanced pancreatic islet related transcription factor network. Moreover, although the roles of miR-124a are well known in pancreatic development and insulin secretion, the inhibition of this miR-124a in trans-differentiation from hBMSCs was never demonstrated. Our results thus establish a link between microRNA inhibitor and islet differentiation from hBMSCs for targeting microRNAs as a new therapeutic avenue to treat diabetes.

5.7. Summary

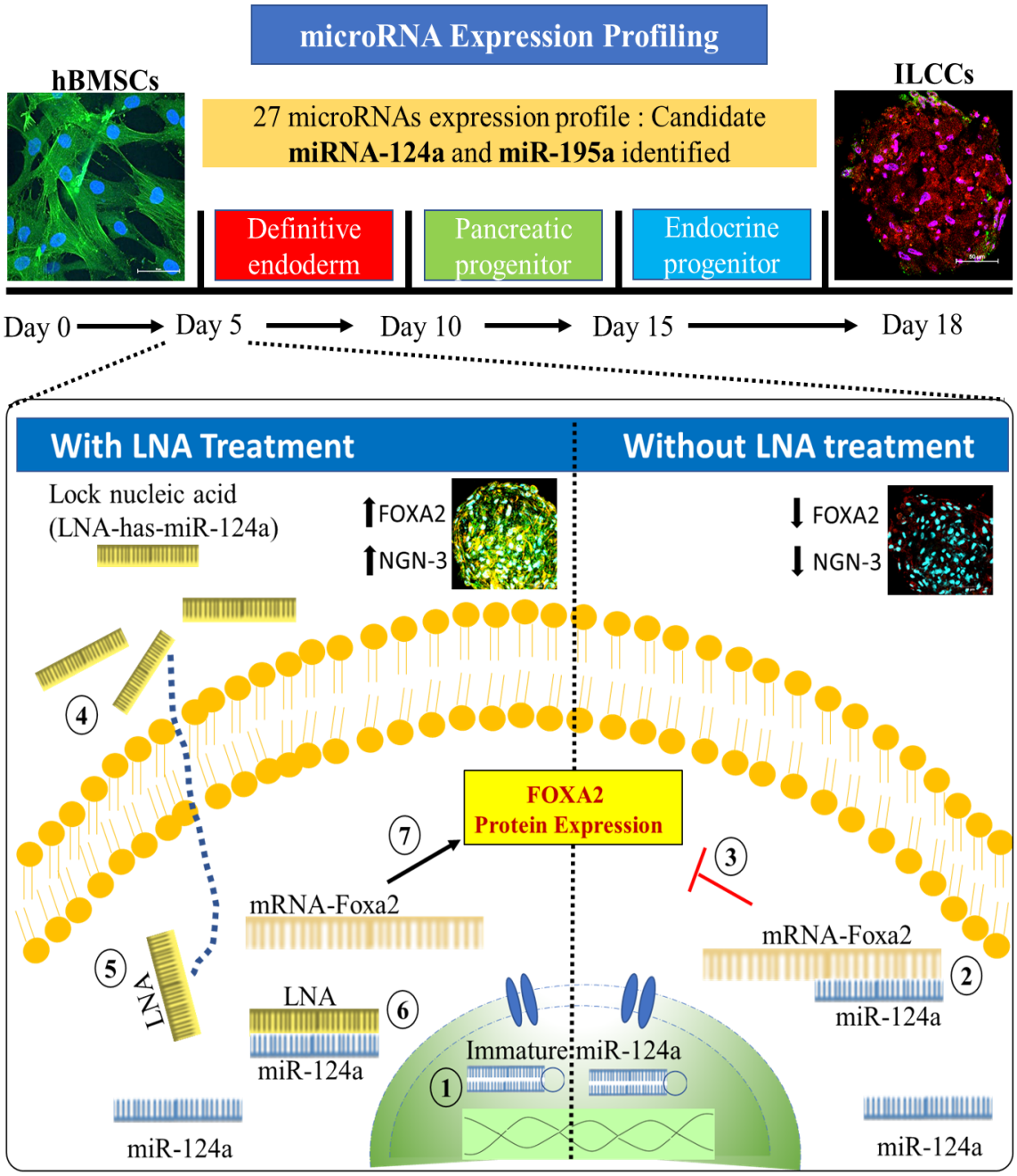


Figure 5. 16 Summary of chapter 5