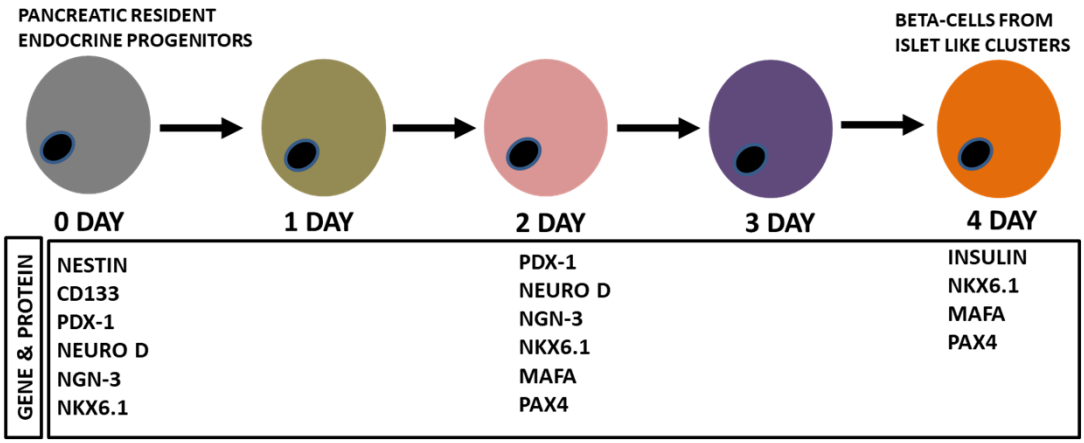
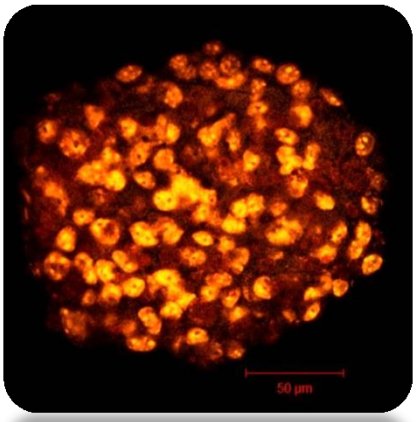
ISOLATION, PURIFICATION & CHARACTERIZATION OF PREPs AND HARNESSING IT'S STEMNESS TO GENERATE FUNCTIONAL INSULIN PRODUCING ISLETS

CHAPTER 3

Chapter 3

Isolation, Purification & Characterization of Pancreatic Resident Endocrine Progenitors (PREPs) and Harnessing it's Stemness to Generate Functional Insulin Producing Islets





3.1. Introduction

Glucose homeostasis is tightly regulated by the hormones of the endocrine pancreas and any disturbance in its function can lead to metabolic disorders causing Diabetes Mellitus, which has become the most prevalent disorder across the globe with the loss of pancreatic β cells as its hallmark (Edlund, 2001; Kim and Hebrok, 2001).

Even after decades of extensive research, there is no cure for diabetes. Drugs have only been sufficient in managing the diabetic condition by keeping the blood glucose levels in-check. In recent years, Islet transplantations have been proved to be, a hopeful therapeutic intervention. However, there is limitation of donor islets creating a dilemma for its therapeutic clinical transition for diabetic patients. It is here that regenerative cellular therapy has shown promise by providing pancreatic islet differentiation using various stem cell sources (Staels et al., 2016).

In order to generate islet clusters, many different types of stem cells both human and animal have been explored extensively. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been explored widely for generating functional islet clusters but there have been distinct hurdles such as absence of certain essential pancreatic markers, having a low index for glucose stimulated insulin secretion (GSIS) and the vast amount of time taken for producing the insulin producing cells. This along with ethical conundrums has compelled researchers to look into other stem cell sources and adult stem cells provide a better platform in terms of clinical and ethical acceptability and availability for islet neogenesis. Adult stem cells both pancreatic and non-pancreatic have been successfully differentiated into functional islet clusters. Non-pancreatic adult stem cells mainly Mesenchymal Stem cells (MSCs) from various sources such as bone marrow, umbilical cord, adipose tissue, dental pulp etc. have been used in *in vitro* studies to increase the islet mass. Literature suggests that there are mainly three distinct sources of Pancreatic adult stem cells or pancreatic progenitors i.e.

progenitors from acinar origin, the ductal progenitors and the intra-islet progenitors which have the potential to generate new insulin producing cells (Afelik and Rovira, 2017; Jiang and Morahan, 2014; Wen et al., 2011). Here in this study, we have focused on adult stem cells from the pancreas and have extensively characterized them as Pancreatic resident endocrine progenitors (PREPs) having MSC characteristics.

Although we recognise the presence of pancreatic progenitors, their potential to replenish the lost beta cell population has not been fully explored. In this study, we emphasise PREPs being the closest and the fastest adult stem cell route available, which can replenish islets of the lost beta cells as they already have the molecular machinery required to form functional islet clusters. This can be achieved by stimulating this machinery using appropriate molecules. Although, the various types of stem cells require a different induction and culture media conditions to be differentiated into functional islet clusters, there are many molecules that can be used for islet neogenesis e.g. Activin A, Betacellulin, KGF, Exendin-4, Nicotinamide etc. (Wong, 2011). In the present study, we have used Swertisin, isolated from an Indian herb *Enicostemma littorale*, a traditional anti-diabetic plant used in traditional medicine, has been characterised as a potent islet differentiating agent. Our group have previously reported Swertisin's islet neogenic potential on NIH3T3 and PANC1 cell lines *in vitro* and in pancreatectomised mice model *in vivo*. Swertisin in these studies was able to ameliorate the diabetic condition effectively (Dadheech et al., 2013; Dadheech et al., 2015). Here, we have tried to emphasize that a small molecule Swertisin along with PREPs with their rapid differentiation capability can provide an incredible therapeutic intervention in the treatment for diabetes mellitus.

3.2. Material and methods

3.2.1 Experimental design:

Pancreas od young adult BALB/c mice were used to isolate islets of Langerhans, which were sequentially passaged to obtain putative pancreatic progenitors. These were characterized extensively for their unique pancreatic progenitor characteristics. These progenitor's inherent capacity to form functional islets was harnessed by using Activin A and Swertisin as islet differentiating agents respectively. Molecular characterization was carried out to identify key factors essential in islet differentiation and understand their expression kinetics. Finally functional characterization of the mature islet clusters was performed to confirm and analyze islet differentiation potential of the isolated PREPs (Fig 3.1).

3.2.2 Chemicals and Plastic ware:

All chemicals and culture media used in this study were purchased from Sigma Aldrich and Invitrogen, ThermoFisher Scientific. The details of the antibodies used are given in Table 1. Molecular biology reagents and cDNA and PCR kits were procured from Invitrogen ThermoFisher Scientific. Cell culture plastic ware was obtained from Nunc (ThermoFisher Scientific). Routine plastic ware was obtained from Axygen, India.

3.2.3 Islet isolation and Culturing:

Islets were isolated from healthy young adult (6 weeks) non-diabetic male BALB/c mice by the method of Xia and Laychock, (Xia and Laychock, 1993), and then subjected to purification. Here, maintaining sterile conditions, pancreas was first excised out and thoroughly minced. The minced tissue was collected and given a wash with cold phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4 and 10 mM Na_2HPO_4). The tissue was put in a 5 ml Hank's balanced salt solution (HBSS) containing 5mg collagenase type V. Digestion was for 15 min in a shaking water bath at 37°C. The chemical digestion was stopped with complete media and islets were washed thrice in cold PBS. The

islets were incubated in RPMI 1640 medium with 10% Fetal Bovine Serum (FBS) South American origin, Gibco and Penstrep (Gibco) for 48 hr and then shifted to DMEM high Glucose with 10% FBS and Penstrep.

