CHAPTER 4

ASSESSMENT OF MOLECULAR MECHANISM IN ISLET DIFFERENTIATION PATHWAY FROM HBMSCS USING BEST COMBINATION OF BIOACTIVE MOLECULES

4.1. Introduction

In the previous chapter, we have effectively standardized the 18 days protocol for *in vitro* differentiation of Human Bone Marrow Mesenchymal Stem Cells (hBMSCs) in Islet-like cell clusters (ILCCs) using novel bioactive molecules cocktail (BMC). However, to understand the regulation of islet differentiation from hBMSCs, here we have provided an analysis of the key transcription factors known to be involved in the pancreatic islet differentiation from hBMSCs. The mass of pancreatic β cells is regulated by compensating between neogenesis, trans-differentiation, proliferation, and apoptosis (Tarabra *et al.* 2012). Bioactive molecules regulate insulin production at various steps in the biosynthetic pathway in addition to its secretion, including the process of the preprohormone, transcription, and translation (Goodge and Hutton 2000). The immediate events to replenish secreted insulin involve the translation of pre-existing mRNA whereas late release of insulin involves gene transcription. Insulin gene transcription is controlled by the combination of various glucose-sensitive transcription factors expressed in pancreatic tissue as shown in figure 4.1. Amongst these, the most essential transcription elements are PDX-1, NEUROD1, MAFA, which activate the insulin gene promoter and insulin gene transcription (Ohneda *et al.* 2000, Melloul *et al.* 2002, Aramata *et al.* 2005, Han *et al.* 2011). NEUROD1 functions primarily in neuroendocrine cells such as adult pancreatic β cells. NGN3 induces NEUROD1 expression in pancreatic development (Sommer *et al.* 1996, Huang *et al.* 2000, Chae *et al.* 2004). Expression of one or more of these transcription factors promotes pancreatic endocrine differentiation from various stem cell sources (Gasa *et al.* 2004, Borowiak and Melton 2009). Additionally, several other reports devised similar pancreatic β cell differentiation methods dealing with the fundamental developmental stages of the definitive endoderm and endocrine pancreas (Blyszczuk and Wobus 2006, Schroeder *et al.* 2006, Jiang *et al.* 2007).

Several reports show that recapitulating *in vitro,* the transcriptional regulation of pancreas development (*in vivo),* is a promising strategy for islet differentiation from stem cells. Such *in vitro* differentiation protocol using human ES cells (hESCs), mimicked the *in vivo* stages of pancreatic development. Thus, the purpose of the present study is to provide sufficient cues to enable mesodermal stem cells transition to definitive endoderm (DE), to pancreatic specific endoderm, and endocrine progenitors finally resulting in hormone-expressing insulin-positive pancreatic islets (D'Amour *et al.* 2005, D'Amour *et al.* 2006, Velazco-Cruz *et al.* 2019) (Figure:4.1). A detailed description of all transcription factors related to islet differentiation

was given in the general introduction and review literature chapter number: 3 (section number: 1.5). Much of what is realized about pancreatic development is acquired from pre-clinical studies, although, there is vital crucial fundamental variation in transcriptional factors network among animal and human pancreatic development. Few other strategies involve ILCCs generation either by reprogramming pluripotent stem cells or adult pancreatic/ extra-pancreatic cell types. This was achieved either by the transfection of pancreatic transcription factors (*PDX-1*, *NGN-3*, *MAFA*, etc.) or by inducing epigenetic changes (Baeyens *et al.* 2018). The liver has been demonstrated to exhibit common embryonic origin and transcriptional factors network similar to the pancreas development (Ber *et al.* 2003, Lee *et al.* 2012). Hence, hepatocytes were used to generate pancreatic insulin-producing cells by overexpression of the *PDX1* gene (Sapir *et al.* 2005, Zalzman *et al.* 2005, Meivar-Levy *et al.* 2007). There are numbers of genes involved in entire processes, and almost all the reports have emphasized on the genes encoding insulin, glucokinase (a key regulator of glycolytic flux in the β cell), *GLUT2* (glucose transporter that is expressed in pancreatic β-cells), islet amyloid polypeptide (IAPP or amylin), and Kir 6.1 (in insulin secretion through ATP-dependent K+ channel). Hence, a better understanding will arise from the profiling of important transcription factors involved in *in vitro* pancreatic islet differentiation of hBMSCs to functional ILCCs.

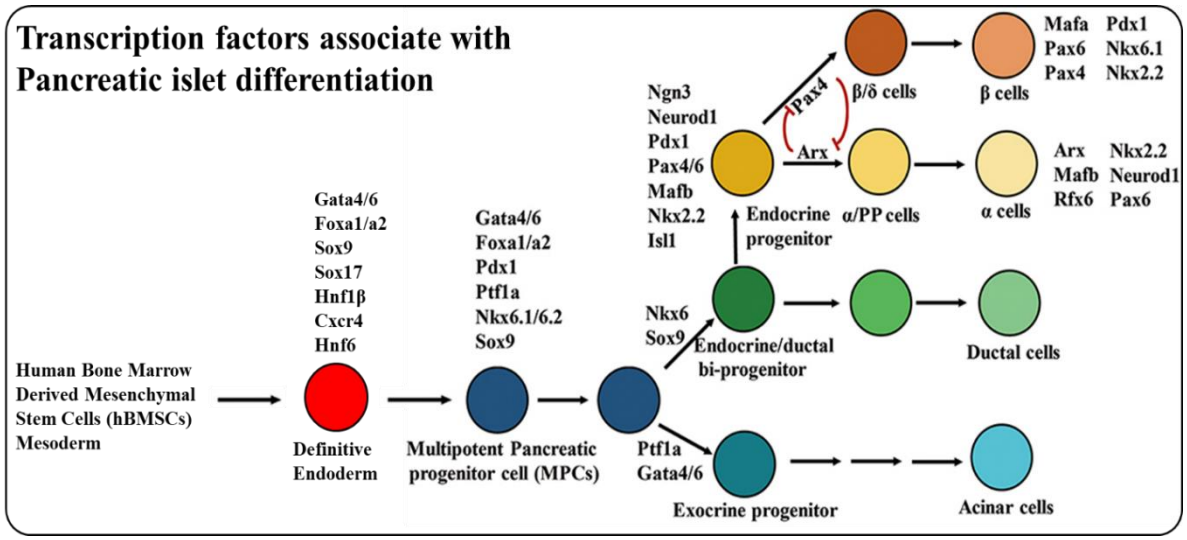


Figure 4. 1: Transcription factors involved in islet differentiation from mesoderm origin stem cell source i.e. hBMSCs. In the representative diagram of islet differentiation, the process of differentiation initiates from mesodermal to definitive endoderm is presented. Further, definitive endoderm cells differentiated into multipotent pancreatic progenitor cells, and this cell eventually differentiated into specializing pancreatic endocrine and exocrine lineage progenitor. This figure is adapted and modified from (Hang and Stein 2011, Jiang and Zhong 2019).

Although bioactive molecules are used in islet differentiation, their exact mechanism has not been elucidated yet (Lee and Jun 2014). One such study by Li *et. al.,* 2010 suggested possible islet differentiation mechanism action of an established differentiating bioactive molecule exendin-4 was to induce important transcription factors i.e. PDX-1 and several intracellular signalling pathways (Movassat *et al.* 2002, Champeris and Jones 2010, Li *et al.* 2010). Based on the results generated so far, in our lab, it is evident that the "swertisin" key differentiating molecule from the "Bioactive molecule cocktail group" facilitate islet differentiation via induction of NGN-3 transcription factor through the TGF-β signalling pathway (Dadheech *et al.* 2015, Srivastava *et al.* 2019).

Considering all the above scientific reports, and our previous chapter's results, we hypothesize that a combination of bioactive molecules might be recapitulating pancreatic development by inducing hBMSCs differentiation *in vitro*. Thus, we decided to investigate the molecular path by screening important transcription factors involved in pancreatic islets development during organogenesis, in the islet differentiation from hBMSCs using the bioactive molecules cocktail (BMC) and activin A. To investigate the unique gene expression and protein expression profile during islet differentiation from hBMSCs the following sub-objective was designed.

(a) Temporal gene expression profile during islet differentiation

The temporal gene expression profile of candidate transcription factors during islet differentiation (0, 5^{th},10^{th},15^{th},18^{th} Day) in BMC and activin A group using qPCR were monitored.

(b) Temporal protein expression profile during islet differentiation

The temporal protein expression profile of crucial transcription factors during islet differentiation in BMC and activin A groups were evaluated using Immunocytochemistry (ICC), Flow cytometry, and Western blotting.

The methodology involved the establishment of the chronological molecular signature of the hBMSCs during islet differentiation is illustrated in the figure:4.1. Further, the experimental design and plan of work is displayed in the figure: 4.2 and figure :4.3 respectively.

4.2. Experimental design of chatper-4

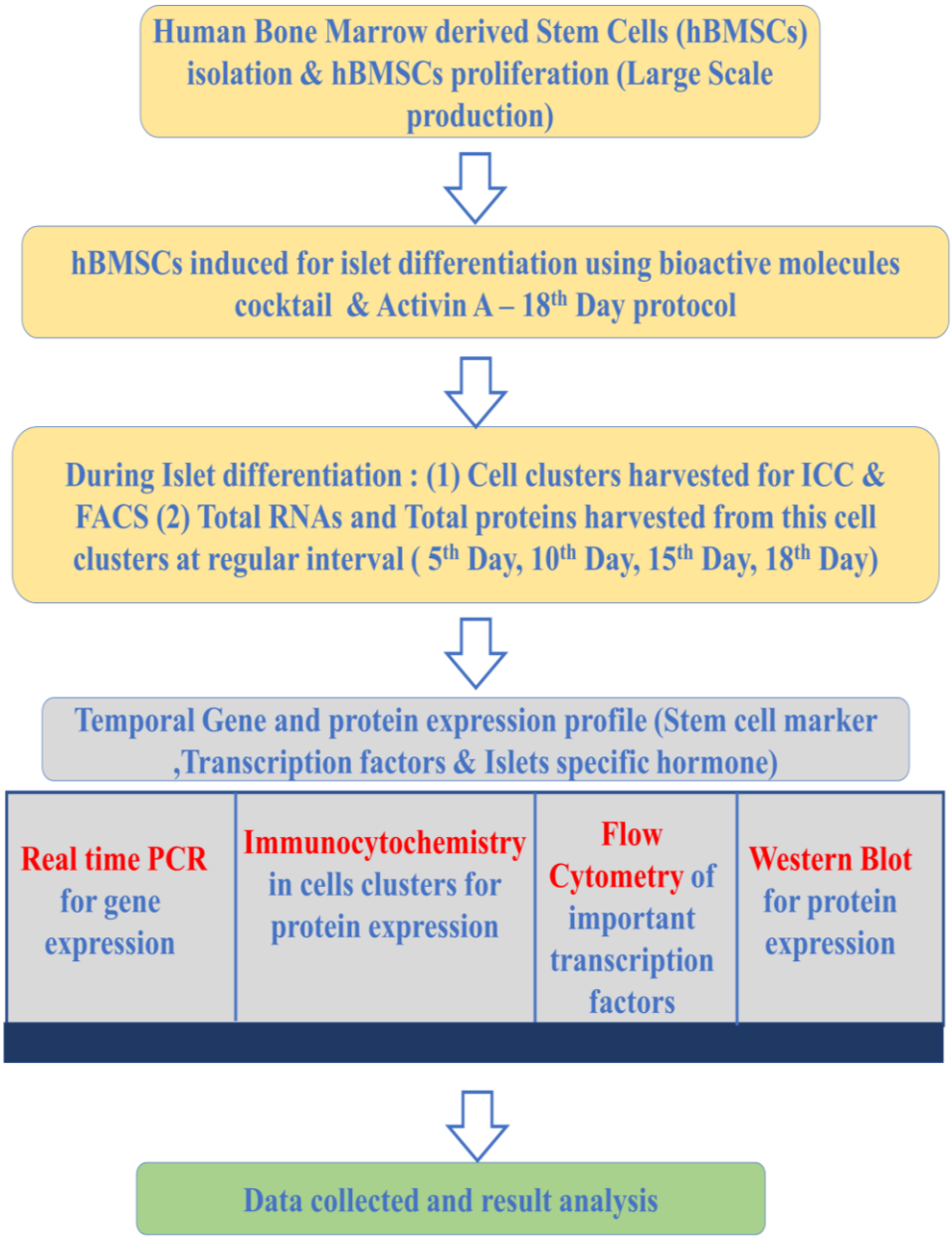


Figure 4. 2: Representative flow chart of experimental design of chapter-4

4.3. Plan of work: Gene and Protein expression profile

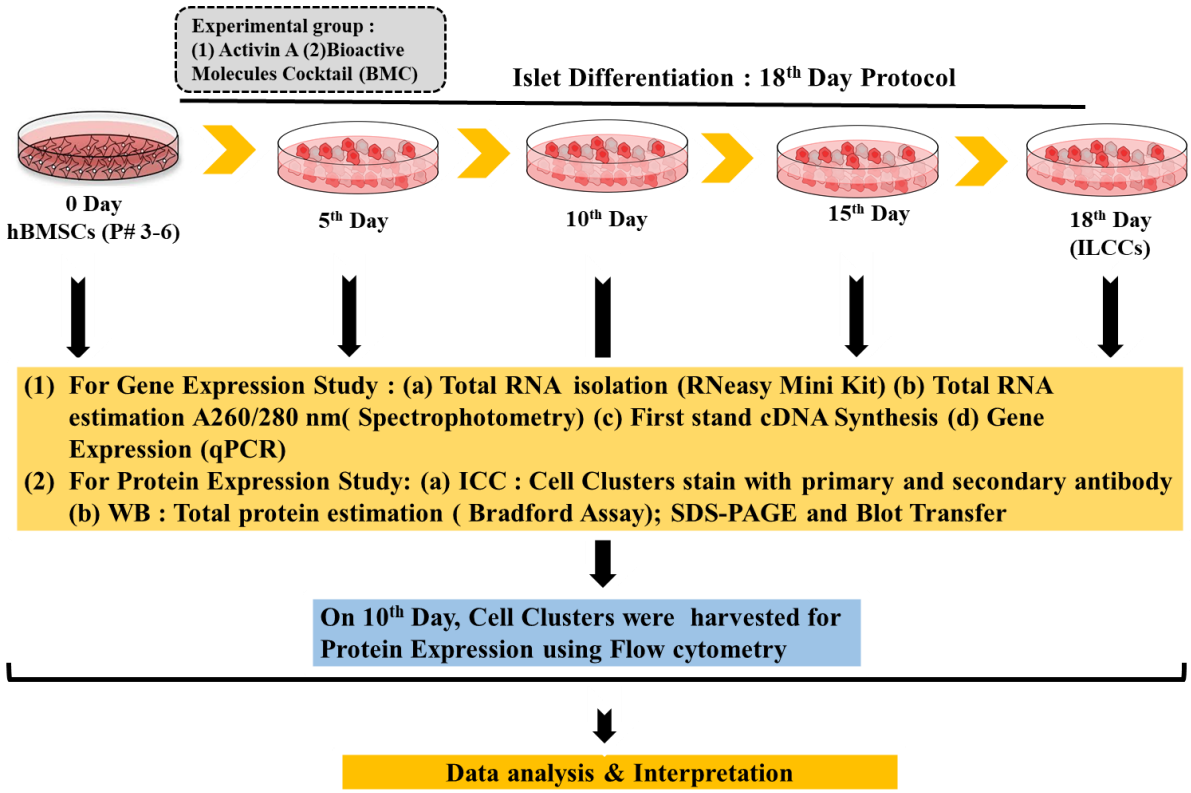


Figure 4. 3: Flow chart of plan of work for chapter-4

4.4. Material & Methods

4.4.1. Materials

All chemicals, media, reagents obtained in this study were purchased from Sigma (USA) and Thermo Scientific (USA). Molecular biology reagents, cDNA synthesis kit, PCR kit were procured from Applied biosystem (ABI), Thermo Scientific, USA. While total RNA isolation kit (RNeasy mini kit) were procured from Qiagen, Germany. All plasticware was purchased from Nunc Inc., (Thermo scientific), USA, and Eppendorf, Germany. The details of all antibodies (Primary and secondary) and PCR primers used in this study are given in appendix).

