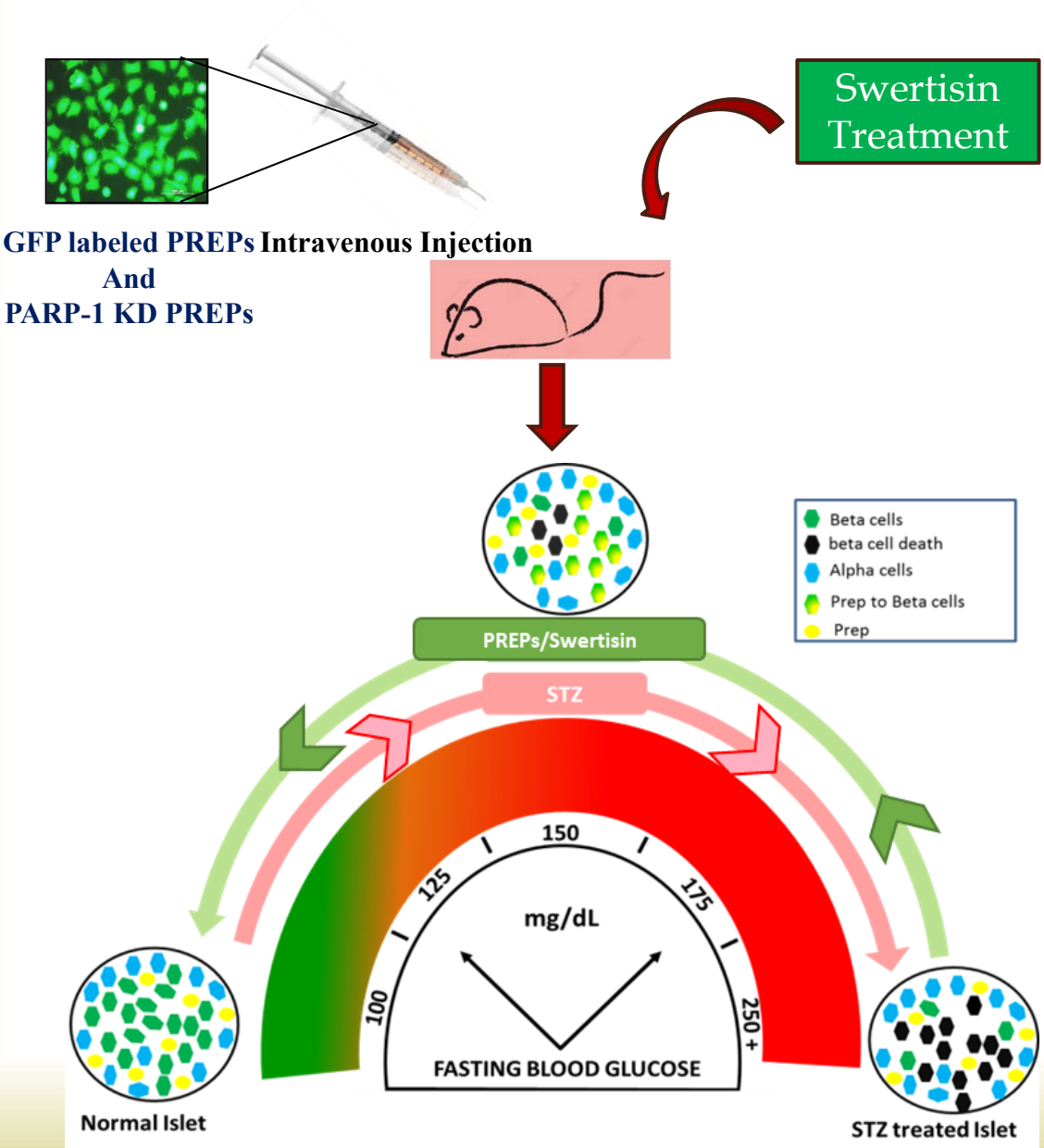
IN VIVO ASSESSMENT OF ROLE OF PARP-1 IN PANCREATIC REGENERATION IN MICE

CHAPTER 5

Chapter 5

In vivo assessment of role of PARP-1 in pancreatic regeneration in mice



5.1.Introduction:

Stem/progenitor cell therapy have been demonstrated to alleviate the diabetic condition post transplantation effectively (Banerjee and Bhonde, 2003a; Ramiya et al., 2000). Popular convection although still controversial, suggests that there exists pancreatic progenitors that migrate and differentiate into islets when needed (El-Gohary et al., 2016). The advantage of pancreatic progenitors is to differentiate very efficiently and very quickly into islets or insulin producing beta cells with minimal *in vitro* manipulation (Noguchi, 2010). It has been previously reported and also demonstrated in earlier chapter, that pancreatic progenitors also possess Mesenchymal stem cells (MSCs) phenotype which is an indication of their self-replicative and potent differentiation potential (Gopurappilly et al., 2013; Zhang et al., 2005).

For over a decade now researchers have been exploring the potential of MSCs in preclinical and clinical studies to explore better therapeutics that can resolve injuries by enhancing endogenous repair, thus opening a new paradigm in cell therapy. Mesenchymal stem cell population is present in almost every organ of our body in a percentage of 0.01% to 0.001%. There are many reports where researchers have purified MSCs from pancreas, bone marrow, skin, hair follicles, umbilical cord, etc. MSCs have also been extensively used in diabetes research owing to their clinical acceptability, versatile source and differentiation flexibility (Pileggi, 2012). Since, islet graft rejection is one of the biggest hurdles in translating a successful regenerative therapy, immuno modulatory and regenerative properties of MSCs makes its use more imperative among the researchers to explore them as potent stem cell cure for Type 1 diabetes. MSCs regulate immune responses which include (i) reducing the generation and differentiation of Dendritic Cells, (ii) increasing the number of Tregs through production of TGF-β, or promoting the generation of regulatory Dendritic Cells producing IL-10, (iii) suppressing effector T cells through various growth factors, inducible nitric oxide synthase (iNOS), hemeoxygenase (HO)-1, prostaglandin, or indoleamine 2,3-dioxygenase

(IDO), (iv) downregulating immunoglobulin production by B cells,(v) inhibiting NK cell cytotoxicity and proliferation (Abdi et al., 2008; Sakata et al., 2011). Also, insulin producing islet like cells clusters differentiated from MSCs have shown to improve hyperglycaemia in diabetic animal models upon transplantation (Lin et al., 2009; Zhao et al., 2012). To understand various regeneration promoting properties of MSCs researchers have used MSCs in combination with pancreatic islets. This lead to couple of very interesting and encouraging observations- when MSCs were co-cultured with pancreatic islets, it was found that Islet behaved as a stem cell niche providing a perfect microenvironment to these cells to differentiate into more insulin producing cells (Karaoz et al., 2011; Rackham et al., 2013). In another report when MSCs were co-transplanted with islets,better improvement in glycaemic index was observed in diabetic animals against islets alone transplantation. Co-transplantation of MSCs with pancreatic islets further demonstrated improved islet graft function by promoting graft vascularization(Figliuzzi et al., 2009; Ito et al., 2010; Rackham et al., 2013). In another study by Park et al, 2010,MSCs has been shown to release several growth factors that support cell survival and when islets were co-cultured with human MSCs significant presence of various trophic factors were found in the medium. These cocultured islets demonstrated lesser ADP/ATP ratio, less apoptosis and better GSIS index with enhanced islet survival and better function after transplantation (Park et al., 2010). There have been reports of using a combination of different MSCs e.g. transplantation of human bone marrow progenitors followed by umbilical cord blood progenitors improved hyperglycaemia and glucose tolerance by increasing beta cell mass associated with the ductal epithelium and augmenting intra-islet capillary densities. Thus, proving that combinatorial human progenitor cell transplantation stimulated both islet-regenerative and revascularization programs (Bell et al., 2011). As we have earlier demonstrated the role of PARP-1 in islet

neogenesis, we further wanted to extrapolate this resultsin the *in vivo* mice model(Akiyama et al., 2001b).

Here, we transplanted Pancreatic Resident Endocrine Progenitors (PREPs) both PARP-1 knockdown with GFP tag and Vector control (shRNA) with GFP tag into STZ treated diabetic BALB/c mice. The key questions which we addressed are:(i) whetherPREPs with MSC characteristics increases homing towards the injured pancreas and depletion of PARP-1 affect the homing potential of PREPs; (ii)whether homed in PREPs can differentiate into insulin producing cells within pancreas and ameliorate the diabetic condition; (iii) whether PARP-1 depleted cells can differentiate in the pancreatic niche without any external stimuli. (iv) whether Swertisin administration along with both normal and PARP-1 depleted PREPs in the STZ diabetic mice model can increase their homing or differentiation potential upon transplantation.

5.2. Materials and methods:

5.2.1.Animal Selection,Induction of Diabetesand Experimental designs:

Adult virgin female mice of BALB/c strain weighing 20-25 g aged 6-8 weeks were kept at animal house with 12 hours light and dark cycle with water and pellet diet *ad libitum*. Gender was selected as per the availability of mice at the animal house at the time of the study. Diabetes was induced with STZ injection (STZ; 65mg/kg body weight) intraperitoneally for 5 days with overnight fasting. Diabetic status of animals was confirmed by monitoring Fasting Blood Glucose using Accucheck Performa glucometer (Accucheck, Roche, USA) at regular intervals as shown in Fig 5.1 and 5.2 till the length of the experiment.

PREP Transplantation Design: 2 x 10^6 GFP labeled PREPs per mice were transplanted intravenously through tail vein with normal saline on Day 10 of the experiment. Animals were sacrificed and analyzed for various parameters on Day 15 of the experiment. Fasting

serum insulin was estimated and compared within the groups on the day of animal sacrifice(Fig 5.1).