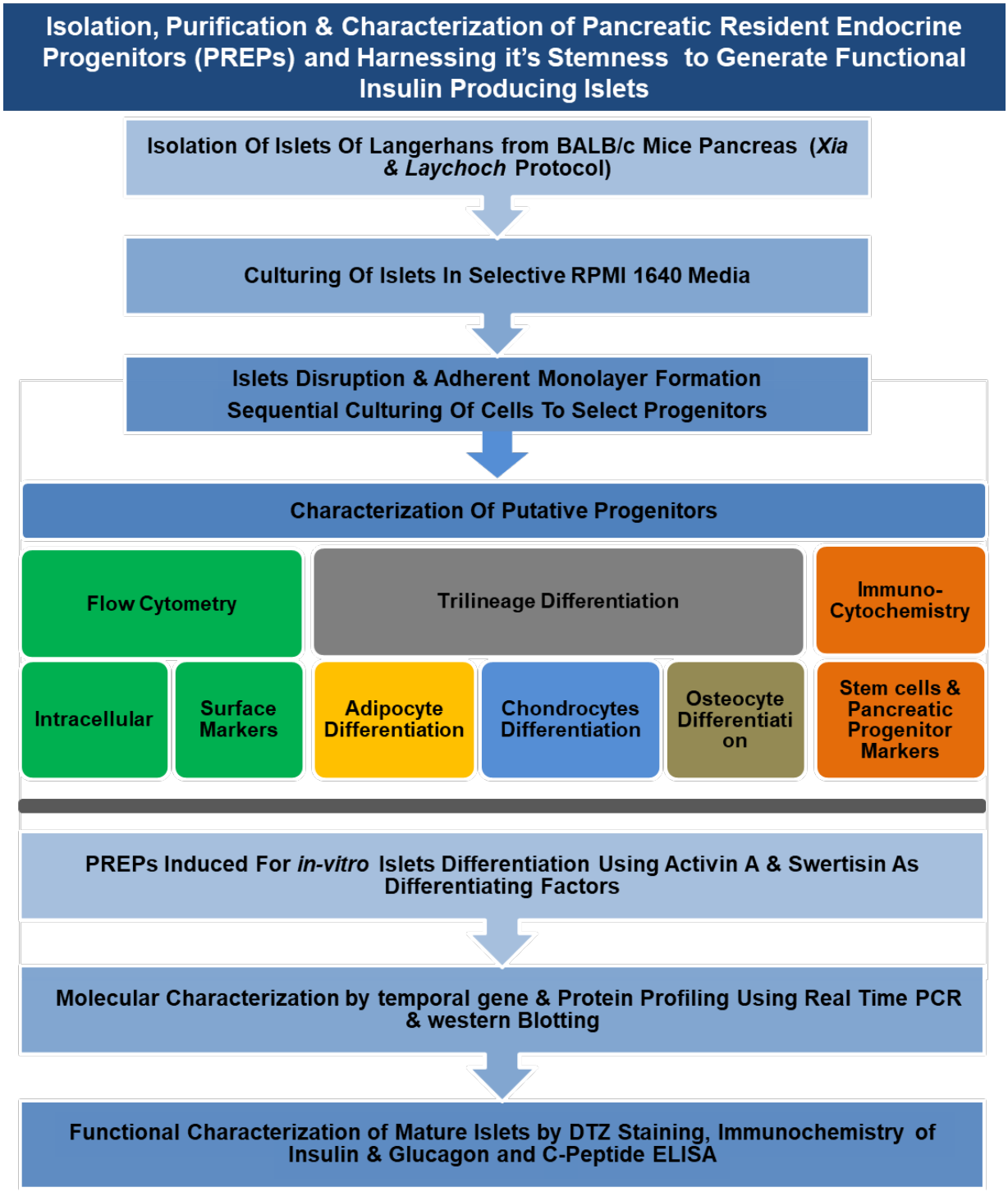


Figure 3.1: Experimental Design for Chapter 3.

3.2.4 Isolation and establishment of mouse pancreatic resident endocrine progenitors (PREPs):

Mouse pancreatic resident endocrine progenitors were isolated from fresh islet preparation followed by digestion of whole mouse pancreas as previously discussed (Dadheech et al., 2015; Srivastava et al., 2016). Two pancreas were used per isolation. Islet preparation was confirmed with 90% dithizone-positive structures. Freshly isolated islets (5000 islets per 25 cm^2 flask) (Nunc, Fisher Scientific, India) were placed and cultured in RPMI-1640 complete media with 10% Fetal bovine serum (FBS) (Gibco, Fisher Scientific, India) for 24 hr and then shifted to DMEM high glucose complete media with 10% serum (Gibco, Fisher Scientific, India) to promote monolayer formation. Cultures were maintained in 95% air/5% CO2 at 37˚C, and the medium was renewed every alternate days. The monolayers were subcultured when the cells had grown to 70% confluence. The derived monolayers were characterized at 8^{th} passage of the culture period before utilizing in the subsequent investigations.

3.2.5 Immunocytochemistry:

Undifferentiated PREPs were assessed for their progenitor nature immunochemically. Immunocytochemistry of the following protein markers: NESTIN, PDX1, NEUROG3, CK-19, KI-67, Insulin and Glucagon were performed. INSULIN, GLUCAGON, NKX6.1 and GLUT2 immunocytochemistry staining was also performed to characterize islet clusters obtained after differentiation from PREPs. Briefly, growing cells or clusters were seeded for overnight on sterile glass coverslips coated were previously and fixed with 4% PFA for 10 min at 4˚C and washed once with PBS, thereafter permeabilized with 0.1% triton X-100 solution in PBS for 5 min at 4˚C. In case of islets they are fixed in absolute chilled methanol for 10 min at room temperature (RT), washed with PBS twice and then in the permeabilization step 0.5% triton X-100 was used. Once permeabilization was done, samples slides were blocked with blocking solution containing 5% BSA in PBS for 1 hr at RT. Slides

were then probed with respective primary antibody at desired dilution as shown in table-1. Followed with 3 x wash in wash buffer (one tenth of blocking buffer), slides were labeled with fluorescent dye conjugated secondary antibody (dilution shown in table-1). Nuclear counterstaining was done with DAPI at 300nM and finally mounted with vectashield mountant for imaging on confocal microscope (Zeiss LSM710).

3.2.6 Flow cytometry

3.2.6.1 Surface marker staining:

Cells were trypsinized and resuspended 0.5 x 10^6 cells per tube in 100 µl of staining media (PBS with 1% BSA). The required antibody as per manufacturer's guidelines in each tube was added. Cells were incubated for 30 min at 4˚C in dark. Further, cells were washed twice with staining media and pelleted by spinning at 2000 rpm for 5 min at 4˚C. Cells were suspended in 500 µl staining media and analyzed on BD FACS ARIAIII with a 70 µM nozzle and 70 PSI pressure conditions. Cells were first gated based on forward and side scatter properties and then compared with unstained negative cells for levels of respective antibodies.

3.2.6.2 Intracellular marker staining:

Cells were trypsinizeds and resuspended 0.5 x 10^6 cells per tube in 100 µl of staining media (PBS with 1% BSA). Cells were fixed with 4% PFA to a final concentration of 2% PFA for 10 min at room temperature. Cells were washed with staining media. Resuspend pellet with vigorous vortexing in 500µl ice-cold methanol per 10^6 cells, incubated at 4˚C for 10 min. Wash with staining media twice. The required antibody as per manufacturer's guidelines in each tube was added. Cells were incubated for 30 min at 4˚C in dark. Further, cells were washed twice with staining media and pelleted by spinning at 2000 rpm for 5 min at 4˚C. Cells were stained with secondary labeled antibody for 15 min at room temperature (RT) in dark as per manufacturers instruction. Cells were washed twice with staining media. Cells

were suspended in 500 µl staining media and 30,000 cells per tube were analyzed on BD FACS ARIAIII with a 70 µM nozzle and 70 PSI pressure conditions. Cells were first gated based on forward and side scatter properties and then compared with unstained negative cells for levels of respective antibodies. Analysis of the data was performed on FlowJo software.

3.2.7 Trilineage Differentiation:

Osteocyte Differentiation: Cells were plated into six- well plate at 10^5 cells/well in the presence of osteocyte reagents (20 mM B-glycerol phosphate, 50 µg/ml ascorbic acid and 10 mM dexamethasone) for 10 days. The culture medium was replaced every 3rd day. Parallel control cells were cultured without osteocyte reagents. Quantification was performed with Alizarin Red staining, which was recovered by 4 M guanidine HCl and estimated at 490 nm.

Adipocyte Differentiation: Adipogenesis was induced by treatment with IBMax (10 mg/ml), 10 mM dexamethasone and 10 mg/l insulin for 8 days. The culture medium was replaced every 3rd day. Parallel control cells were cultured without adipocyte differentiation medium. Quantification was done by staining with Oil Red O staining, which was recovered using isopropanol and estimated at 520nm.

Chondrocyte Differentiation: Chondrocyte differentiation was induced by treatment with 10 mM dexamethasone and 10 mg/l insulin for 20 days. The culture medium was replaced every 5th day. Parallel control cells were cultured without chondrocyte differentiation medium. Quantification was performed with Alcian blue staining, which was recovered by ethanol and estimated at 595 nm. The cells were observed under phase contrast microscope at 20x objective magnification.

3.2.8 Islet Differentiation:

Initially PREPs were differentiated into insulin producing cells in DMEM F12 as basal media along with other differentiating factors. Later on the basal media was changed to DMEM

knockout keeping the other conditions same. The cells were allowed for differentiation in presence of Serum free (1% BSA) DMEM Ham's F-12 (1:1, 8 mM Glucose) (tried initially) and later replaced by DMEM knockout media (Invitrogen, ThermoFisher Scientific), Activin-A (Sigma, Aldrich USA) and Swertisin as previously described (Dadheech et al., 2013; Dadheech et al., 2015). PREPs were differentiated using Activin-A at 5 ng/ml and Swertisin at 15 µg/ml concentrations as differentiating agents in a four day differentiation protocol with insulin (5 µg/ml) transferrin (5 µg/ml) and selenite (5 ng/ml) cocktail. Differentiated islet clusters were washed three times with ice cold Phosphate buffered saline and stained with DTZ stain to final concentration of 0.1mg/ml from a 100x stock made in DMSO. Bright crimson red colored clusters were observed in light microscope and photographed.

3.2.9 Protein extraction and Western blotting:

Undifferentiated PREPs and differentiated islet like clusters from *in vitro* experiments were harvested in Laemmli SDS sample buffer and sonicated at 45% amplitude for 20 sec with 0.2 sec pulse on/off. 20 µg of total protein as estimated by Bradford's method was resolved on SDS-PAGE Tris-glycine gels and transferred to nitrocellulose membrane. Non-specific binding was blocked by incubating the membranes in 5% fat free skimmed milk with 0.1% Tween-20 in PBS/TBS for 1 h at RT. The blots were subsequently incubated with primary antibodies against the following proteins: Nestin, CD133, Pdx-1, Neurog-3, NeuroD, Pax4, MafA, Nkx6.1, P38mapk, Phospho-P38mapk, Smad2/3, Smad-7, pSmad-3 and β-Actin proteins overnight at 4 °C, with gentle agitation. Blots were washed with TBS/PBS containing 0.1% Tween (TBS/PBS-T) (4 × 15 min) and then incubated with respective secondary antibodies conjugated with HRP for 1 h at RT with gentle agitation (Table 4.2). After four washes with PBS/TBS-T and two washes with PBS/TBS; specific bands of immune-reactive proteins were visualized using Ultrasensitive enhanced chemiluminiscence

reagent (Millipore, USA) and images were captured on chemigenious gel documentation system (Uvitech, Cambridge).