4.4.2. Islet differentiation from hBMSCs

Characterized hBMSCs were induced for the formation of ILCCs using activin A and BMC. The method for islet differentiation was pursued the same as specified earlier in chapter number: 3 (section: 3.3.2-H).

4.4.3. Gene expression study:

Cell clusters were harvested at different time points (0,5th,10th,15th,18th day of differentiation) during islet differentiation and total RNAs was extracted as per kit manufacturers' instruction (RNeasy total RNA isolation kit, Qiagen, Germany). Further, RNA quantification, cDNA synthesis, and qPCR were followed the same way as mentioned previously in chapter number: 3 (section number: 3.8).

4.4.4. Immunocytochemistry study

Cell clusters were harvested at different time points (0,5th,10th,15th,18th day of differentiation) during islet differentiation by centrifuge at 1000 rpm (Eppendorf, Germany). Cell clusters were kept for adherence to specialized chamber slide (SPL life science co Ltd., Korea) with complete media for 5-6 hrs. Upon attachment, cell clusters were immediately fixed with 4 % PFA solution for 10 min and then stained for various transcription factors/islet markers with a method as explained previously in chapter 3 (section:3.32-D).

[**Note:** -For single cells ILCC study. Cell clusters were incubated with an enzyme (mild trypsinization) for cell dispersion and re-cultured in a specialized chamber slide (SPL life science co Ltd., Korea) for 24-48 hrs., ICC was performed from single cells from cell clusters in the same manner as described above].

4.4.5. Flow cytometry study:

10th-day cell clusters were harvested and incubated with an enzyme to generate single cells from cell clusters and further flow cytometry staining protocol was performed, as described earlier in chapter number 3 (section number:3.3.2-C).

4.4.6. Western blot study:

Cell clusters were harvested during islet differentiation (0,5th,10th,15th,18th day of differentiation) in activin A and BMC group to detect the protein expression of all-important transcription factors. Further, protein extraction and western blotting were performed similarly as previously depicted in chapter number: 3 (section number:3.3.2-E).

4.5. Results

To reveal insight into a possible molecular mechanism of action and the effectiveness of our islet differentiation strategy, we used three different human bone marrow-derived mesenchymal stem cells samples and scrutinized the temporal expression of most important

transcription factors during differentiation (associated with the pancreatic developments). The islet differentiation process has been evaluated in four important steps i.e. formation of (a) definitive endoderm (b) pancreatic progenitor (c) Pancreatic endocrine progenitors and (d) functional pancreatic islets. Above mentioned events were analyzed by gene expression (qPCR) and protein expression (ICC, flow cytometer, western blotting).

4.5.1. Temporal gene expression during islet differentiation using Real-time qPCR

To determine whether the hBMSCs had undergone step-wise pancreatic islet differentiation, gene expression profile for pancreatic islet differentiation markers were assessed at different time points (0 Day, 5 Day,10 Day, 15 Day, and 18 Day) using qPCR (Real-Time PCR). There was no basal expression of any pancreatic development markers in the undifferentiated hBMSCs signifying the role of bioactive molecules cocktail (BMC) in pushing these cells towards islet differentiation.

A. Definitive Endocrine genes:

We examined a panel of definitive endoderm specific transcription factors, such as *GATA-6, GATA-4, SOX17, SOX-9, FOXA2,* and *HNF-1β*. These markers were detected by qPCR in both condition activin A and BMC on 5^{th}, 10^{th}, 15^{th}, and 18^{th} day of differentiation. As shown in the figure:4.4, *GATA6* was detected on 5^{th} day of differentiation and its expression increased gradually till 10^{th} day in both groups. The increasing temporal expression of *GATA6 & GATA4* during the early phase of differentiation indicated the entry of hBMSCs into a definitive endoderm lineage. *GATA4* increased even after 15^{th} day of the differentiation process in both groups. The expression of *HNF1-β* a transcription factor involved in the maintenance of definitive endoderm showed a peak at day 15 in activin A group but on 10^{th} day in the BMC group. The gradual decrease in the *HNF-1 β* expression post 15^{th} day was essential for the induction of *NGN-3* a pancreatic endocrine marker. *FOXA2* gene is also an essential component for marking and maintaining the definitive endoderm formation. It was detected on 5^{th} day with higher expression in the BMC group as compared to activin A group, suggesting quick islet differentiation progression in BMC group. Interestingly, we also observed continuous expression of *SOX17* and *SOX9* genes had throughout the differentiation process in both groups.

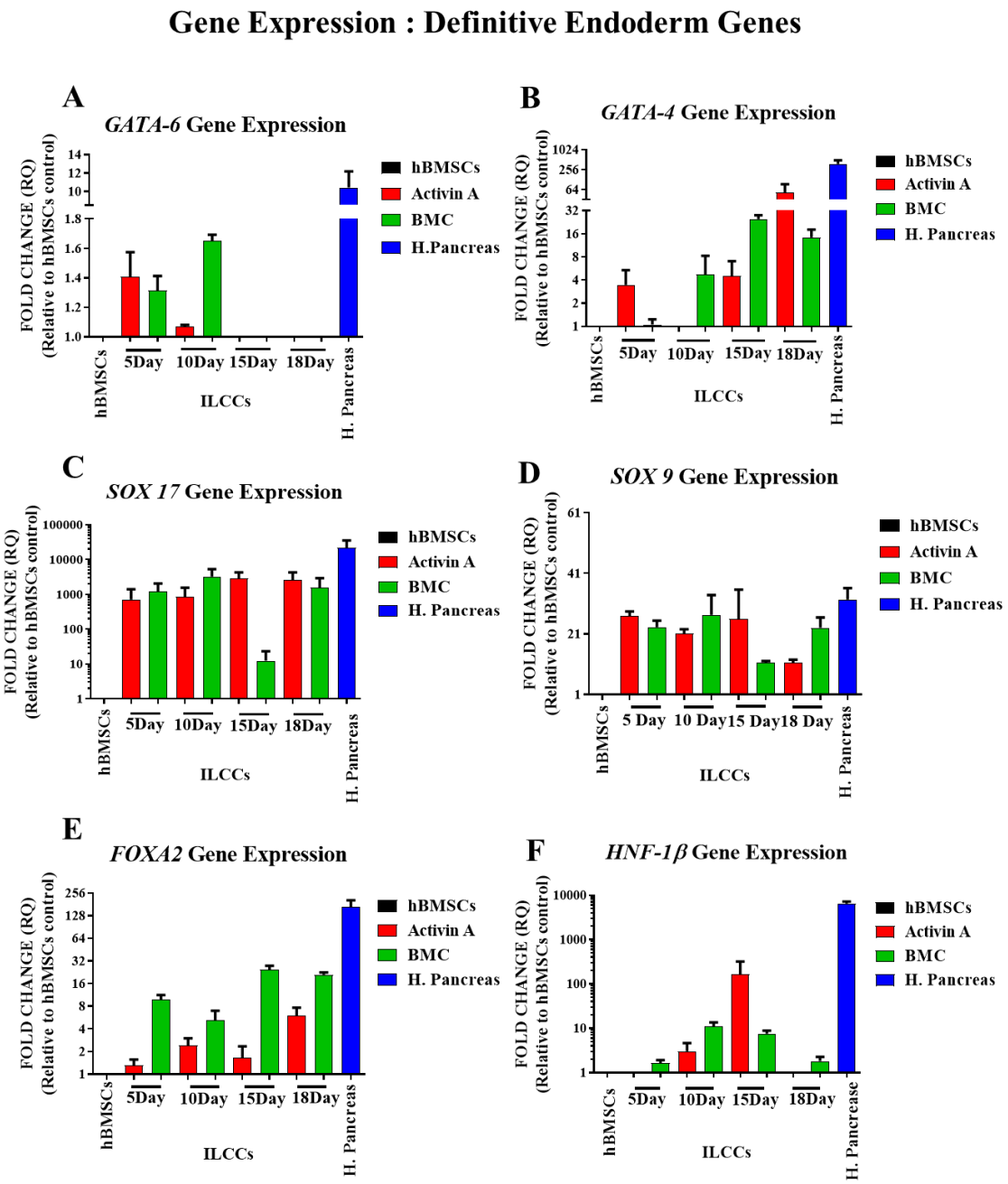


Figure 4. 4: qPCR detection temporal dynamics of gene expression of definitive endoderm marker from hBMSCs derived ILCCs. Cell and clusters were collected at day 0, 5,10, 15, and 18 were analysed by qPCR for *GATA6*(A), *GATA4*(B), *SOX17*(C), *SOX9*(D), *FOXA2*(E), and *HNF-1β*(F) gene expression from both group, such as BMC and activin A. For each test group, relative expression was normalized to hBMSCs (Undifferentiated – 0 Day). The human total pancreas cDNA was used as an experimental positive control. β-actin was utilized as the endogenous control. The transcript abundance value (fold change) is represented graphically as Mean ± SEM (N=3).

B. Pancreatic progenitor genes:

To track the progression from definitive endoderm towards pancreatic progeny, we further analysed a panel of pancreatic progenitor's specific transcription factors, such as *PDX-1*, *NKX6.1*, and *NKX2.2*. RNA profiling demonstrated the presence of all these markers during differentiation in both activin A and BMC group (Figure: 4.5). *PDX-1* gene was detected on 5th day of cell clusters in BMC while on 10th day in activin A group, with a gradual increase showing a peak at day 15, and further decline on day 18. The expression of the *NKX6.1* gene was first noticed on 10th day of cell clusters and was maintained till the end of islet differentiation in both groups, whereas the expression of the *NKX2.2* gene was detected early on 5th day and showed a peak at day 15 in both groups.

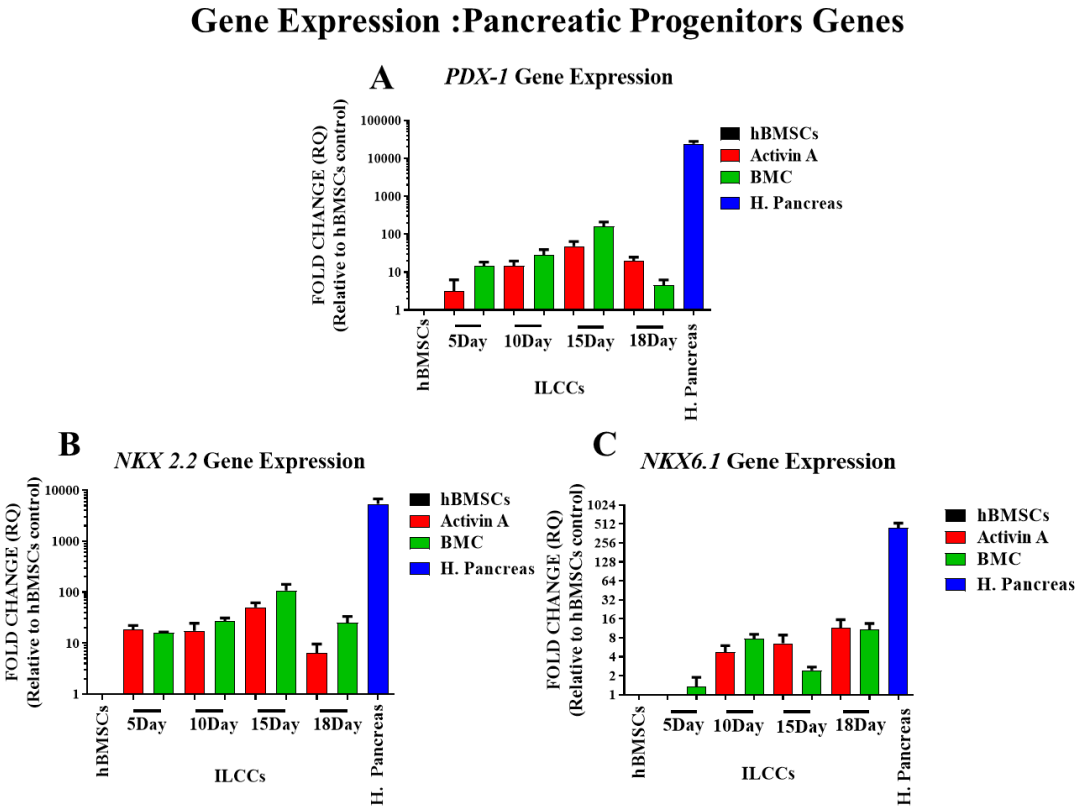
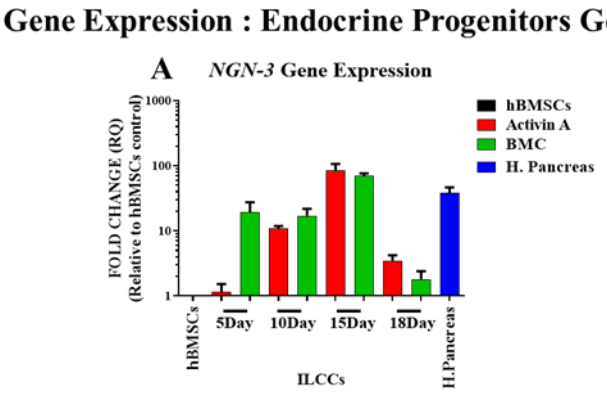


Figure 4. 5: qPCR detection temporal dynamics of gene expression of pancreatic progenitors' marker from hBMSCs derived ILCCs. Cell and clusters were collected at day 0, 5,10, 15, and 18 were analyzed by qPCR for *PDX-1* (A), *NKX2.2* (B) and *NKX1.6* (C) gene expression from both group, such as BMC and activin A. For each test group, relative expression was normalized to hBMSCs. The human total pancreas cDNA was utilized as an experimental positive control. β-actin was used as the endogenous control. The transcript abundance value (fold change) is represented graphically as Mean ± SEM (N=3).