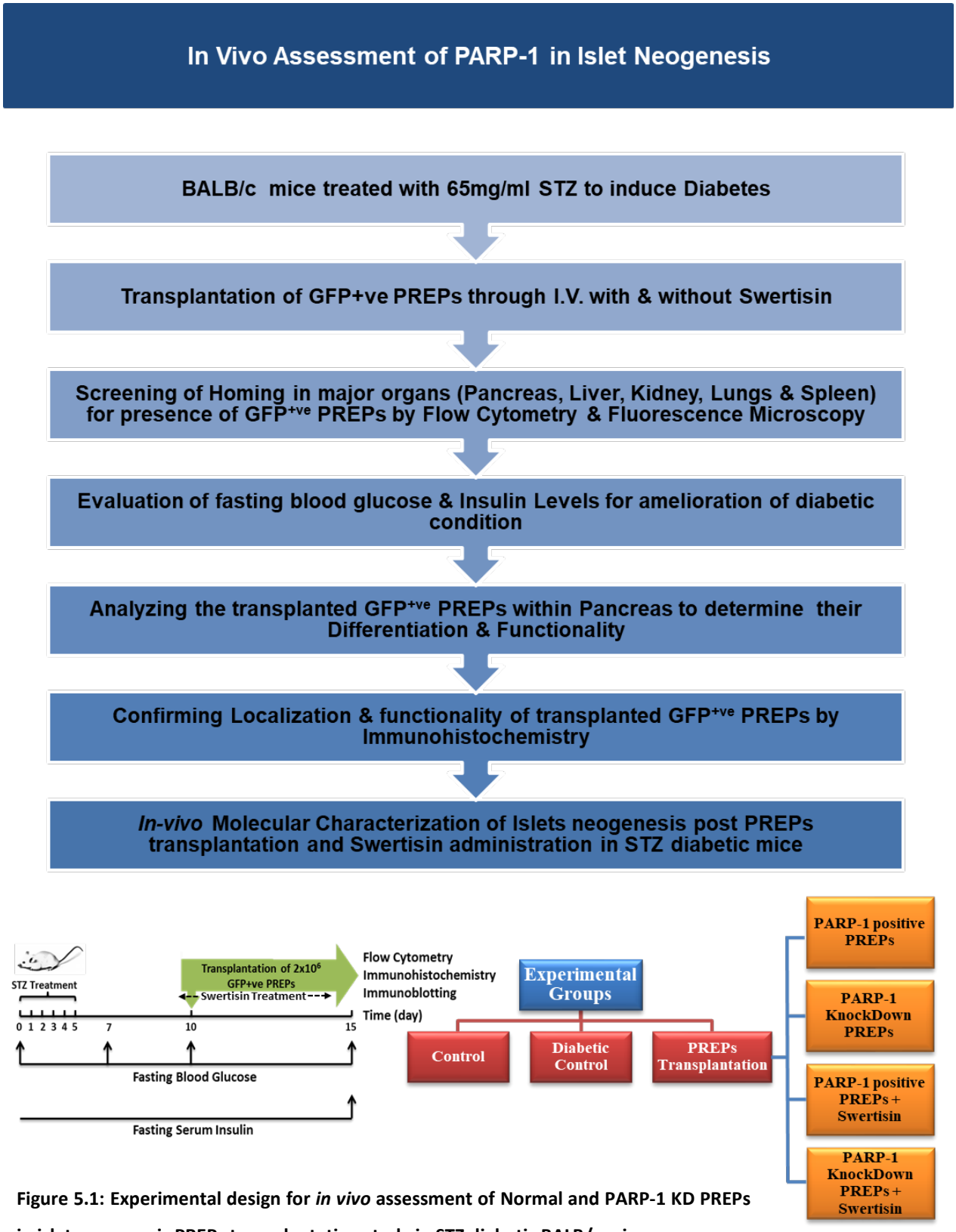


Figure 5.1: Experimental design for *in vivo* assessment of Normal and PARP-1 KD PREPs in islet neogenesis PREPs transplantation study in STZ diabetic BALB/c mice.

Long term Swertisin administration Design: The mice were treated with Swertisin (2.5mg/kg body wt.) from 14th day of experiment till 30th day when the mice were sacrificed. Swertisin was administered with saline intravenously through tail vein. Fasting serum insulin was estimated and compared within the groups on the day of animal sacrifice. Other parameter for amelioration of the diabetic condition and islet neogenesis were assessed (Fig 5.2).

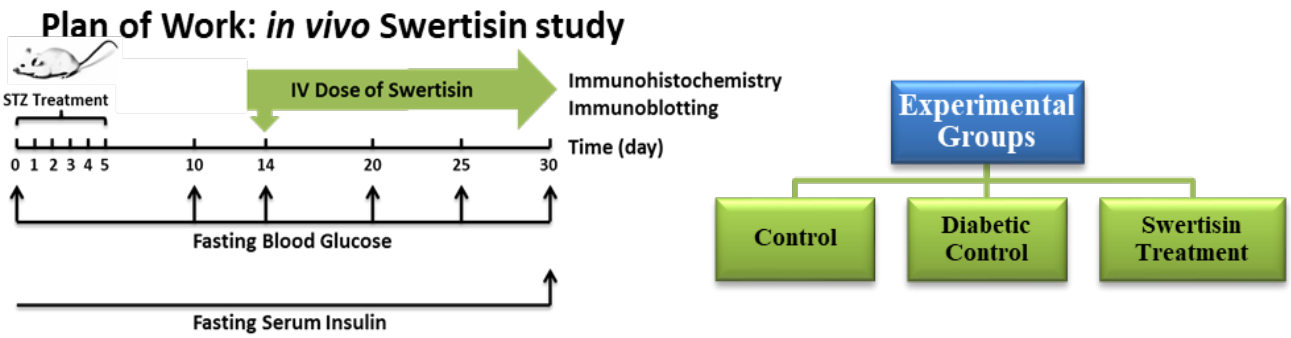


Figure 5.2: Experimental design for Swertisin treatment for extended time period: Schematic representation of the work flow for the *in vivo*Swertisin treatment in STZ diabetic BALB/c mice.

These studies were carried out in strict accordance as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals, India (CPCSEA) after approval from CPCSEA of Department of Biochemistry, The M. S. University of Baroda. Post experiment animals were euthanatized using xylazine (10 mg/kg) and ketamine (150mg/kg) injection followed by cervical dislocation ensuring death.

5.2.2. Chemicals:

As described in chapter 3, section 3.2.2.

5.2.2Transfection and GFP labeling:

As described in chapter 4, section 4.2.4.

5.2.3Flow cytometry for Surface markers from pancreas and liver tissues:

Tissues were minced thoroughly and washed with cold PBS to remove fat. The minced tissue was subjected to chemical digestion by using 0.1% Trypsin EDTA solution with mechanical shaking for 10 min. The digested tissue was washed thrice with cold PBS and filtered through a 70 micron cell strainer.Cell were directly analyzed for GFP. For CD44 staining, 0.5 x 10^6 cells were resuspended per tube in 100 µl of staining media (PBS with 1% BSA) containing antibody with concentration mentioned as per manufacturers guidelines. Cells were incubated for 30 min at 4˚C in dark followed by washing with staining media twice. Further, the cells were suspended in 500 µl staining media and 1 x 10^5 cells were analyzed per tube 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.

5.2.4.Flow cytometry for Intracellular markers:

As described earlier in chapter 3, section3.2.6. The cell suspension was passed through a 70µ strainer and proceeded for GFP acquisition or staining as described above. For GFP and GFP-Insulin dual positive acquisition 100,000 cells were acquired by flow cytometry per tube.

5.2.5.Protein extraction and Western blotting:

Tissue were harvested and kept on ice. They can be stored at -80˚C at this stage. Tissue were minced with the help of a mortar and pestle in liquid nitrogen on ice. The mashed tissue powder was resuspended in Laemmli buffer with 4 M urea and sonicated on ice for 7 cycles of 20 sec with 2 sec on and 0.2 sec off sequence with 2 min on ice between every cycle. The rest of the protocol is as described in chapter 3, section 3.2.9.

5.2.6.Insulin ELISA:

Mice fasting serum samples were collected at various time point from PREPs transplanted and Swertisin administered animals,and were analyzed using Insulin ELISA kit as per manufacturer's protocol (MercodiaInc, USA).

5.2.7.Cryosectioning and Immunohistochemistry:

After treatment period, mice were sacrificed, splenic pancreas were dissected out and fixed by immersion in 4% paraformaldehyde overnight at 4˚C and then cryo-protected in 15% and 30% sucrose solutions in 0.1 M sodium phosphate buffer (pH 7.4). These tissues were then embedded in tissue freezing medium (OCT, Leica), and frozen. Pancreatic tissue sections were mounted on Poly-L-lysine coated slides. Cryosectioning was performed on a cryostat (Leica CM1520) at 20 μm intervals. Sections were incubated in blocking buffer [2% fetal bovine serum, 2% bovine serum albumin, 0.1% Triton X-100 in Phosphate Buffer Saline (PBS) with pH 7.4] followed by incubation in primary antibody overnight at 4˚C (table 1). After incubation, sections were rinsed in washing buffer (ten times diluted blocking buffer in PBS) and then incubated respective secondary antibody (table 1). Further, sections were counterstained with the DNA stain DAPI, washed with PBS, mounted with coverslips and finally sealed with transparent nail paint. Immuno-stained sections were viewed under confocal microscope (Zeiss LSM 710) and the fluorescence above the negative slides (only secondary antibody treated) were captured.

5.2.8.H&E staining:

Tissues were harvested and fixed in 4% paraformaldehyde (PFA) in PBS solution.Fixed tissue sections were stained with H&E staining kit (Rapid H & E Kit, Bio Lab Diagonstics), and observations were recorded under (10x magnification) a light microscope.

5.2.9.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).