3.2.10 RNA extraction, First Stand c-DNA synthesis and Real time PCR (qRT-PCR):

Undifferentiated PREPs and differentiated islet like clusters were subjected to RNA isolation using TriSoln (Sigma Alrich, USA) followed by its quantification on Shimadzu Nanospectrophotometer (Shimadzu, Japan) at lambda 260 and 280 nm. 2 µg of total RNA was reverse transcribed into cDNA using first strand c-DNA synthesis kit (Fermentas INC., USA) as per manufacturer's instruction for gene expression profiling. qRT-PCR was performed with optimal conditions for *Pdx-1, Neurog3, NeuroD, Nkx6.1, Insulin and Glucagon* genes against *β-Actin* as endogenous control by running real time PCR performed at annealing temperature 60˚C (Table 3.1). One µl of ten times diluted cDNA product was used to amplify genes using Power SYBR green 2X Master Mix (Invitrogen, USA) on 7500 real time PCR system (Applied Biosystems, USA).

3.2.11 C-Peptide ELISA:

Glucose stimulated c-peptide release assay: Differentiated islet clusters were initially incubated for 15 min in glucose free Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 0.5% bovine serum albumin and then induced with 5.5mM and 20mM glucose in KRBH respectively for additional 2 hr on constant shaking at 200 rpm condition at 5% CO^2 and 95% O^2 at 37˚C. After brief centrifugation, the supernatant was collected and frozen at –70∘C until further analysis and the pellet was used for protein estimation for normalization. C-peptide assay was performed using mouse-c-peptide ELISA (ALPCO Immunoassays, USA) as per manufacturer's protocol.

3.2.12 Statistical Analysis:

The data is presented as mean ± SEM. The significance of difference was evaluated by the paired Student's t-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA).

3.3. Results:

3.3.1 Isolation of Islets from mouse (BALB/c) Pancreas and Purification of Pancreatic resident endocrine progenitors (PREPs):

CIslets were isolated and purified from BALB/c mice using collagenase Type-V digestion. These islets were cultured in DMEM high glucose complete medium for islet cluster disaggregation into a monolayer culture, which further lead to isolation and culturing of Pancreatic progenitors. We obtained a homogenous population of cells with sequential passaging (Fig 3.2). The purified cells were than characterized at passage 10 for the presence of stem/progenitor along with pancreatic endocrine markers.

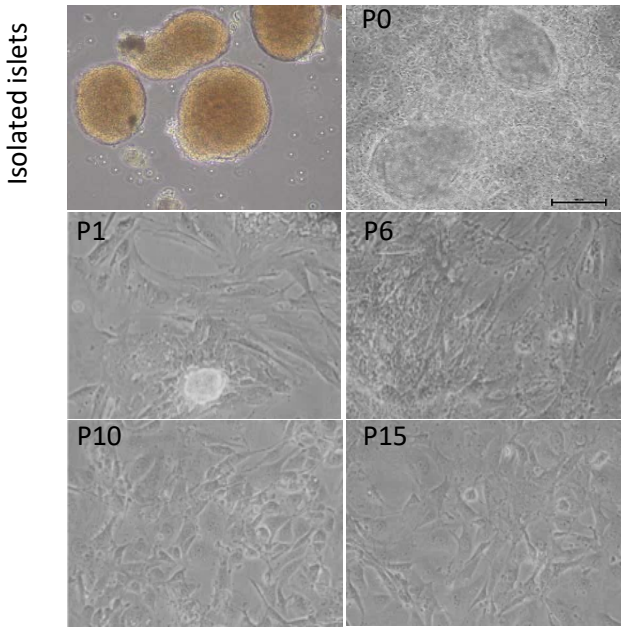


Fig 3.2: Isolation of Pancreatic resident endocrine progenitors: Purification of PREPs by sequential passaging of endocrine pancreas from P_0 to P_{15}, where a homogeneous population of mesenchymal cells were observed to be purified with sequential passaging (N=6). The scale bar represents 100 microns.

3.3.2 Characterization of PREPs for their adult stem cell nature:

The immunocytochemistry panel at passage 10 in Fig 3.2 defines these cells for their pancreatic neuroendocrine lineage by identifying respective protein markers (Table 4.2). The presence of Nestin in the cytoplasm specified that the purified cells were positive for

stem/progenitor phenotype. PDX-1 and Neurogenin3 are important transcription factors, localized in the nucleus confirming their pancreatic and pancreatic endocrine lineage respectively. Existence of Cytokeratin-19 (Ck-19) suggested that these cells have pancreatic ductal origin, which descended within Islets of Langerhans. These cells were absent for Insulin and Glucagon. Also, the Insulin and Glucagon transcript levels were significantly lower when compared to mice pancreas. This abolished the possibility of these cells to be terminally differentiated β or α cells respectively (Fig 3.3 & 3.4).

These cells were further characterised for pluripotency markers viz. Oct3/4, Sox2 and Nanog, which were not expressed in these cells suggesting that these cells are not pluripotent. They were further screened for cluster of designation (CD) surface markers for the MSC phenotype viz. CD90, CD44, CD34, CD133, CD45 and Glut2 along with Vimentin and PDX-1. We observed the cells to be highly positive for CD90, CD44, CD34, CD133, PDX-1, Vimentin & Glut2 and negative for CD45. This confirmed their pancreatic endocrine progenitor and Mesenchymal stem cell phenotype (Fig 3.5).

3.3.3 Trilineage Differentiation of Mouse PREPs:

Mesenchymal stem cell nature of PREPs was further confirmed by performing Trilineage differentiation.

3.3.3.i Differentiation into Adipocyte Differentiation:

PREPs were kept in the adipocyte differentiation media for a period of eight days. Adipocyte differentiation was confirmed by the presence of oil droplet formation in the cytoplasm. These oil droplets were stained red in colour when stained with the oil o red stain and the staining for adipogenesis was highly significant when compared to undifferentiated PREPs (Fig 3.6A and 3.6Bi).

3.3.3.ii Osteocyte Differentiation:

The next step taken towards the mesodermal lineage was to differentiate the PREPs into osteocytes. PREPs were kept for osteocyte differentiation for a period of twelve days. A distinct change in the cell morphology was observed viz. cells became more compact along with considerable mineralization around the cell surface. This mineralization was confirmed by staining with Alizarin Red S dye which stains the calcium deposited by osteocytes, thus confirming the differentiation (Fig 3.6A and 3.6Bii).

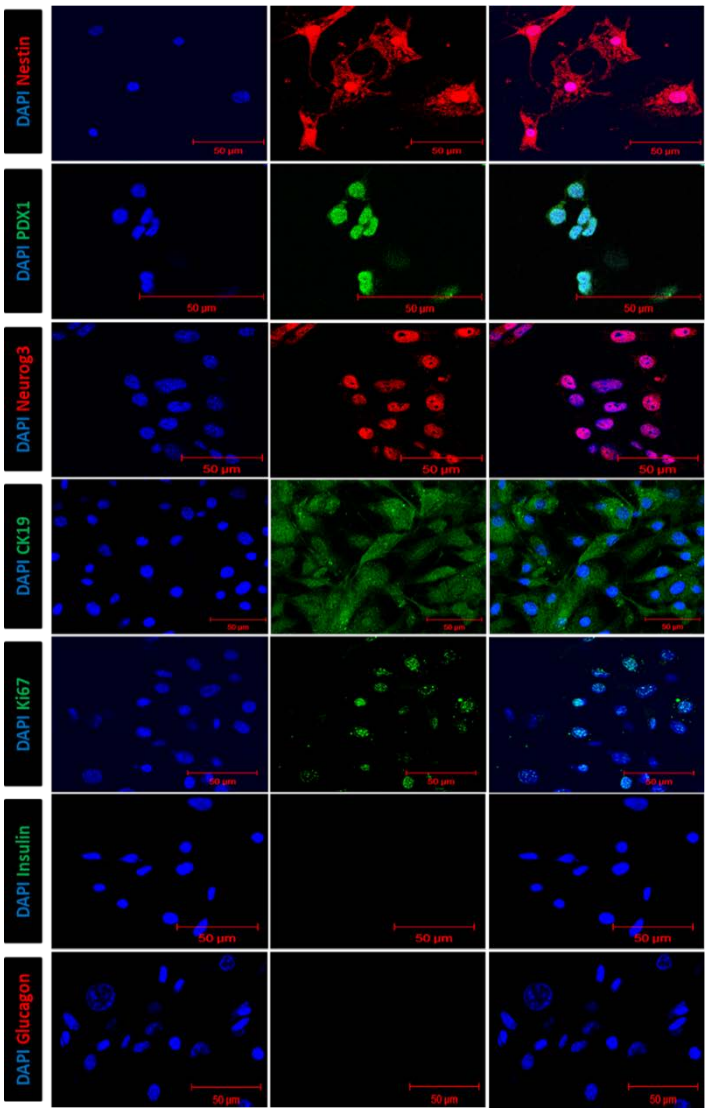


Fig 3.3: Characterisation of Pancreatic resident endocrine progenitors using Immunocytochemistry: Characterisation of PREPs using immunocytochemistry was performed to identify their proliferative endocrine pancreatic stem cell origin and to confirm the absence of pancreatic hormones. Here the markers are as follows: NESTIN (red); PDX1 (green); NEUROG3 (red); CK19 (green); Ki67 (green); INSULIN (green) and GLUCAGON (red) (N=3).

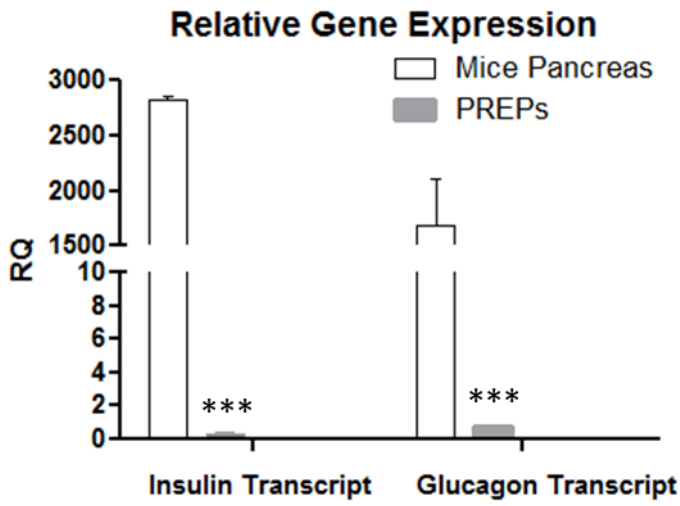


Fig 3.4: Relative *Insulin* Expression: Comparison between PREPs' and mice pancreas Insulin and Glucagon transcript levels. The graphs are plotted with mean values ± SEM. *** p≤0.001undifferentiated vs mice pancreas (N=3).