C. Pancreatic Endocrine Progenitors:

Further, in order to map the progression of islet differentiation, we analyzed crucial pancreatic endocrine progenitor markers and pancreatic islet precursor marker, like *NGN-3*, *NEUROD-1*, *RFX-6*, *PAX-6*, *PAX-4*, *MAF-A* and *MAF-B* which were identified by qPCR in both groups (Figure: 4.6). Among all, *NGN-3* is key master endocrine regulators and it was spotted promptly at 5th day of cell clusters in BMC, while on 10th day in activin A group, its expression was boosted on day 15 day, then expectedly decreased at day 18 in both groups. This indicated the accelerated islet differentiation process in the BMC group as compared to activin A. Another important endocrine specific transcription factor *NEUROD1* was detected at 5th day elevated early on 10th day and 15th day in BMC and activin A group respectively, then gradually declined on 18th day in both groups. Similarly, *RFX-6* gene expression was noted on 5th day maintained till day 15 and slightly increased at 18th day in both groups suggesting the sustenance of the functional identity of pancreatic β cells. Surprisingly, *PAX-6* gene expression was observed only day 15 and moderately reduced on 18th day in both groups. Further, *PAX-4* gene expression was noticed at 5th day and slightly increased at day 15 and day 18 in BMC and activin A group respectively, indicating the functional integrity of pancreatic α- cells. Furthermore, we confirmed pancreatic islet-specific β and α cells marker by analyzing gene expression of *MAF-A* and *MAF-B* respectively. *MAF-A* gene expression was observed on 5th day and gradually upregulated with an increasing day of differentiation whereas, *MAF-B* gene expression was more elevated in BMC as compared to activin A group in each time point of islet differentiation.



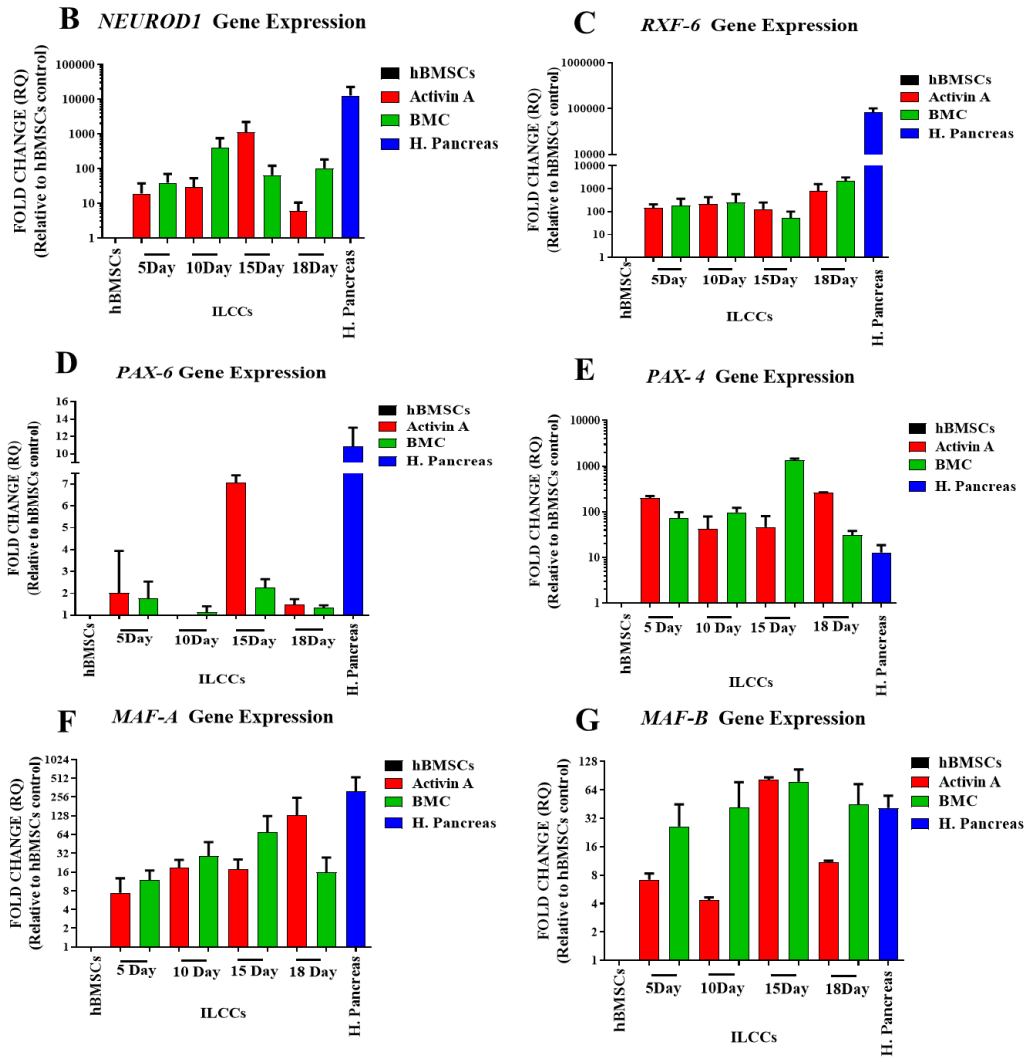


Figure 4. 6: qPCR detection temporal dynamics of gene expression of endocrine progenitors' marker from hBMSCs derived ILCCs. Cell and clusters were collected at day 0, 5,10, 15, and 18 were analysed by qPCR for *NGN-3*(A), *NEUROD1*(B), *RFX-6*(C), *PAX-6*(D), *PAX-4*(E), *MAF-A*(F) and *MAF-B*(G) gene expression from both group, such as BMC and activin A. For each test group, relative expression was normalized to hBMSCs. The human total pancreas cDNA was used as an experimental positive control. β -actin was utilized as the endogenous control. The transcript abundance value (fold change) is represented graphically as Mean ± SEM (N=3).

D. Functional pancreatic islets genes

Lastly, we studied a panel of mature pancreatic islet-specific symbolic genes, like *INS, GCG, SST, PPY, PSCK1, and GLUT2*. Insulin & glucagon hormone transcript, an ultimate marker of terminal differentiated pancreatic islets were early identified at day 10 and considerably enhanced at the late phase of the islet differentiation protocol (Day 18) in both groups. Moreover, somatostatin *(SST)* and pancreatic polypeptide *(PPY)* gene expression were detected

at a very low level on day 5 in BMC and gradually upregulated with the increasing days of islet differentiation in both groups, which indicated modest amount of δ cell and PP cell population in ILCCs. Surprisingly, *PCSK1* gene expression was detected on 5th day and progressively increased during islet differentiation in both groups. Finally, Glucose transporter *GLUT-2* gene expression was noticed on 10th day in both groups while in BMC, we observed a sudden increase on 15th day and moderate expression on 18th day in both groups (Figure: 4.7).

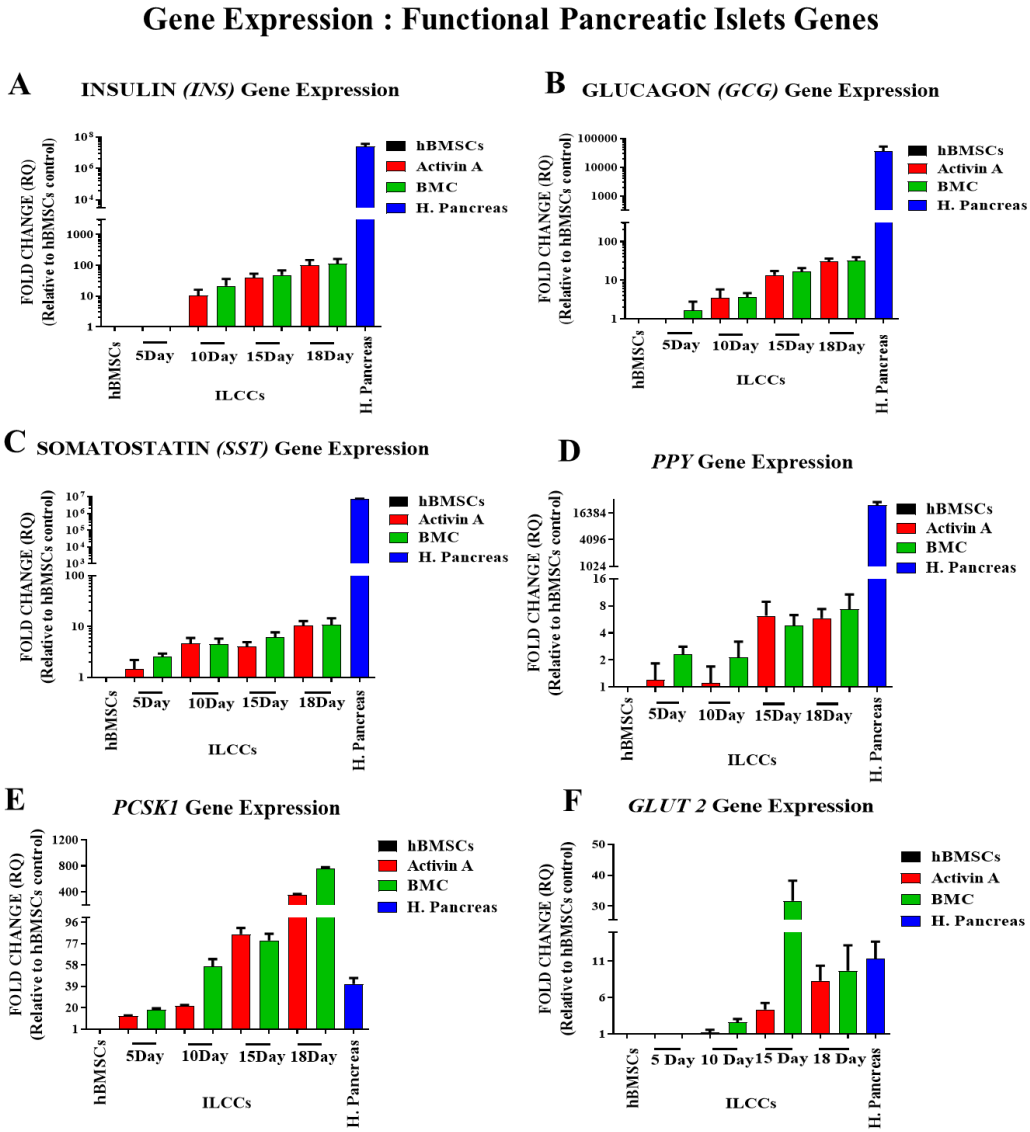


Figure 4. 7: qPCR detection temporal dynamics of gene expression of pancreatic islets specific marker from hBMSCs derived ILCCs. Cell and clusters were collected at day 0, 5,10, 15, and 18 were analyzed by qPCR for *INS*(A), *GCG*(B), *SST*(C), *PPY*(D), *PCSK1*(E), and *GLUT2*(F) gene expression from both group, such as BMC and activin A. For each test group, relative expression was normalized to hBMSCs. The human total pancreas cDNA was used as an experimental

positive control. β -actin was utilized as the endogenous control. The transcript abundance value (fold change) is represented graphically as Mean ± SEM (N=3).

The human pancreas tissue was used as a positive control to validate our data. Gene expression analysis in hBMSCs derived ILCCs was similar to that in human pancreas tissue and confirmed the differentiation of hBMSCs into ILCCs *in vitro* upon treatment with activin A and BMC condition. A preliminary observation of real-time PCR for many important genes like insulin, glucagon, somatostatin, *PPY* expression was lower at < 1 % of that in the human pancreas tissue (positive control). More importantly, the expression of functional pancreatic islets hormone genes such as *INS, GCG, SST, PPY,* and *PSCK1* was extremely enhanced at 18th Day ILCCs. Hence, differentiated cell clusters were termed as "Islet-like cell clusters (ILCCs)" rather than "insulin-producing cell clusters (IPCs)". Overall, qPCR results suggested the dynamic expression of crucial transcription factors genes. It permitted better recognition of the diverse and consecutive pancreatic developmental phases that could be effectively recapitulated by steps-wise islets differentiation protocol. The islet differentiation was further confirmed by immunocytochemistry analysis using confocal microscopy.

4.5.2. Temporal protein expression during islet differentiation using Immunocytochemistry (ICC) assay:

To determine whether the hBMSCs had undergone step-wise pancreatic islet differentiation, protein expression profile for pancreatic islet differentiation markers and pancreatic islet hormones were assessed at different time points (5 Day,10 Day, 15 Day, and 18 Day) by immunocytochemistry.

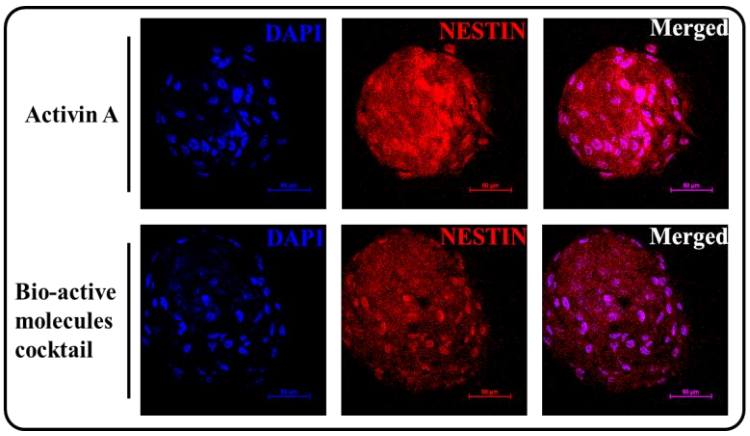


Figure 4. 8: Immunofluorescent images (confocal microscopy) of nestin protein expression in 5th day cell clusters derived from hBMSCs using activin A and BMC. Nestin is a stem cell marker

and Nestin positive stem cells are cardinal for initiating the islet differentiation process. DAPI was used as nuclear counterstain; Red and blue color represent PE and DAPI respectively. [Magnification 60 X; scale bar: 50 µM]

A. 5th Day Cell Clusters:

To confirm pancreatic differentiation of hBMSCs into pancreatic endocrine progenitors, immunostaining of Nestin, REG-1, FOXA2, PDX-1, NGN-3, NKX6.1, and GLUT-2 was performed for 5th day cell cluster. The nestin-positive stem cell is a well-defined population of cells that contribute in the neogenesis of pancreatic endocrine cells**.** Hence, first of all, we focused on nestin expression in cell clusters. The results demonstrated that after the first-stage induction (5th day), nestin protein was slightly higher in activin A groups as compared to the BMC (Figure: 4.8).