5.3.Results:

5.3.1.Validation of STZ induced diabetic mice model:

BALB/c mice were treated with low dose STZ (65mg/kg b wt) for a period of 5 days. It was observed that fasting blood glucose of the STZ treated mice were all elevated across groups to >200mg/dl after five days of dosing against the normal control mice which maintained at between 80 to 120 mg/dl. Pancreatic tissue was screened for histological changes and observed that the islets of Langerhans in STZ treated pancreas had shrunk in size significantly compared to the normal control mice pancreas. Thus, the histology coupled with elevated fasting blood glucose confirmed their diabetic pathophysiology (Fig5.3).

5.3.2Co-Transfecting PREPs with PARP-1 Knockdown shRNA vector (pSIP912), control vector and GFP positive vector (pEGFPN1) and its vector control:

PREPs were co-transfected with pSIP912 vector and pEGFPN1 vector and its vector control (pU6) using NEON Electroporation system to generate stable GFP+vePREP clones fortransplantation studies. Pure GFP+ve colonies can be observed for both transfections which were validated using western blots as described in chapter 4, section 4.3.1.

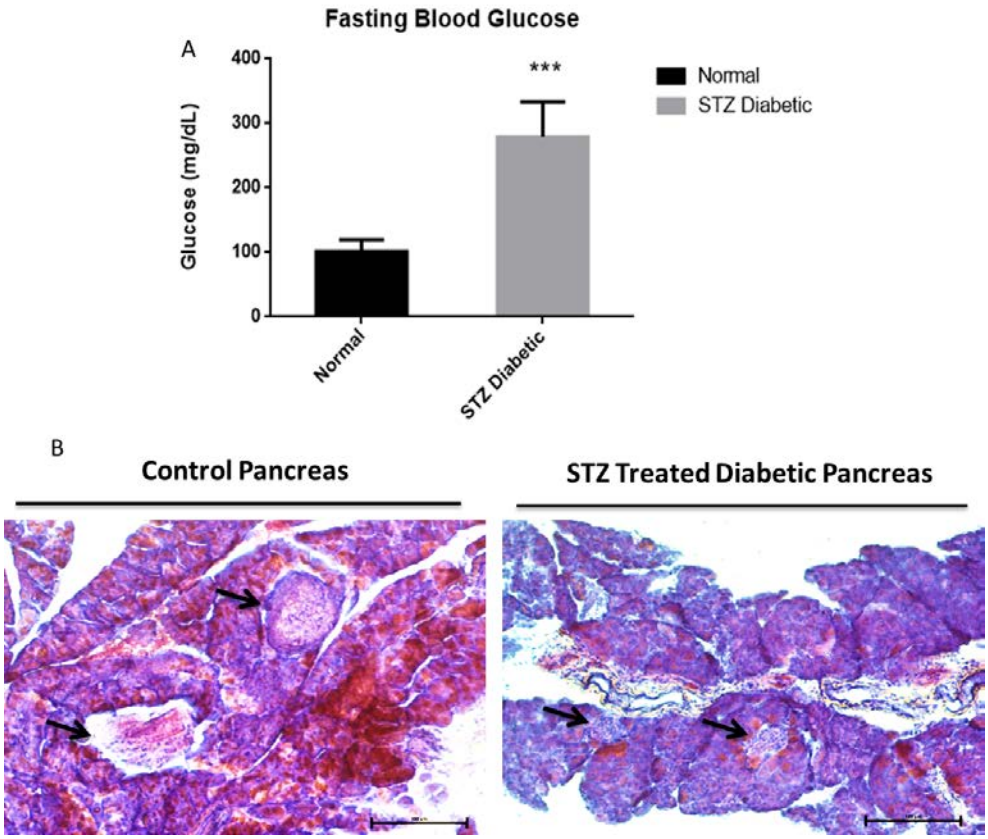


Figure 5.3: Validation of STZ BALB/c mice model: (A) Comparative fasting blood glucose levels between the control and the diabetic mice after STZ treatment. (B) H & E staining of pancreatic sections demonstrating morphology of Islet of Langerhans in control and STZ treated diabetic pancreas. ***p≤0.001 vs Normal. N=8.

5.3.3Mapping the Homing potential of Transplanted GFP positivePARP-1 positive and knockdownPREPs in the pancreas of STZ treated Type 1 Diabetic mice:

It is classically observed that homing of MSCs takes place towards the damaged tissue which in this case is the pancreas due to the STZ treatment. The liver and pancreas of the diabetic mouse model were harvested respectively after transplantation and their single cell suspensions were screened for GFP and PARP-1 positive and PARP-1 knock down PREPs through Flow Cytometry. Samples were acquired and analysed. The graphs determine the percentage of GFP positive population in the pancreatic and liver single cell suspension. The X-axis shows intensity of GFP fluorescence and Y-axis gives the cell count.The other major organs viz. spleen, lungs and kidneys were screened for GFP positive population with fluorescent microscopy which was found nil.

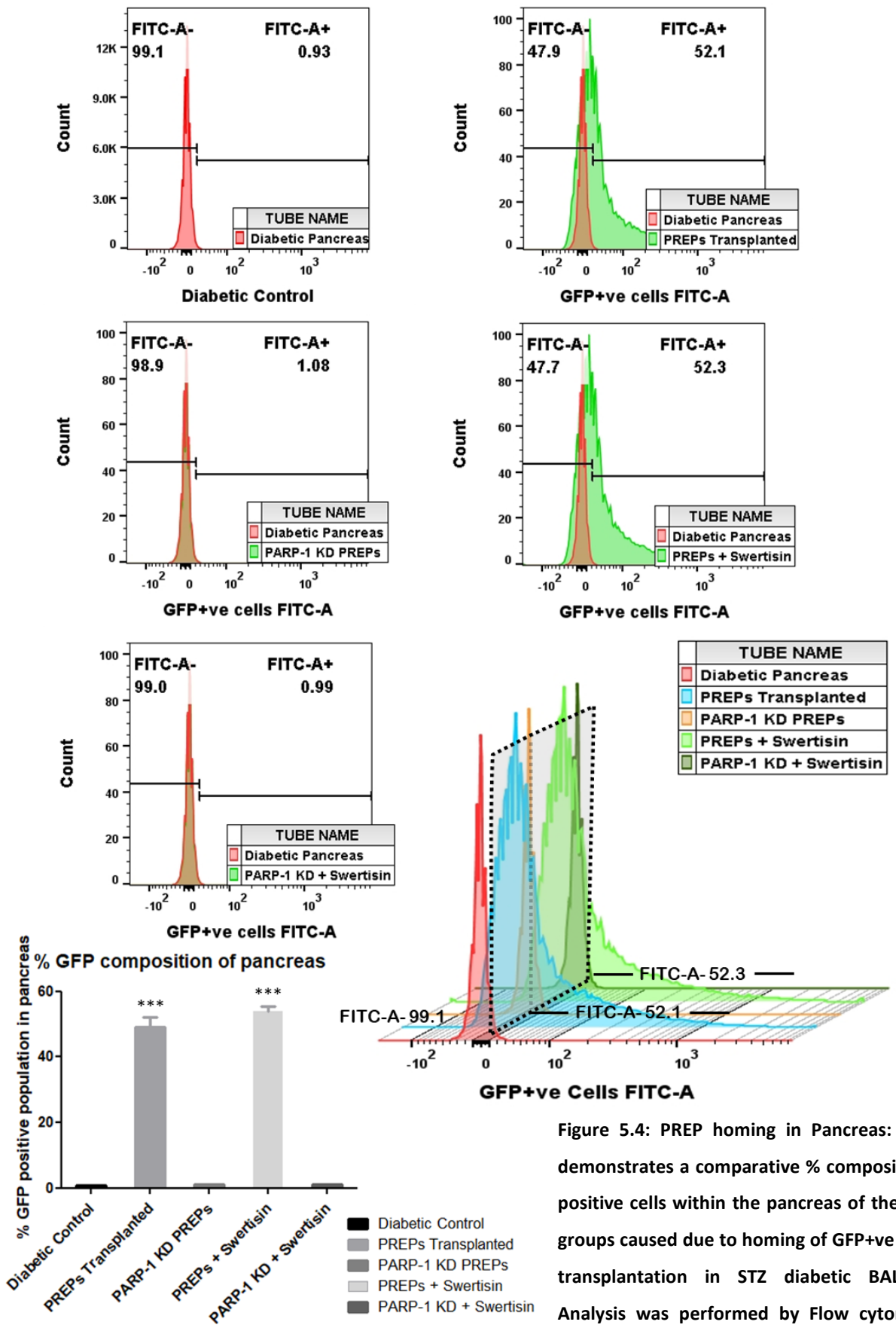


Figure 5.4: PREP homing in Pancreas: The figure demonstrates a comparative % composition of GFP positive cells within the pancreas of the respective groups caused due to homing of GFP+ve PREPs post transplantation in STZ diabetic BALB/c mice. Analysis was performed by Flow cytometry. The graph plotted is with mean values ± SEM. *** p≤0.001 vs diabetic control. N=3.