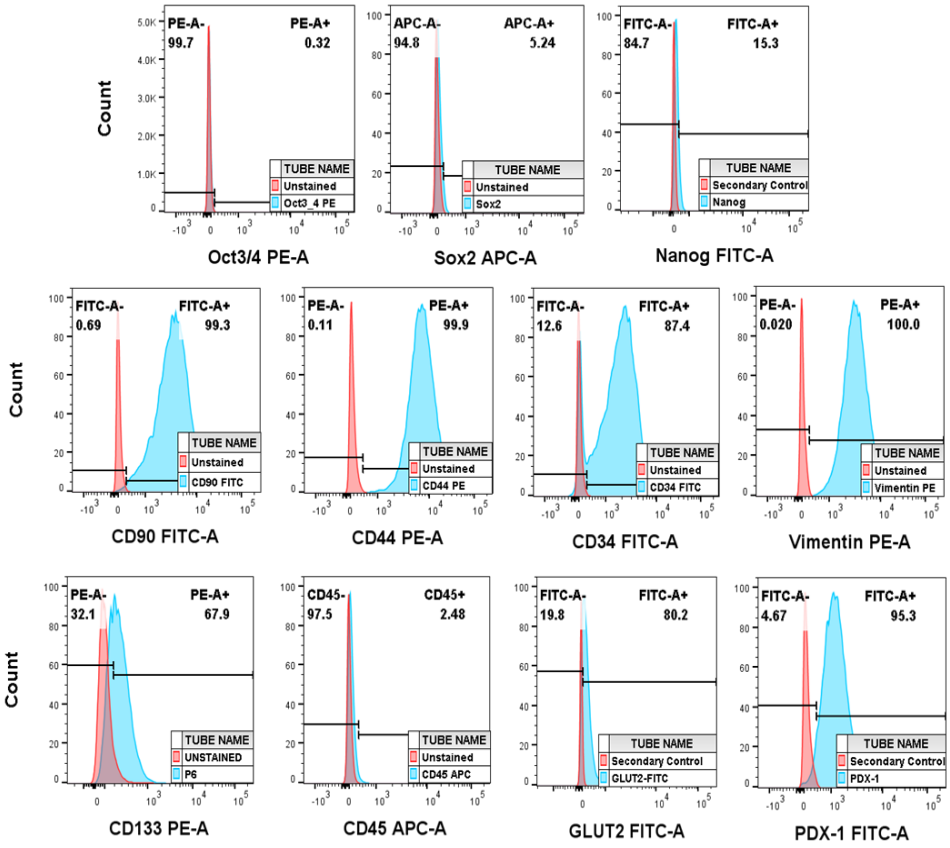


Fig 3.5: Characterisation of Pancreatic resident endocrine progenitors using Flow Cytometry: PREPs were negative for pluripotency markers. However, MSC markers such as CD90, CD44 and Vimentin were positive and negative for CD45. PREPs were also positive for CD34 (a progenitor marker) along with markers like GLUT2 and PDX1 (N=3).

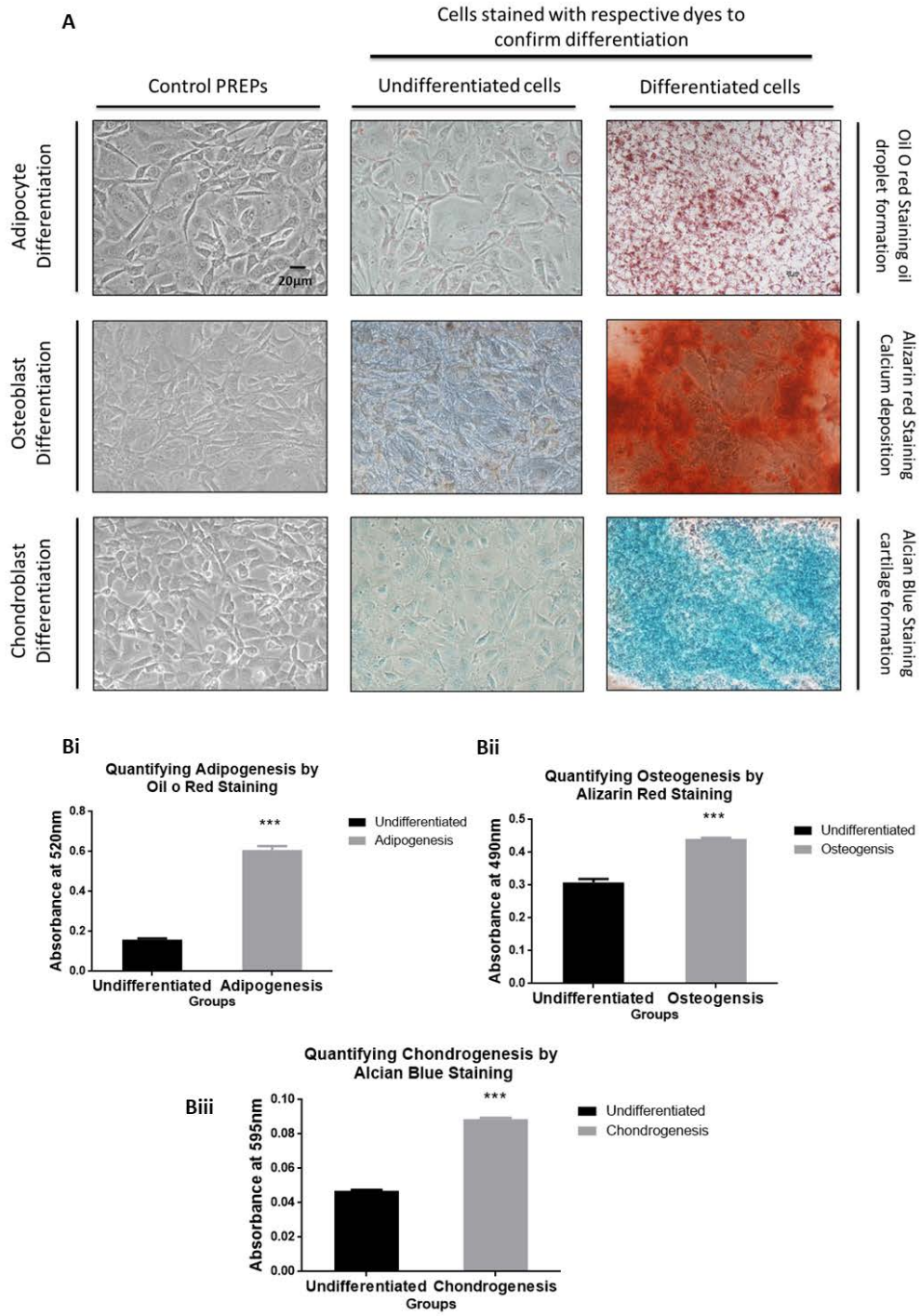


Figure 3.6: Characterisation of PREPs into mesenchymal stem cells by trilineage differentiation: PREPs were induced for Adipogenic, Osteogenic and Chondrogenic differentiation and finally confirmed by staining with respective dyes. Comparative, statistical analysis of extent of trilineage differentiation is depicted by graphs of extracted stains from undifferentiated and differentiated cells from Adipogenesis, Osteogenesis and Chodrogenesis respectively. The graphs are plotted with mean values ± SEM. *** p≤0.001undifferentiated vs differentiated (N=3).

3.3.3.iii Chondrocyte Differentiation:

Finally, in mesodermal lineage differentiation of PREPs to chondrocytes was performed. In this experiment, PREPs were kept in the chondrocyte differentiation media for exactly 30 days. These cells were stained with alcian blue stain to seek the presence of proteoglycan aggrecan, which is an indicator of cartilage formation. The blue stain confirmed chondrocyte differentiation (Fig 3.6A and 3.6Biii).

All the data presented for immunocytochemistry, flow cytometry and trilineage differentiation provided us substantial evidences that PREPs are of pancreatic endocrine origin that possess MSC characteristics. Hence, these cells were defined as mice pancreatic resident endocrine progenitor cells (PREPs).

3.3.4.1 Differentiation of PREPs into islet like cell clusters in DMEM Ham's F-12 (1:1 8 mM Glucose):

The purified and characterised PREPs were subjected to islet differentiation using Swertisin and Activin A respectively as described in the timelines. The initial sets of experiments were performed with DMEM Ham's F-12 (1:1, 8 mM Glucose) as a basal media in a serum free condition along with other differentiating factors. Here, we observed that it took 8 days for PREPs to differentiate into mature islet morphology, which we then confirmed by DTZ staining. We further performed functional and molecular characterization of these islets by immunecytochemically staining them with Insulin and Glucagon, which were positive in these islets followed by a temporal protein profile which provided us with the evidence that Swertisin followed a similar signaling cascade as Activin A (Fig 3.7).