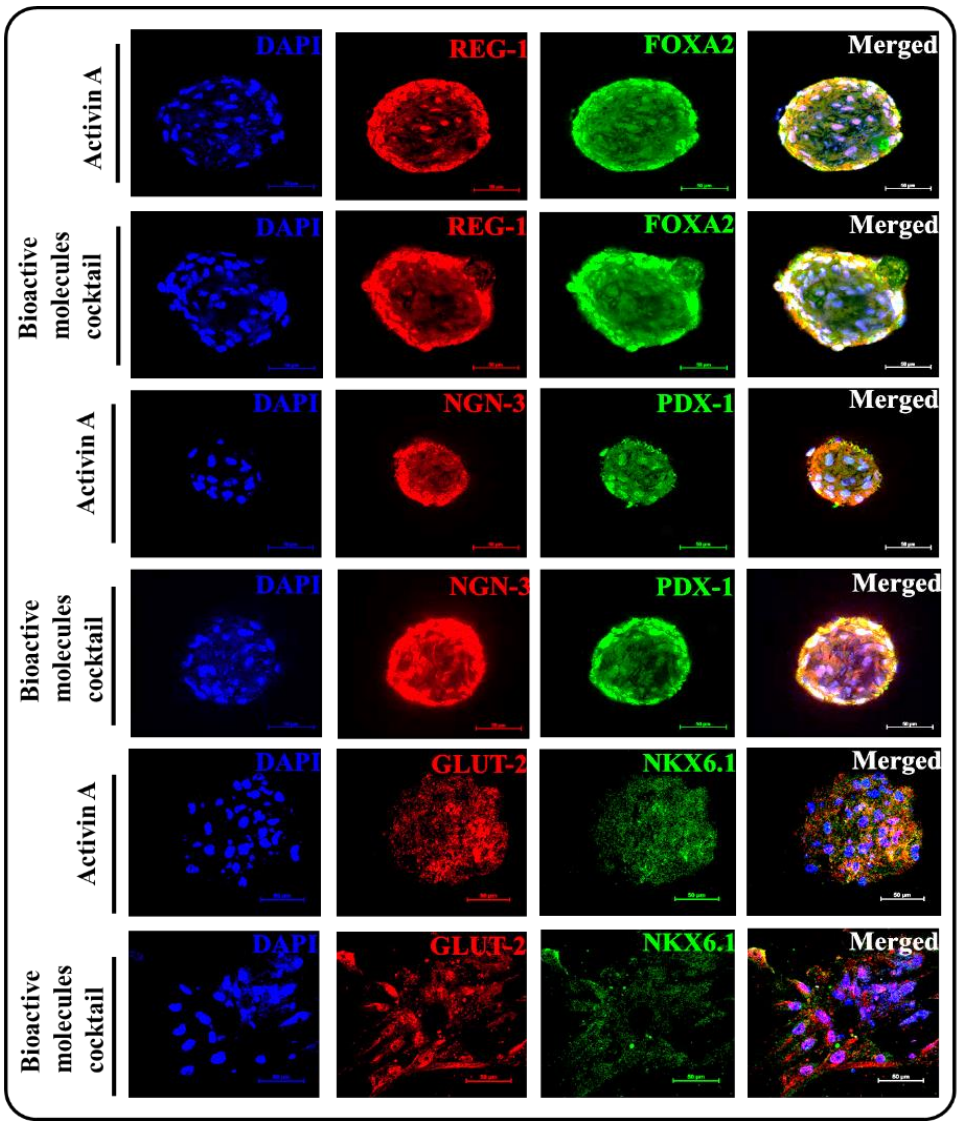


Figure 4. 9: Comparative confocal immunofluorescence expression of definite endoderm markers such as FOXA2(Green), REG-1(Red) and pancreatic progenitor as well as pancreatic endocrine markers such as PDX-1 (Green), NKX6.1(Green), NGN-3(Red), GLUT-2(Red) protein expression in 5th Day cell clusters derived from hBMSCs using activin A and BMC. Definitive endoderm transcription factors FOXA2 and REG-1 showed higher expression as compared to mature pancreatic islet marker such as GLUT2 in 5th Day (First stage) of cell clusters. More interestingly, NGN-3 protein strongly expressed in a very short period of induction (5th Day) in the BMC group as compare to activin A group. DAPI was used as nuclear counterstain (blue); REG-1 (AF555), FOXA2 (FITC), PDX-1 (FITC), NGN-3 (AF555), NKX6.1 (FITC) and GLUT-2(AF555). Red and Green represent AF555 and FITC conjugates, respectively [Magnification 60 X; scale bar: 50 µM].

Next, definitive endoderm markers REG-1 and FOXA2 protein were immensely expressed in 5th day cell clusters in both groups, suggesting hBMSCs' entry into definitive endoderm differentiation.

Very interestingly, early pancreatic progenitor marker and endocrine progenitor markers such as PDX-1, and NGN-3 (respectively) protein were strongly expressed in BMC as compared to Activin A group, which was indicating accelerated islet differentiation process in BMC group further supporting gene expression data. As expected, a mature endocrine marker such as NKX6.1 and GLUT-2 protein was weakly expressed at 5th day cell clusters in both groups (Figure: 4.9).

B. 10th Day Cell Clusters:

Our previous gene expression results clearly indicated that 10th day time point was very crucial in the entire islet differentiation process. Hence, we focused on day 10 and corroborated the status of definitive endoderm (FOXA2), pancreatic progenitors (PDX-1, NKX6.1, NKX2-2), pancreatic endocrine progenitors (NGN-3, NEUROD-1, ARX, PAX-4), and mature pancreatic islet (c-peptide and Glucagon) markers by immunostaining to that of gene expression. Firstly, nestin and FOXA2 proteins were co-localized with stable expression of FOXA-2 in both groups. The double immunostaining of PDX-1 and NGN-3 demonstrated intense high expression of NGN-3 whereas, a moderate level of PDX-1 protein expression in both groups, which suggested that most of the cells from clusters progress towards endocrine progenitors. Also, we noticed that cell clusters in both groups were strongly positive for NEUROD-1, which

is the second important transcription factor (after NGN-3) in the endocrine progenitor marker (Figure 4.10).

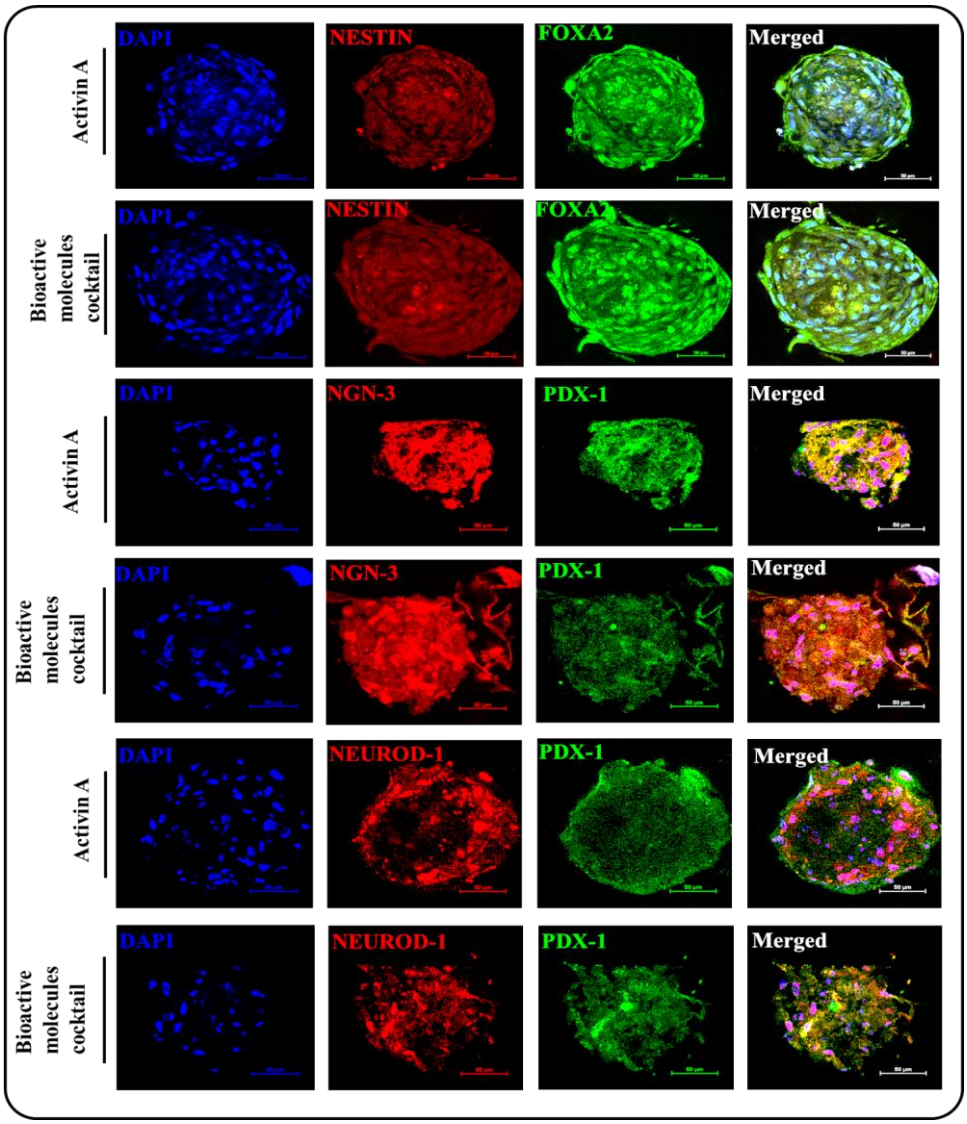


Figure 4. 10: Comparative confocal immunofluorescence expression of definite endoderm markers such as FOXA2(Green) and pancreatic progenitor as well as pancreatic endocrine markers such as PDX-1 (Green), NGN-3(Red), NEUROD-1(Red) protein expression in 10th day cell clusters (second stage) derived from hBMSCs using activin A and BMC. Definitive endoderm transcription factors FOXA2 and early pancreatic and endocrine progenitor showed higher expression as compared to stem cell markers such as nestin in 10th day (Second stage) of cell clusters. More surprisingly, FOXA2 protein strongly expressed in the second stage of induction (10th Day) also in activin A and BMC group. As expected, NGN-3 protein prominent expressed in the second stage of induction (10th Day) in both groups. DAPI was used as nuclear counterstain (blue); Nestin (Red), FOXA2 (FITC), PDX-1 (FITC), NGN-3 (AF555), and NEUROD-1 (AF555).

Red and Green represent AF555 and FITC conjugates, respectively [Magnification 60 X; scale bar: 50 µM].

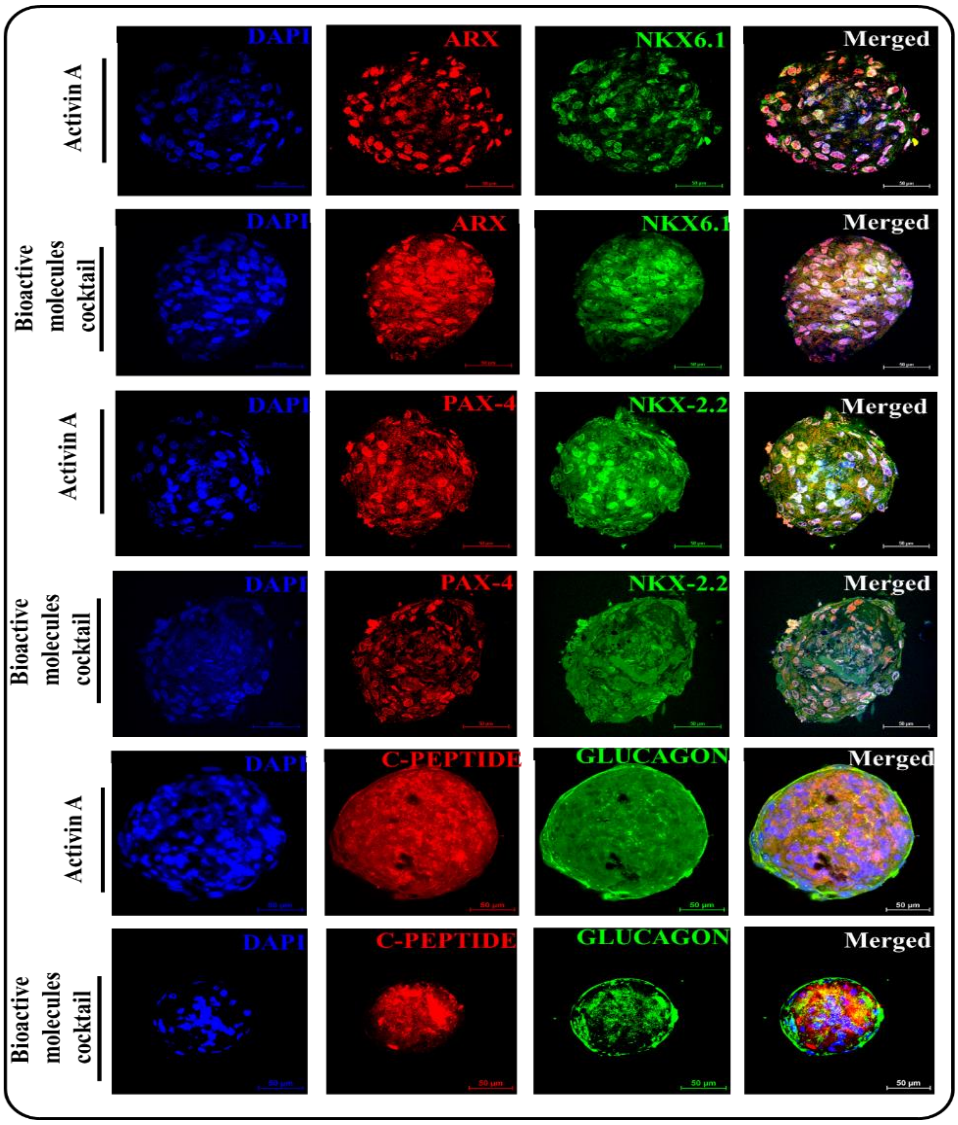


Figure 4. 11: Comparative confocal immunofluorescence expression of mature pancreatic progenitor markers such as NKX6.1(Green), NKX2.2 (Green) and mature pancreatic endocrine progenitor markers such as ARX (Red), PAX-4 (Red), NEUROD-1(Red) and terminal differentiated pancreatic islet hormones such as c-peptide and glucagon protein expression in 10th day cell clusters (Second stage) derived from hBMSCs using activin A and BMC. Expression of ARX and PAX-4, which are two crucial mature endocrine progenitor transcription factors, induce the differentiation of islet precursor cells into β/δ and α/PP cells. we obtained human c-peptide and glucagon co-expressing (poly-hormonal) cell clusters at the end of second stage induction (10th Day) in both groups, which suggests that immature cell clusters. DAPI was used as a nuclear counterstain (blue); ARX (AF555), NKX6.1 (FITC), PAX-4 (AF555), NKX2.2

(FITC), C-peptide (TRITC) and Glucagon (FITC). Red and Green represent AF555/TRITC and FITC conjugates, respectively [Magnification 60X; scale bar: 50 µM].