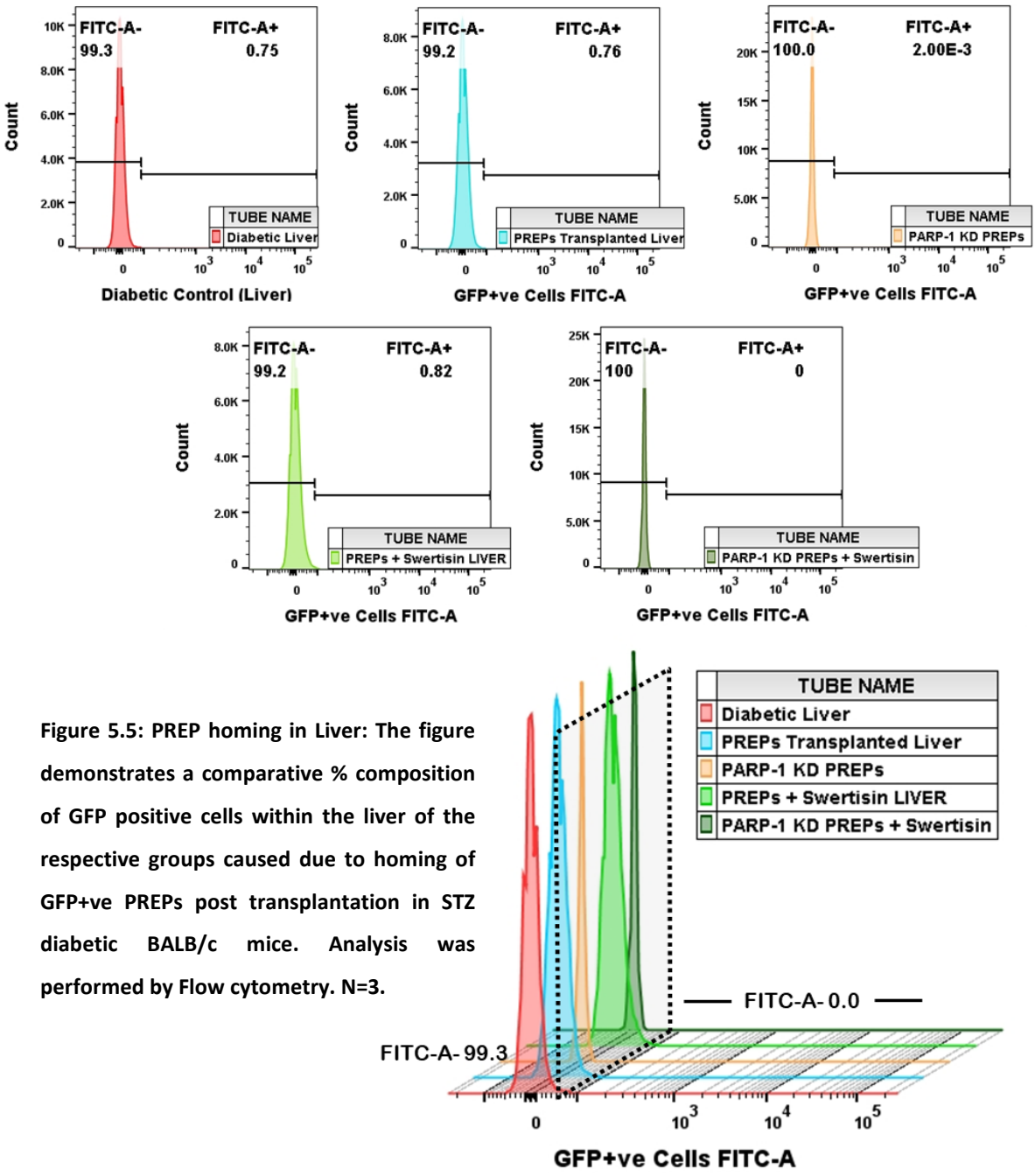


Figure 5.5: PREP homing in Liver: The figure demonstrates a comparative % composition of GFP positive cells within the liver of the respective groups caused due to homing of GFP+ve PREPs post transplantation in STZ diabetic BALB/c mice. Analysis was performed by Flow cytometry. N=3.

5.3.3.1. Flow cytometric analysis of GFP positive PREPs in the Pancreas of STZ Diabetic mice:

The graphs of Flow Cytometry analysis (Fig5.4) demonstrate the localization and composition of GFP positive cells within the analysed pancreatic tissues. It was observed that in the PREPs (PARP-1 positive) transplanted groups ~52.1% GFP positive population was detected and~52.3% was observed in the PREPs (PARP-1 positive) transplanted with Swertisin. However, there was no GFP positive cell localization observed in the groups

transplanted with PARP-1 KD PREPs. These results suggest that PARP-1 may plays a role in homing of PREPs to the damaged tissue i.e. pancreas with no further enhancement by swertisin in homing.

5.3.3.2.Homing of GFP positive PREPs in the liver of STZtreated Type 1 Diabetic mice:

The X-axis shows intensity of GFP fluorescence and Y-axis gives the cell count. According to flow cytometry of liver (Fig 5.5) there was no localization of GFP positive PREPs across all experimental groups.

5.3.3.3.Homing of GFP positive PREPs in the kidney, lungs and spleen of Type 1 Diabetic mice:

Kidney, Lungs and Spleen were also screened for localization of GFP positive PREPs by Fluorescence microscopy. The results did not show GFP positive population in the histological sections of Kidney, Lungs and Spleen of all the 5 experimental groups (Fig 5.6).

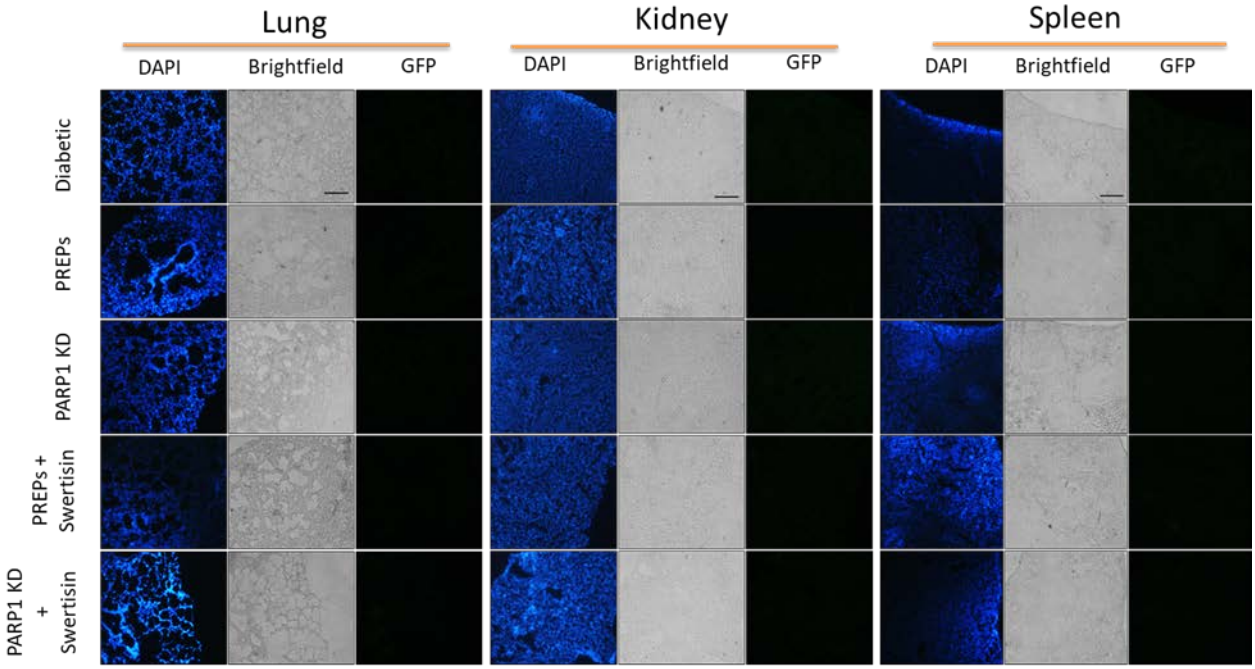


Figure 5.6: PREPs homing in Lung, Kidney & Spleen: The figure demonstrates a presence of GFP positive cells within the lung, kidney and spleen of the respective groups caused due to trapping of GFP+ve PREPs post transplantation in STZ diabetic BALB/c mice. Analysis was performed by fluorescence microscopy. Scale bar measures 100 µm. N=3.

5.3.4.Fasting Blood Glucose and Insulin to evaluate the diabetic conditionbefore and after transplantation:

Fasting Blood Glucose levels were monitored before PREPs transplantation and after transplantation of PREPs in STZ diabetic BALB/c mice.The fasting blood glucose levels indicated the status of the severity of diabetic condition induced in the BALB/c mice. A 2.5 times increase in the blood glucose was observed in the experimental groups demonstrating that they become diabetic (Fig 5.7A). These diabetic mice were then treated for diabetes by transplanting (PARP-1 positive and knockdown) PREPs alone and with Swertisin treatment. After transplantation it was observed that the group transplanted with PARP-1 positive PREPs reverted back to normoglycaemic condition (100±20 mg/dL) whereas there was a profound increase in the fasting blood glucose levels in the group transplanted with PARP-1 KD (499±117 mg/dL) PREPs. However, in swertisin treated groups, PARP-1 positive and PARP-1 KD PREPs both significantly lowered the blood glucose levels (162±79 mg/dL) as compared to before transplantation (209±45 mg/dL), highlighting Swertisin's inherent potential of lowering blood glucoseloweing (Fig 5.7B).Further, it was observed that fasting serum insulin decreased significantly in the diabetic mice while recovered back significantly after PREPs transplantation to STZ diabetic mice (Fig 5.7C).

5.3.5.Flow Cytometric analysis to determine differentiation and functionality of transplanted PREPs*in vivo*:

Flow cytometry was performed to verify if the transplanted PREPs were able to differentiate into insulin producing cells in the pancreatic niche. We assessed population which was dual positive for GPF and Insulin, to confirm the populationthat differentiated to β cells post transplantation.