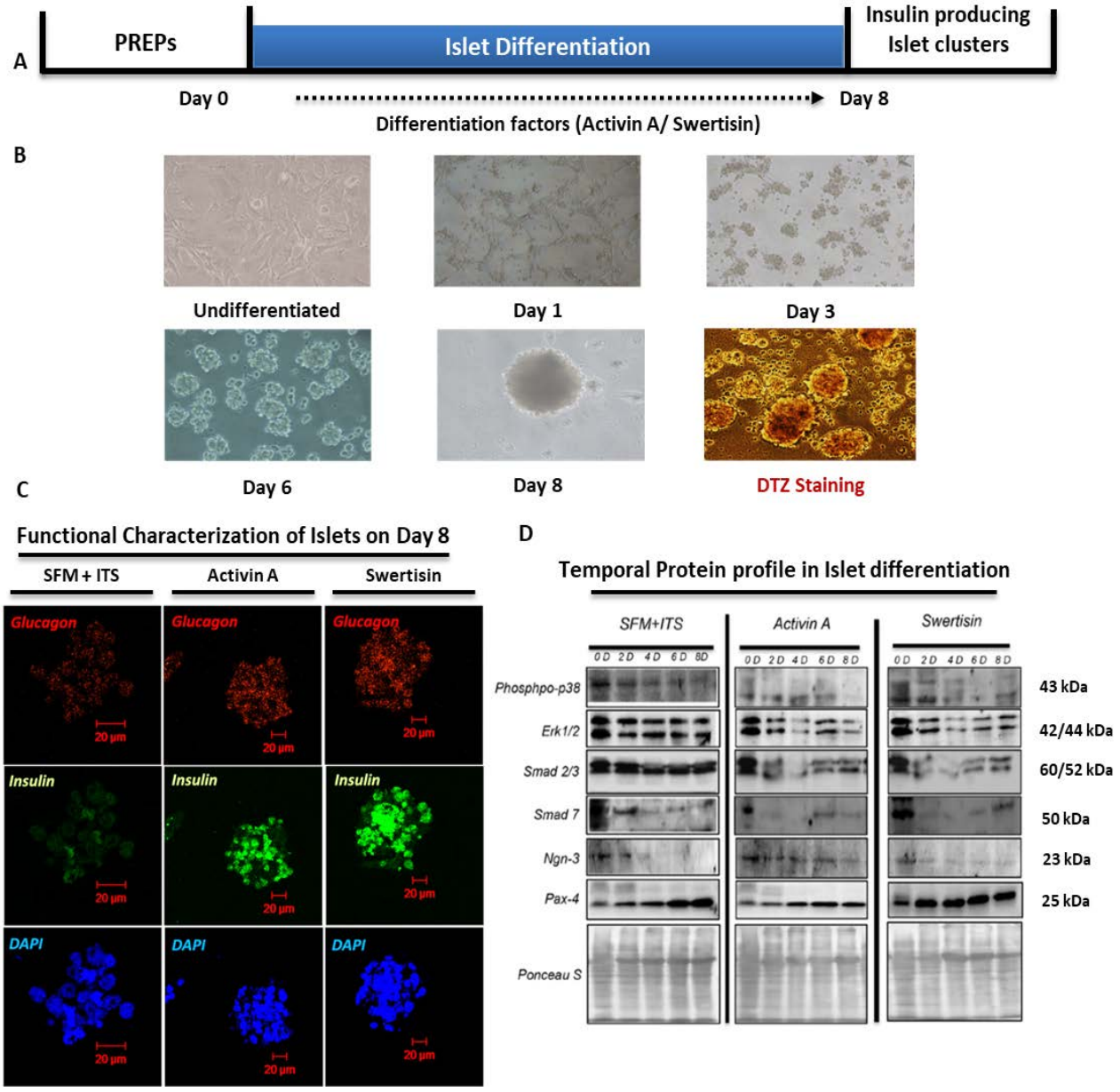


Fig 3.7: Islet Differentiation from PREPs in DMEM Ham's F-12 (1:1, 8 mM Glucose): (A) Islet Differentiation timeline is described. (B) Representative Microscopic morphological observed images are demonstrated during islet differentiation. It can be observed that cells begun to migrate to form clusters which slowly form mature islets at day 8 of differentiation. To confirm mature islet formation DTZ staining was performed. (C) A fluorescent image represents immunostaining for insulin (green) and glucagon (red) in clusters from SFM/ITS, Activin-A and Swertisin groups. DAPI was used as nuclear stain. (D) Demonstrates immunoblotting of key differentiation pathway parameters that indicate the conversion of PREPs into islet like clusters. Key transcription factors and MAP Kinase pathway proteins like P-p38, Erk1/2 Ngn-3, Pax-4, and Smad proteins under differentiation were monitored. Ponceau S staining was used as loading control (N=3).

3.3.4.2 Differentiation of PREPs into islet like cell clusters in DMEM KO (25 mM Glucose):

Later on in order to optimize the islet differentiation culture conditions, we switched to DMEM Knockout as the basal media with serum free condition along with the other differentiating factors on non-cell culture treated plates. We observed completely mature islets at day four of differentiation in both groups, whereas the serum free media group (SFM) resulted in immature loose small cell clusters, which served as the negative control with respect to the activity of Swertisin and Activin A. In the course of differentiation, cells migrated towards a central zone to form spherical islet cell clusters but here we observed cluster formation from day one itself. To confirm positive islet differentiation, these clusters were stained with diathiazone (DTZ), which stains the zinc present within the mature islets bound to insulin. We observed a positive staining of DTZ in the islet clusters from in both Swertisin and Activin A groups, whereas there were no islet clusters that positively stained with DTZ in the SFM group. This confirmed the formation of mature islets in both Swertisin and Activin A treated groups and incomplete differentiation with immature islets in the SFM group (Fig 3.8). Also, with DMEM KO PREPs took shortest route to islet differentiation i.e. 4 days against DMEM Ham's F-12 where islet formation took 8 days.

We observed that the clusters formed in both Swertisin and Activin A treated groups were well compact and spherical with their size mainly between 100 to 300 microns. Whereas in comparison to the SFM group which had mostly smaller and loose clusters, the islet sizes were significantly less in the range of 100 to 300 microns and were mostly smaller than 100 microns, which suggested that the SFM group alone was insufficient to completely differentiate PREPs into Islet of Langerhans. Also, the efficiency of islet formation was significantly better in the Swertisin group when compared to Activin A group (Fig 3.9).

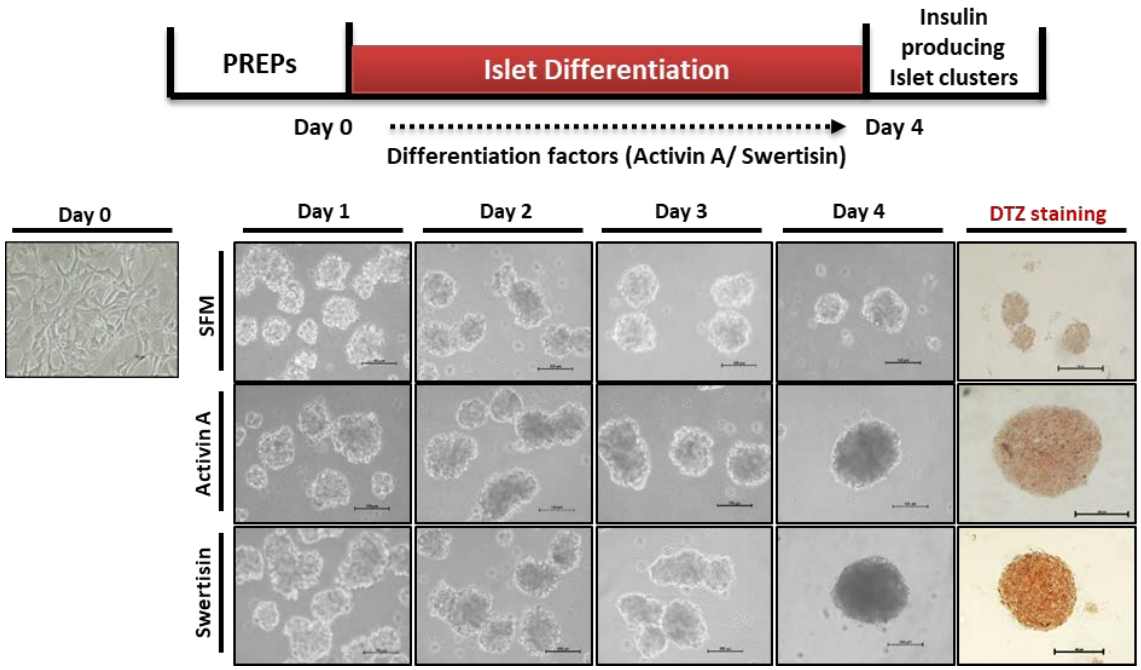


Fig 3.8: Islet Differentiation from PREPs in DMEM KO: Islet Differentiation timeline is described. Microscopic morphological changes were observed during islet differentiation by using both Swertisin and Activin A as differentiating agents. It can be observed that Islet clusters begin to form from day 1 itself which are then allowed to mature till day 4 of differentiation. To confirm mature islet formation DTZ staining was performed. Islets generated in the Activin A and Swertisin groups stained positive for DTZ confirming presence of insulin within, whereas SFM islets were negatively stained for DTZ suggesting incomplete or no differentiation (N=6). Scale bar represents 20 at day 0 and 100 microns everywhere else.

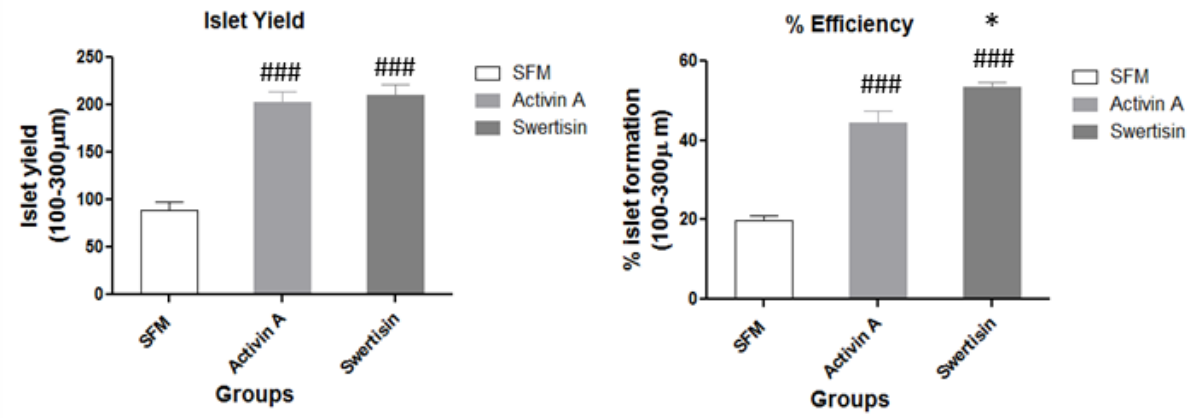


Fig 3.9: Islet yield and percentage efficiency: A comparison between total numbers of islet clusters formed between 100-300 µM diameter across the groups were made, to understand the effective islet yield after differentiation. The second graph signifies the percentage efficiency of islet formation across groups. The graph plotted is with mean values ± SEM. ### p≤0.001 SFM vs Activin A and Swertisin; *p≤0.05 Activin vs Swertisin (N=3).