Moreover, we observed that cell clusters in the BMC group were highly positive for ARX (α-cell progenitor marker) and NKX6.1 (β-cell progenitor marker) protein as compared to activin A. We further examined these cell clusters for the presence of mature endocrine markers such as PAX-6 and NKX2.2. PAX-4 and NKX2.2 protein which was high in activin A group as compared to BMC groups. Finally, we investigated cell clusters for the presence of c-peptide and glucagon. We evidenced a perfect presence of c-peptide and glucagon in cytoplasmic granules of activin A and BMC, which was suggesting that most of the cells (from cell clusters) were at poly-hormonal and immature stages (Figure: 4.11). These results specified that differentiated cell clusters synthesized insulin de novo as indicated by the presence of c-peptide-protein expression.

C. 15th Day Cell Clusters:

After evaluation on 10th day of differentiation, we evaluated the status of definitive endoderm markers (REG-1, FOXA2), pancreatic and endocrine progenitor markers (PDX-1, NGN-3, NEUROD-1, and GLUT2) at 15th day of cell clusters in both groups.

Cell clusters were immune-negative for the expression of nestin on 15th day in both groups. Additionally, we also observed very less expression of FOXA2 and REG-1 protein, which suggested the islet differentiation process passed through the definitive endoderm marker stage and entered into the terminal phase (Figure: 4.12). Further, we recognized moderate expression of PDX-1 and prominent expression of NGN-3 and NEUROD-1, endocrine progenitor markers in both groups, suggesting the cell clusters are at pancreatic endoderm progenitor stages (Figure:4.13). Lastly, we carried out immunostaining experiments to evaluate the status of early pancreatic progenitor as well as early and late endocrine progenitor marker (PDX-1, NKX6.1, NGN-3, MAF-A) alone with a pancreatic mature marker (GLUT-2, Glucagon).

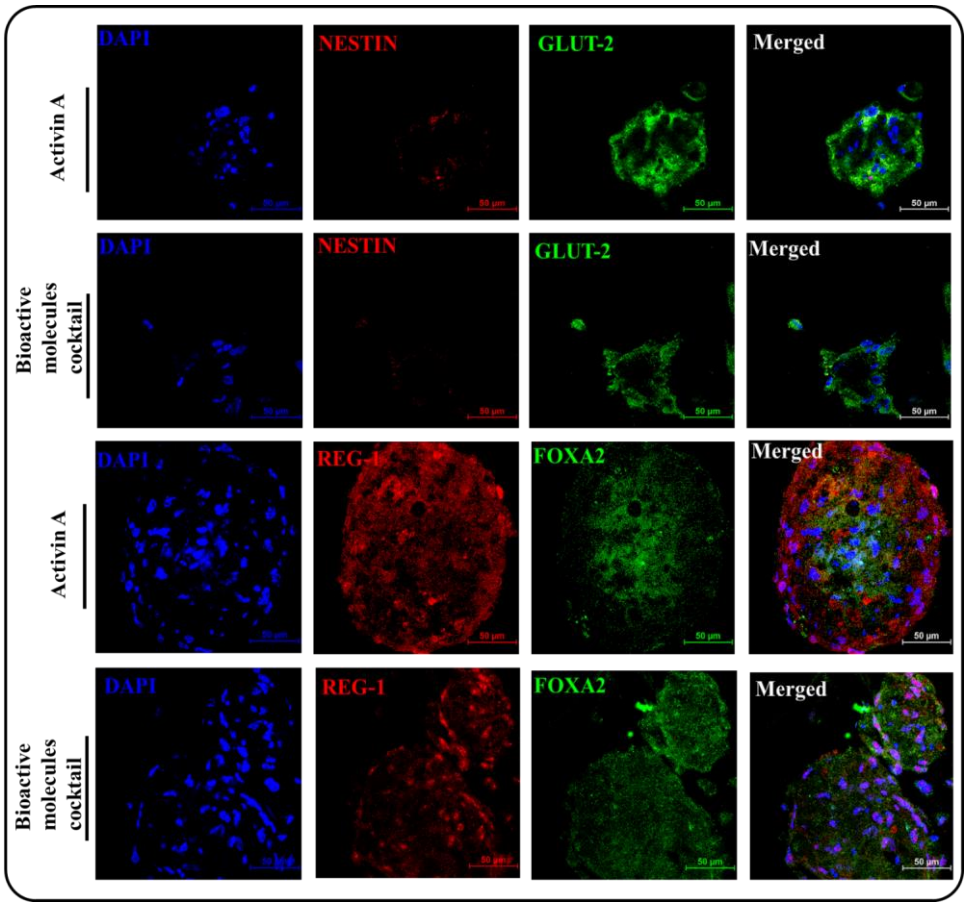


Figure 4. 12: Comparative confocal immunofluorescence expression of definitive endoderm marker like FOXA2 (Green), REG-1(Red) and stem cell marker nestin (Red) along with mature pancreatic islet markers such as GLUT-2 (Green) protein expression in 15th day cell clusters (Third stage) derived from hBMSCs using activin A and BMC. Co-expression of nestin and GLUT-2 certainly displayed a reduced expression of nestin and drastically upregulated expression of GLUT-2, indicating a positive sign of the islet differentiation process. Definitive endoderm transcription factors REG-1 showed higher expression in activin A group as compare to BMC group, whereas no difference was observed in FOXA2 protein expression between both groups. DAPI was used as a nuclear counterstain (blue); Nestin (PE), GLUT-2(FITC), REG-1(TRITC), and FOXA2(FITC). Red and green color represents PE/TRITC and FITC conjugates, respectively [Magnification 60 X; scale bar: 50 µM].

D. 18th Day Cell Cluster:

As expected, NGN-3 and PDX-1 were moderately expressed on 18thday of ILCCs in both groups. Additionally, GLUT-2, and NKX6.1 protein, which is a marker for glucose membrane transporter and terminally differentiated insulin-producing β- cells respectively, was strongly positive at 18th day ILCCs in both groups. The activin, A group showed a moderately higher

expression of GLUT-2 protein as compared to the BMC group. The protein expression of GLUT-2 suggested that the acquired ILCCs signify a definitive step in the direction of committed pancreatic β-cells, response to a glucose challenge. Moreover, a co-expression study revealed that mature pancreatic β cell progenitors, MAF-A, and mature pancreatic α- cells marker, glucagon, were separately expressed in almost all at 18thday ILCCs in both groups suggesting more efficient differentiation and matured ILCCs (Figure:4.14). Thus, our novel differentiation protocol generated a highly pure population of pancreatic progenitors & endocrine progenitors cells expressing the PDX-1, NGN-3, NKX6.1, and MAF-A markers by 18th day of differentiation. Ultimately, all the transcription factors viz. REG-1, FOXA2, PDX-1, NGN-3, NEUROD-1, NKX6.1, NKX2.2, ARX, PAX-4, and MAF-A, which are necessary for endocrine pancreatic development and its function, were temporally up-regulated in the course of islet differentiation.

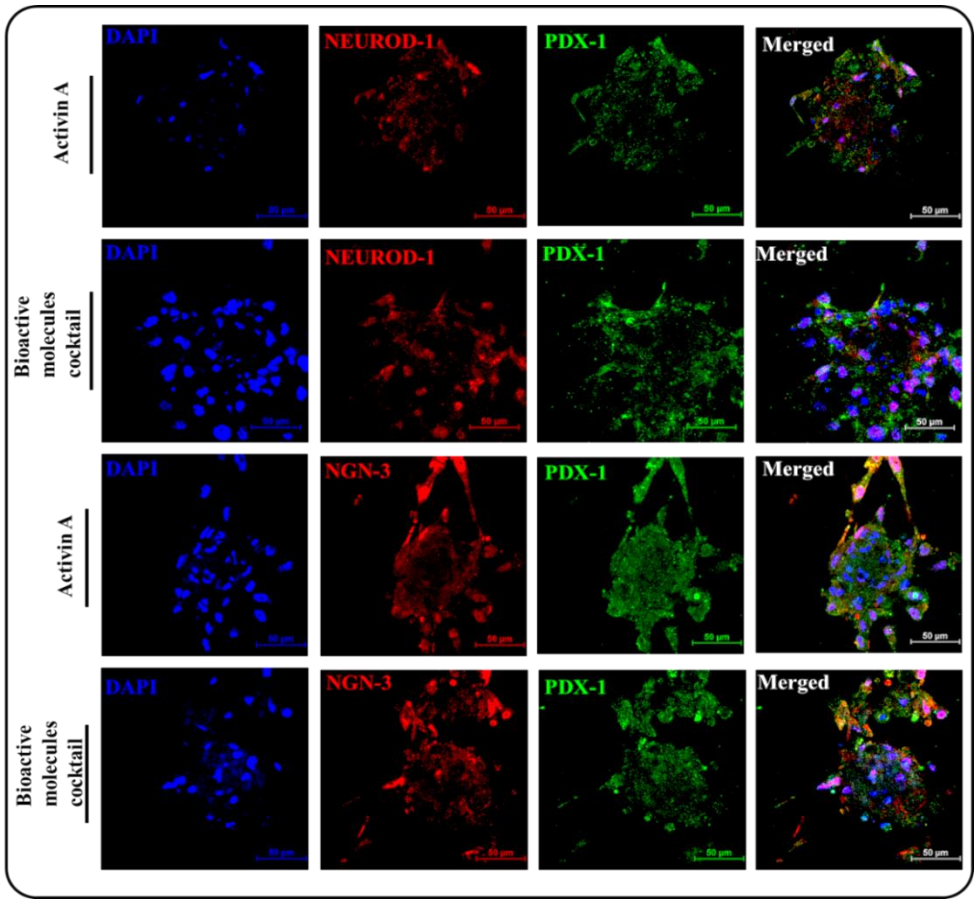


Figure 4. 13: Comparative confocal immunofluorescence expression of centrally important pancreatic progenitor markers like PDX-1(Green) and endocrine progenitor-like NGN-3(Red), NEUROD1(Green) protein expression in 15th day cell clusters (Third stage) derived from hBMSCs using activin A and BMC. As expected, co-expression of NEUROD-1 and PDX-1 showed

moderate expression while NGN-3 expression demonstrated above baseline expression, which suggests that the islet differentiation process towards the terminal phase (Islet maturation process). DAPI was used as a nuclear counterstain (blue); NEUROD-1 (TRITC), PDX-1 (FITC), and NGN-3(TRITC). Red and green color represent TRITC and FITC conjugates, respectively [Magnification 60X; scale bar: 50 µM].

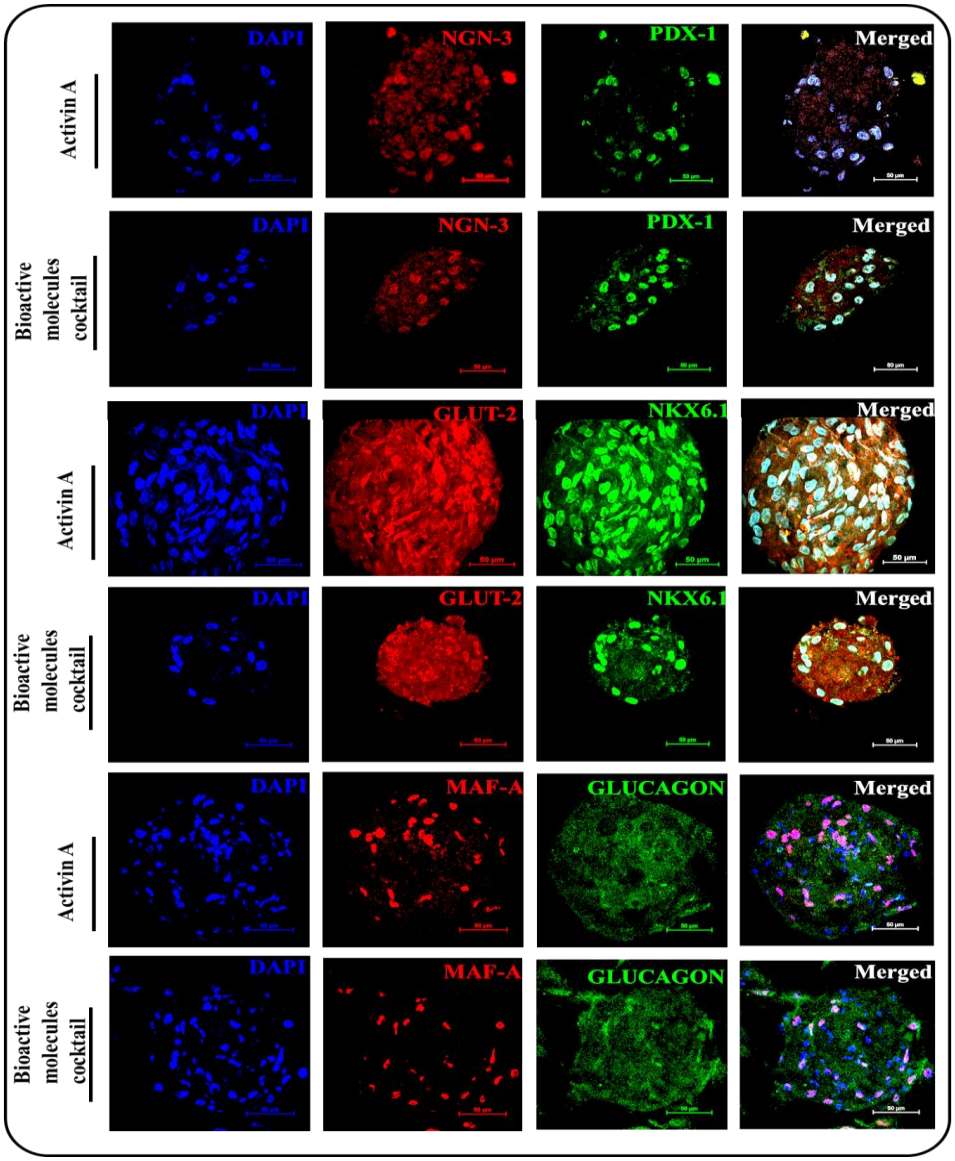


Figure 4. 14: Comparative confocal immunofluorescence expression of pancreatic and endocrine progenitor such as PDX-1(Green), NGN-3(Red) as well as terminal pancreatic differentiated markers such as NKX6.1 (Green), MAF-A(Red), GLUT-2 (Red), Glucagon (Green) protein expression in 18th day ILCCs (Four stages) derived from hBMSCs using activin A and BMC. Early pancreatic and endocrine progenitor marker PDX-1, NGN-3 showed intermediate expression at the final stage of differentiation, which necessary for maintaining pancreatic and endocrine progenitor pool within pancreatic islets. Further, β-cell maintenance progenitor

marker like NKX6.1 and MAF-A protein strongly expressed and pancreatic α- cell marker glucagon protein moderate expressed at the end of the four-stage of induction (18th Day) in activin A and BMC. Interestingly, we noticed that still, small population cells from clusters showed poly-hormonal stage based on co-expression of MAF-A and glucagon protein. DAPI was used as a nuclear counterstain (blue); NGN-3 (TRITC), PDX-1 (FITC), NKX6.1 (FITC), GLUT-2 (TRITC), MAF-A (AF555) and Glucagon (FITC). Red and green represent TRITC/ AF555 and FITC conjugates, respectively [Magnification 60X; scale bar: 50 µM].