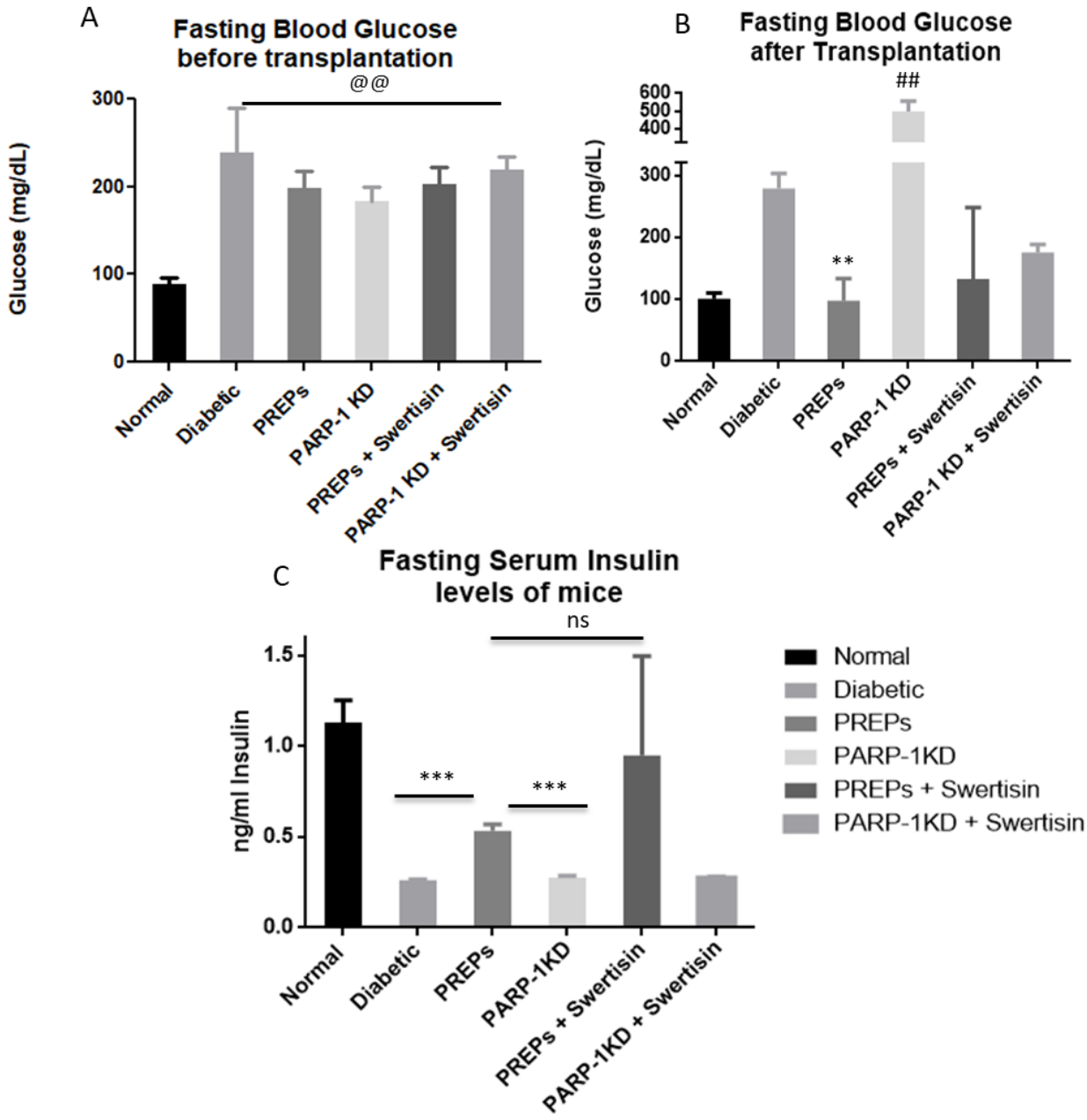


Figure 5.7: Fasting blood glucose to evaluate the diabetic condition: (A) The figure demonstrates elevated fasting blood glucose levels which are hyperglycaemic when compared to control just before transplantation of PREPs in STZ diabetic mice. (B) The figure demonstrates a comparative fasting blood glucose across all groups at 5th day after transplantation. The graph plotted is with mean values ± SEM. ** p≤0.01 vs diabetic control; ##- p value ≤ 0.01 for vs PREPs; @@- p value ≤ 0.01 vs Normal. N=3. (C) The graph demonstrates a comparative fasting serum insulin levels between the experimental groups. The graph plotted is with mean values ± SEM. *** p≤0.001 vs PREPs. N=3.

The cytometry graphs indicated the percentage of cells within Pancreas which were dual positive for GFP and Insulin along with their individual signals for GFP and Insulin respectively. The X-axis in Fig 4f represented percentage of Insulin positive cells and Y-axis represents percentage of GFP positive cells. The graph has been divided into 4 quadrants namely Q1, Q2, Q3 and Q4. Q1 represented cells positive only for GFP. Q2 represented cells positive for GFP as well as insulin i.e. cells are dual positive. Q3 represented cells positive for insulin only. Q4 represented the population negative for both the parameters being monitored i.e. dual negative population.

The graphs of flow cytometry (Fig5.8) show the population of cells dual positive for GFP and Insulin (~46.3%) confirming the differentiation of transplanted GFP positive PARP-1 positive PREPs into functional β cells. The groups transplanted with PARP-1 KD PREPs did not observe any dual positive cells, which was expected as they did not show any localization within the pancreas previously (Fig 4.4a). The groups treated with Swertisin along with transplantation of PARP-1 +ve and PARP-1 KD PREPs (Fig 5.8) depicted similar results (~41%) as compared to the groups transplanted with PREPs alone. Thus, suggesting that there was no synergistic effect of Swertisin in *in vivo* differentiation of PREPs after transplantation.

Screening of dual positive population of GFP and CD 44 (mesenchymal stem cell marker) was performed.The graphs indicated the percentage of cells in the pancreas which were dual positive for GFP and CD44 and individually for GFP and CD44 respectively. The X-axis in Fig 5.9determines number of CD44 positive cells and Y-axis determines the number of cells positive for GFP. The graph is divided into 4 quadrants namely Q1, Q2, Q3 and Q4. Q1 represented cells positive only for GFP. Q2 represented cells positive for GFP as well as

CD44 i.e. cells are dual positive. Q3 represented cells positive for CD44. Q4 represented the population negative for both the parameters being monitored i.e. dual negative population.

Cells dual positive for CD 44(Fig 5.9) were observed in groups transplanted with PARP-1 positive PREPs (~18.9%) and PREP + Swertisin group (~28%) but not in those transplanted with PARP-1 KD PREPs with and without Swertisin. We observed a subset of CD44 positive population which was also GFP negative indicating a different subset of cells having present both in the PREPs transplanted and PREPs with Swertisin transplanted groups (Fig 5.9).

5.3.6.Immunohistochemistry to confirm *in vivo* differentiation of transplanted PREPs by observing co-localization of GFP with c-peptide within pancreatic tissue:

To further confirm the differentiation of transplanted PREPs to from Neoislets,pancreatic sections of all groups were subjected to immunohistochemistry. We observed small clusters which had Co-localization of GFP with c-peptide, which is a terminal differentiation marker ofβ cells confirming insulin biosynthesis(Fig 5.10). DAPI (4',6-diamidino-2-phenylindole)was used to stain the nuclei of all cells in the sections,depicted by blue colour. The GFP positive cells were observed, showing green fluorescence and c-peptide was stained with TRITC (Tetramethylrhodamine) which showed red fluorescence. The yellow fluorescence was observed in the last column which was due to the overlapping of the fluorescence of GFP and TRITC (c-peptide). The confocal imaging showed the co-localisation in the groups transplanted with PARP-1 positive PREPs and no co-localisation in the groups transplanted with PARP-1 KD PREPs.

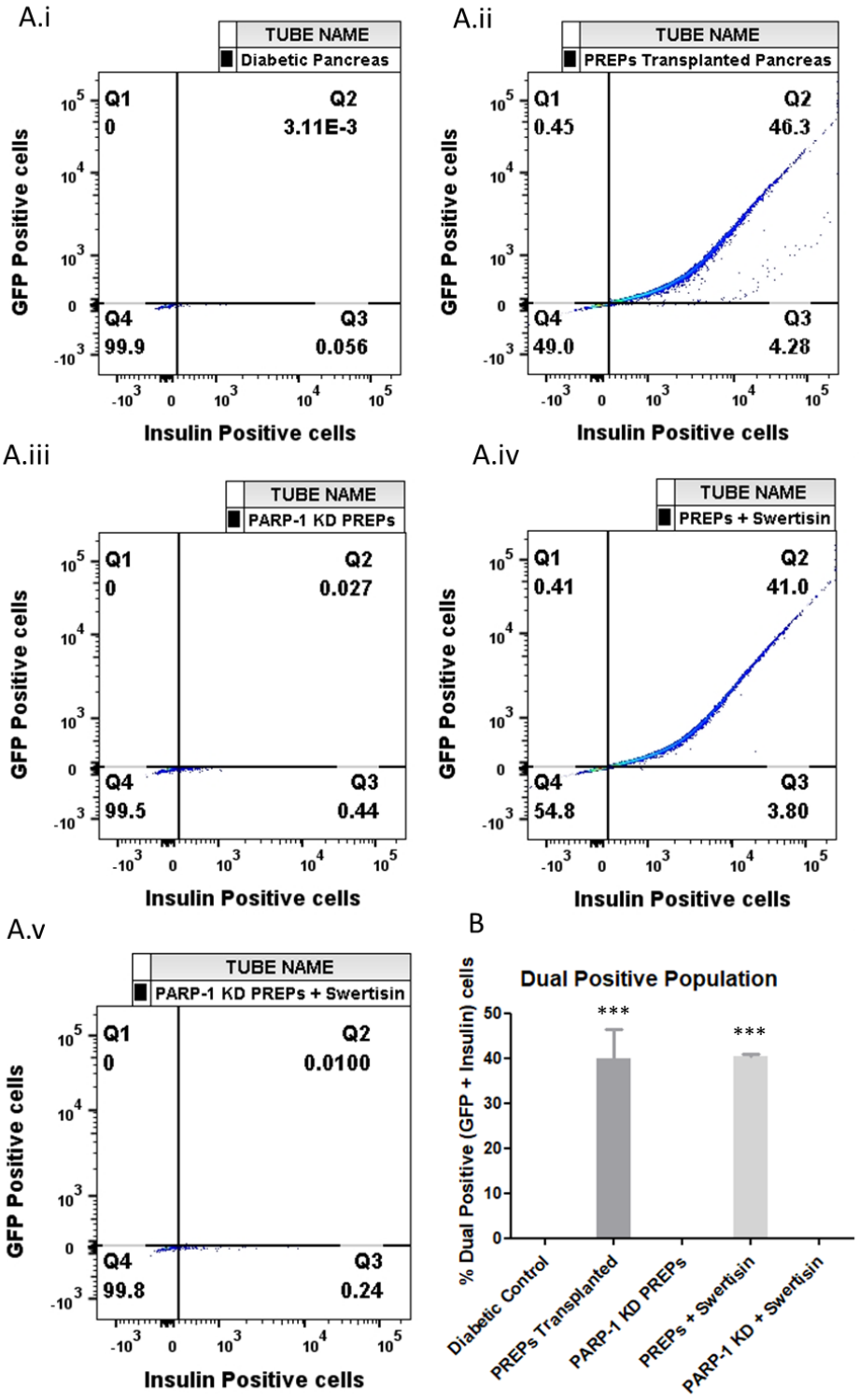


Figure 5.8: Analysing differentiation and functional assessment of Transplanted PREPs in pancreas: (A.i to A.v) The flow cytometric graphs demonstrate distribution of cells as dual positive GFP and Insulin positive (Q2), dual negative (Q4), only GFP positive (Q1) and only Insulin positive (Q3) cells. (B) The graph demonstrates statistical significance of the dual positive population in respective groups. The graph plotted is with mean values ± SEM. *** p≤0.001 vs diabetic control. N=3.