3.3.5 Functional Characterisation of differentiated islet clusters:

The SFM, Activin A and Swertisin islet clusters were then evaluated for the presence of insulin (red) and glucagon (yellow-pseudo colour) to identify the incidence of terminally differentiated beta and alpha cells respectively by immunocytochemistry using a confocal microscopy. We observed the presence of both insulin and glucagon in the differentiated islets of both Activin A and Swertisin groups. We also observed intense staining for Nkx6.1 (green) and Glut2 (red) in these islet clusters. However, in the SFM group we observed very less Insulin, Nkx6.1 and Glut2 and more of Glucagon suggesting incomplete differentiation. These observations confirmed the formation of mature islet clusters synthesizing pancreatic endocrine hormones and glucose transporters, which are all necessary for glucose homeostasis in the Activin A and Swertisin groups (Fig 3.10).

Finally, Islets of Langerhans must sense increase in glucose for their proper functioning. We challenged these islet clusters with 5mM and 20mM glucose concentration and measured their c-peptide release using c-peptide ELISA. We observed a significantly more c-peptide release in 20mM Glucose exposure compared to 5 mM in both Activin A and Swertisin treated groups with respect to the SFM group. Swertisin islet clusters released significantly more c-peptide than Activin treated, which again complements all our above data. Hence, this confirmed that the islets generated using Swertisin were mature and completely functional (Fig 3.11).

3.3.6 Time dependent gene and protein profiling of Islet Clusters differentiated from PREPs:

In an earlier report we have demonstrated Swertisin's molecular mechanism in islet differentiation both *in vitro* and *in vivo.* Here we observed that Swertisin like Activin A follows AKT-MEPK-TKK pathway (Dadheech et al., 2015).

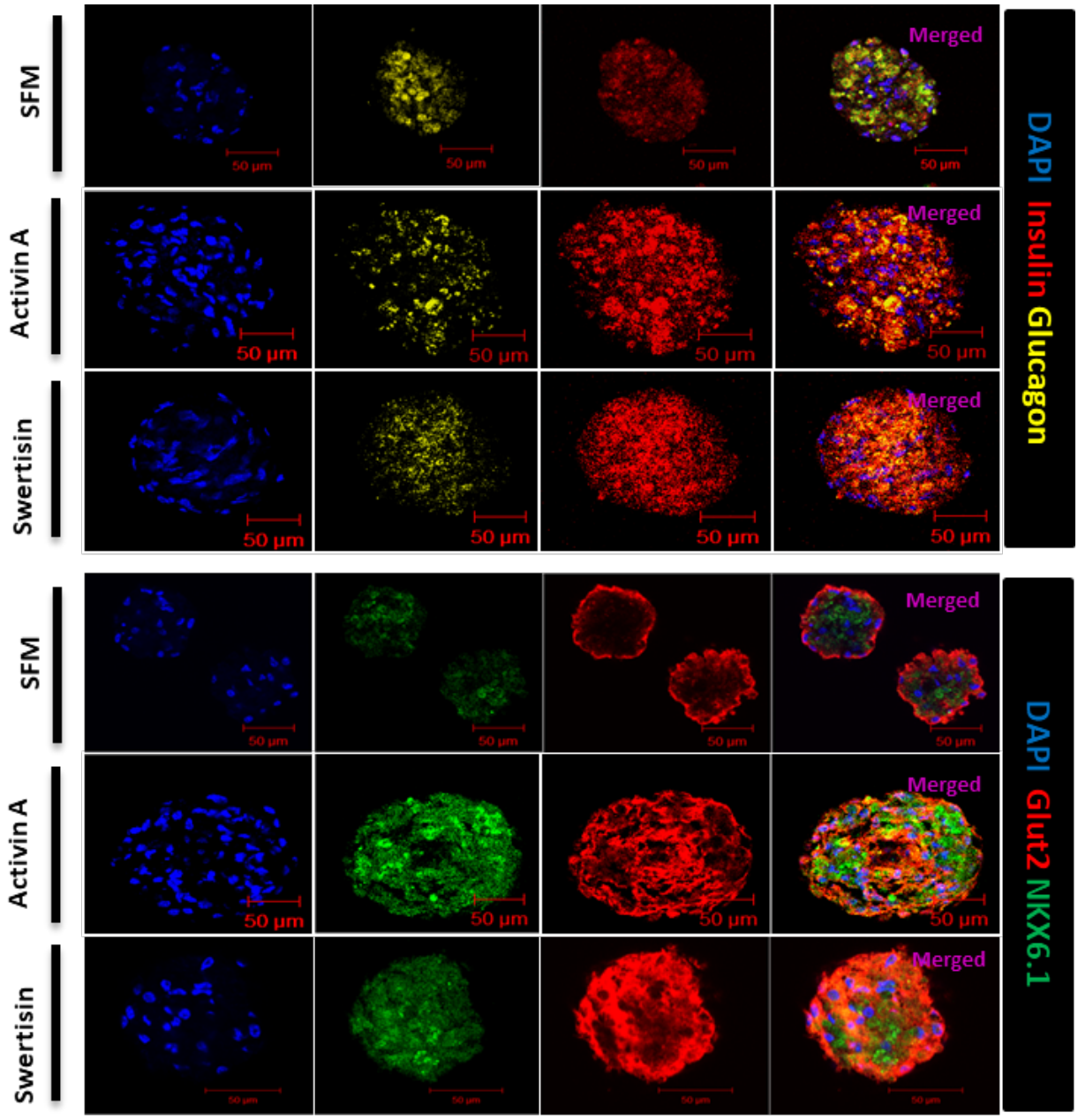


Fig 3.10: Functional Characterization of mature Islets differentiated from PREPs: Immunocytochemistry of mature islets demonstrating the presence of Insulin (red) along with glucagon (yellow-pseudocolor) and nkx6.1 (green) along with Glut2 (red) in differentiated islets in SFM, Activin A and Swertisin groups. Nuclei were stained with DAPI (blue) (N=3).

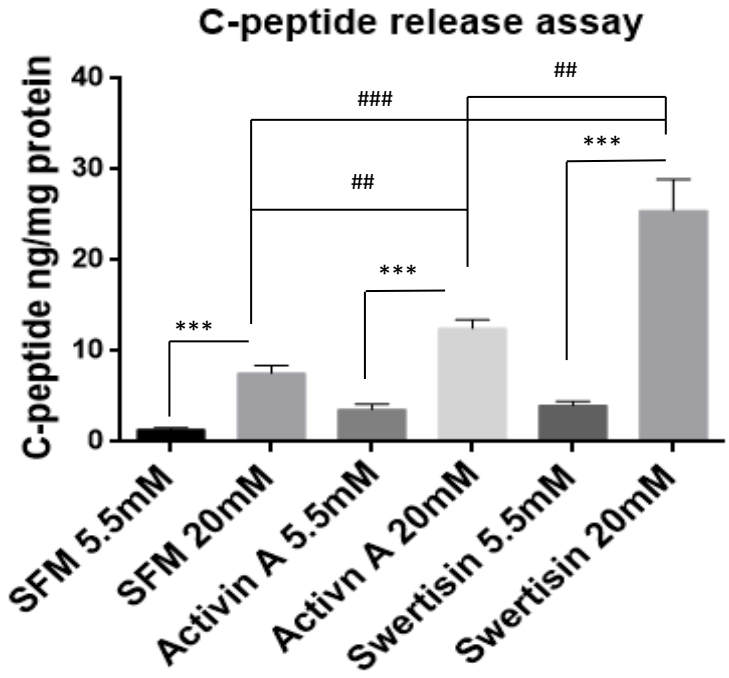


Fig 3.11: Functional Characterization of Islets: C-peptide release assay was performed to confirm the responsiveness of differentiated islets to the presence of glucose. The graphs are plotted with mean values ± SEM. *** p≤0.001 5.5mM SFM/Activin A/Swertisin vs 20mM SFM/Activin A/Swertisin. ## p≤0.01 20mM SFM vs 20mM Activin A and 20mM Activin A vs 20mM Swertisin; ### p≤0.001 20mM SFM vs 20mM Swertisin (N=3).

Further, to trace temporal expression of various genes during the process of islet differentiation, PREPs were introduced to Islet differentiation media (DMEM KO) with respective differentiating agents. The differentiated islet samples were harvested every day across groups for a period of four days, which were analysed for their gene and protein expression by qRT-PCR and western blotting respectively (Table 3.1).

Gene expression profile for the important transcription factors (TFs) viz. *Pdx1, Neurog3, NeuroD and Nkx6.1* along with hormones *Insulin and Glucagon* was performed. With respect to TFs a general pattern was observed from the undifferentiated PREPs at Day 0 to the mature islets at Day 4. A gradual increase in all the TFs and the two hormones was observed throughout the gene expression plots. Also, the expression of TFs in the SFM group was lower than Activin A and Swertisin. Interestingly, we observed an increase in the insulin transcript levels in the Swertisin group compared to Activin A and SFM groups at Day 4 with upregulated Glucagon levels in the SFM groups in the later time points compared to other groups (Fig 3.12a-f).