4.5.3. Immunocytochemistry assay confirms protein expression in single cells (Isolated from cell clusters):

Due to the 3-dimensional nature of the islet differentiation planer culture, the true extent of transcription factors expression is difficult to glean from 3-D cell cluster's images with immunostaining. Hence, we performed an additional experiment related to immunocytochemistry in which, single-cell analyzed for essential transcription factors and pancreatic hormones expression in ILCCs treated with bioactive molecules cocktail. The single cells from the digestion of cell clusters/ILCCs following the multi-stage induction adhered during 24-48 hours of subculture and then were fixed and immuno-stained for c-peptide, glucagon, GLUT-2 and crucial pancreatic islet developmental marker such as definitive endoderm, pancreatic progenitor, endocrine progenitors. Differentiated cells derived from hBMSCs exhibited abundant expression of FOXA2 and REG-1 in the nucleus. More surprisingly, the expression of FOXA2 protein was also detected in cytoplasm in few differentiated cells. Additionally, REG-1 protein-expressing differentiated cells were magnified in order to identify perfect localization in cells (Figure: 4.15). Further, we observed PDX-1 and NGN-3 protein expression in the nucleus. Interestingly, partial expression of PDX-1 protein was also identified in the cytoplasm in minor differentiated cells, which indicated that PDX-1 protein has inactive form in cytoplasm and translocate into the nucleus when needed. Transcription factors NGN-3 was abundantly present in the nucleus of differentiated cells. Moreover, we demonstrated NEUROD1 protein accumulate in the nucleus with support of the magnified image of the cell's nucleus (Figure: 4.16). Next, we identified important transcription factors expression such as ARX, PAX-4, NKX6.1, and MAFA predominantly in the nucleus (Figure: 4.17). These observations strongly suggested BMC induced all-important "Transcription factors" expression in differentiated cells with perfect localization which has not been reported until recently.

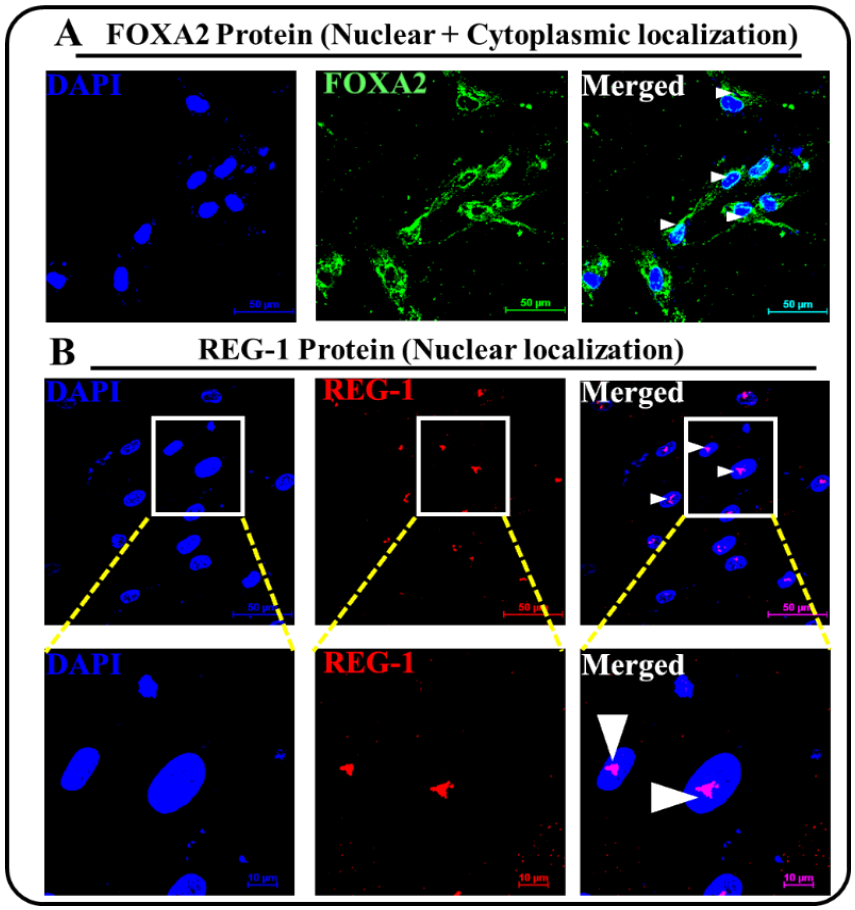


Figure 4. 15: Immunocytochemistry assay (confocal microscopy) of definitive endoderm transcription factors expression in single cells from ILCCs derived from hBMSCs using BMC. (A) the single cells expressed definitive endoderm marker transcription factors FOXA2 (Nuclear as well cytoskeletal/ cytoplasmic localization in cells), (B) another equally important endoderm marker transcription factors REG-1 (Exclusively nuclear localization), also magnified images shows in lower penal ("White Arrow" for localization). DAPI was used as nuclear counterstain; Green and red represent FITC and TRITC respectively. [Magnification 60 X; scale bar: 50 µM in all images while in the magnified view of REG-1 show scale bar: 10 µm].

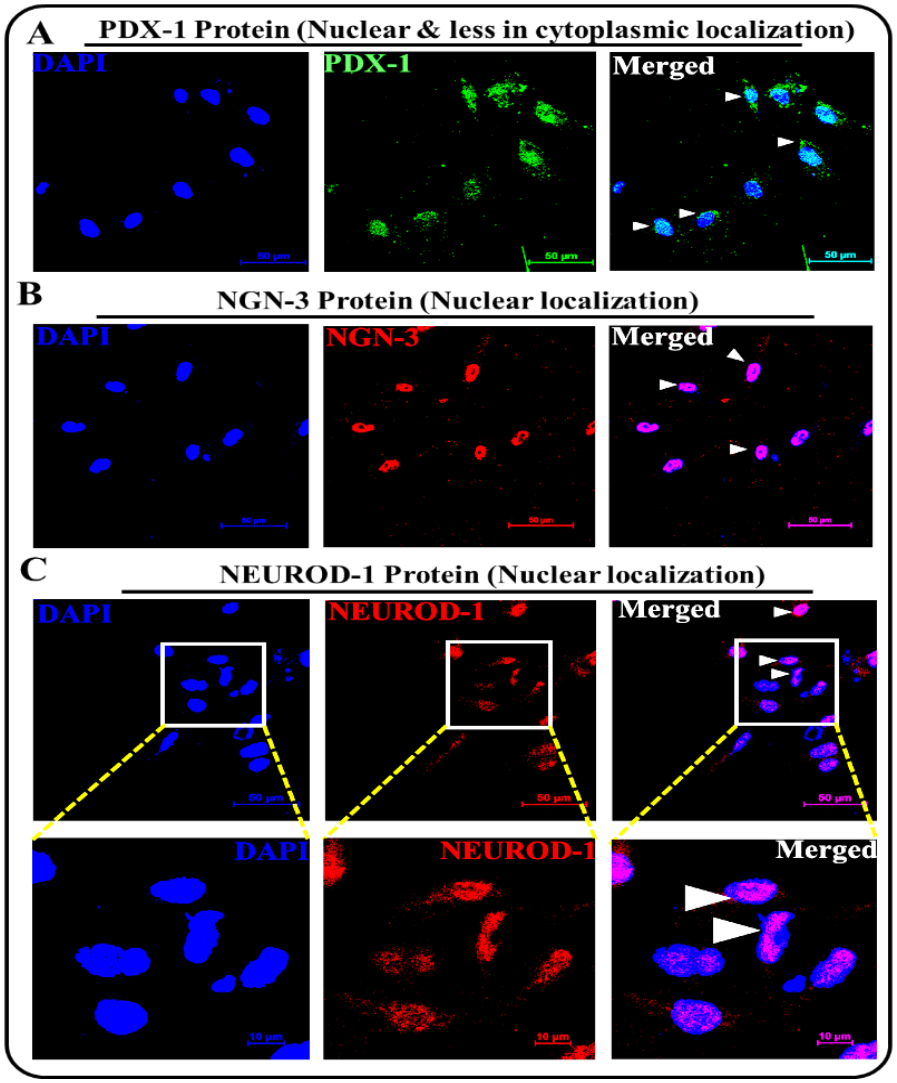


Figure 4. 16: Immunocytochemistry assay (confocal microscopy) of transcription factors expression in single cells from ILCCs derived from hBMSCs using BMC. (A) the single cells expressed important pancreatic progenitor marker transcription factor PDX-1 (Nuclear as well cytoskeletal/ cytoplasmic localization in cells), and (B) master regulator of endocrine progenitor marker transcription factor NGN-3 (Exclusively nuclear localization), (C) another second crucial endocrine progenitor marker transcription factor NEUROD1 (nuclear localization), also magnified images shows in lower penal ("White Arrow" use for localization). DAPI was used as nuclear counterstain; Green and red represent FITC and TRITC respectively. [Magnification 60 X; scale bar: 50 µM in all images while in the magnified view of REG-1 show scale bar: 10 µm].

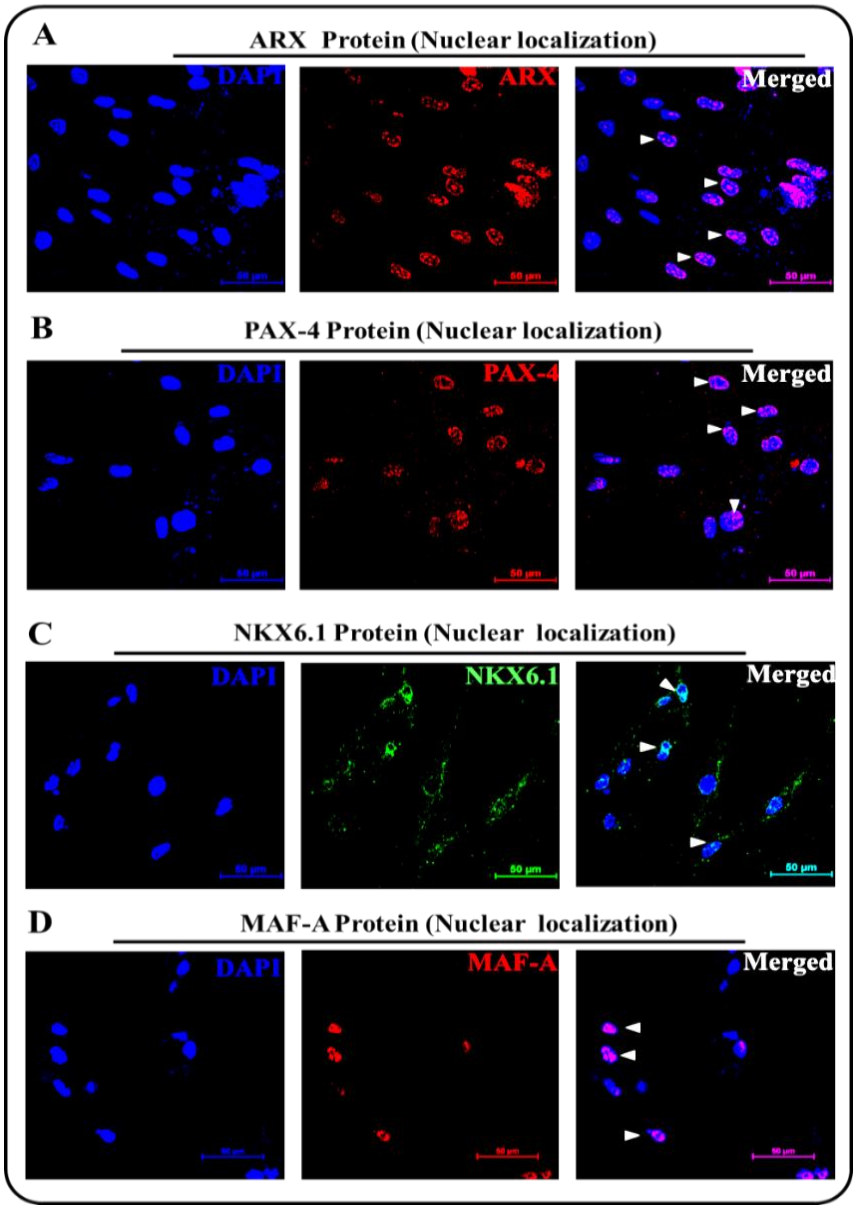


Figure 4. 17: Immunocytochemistry assay (confocal microscopy) of transcription factors expression in single cells from ILCCs derived from hBMSCs using BMC. The single cells expressed in nuclear localization ("White Arrow" use for cellular localization) of important α cell-specific pancreatic progenitor marker transcription factor ARX (A), and β cell-specific pancreatic marker transcription factors PAX-4 (B), NKX6.1 (C), MAF-A (D). DAPI was used as nuclear counterstain; Green and red represent FITC and TRITC respectively. [Magnification 60 X; scale bar: 50 µM].