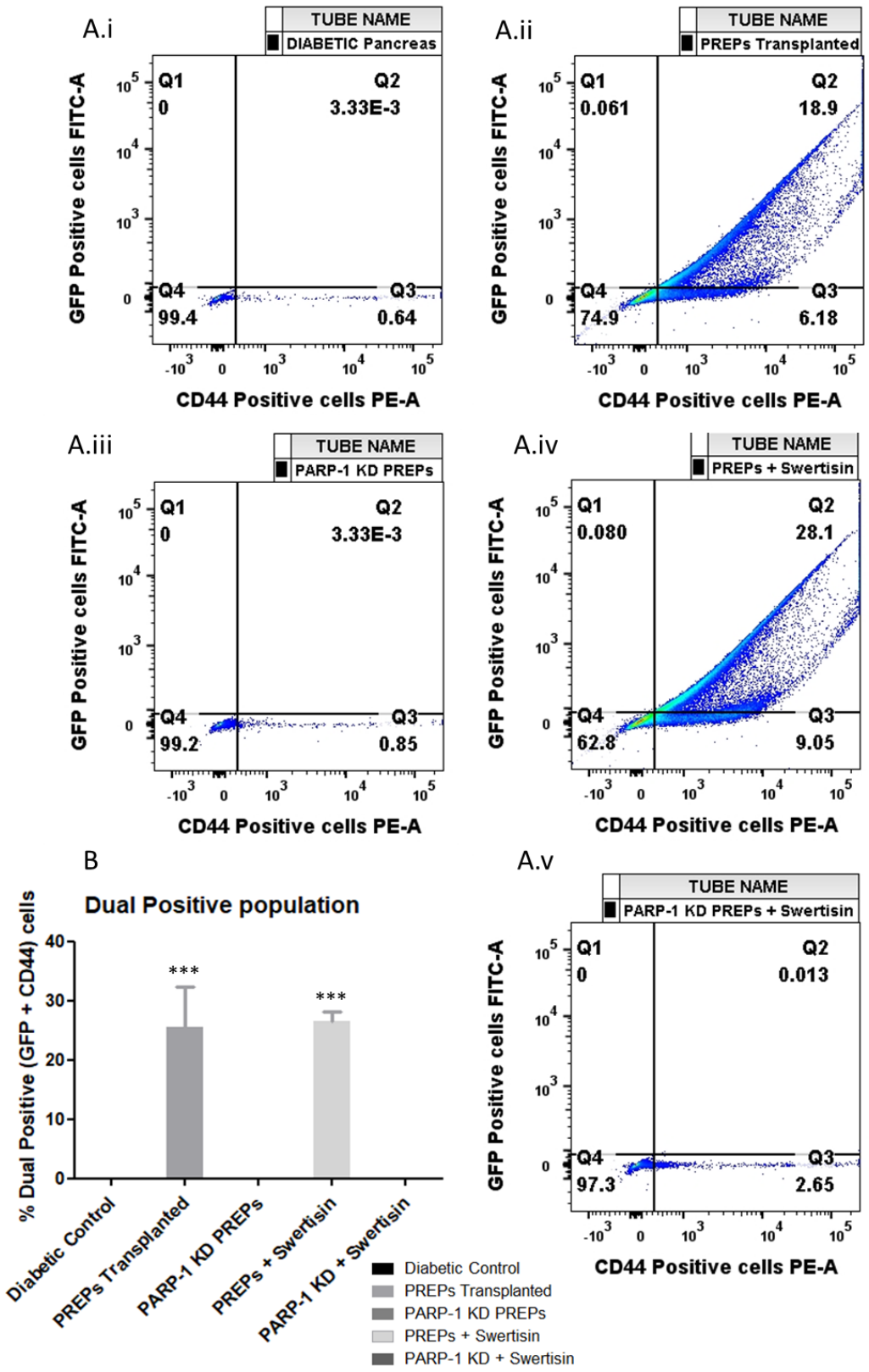


Figure 5.9: Analysing differentiation of Transplanted PREPs: (A.i to A.v) The flow cytometric graphs demonstrate distribution of cells as dual positive GFP + CD44 positive (Q2), dual negative (Q4), only GFP positive (Q1) and only CD44 positive (Q3) cells. (B) The graph demonstrates statistical significance of the dual positive population in respective groups. The graph plotted is with mean values ± SEM. *** p≤0.001 vs diabetic control. N=3.

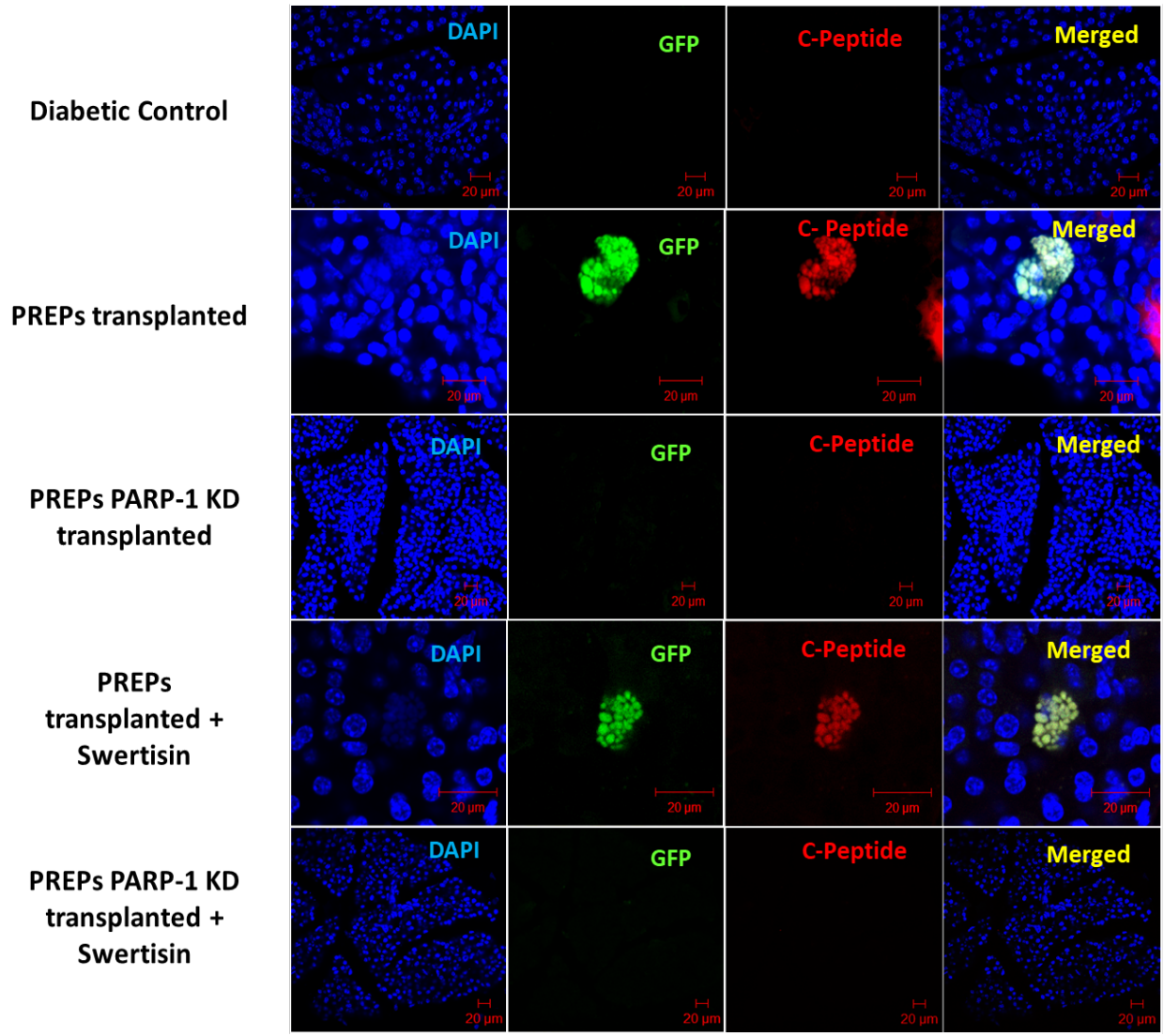


Figure 5.10: Functional Assessment of Transplanted PREPs in pancreas: The figure demonstrates co-localization (yellow) of GFP (green) with C-peptide (red) within the pancreas of PREPs transplanted and PREPs + Swertisin groups. Dapi (blue) stained the nucleus. N=3.