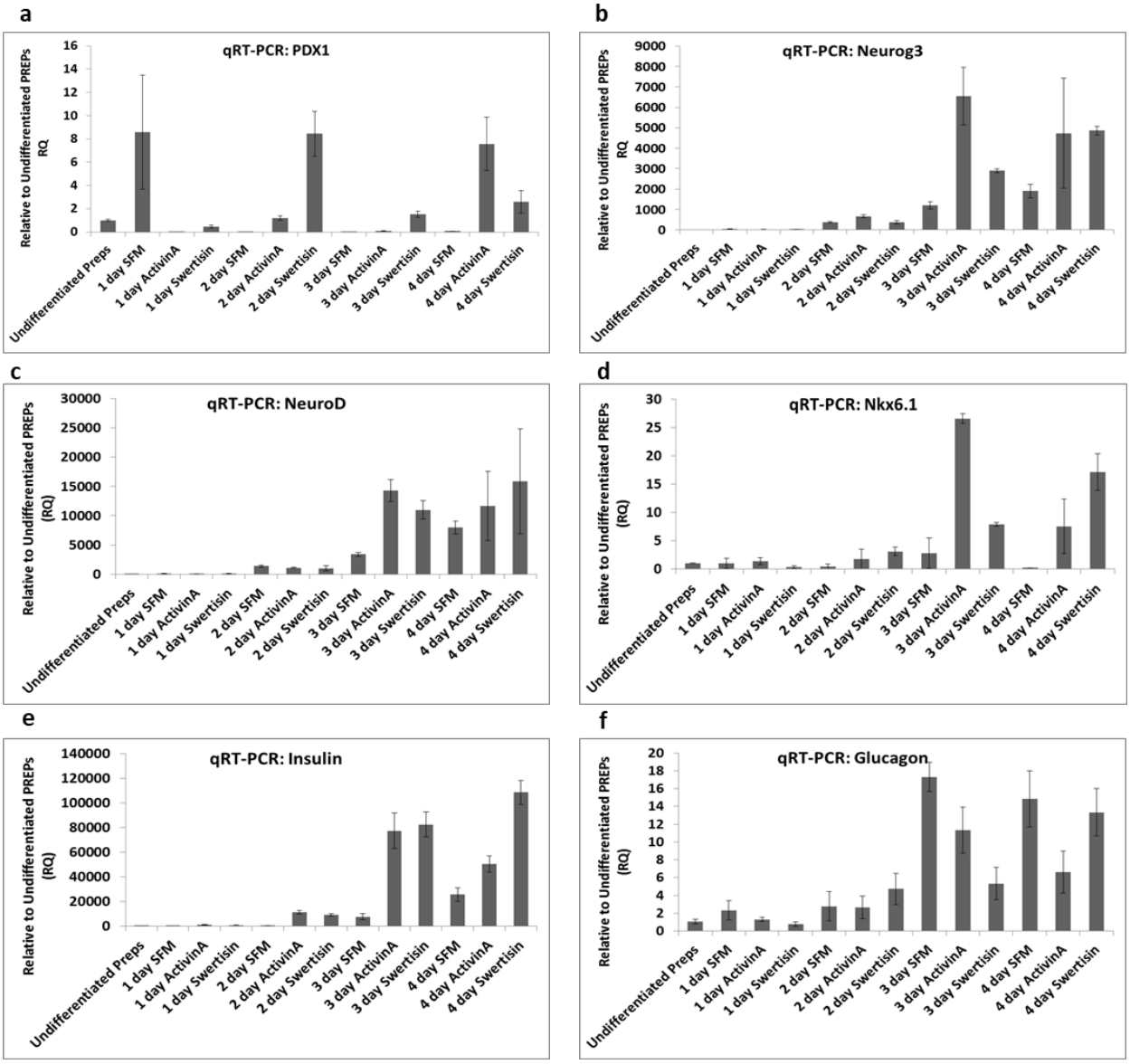


Fig 3.12a-f: Temporal gene and protein profiling in Islet Differentiation from PREPs:(a) Temporal day wise Pdx1 expression during islet differentiation from PREPs for SFM, Activin A and Swertisin groups. (b) Temporal day wise Neurog3 expression during islet differentiation from PREPs for SFM, Activin A and Swertisin groups. (c) Temporal day wise NeuroD expression during islet differentiation from PREPs for SFM, Activin A and Swertisin groups. (d) Temporal day wise Nkx6.1 expression during islet differentiation from PREPs for SFM, Activin A and Swertisin groups. (e) Temporal day wise Insulin expression during islet differentiation from PREPs for SFM, Activin A and Swertisin groups. (f) Temporal day wise Glucagon expression during islet differentiation from PREPs for SFM, Activin A and Swertisin groups. The data has been normalized to the expression of β-Actin which was taken as endogenous control. N=3.

The western blot analysis painted a unique ability of these cells, which help them differentiate so rapidly and make their route to the islet differentiation the shortest. The first important observation was the presence of pancreatic transcription factors like PDX1, Neurog3, NeuroD, MafA, Pax4 and Nkx6.1 in the undifferentiated PREPs along with along with expression of phosphorylated Smad3. This observation lead us to believe that these cells already possessed the machinery required for them to form islet clusters and produce insulin in mere four days, which would be the shortest reported time for mature islet differentiation from stem/progenitors. The second observation was that the expression of stem cell markers both Nestin and CD133 for Activin A and Swertisin treated groups decreased rapidly and significantly, which ultimately by day four was completely abolished confirming PREPs losing their progenitor phenotype and becoming terminally differentiated. However, in the SFM group persistent expression of Nestin and CD133 suggested PREPs inability to differentiate into islet clusters complementing the microscopic and DTZ staining data (Fig 3.12). Thirdly, the signalling molecules in the AKT-MEPK-TKK pathway were activated on induction with Activin A and Swertisin. In both these groups, we observed elevated phosphorylation of smad3 and increased expression of smad4 along with decreased expression of inhibitory smad7. Phosphorylation of p38MAPK was also observed to be elevated. All these regulatory signalling protein activations were observed to be more elevated in the Swertisin group when compared to the ActvinA treated group (Fig 2p). Lastly, all the transcription factors viz. PDX1, Neurog3, NeuroD, Pax4, MafA and Nkx6.1, which are absolutely necessary for the development of endocrine pancreas and its functioning, were temporally up regulated in the course of islet differentiation. Also, if we focus on Nkx6.1 alone, which is the marker for terminally differentiated insulin producing beta cells, it was observed to be weakly expressed in the SFM group suggesting poor or incomplete differentiation, where as its expression is significantly elevated in the other two groups.

Nkx6.1 expression at day 4 in the Swertisin treated group was significantly more than the Activin treated group suggesting more efficient differentiation. The endogenous control taken was beta actin (Fig 3.13).

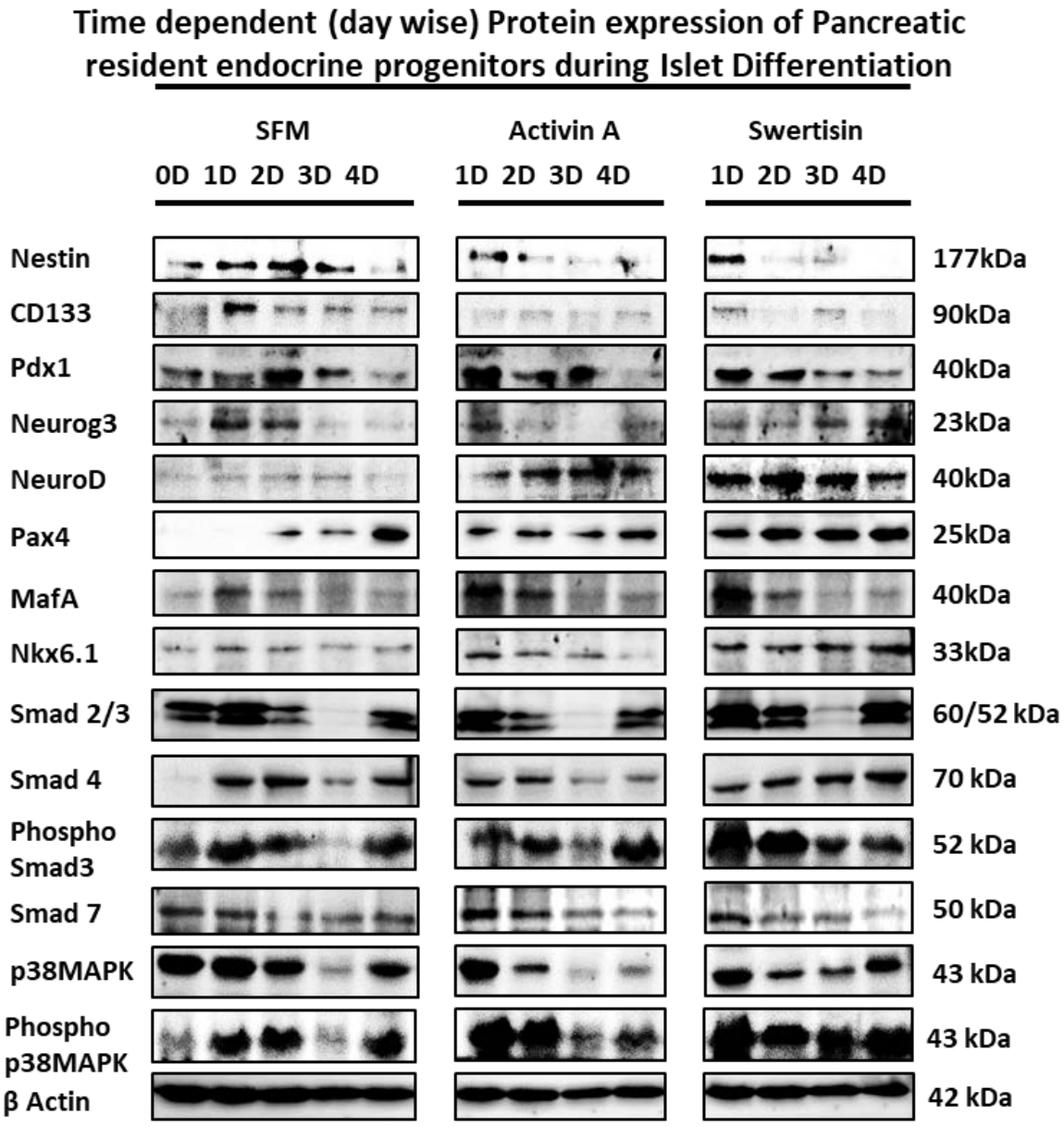


Fig 3.13: Protein profiling in Islet differentiation from PREPs: Temporal protein profiling was performed from undifferentiated state to day 4 of islet differentiation, which demonstrated the kinetics of various stem cell markers and key transcription factors along with kinetics of various TGF-β signaling molecules islet differentiation and β-actin as endogenous control (N=3).