We also noted GLUT2 protein expression in the cell membrane. With immunocytochemistry, the presence of insulin-containing granules within the cytoplasm of the c-peptide positive cells was seen. Similarly, we detected glucagon expression in the cytoplasm in differentiated cells

(Figure: 4.18). Further, immunofluorescent analysis of both 2ºA control (TRITC/AF555, FITC) was performed in the absence of primary antibody. All secondary antibody control was captured under the same acquisition parameters by confocal microscope Zeiss LSM 710 /ECLIPSE Ti2 Nikon and analyzed by software (NIS Elements- AR 5.11.01, Nikon, Japan). [As showed in chapter number :3 & 5; figure number: 3.21, figure number: 5.15 respectively]

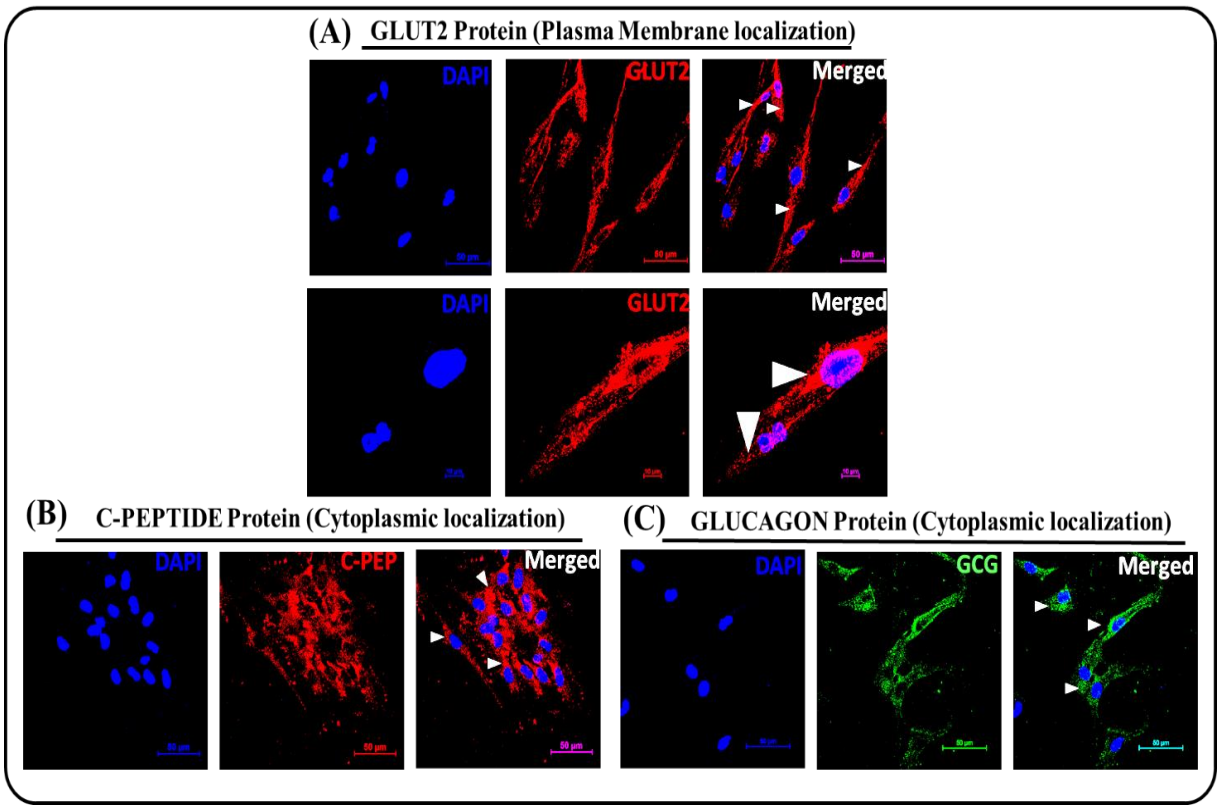


Figure 4. 18: immunocytochemistry assay of single cells from ILCCs: the single cells expressed mature pancreatic marker GLUT2 (Glucose transporter), also magnified images shows in lower penal ("White Arrow" for localization) (A). C-peptide expressed in cytoskeletal/ cytoplasmic localization in cells (B) and glucagon also expressed in cytoskeletal/ cytoplasmic localization in cells(C). DAPI was used as nuclear counterstain; Green and Red represent FITC and TRITC respectively. [Magnification 60 X; scale bar: 50 µM in all images while in the magnified view of GLUT2 show scale bar: 10 µm)]

4.5.4. Flow cytometry analysis support protein expression during islet differentiation

Based on our immunostaining data, day 10 appeared to be an important stage for quantification of certain crucial transcription factors, cytofluorimetry studies at day 10 showed approx. 34% FOXA2 expression in the BMC, i.e. 9% increase in comparison to activin A group. Further,

PDX-1, NEUROD-1, NGN-3, NKX2.2 demonstrated 78%, 80%, 53%, 27% and 75 %, 58 %, 62 %, 24 % expression in BMC and activin A groups respectively (Figure: 4.19). Interestingly, the BMC group (80%) displayed significantly high protein expression of NEUROD1 as compare to activin A group (59%), which suggests a rapid endocrine differentiation process. Unfortunately, the protein expression of NGN-3 showed minor differences within both activin A (62%) and BMC (53%) groups (Figure:4.19).

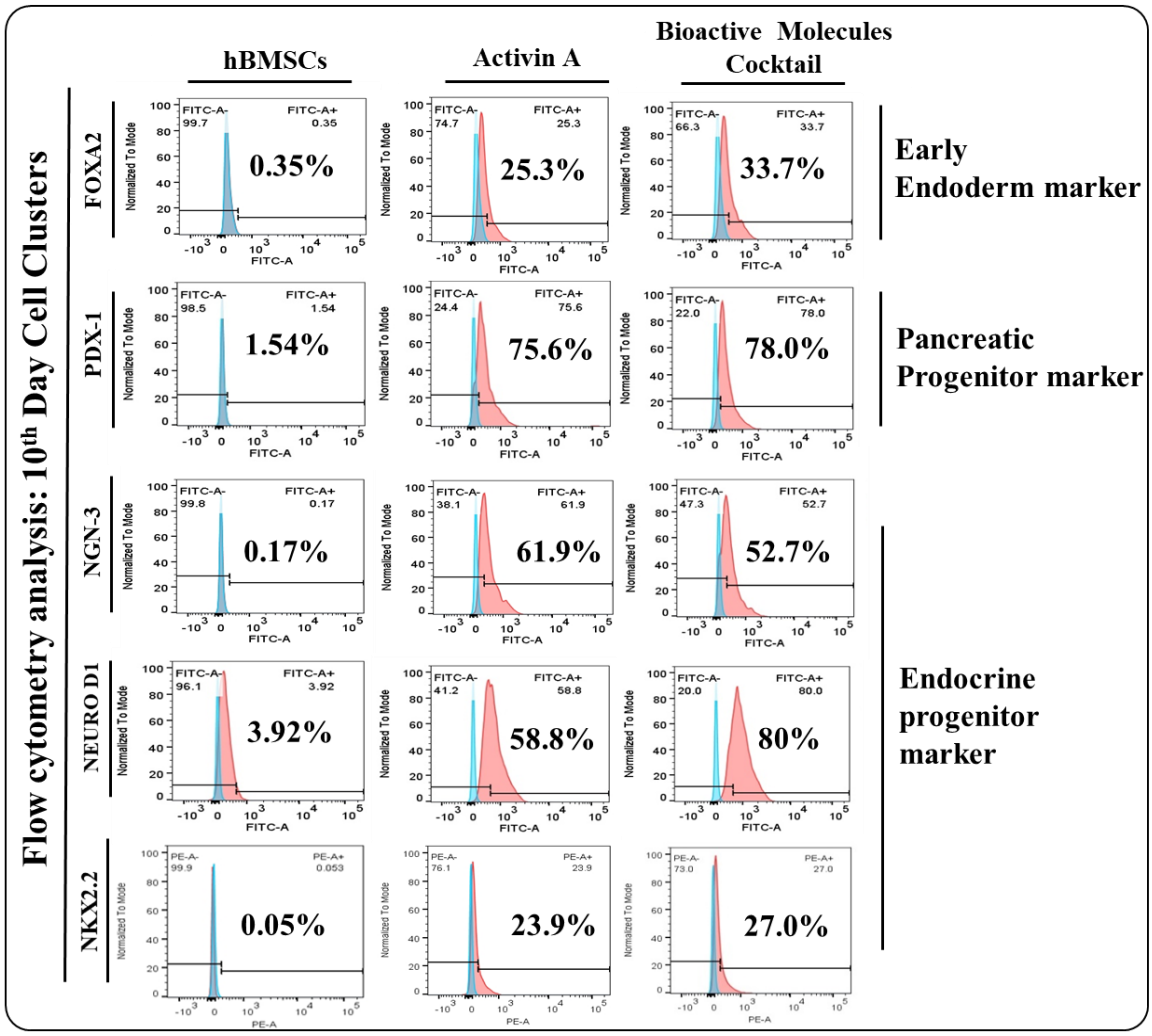


Figure 4. 19: Flow cytometry analysis of important pancreatic islets differentiation-related transcription factors on 10th day cell clusters. Representative FACS analysis shows that 10th cell clusters were demonstrated expression definitive endoderm marker (FOXA2), Pancreatic progenitor marker (PDX-1), and endocrine progenitor marker (NGN-3, NEUROD-1, NKX2.2) in both groups (activin A and BMC). Isotype matched secondary antibody control (FITC- FOXA2, PDX-1, NGN-3, NEUROD-1; PE(TRITC)- NKX2.2) was used to eliminate background staining.

4.5.5. Temporal protein expression during islet differentiation using western blotting

To confirm the data obtained by immunocytochemistry and flow cytometry, a new set of experiment was planned to monitor protein expression by western blotting technique. The results of these experiments are reported in Figure: 4.20.

Western blot analysis showed greater expression of nestin (Stemness marker) at Day 0 (hBMSCs) which gradually decreases as the process of differentiation progresses. Pancreatic islet markers such as REG1 (Regenerative islet-derived 1) are highly expressed on day 5 declining from day 10 onwards and completely abolished at end of islet differentiation in both groups, suggesting increase hBMSCs participation rate during islet differentiation. FOXA2 (definitive endodermal marker) is highly expressed on day 5 as compared to other stages in the process of differentiation, indicating mesenchymal stem cells progression towards definitive endoderm in activin A and BMC groups. In both groups, we observed PDX-1 expression gradually increases from day 5 onwards, suggesting pancreatic progenitors' formation. We also checked the expression of NGN3, endocrine progenitor marker distinctly increases from day 10 of the differentiation process, which suggested hBMSCs ability to differentiate into pancreatic endocrine progenitors complementing the immunocytochemistry and flow cytometry data. NEUROD1, again an endocrine progenitor marker is markedly expressed on day 5 as well as day 10 of the differentiation process in both groups. Additionally, we also performed GLUT2 protein expression during islet differentiation. More surprisingly, GLUT2 expression detected at 5^{th} day and 10^{th} day of cell clusters and again reappear at 18^{th} day of ILCCs, indicating the formation of glucose sensing pancreatic β-cells on day 18 of the differentiation protocol. Non-differentiated hBMSCs expressed very low levels of REG-1, PDX1, and NEUROD1. All these regulatory transcription factors activation was observed to be similar in both activin A and BMC groups.

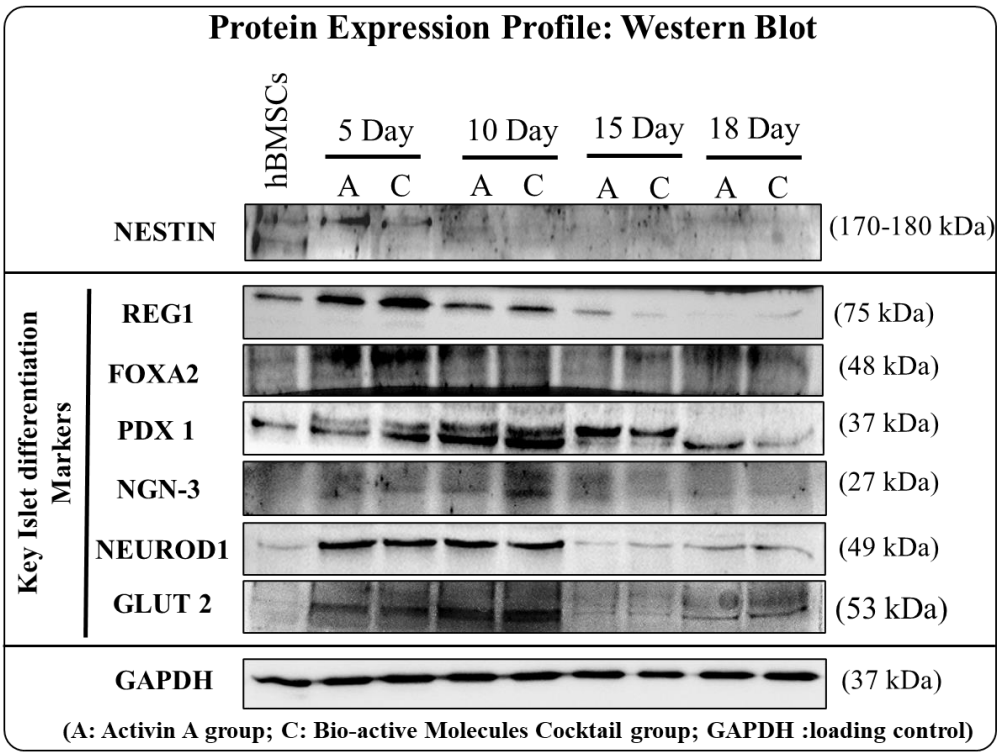


Figure 4. 20: Protein profiling during islet differentiation from hBMSCs using western blot analysis. Temporal protein profiling was performed from 0 Day (undifferentiated hBMSCs), 5 Day (First stage), 10 Day (Second stage), 15 Day (Third stage), and 18 Day (four-stage) of islet differentiation, which demonstrated the kinetics of stem cell marker (NESTIN) and various key islet differentiation-related transcription factors (REG-1, FOXA2, PDX-1, NGN-3, NEUROD1) and Islet functionality marker GLUT-2. GAPDH as an endogenous control [N=2].

To sum up, these experiments showed temporal protein expression quite similar to the conventional protein profile of the pancreas developmental pathway. These results confirmed the efficacy and reproducibility of our augmented multi-step islet differentiation protocol for the achievement of ILCCs from hBMSCs using BMC and activin A. To our knowledge this report is the first of its kind to demonstrate gene as well as protein expression of key lineage-specific markers in islet differentiation, using hBMSCs as the stem source. This indicates that hBMSCs have great potential for diabetic therapy. Efficient and rapid ILCCs formation by BMC similar to activin A (Positive control) suggesting the presence of some strong differentiating agents which is facilitating ILCCs generation and enforcing hBMSCs to differentiate into pancreatic islet hormone-positive cells.

4.6. Discussion

Considerable advancement has been made on pancreatic developmental biology including a transcriptional regulation network of pancreatic progenitor and pancreatic endocrine specification, growth, and lineage allocation. However, the differentiation mechanisms of pancreatic β-cells remain elusive, and in-depth understandings towards this field will contribute to enhancing our knowledge of both the quality and the quantity of newly differentiated β-cells from different sources. To achieve this, we explored development-related transcription factors during islet differentiation from hBMSCs as stem cell sources and various bioactive molecules as a differentiation cocktail using diverse methods. Further, we compared the temporal profile of transcription factors playing an inevitable role in islet differentiation with the ILCCs generated from the herbal bioactive-based approach including islet promoting factors and activin A.

Limited articles demonstrated pancreatic islet differentiation using herbal bioactive compounds. Such as Ogata *et al.*,2004 showed islet differentiation from rat progenitor using betacellulin- δ4 and bioactive molecule conophylline isolated from plant *Ervatamia microphylla* (Ogata *et al.* 2004, Kitamura *et al.* 2007). Nonetheless, the involvement of the transcription factors underlying the differentiation of hBMSCs to pancreatic islets in response to bioactive compounds remains unclear. Several strategies have been reported the pancreatic transcription factors expression during pancreatic islet differentiation from various stem cell sources (Kobinger *et al.* 2005, Zhou *et al.* 2008, Zhang *et al.* 2009) such as embryonic stem cell-derived (Kroon *et al.* 2008), hBMSCs (Jafarian *et al.* 2014).

Thus, we proposed to mimic the *in vivo* pancreatic islet development stages to that of an *in vitro* multistage islet differentiation from mesoderm origin stem cells source i.e. hBMSCs. This multistage protocol involves a series of endodermal intermediates such as definitive endoderm, pancreatic progenitor, pancreatic endocrine progenitor and eventually resembling those that occur during pancreatic development *in vivo*.

The initial essential step in the differentiation of hBMSCs against the pancreatic lineage is the stimulation of definitive endoderm (DE). The principal essential advance in the separation of hBMSCs toward the pancreatic heredity is the detail of authoritative endoderm (DE).