5.3.7.Protein expression levels of signaling molecules and transcription factors of the islet neogenesis pathway

The level of expression of the transcription factors of the Islet Neogenic pathway were screened by Western blotting to understand the kinetics of key transcription factors involved in islet neogenesis.

There were no significant changes observed in the expression of PARP-1 and SMAD2/3 protein levels. However, we observed an elevated expression of phosphoSMAD3 levels in the PAPR1+ve PREPs transplanted and Swertisin treated groups. Pdx-1 is a transcription factor

required to maintain the functionality of β cells and is expressed early in the Islet Neogenic pathway, which was observed to have upregulated in the PREPs (PARP-1+ve) transplanted and Swertisin treated groups and decreased expression was observed in the diabetic control and PARP-1 KD PREPs transplanted groups. We observed similar profile in the other key transcription factors viz. Neurog3, Pax4 and Nkx 6.1. Nkx6.1 is specific for β cells. β-Actin was used as an endogenous control (Fig 5.11).

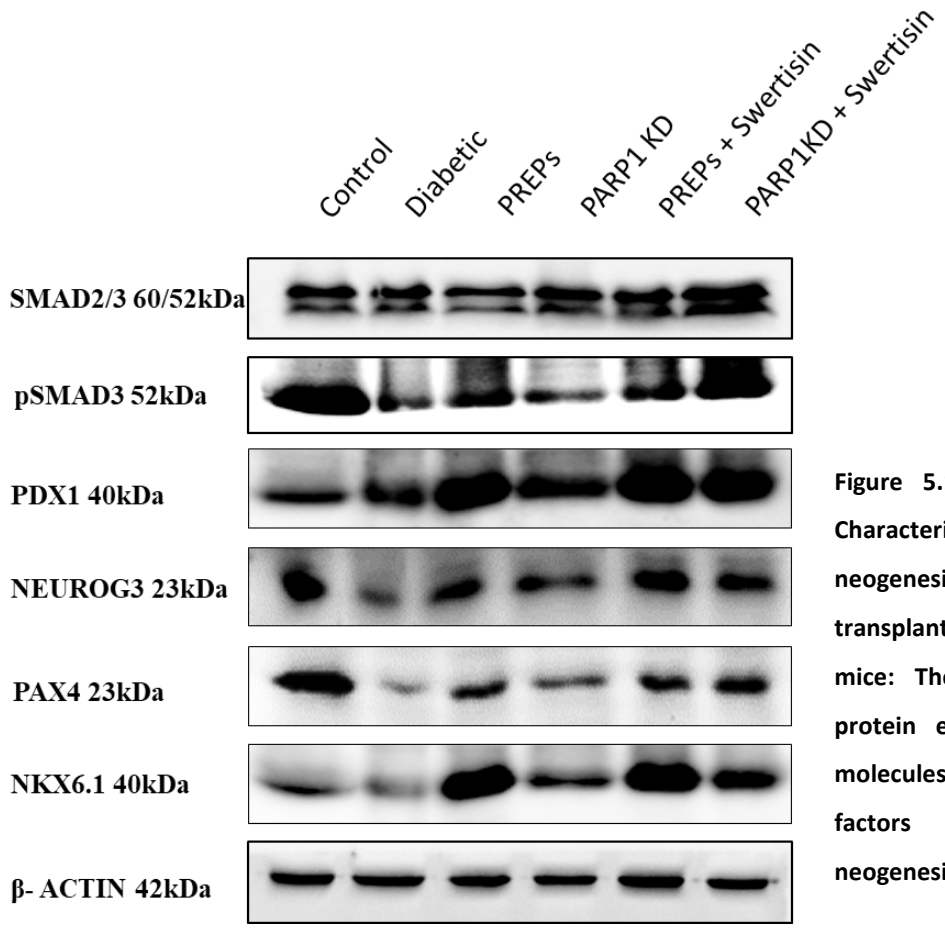


Figure 5.11: *In vivo* molecular Characterization of islet neogenesis post PREPs transplantation in STZ diabetic mice: The figure demonstrates protein expression of signaling molecules and key transcription factors involved in islet neogenesis. N=3.

5.3.8.Immunohistochemistry to confirm *in vivo* differentiation of transplanted PREPs into β cells by observing co-localization of GFP with Nkx6.1 within pancreatic tissue

In order to augment our western data we stained the pancreatic sections and observed for GFP and Nkx6.1 co-localization. We again observed small GFP positive clusters which had co-localized with Nkx6.1, which is a specific marker for β cells (Fig 5.12). Therefore,

confirmed functionality of transplanted GFP+vePREPs that had homed into the STZ treated damaged pancreas and further differentiated into β cells. DAPI was used to stain the nuclei of all cells in the sections which was blue in colour. The GFP positive cells were observed, showing green fluorescence and Nkx6.1 was stained with TRITC (Tetramethylrhodamine) which showed red fluorescence. The yellow fluorescence was observed in the last column which was due to the overlapping of the fluorescence of GFP and TRITC (Nkx6.1). The confocal imaging showed the co-localisation in the groups transplanted with PARP-1 positive PREPs and no co-localisation in the groups transplanted with PARP-1 KD PREPs.

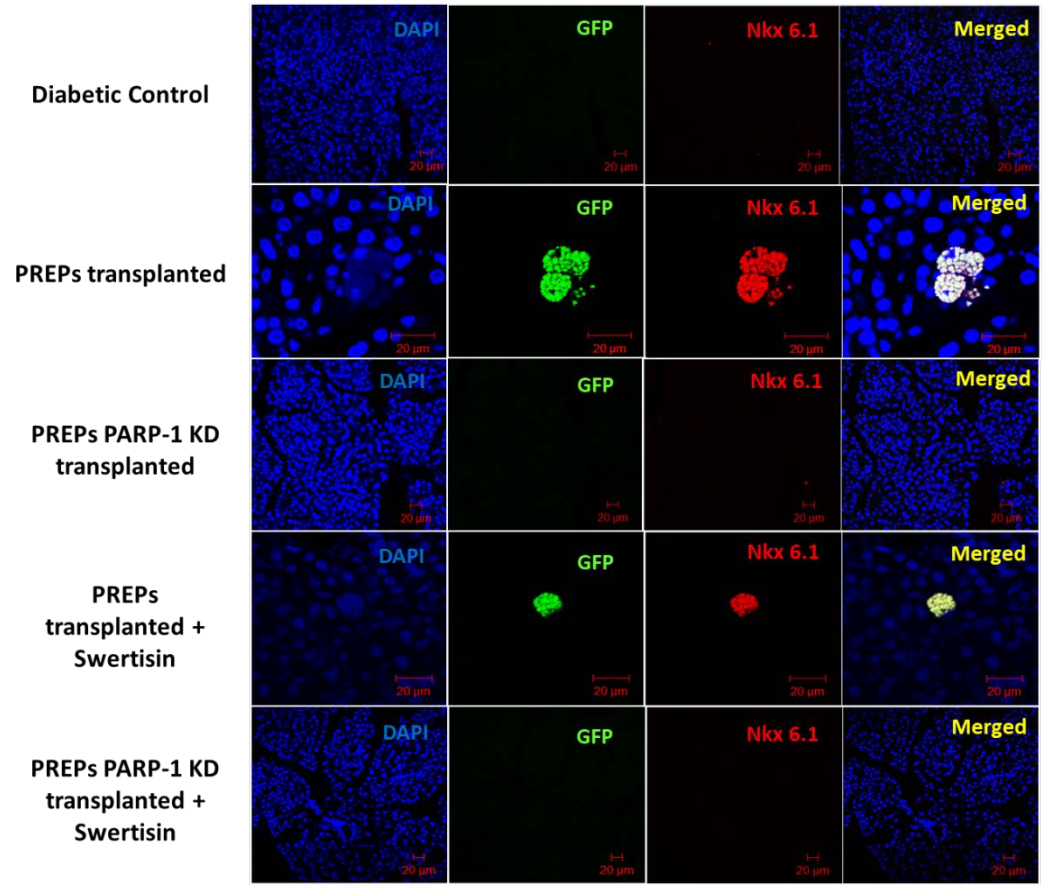


Figure 5.12: Functional Assessment of Transplanted PREPs in pancreas: The figure demonstrates co-localization (yellow) of GFP (green) with Nkx6.1 (red) within the pancreas of PREPs transplanted and PREPs + Swertisin groups. Dapi (blue) stained the nucleus. N=3.

5.3.9.*In vivo* assessment of Swertisin induced amelioration in STZ treated diabetic BALB/c mice:

STZ treated diabetic BALB/c mice were treated with Swertisin and analysed for amelioration for their diabetic condition. STZ treatment caused a significant increase in the fasting blood glucose (FBG) levels and decrease in fasting serum insulin levels in the diabetic BALB/c mice. We observed that with the treatment of Swertisin there was a decrease in the FBG levels, which came back to normal range in a week's time and persisted till the end of Swertisin treatment after which the mice were sacrificed. Further, the fasting serum insulin levels were significantly increased after the Swertisin treatment to diabetic BALB/c mice. Acquiring normoglycemia and increase in the insulin levels were clear indication of gain of function of mice endocrine pancreas (Fig 5.13).

Mice were sacrificed post Swertisin treatment and their pancreas were harvested for protein expression analysis by western blotting and immunohistochemistry. Western blotting data gave us a comprehensive understanding with respect to recovery of diabetic mice post Swertisin treatment. We observed that all the key transcription factors viz. PDX1, NEUROG3, MAFA, NKX6.1 and GLUT2 essential for endocrine pancreatic development, regeneration and function were significantly elevated in the Swertisin treated group which were extensively abated during the diabetic condition. This suggested that the diabetic pancreas when treated with Swertisin was able to recover its function. Beta Actin served as endogenous control (Fig 5.14).