3.4. Discussion

Our study here was an attempt to understand the fidelity of pancreatic progenitors in treating diabetes mellitus type I, which could be extrapolated to the chronic type II DM condition with prominent beta cell death. In the present study, we have also attempted to comprehend the characteristics of pancreatic resident endocrine progenitors. We believe that with the current advancement in regenerative medicine, the focus has been shifted more towards Embryonic (ESCs) and induced pluripotent (iPSCs) stem cells, which have developed a blind spot for the body's own inherent capacity to regenerate. Hence, we wanted to explore the islet neogenic potential of pancreatic resident endocrine progenitors under influence of certain bioactives viz. Swertisin and Activin A in the *in vitro* conditions.

We isolated PREPs from BALB/c mouse pancreas. Islets were first isolated and cultured in RPMI1640 complete media for 48 hr. and then transferred to DMEM high glucose complete media. The islets after adhering disaggregated and formed a monolayer. This monolayer was sequentially passaged until a homogenous cell population was achieved. These cells exhibited characteristics of previously reported pancreatic progenitors with certain profound discrepancies from the reported literature. These cells were positive for Nestin, Pdx1, Neurog3, and negative for Insulin and Glucagon. This is a classical pancreatic progenitor signature (Banerjee and Bhonde, 2003; Zulewski et al., 2001). However, we also observed presence of markers such as Ki67 and CK19. Ki67 presence indicates the proliferative nature of these cells (Jiang et al., 2011). Finally, presence of CK19 suggested their pancreatic ductal origin. Hence, we believe that these cells which were positive for both Nestin and CK19 are pancreatic progenitors that had a ductal origin but have migrated into an islet niche for neogenesis (Zulewski et al., 2001). Further, these progenitors were positive for mesenchymal stem cell markers like CD44, CD90 and Vimentin, which has been found positive in Nestin positive progenitors and negative for CD45. However, these cells were unique in expressing

CD34. All the previous data on pancreatic progenitors have suggested its absence but we observed a robust presence of CD34 in these progenitors (Zanini et al., 2011; Zhang et al., 2005). There has been a growing belief among the scientific community that CD34 positivity is a prominent characteristics of cells with progenitor activity (Sidney et al., 2014). CD133 is another progenitor marker present abundantly in these cells. It has been reported as a maker that can be used to enrich the pancreatic progenitor population (Sugiyama and Kim, 2008). Finally, Glut2 was abundantly present in this population. Presence of Glut2 in pancreatic progenitor originating from duct has been previously reported (Pang et al., 1994). Presence of Glut2 positive progenitor population was observed in STZ treated normoglycaemic mice attained after insulin treatment. They also suggested that hyperglycaemic conditions decreased Glut2 expression, which was not in our case as we have isolated and cultured these cells strictly in proliferative media containing 25 mM glucose (Guz et al., 2001). Hence, the two unique markers for these cells were CD34 and GLUT2, which may be used for enriching and purifying these cells. These cells demonstrated Adipogenic, Osteogenic and Chondrogenic ability establishing their multipotent characteristic, which was reported previously (Zanini et al., 2011).

It has been observed that beta cells within the Islets of Langerhans succumb to ROS produced due to glucotoxic environment as they lack effective ROS scavenging mechanism (Gorasia et al., 2015). The Progenitors isolated were throughout cultured and differentiated in a media (DMEM high Glucose and DMEM Knockout respectively) with 25mM glucose hence constantly maintained in high glucose conditions mimicking the diabetic sugar environment. There have been many reports that suggest that the pancreatic progenitor population not only thrives in the high glucose environment but these conditions stimulate islet neogenesis (Kim et al., 2013). On subjecting these cells to *in vitro* islet differentiation, they started forming clusters from the first day of differentiation and matured into islet clusters in a span of mere

four days. We suspected that this impressively fast differentiation capability of PREPs was the result of them taking the shortest route for islet differentiation under the specific conditions provided, which was not observed with DMEM Ham's F-12 (1:1, 8 mM Glucose) media having 8 mM glucose (Gopurappilly et al., 2013; Venkatesan et al., 2011). Further, the rapid differentiation was observed in both Activin A and Swertisin induced groups. Our lab previously reported that both Activin A and Swertisin induced islet differentiation follow AKT-MEPK-TKK pathway, where Swertisin's effective treatment demonstrated triggering of TGF-β pathways for islet neogenesis in 70% pancreatectomised diabetic mice model (Dadheech et al., 2015). The temporal protein expression with respect to islet differentiation from undifferentiated cells at day zero to completely mature islet clusters at day four evidently confirmed high islet forming fidelity. The undifferentiated progenitors demonstrated basal expression of the key transcription factors viz. Pdx1, Neurog3, NeuroD, MafA and Nkx6.1 required for endocrine pancreatic development and function along with activation of key signalling molecules like pSMAD3 and pP38MAPK (Kim and Hebrok, 2001; Murtaugh, 2007). This gave us a clear understanding of the inherent potential of these cells to differentiate into islet clusters, justifying their name as pancreatic resident endocrine progenitors (PREPs). Further, TGF-β signaling has also been reported to enhance islet function by increasing their glucose responsiveness (Bertolino et al., 2008). It has been demonstrated to upregulate Insulin and Glut2 expression along with key transcription factors involved in islet neogenesis and homeostasis i.e. Pdx1, NeuroD, Pax4 and MafA (Matsuoka et al., 2007; Sosa-Pineda, 2004). Hence, it not only aids in beta cells differentiation but also regulates beta cell mass. Further, it has been observed that Activin A via Smad signaling also downregulates glucagon gene expression in alpha cells in human islets (Mamin and Philippe, 2007; Yasuda et al., 1993). This supports our results as we observe similar results with both Activin A and Swertisin groups while in the SFM group we observe that most of the cells

were glucagon positive where both gene and protein expression of glucagon was highly upregulated. Also, we have previously demonstrated that blocking Activin signaling in vitro during islet differentiation from stem cells completely abolished islet formation (Dadheech et al., 2015). Thus, TGF-β signaling is imperative for islet neogenesis, regulating β-cell mass and/or proper insulin release in response to glucose. Also, if we focus on the differentiation of these cells, it can be observed that SMAD7, which is an inhibitory SMAD, was expressed highly in the undifferentiated cells thus inhibiting the islet differentiation but with the induction and progression of islet differentiation its expression subsides with increasing expression of regulatory Smad4 and phosphorylation of SMAD3. Further, phosphorylation of pMAPK also increases, which propelled the differentiation forward by upregulating the above-mentioned key transcription factors. Since, the machinery required for islet differentiation was inherently present in these cells, stimulating them with islet differentiating agents and providing the right conditions induced the shortest route ever reported for any stem/progenitor population for the same (Lina Sui, 2013; Wen et al., 2011). There have been many protocols developed over the years using Embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and various mesenchymal stem cells (MSCs) using various growth factors and differentiating agents which extends for several weeks with various stages in differentiation making the process increasingly complex and cumbersome (Wong, 2011). Taking into account the process, time taken and results obtained, we describe the shortest differentiation process with effective and desirable outcome. Complete differentiation was marked by the abatement of stem cell markers viz. Nestin and CD133 at day four of differentiation. Islet differentiation was significantly more efficient in the Swertisin induced group as compared to the SFM and Activin A groups. However, with SFM stem cell markers were persistent throughout the differentiation indicating incomplete or no differentiation. These islets were further functionally characterised. Although, the islets generated from both

the groups were functional i.e. expressed insulin transcript, were substantially positive for hormones insulin and glucagon, expressed Glut2 and Nkx6.1 (beta cell marker) and demonstrated glucose stimulated c-peptide secretion; we observed that Swertisin induced islets were significantly superior across all the functional parameters, thus generating better quality of Islets of Langerhans.

Sr. No.	Gene Name (Mus musculus)	Gene Accession no.	Primer Forward seq.	Primer Reverse seq.	PCR Condition (Tm)
1	Pdx1	NM_008814	5'CAAAGCTCACGCGTGG AAAG 3'	5'TGATGTGTCTCTCGG TCAAG 3'	60
2	Neurog3	NM_009719	5'GCGCAATCGAATGCAC AACCTCAA 3'	5'TTGAGTCAGCGCCCA GATGTAGTT 3'	60
3	NeuroD	NM_010894	5'TCTTTCAAACACGAACC GTCC 3'	5'AGATTGATCCGTGGC TTTGG 3'	60
4	Nkx6.1	NM_144955	5'GCCTGTACCCCCCATCA AG 3'	5'ACGTGGGTCTGGTGT GTTTTC 3'	60
5	Insulin (Ins1)	NM_008386	5'TGG CTT CTT CTA CAC ACC CAA3'	5'ACA ATG CCA CGC TTC TGC 3'	60
6	Glucagon (Gcg)	NM_008100	5'ATG AAG ACC ATT TAC TTT GTG GCT3'	5'GGT GTT CAT CAA CCA CTG CAC3'	60
7	β-Actin	NM_007393	5'GGT GTG GTG CCA GAT CTT GC3'	5'ACG AGG CCC AGA GCA AGA3'	60

Table 3.1: List of Primers for qRT-PCR.