Recent reports demonstrated that nestin performs essential roles as an intermediate regulator, directing the differentiation of MSCs into ILCCs (Kim *et al.* 2010). In the current investigation, we effectively induced nestin-positive hBMSCs population into definitive endoderm lineage, then into pancreatic/endocrine progenitor and eventually into well-functioning ILCCs using the four-stage induction procedure.

Although, the qualities of nestin-expressing MSC are remain debatable (Blyszczuk *et al.* 2004). Several reports suggest that nestin-expressing stem cells could be a useful strategy to trigger pancreatic islet differentiation (Zulewski *et al.* 2001, Milanesi *et al.* 2011). Additionally, our temporal protein expression data revealed that the undifferentiated hBMSCs demonstrated moderate expression of nestin (stem cell markers) which was gradually decreasing with an increasing day of the islet differentiation process. This gave us a clear understanding of undifferentiated stem cells diminishing stemness properties and differentiating towards pancreatic lineages.

Earlier *in vivo* examinations had proposed TGF-β and Wnt signals as essential signals that prompt definitive endoderm formation (Conlon *et al.* 1994, Haegel *et al.* 1995, Liu *et al.* 1999, Brennan *et al.* 2001, Kelly *et al.* 2004). Hence, activin A, a member from the TGF-β family, was utilized at high concentration in combination along with Wnt3a to efficiently induce definitive endoderm from an hESCs *in vitro* (D'Amour *et al.* 2006, Yao *et al.* 2006, Kroon *et al.* 2008). Other transcription factors such as SOX17, SOX9, GATA4, GATA6, and FOXA2 are among the most prominent players of the pancreatic transcriptional network hierarchy controlling pancreas developments (Carrasco *et al.* 2012). We reported high expression of GATA4/6, SOX17/9, HNF1β during an early stage of islet differentiation in the presence of activin a or bioactive molecule. In the present study, we focused more on the FOXA2 transcription factor because it has been postulated to play a central regulator role in pancreatic β-cell differentiation due to its ability to directly bind and trans-active PDX-1 cis-regulatory elements (Wu *et al.* 1997, Ben-Shushan *et al.* 2001). Additionally, Foxa2 has a crucial role in glucose uptake and insulin secretion, controlling several genes such as the ATP-sensitive K+ channel, Kir6.2 (potassium channel), and Sur-1 (sulfonyl urea receptor) (Lee *et al.* 2002, Wang *et al.* 2002, Lantz *et al.* 2004, Gao *et al.* 2007). In our protocol, the FOXA2 gene and protein expression were observed throughout the islet differentiation process. Further, Manuel Carrasco *et. al.,* 2012 demonstrated that GATA-6 and GATA-4 transcription factors also

regulate PDX-1 expression, essential for maturation and differentiation of common pancreatic precursor cells in islet differentiation (Fujimoto and Polonsky 2009, Carrasco *et al.* 2012).

Next, the second important step in the differentiation of hBMSCs in the direction of the functional pancreatic is the pancreatic progenitors. Pancreatic progenitor markers, such as PDX-1, NKX2.2, and NKX6.1, were remarkably expressed in the middle stage of differentiated cell clusters at mRNA and protein levels. NKX2.2 is required in early pancreatic progenitors' development and essential for the regulation of downstream NGN-3 positive endocrine progenitor population (Churchill *et al.* 2017). Further, Junfeng Wang *et. al.*,2004 demonstrated NKX2.2, PAX6, and PAX4 as an important regulator of differentiating pancreatic progenitor into pancreatic endocrine precursor (Wang *et al.* 2004). Similarly, we found co-expression of mature pancreatic progenitor's transcription marker i.e. NKX2.2 and PAX-4 during islet differentiation. NKX6.1, downstream of NKX2.2, is essential for the maturation of β-cell progenitors during the second wave of pancreatic β-cell development and suppresses glucagon expression in α-cells (Sander *et al.* 2000, Schisler *et al.* 2005). In the present study, we detected a high level of PDX-1 protein expression at the middle stage of islet differentiation may trigger a cascade of events leading to mature islet hormone responding transcription factors protein expression such as NGN-3, MAFA. The progress of different pancreatic β-cells cell types in close juxtaposition to each other in cell clusters increases the feasibility that pancreatic hormone-positive cells may differentiate from a mutual progenitor, but not certainly single progenitor. Recently, Ting Zhang *et. al.*, 2019 demonstrated PAX-4 act with PDX-1, NGN-3, MAFA in the islet differentiation process (Zhang *et al.* 2019).

Further, the third essential step in the differentiation of hBMSCs toward the mature pancreatic lineage is the pancreatic endocrine progenitors. Similar to PDX1, NGN3 is involved in the recruitment of progenitor cells and drives cells towards an endocrine lineage by assisting the second wave of expression, PAX4, NEUROD1/BETA2, and MAFA which acts as β-cell differentiation factors. NGN3 triggers NEUROD1 expression and suppresses cell division by inducing of cyclin-dependent kinase inhibitor in pancreatic precursors. NGN3 arises in an early stage of islet differentiation before the distinction between α and β cells population; in contrast to some of the other transcription factors, NGN3 becomes undetectable in the human adult pancreas (Huang *et al.* 2000, Schwitzgebel *et al.* 2000). Similarly, the *NGN-3* gene and protein expression pattern were found in our islet differentiation protocol. Importantly, in the present study, *NGN-3* gene expression was early detected in the BMC group as compared to activin A

groups, suggesting rapid induction of islet differentiation due to the presence of "swertisin" as a key bioactive molecule in the bioactive molecule cocktail group. Our lab reports have previously demonstrated that swertisin can potentially trigger NGN-3 expression in the islet differentiation process from mouse pancreatic residential endocrine progenitors (Srivastava *et al.* 2019). During embryogenesis, only cells expressing NGN-3 are committed to islet progenitors (Desgraz and Herrera 2009) and Guoqiang Gu, *et.al.,*2002 have shown that cells co-expressing PDX-1 and NGN-3 eventually become pancreatic hormone-producing cells (Gu *et al.* 2002). Thus, we have demonstrated the expression of the NGN-3 with PDX-1 transcription factor in cell clusters during islet differentiation. Recent studies support that genetic manipulation or small molecules lead to differentiation of hBMSCs into functional pancreatic islets. Atsushi Kubo *et. al.,*2011 demonstrated pancreatic islet related transcription factors, being upregulated during islet differentiation by genetic manipulation using PDX-1, NGN-3 overexpression (Karnieli *et al.* 2007, Rukstalis and Habener 2009, Kubo *et al.* 2011). Baeyens et al., in 2006 showed that *in vitro* differentiation factors stimulation could recapitulate an embryonic pancreas differentiation pathways, in trans-differentiation of exocrine cells via high NGN-3 expression using transfection method (Baeyens *et al.* 2006). Additionally, reports suggested that hBMSCs differentiated into an insulin-producing cell by transfecting three genes - *PDX-1, NEUROD, NGN-3*, but they lacked glucose-responsive insulin secretion (Karnieli *et al.* 2007), However, the protocol developed in our study using bioactive were capable to induce *PDX-1*, *NEUROD1*, and *NGN-3* gene expression, as well as glucose-responsive insulin expression without any gene transfection and thus strongly, resemble pancreatic endocrine progenitors' cells. This has been endorsed by our dual staining immunocytochemical results. However, none of the studies have shown a lineage-specific transcription factor localization in cells through immunocytochemistry during the islet differentiation process from hBMSCs.

Additionally, reports suggested that the induction of both NEUROD1 and NGN3 can drive islet differentiation and further induce NKX2.2, PAX-4 (Gasa *et al.* 2008, White *et al.* 2008, Gu *et al.* 2010), which are required for expansion of pancreatic the β-cell lineage in pancreatic development (Sosa-Pineda *et al.* 1997, Smith *et al.* 2000, Smith *et al.* 2003, Brun and Gauthier 2008). Whereas in the mature β- cell PDX1, MAFA, NEUROD1, and NKX2.2 play important roles in regulating the expression of insulin and to some extent for maintaining β-cell function. Pax4 (and possibly HNF1α and Hnf4α) is important in the proliferation of β-cells in the adult pancreas. Interestingly, in our gene study, all-important transcription factors such as

NEUROD1, NKX2.2, and *PAX-4* expression were detected in an early stage of islet differentiation in both groups, indicating the positive sign of initiating islet differentiation process. Further, NKX2.2 directly regulates MAFA which regulates glucokinase, GLUT2, and prohormone convertase, which proceeding proinsulin to insulin (Wang *et al.* 2007).

The presence of PDX1 positive population inside differentiating cells clusters and co-expression in hormone-positive cells with expression of MAFA is, steady with pancreatic β cell lineages defined in ordinary pancreas development (Jensen *et al.* 2000). However, in the present study, hBMSCs derived cell clusters exhibited poly- hormonal expression, heterogeneous populations such as insulin and glucagon expression, which is suggesting fetal islets like functional signatures (Bruin *et al.* 2014, Hrvatin *et al.* 2014). Double-immunostaining for MAFA and glucagon has confirmed the low numbers of glucagon expression α-cell-containing in ILCCs. Moreover, we demonstrated transcription factor ARX to be present at the middle stages of differentiation which is required for the pancreatic development of cells towards α cell lineage.

Collectively, it seems that BMC and activin A group can induce several stages in the complex islet differentiation process, including the early stages and middle stage exemplified by GATA6, SOX17, and PDX-1, NGN-3 respectively, in addition to late stages of insulin & glucagon secretion exemplified by MAFA, MAFB. These hBMSCs-derived ILCCs resembled a mid-gestation human fetal pancreas and thus it may be also a useful model for studying human pancreas development.

Lastly, the fourth essential step in the differentiation of hBMSCs toward the mature pancreatic lineage is the functional pancreatic islet marker. In our present study, we detected all-important mature pancreatic markers transcripts such as *INS, GCG, SST, PPY, PCSK1,* and *GLUT2* in the last phase of islet differentiation. Similarly, Daniela pezzolla *et. al.,*2015 demonstrated that resveratrol promotes islet differentiation, proliferation, and increases mature endocrine pancreatic markers such as insulin, glucagon, gene expression content of ILCCs (Pezzolla *et al.* 2015). In the present study, the addition and withdrawal of a combination of small bioactive molecules in a stage-wise manner are required for pancreatic transcription factors upregulation and downregulation in order to generate perfect ILCCs. To our knowledge, very few studies have demonstrated the protein expression profile of transcription factors during pancreatic islet differentiation from hBMSCs by western blot, flow cytometry owing to its scanty and transient

expression (Gabr *et al.* 2013, Gabr *et al.* 2014, Jafarian *et al.* 2014). We believed that this is the first report for addressing an islet differentiation event induced by the ectopic expression of important transcription factors using bioactive molecules from hBMSCs. Finally, transcription factors machinery findings open up the door for more warranted investigations to gain a more in-depth understanding of some obscure parts of the story of differentiation of stem cells into ILCCs, and the mechanisms of action of various extrinsic differentiating factors. These hidden transcription factors, if unravelled, will surely be advantageous. Hence in the next chapter, we studied the microRNAs profile to enhance our overall understanding of the islet differentiation from hBMSCs.

4.7. Summary of Chapter-4

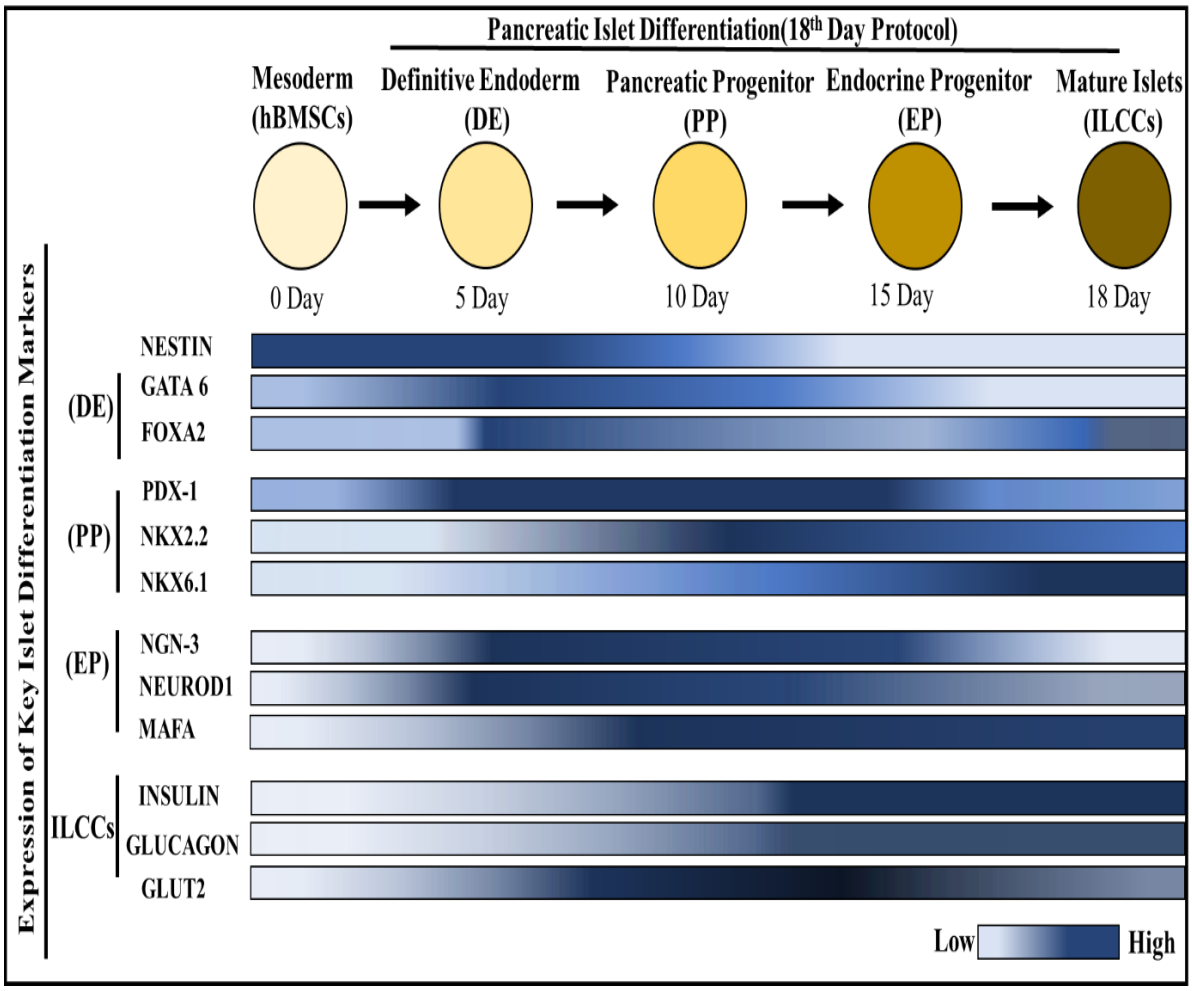


Figure 4. 21: Summary of chapter-4