Finally, in the immunohistochemistry data we have compared the diabetic mice pancreas to the Swertisin treated mice pancreas. Here, we observed an increased expression of NESTIN and NEUROG3 double positive cells compared to the islets in the diabetic mice pancreas which demonstrated weak NESTIN & no NEUROG3 staining. Also, the islets in the diabetic pancreas appeared to be hollowed due to the impact of STZ whereas that was not the case

with Swertisin treated mice pancreas which seemed to have recovered significantly. We further observed increased NESTIN and GLUT2 expression and co-localisation. Presence of c-peptide was observed more prominently in the Swertisin treated mice pancreas. This data suggested that Swertisin treatment was capable of triggering the resident endocrine progenitor population within the pancreas to regenerate the damaged beta cells and thus recovering the endocrine pancreatic function (Fig 5.14).

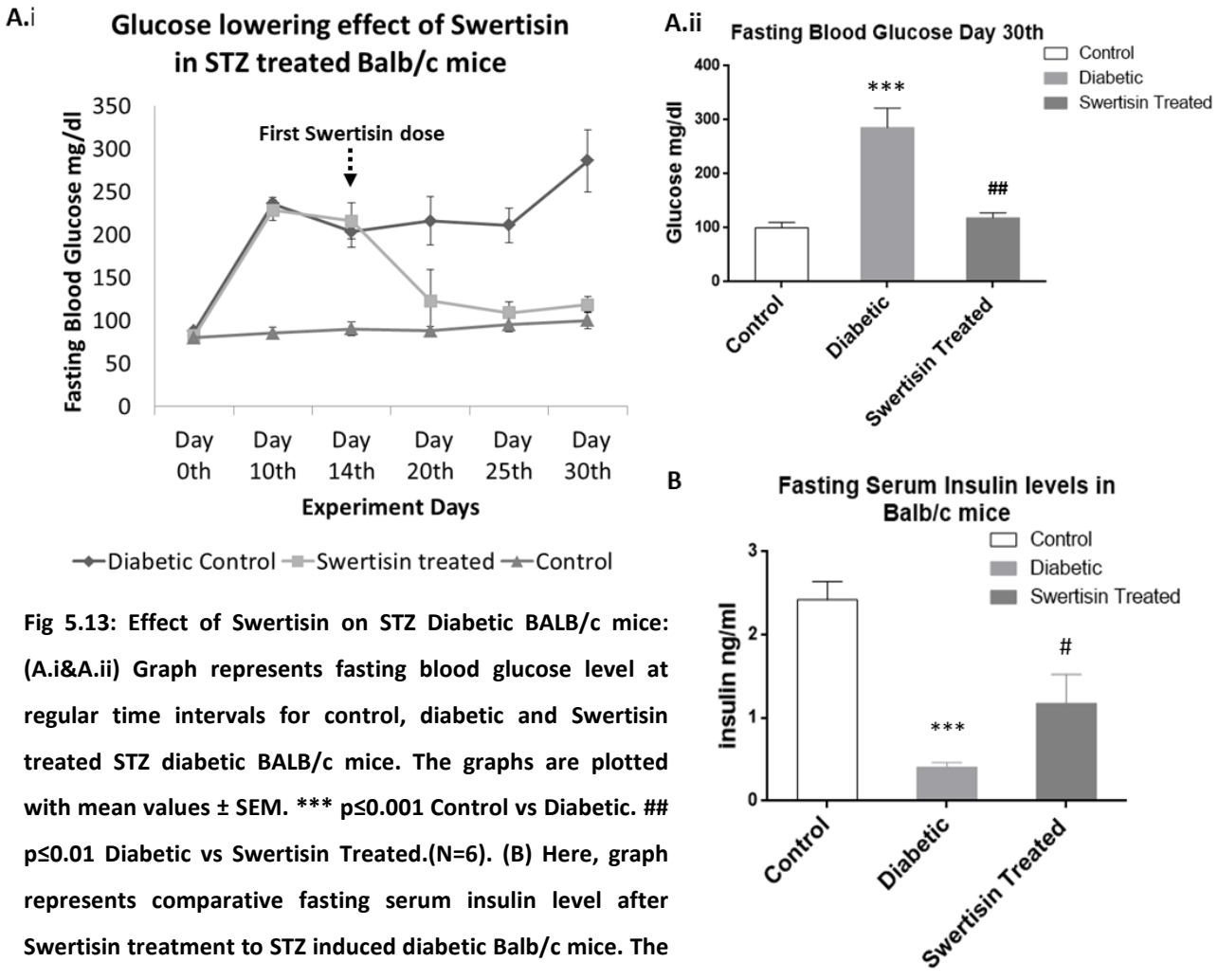


Fig 5.13: Effect of Swertisin on STZ Diabetic BALB/c mice: (A.i&A.ii) Graph represents fasting blood glucose level at regular time intervals for control, diabetic and Swertisin treated STZ diabetic BALB/c mice. The graphs are plotted with mean values ± SEM. *** p≤0.001 Control vs Diabetic. ## p≤0.01 Diabetic vs Swertisin Treated.(N=6). (B) Here, graph represents comparative fasting serum insulin level after Swertisin treatment to STZ induced diabetic Balb/c mice. The graphs are plotted with mean values ± SEM. *** p≤0.001 Control vs Diabetic. #p≤0.05 Diabetic vs Swertisin Treated (N=3).

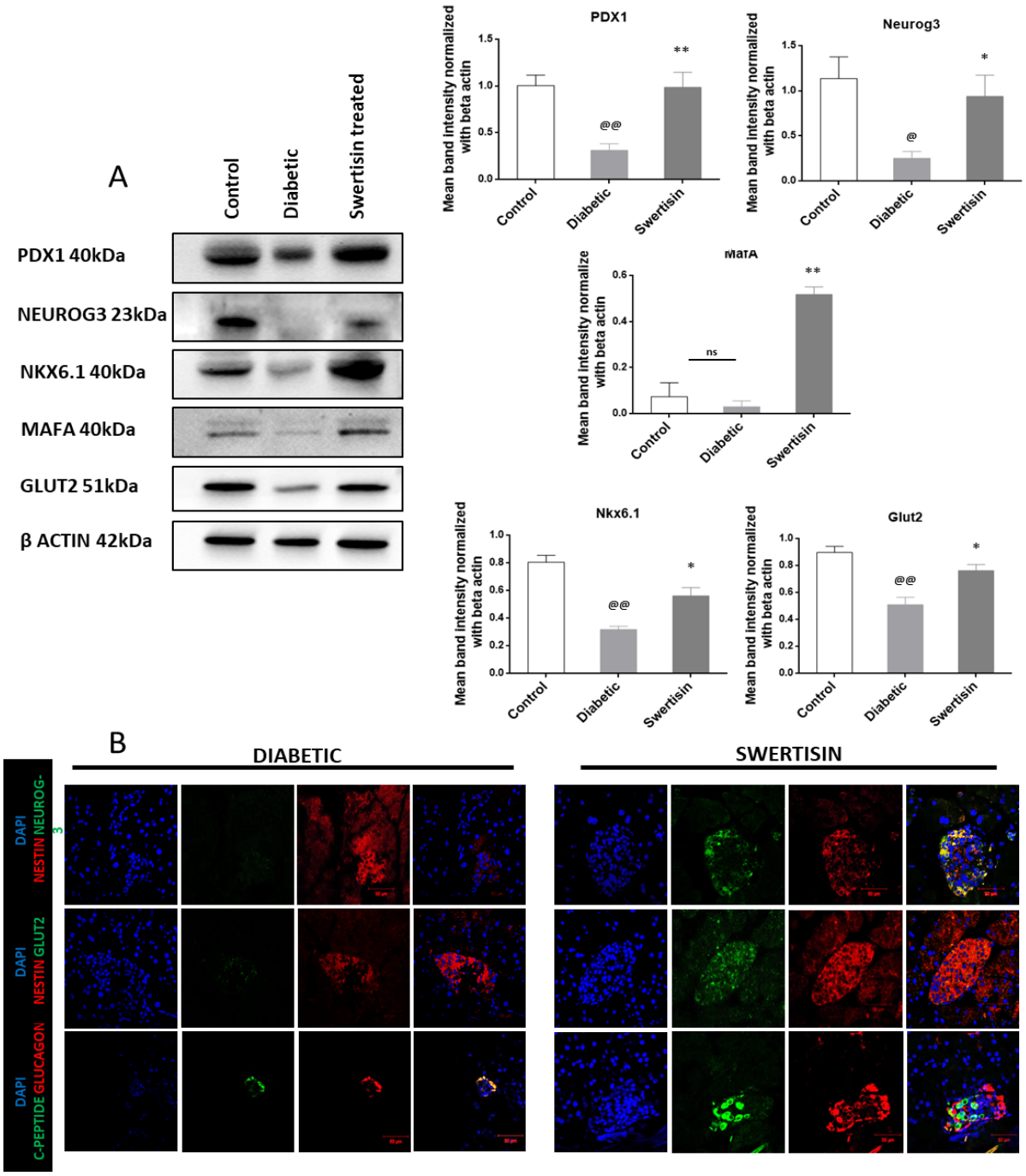


Fig 5.14: Pancreatic Protein expression in Swertisin treated STZ diabetic mice: (A) Comparative protein profile for the key transcription factors and markers essential for endocrine pancreatic regeneration using immunoblotting has been demonstrated. The transcription factors are as follows: PDX1, NEUROG3, MAFA, NKX6.1, GLUT2 and BETA ACTIN as endogenous control. Densitometric graphs are plotted with mean±SEM band intensity normalized with BETA ACTIN levels for respective proteins, @=Control vs Diabetic (@@≤0.005 p value and @≤0.05 p value) and *=Diabetic vs Swertisin (≤0.005 p value and *≤0.05 p value) (B) Comparative immunohistochemistry of Diabetic mice pancreas treated with and without Swertisin. A combination of NEUROG3 (green) along with NESTIN (red), GLUT2 (green) along with NESTIN (red) and C-PEPTIDE (green) along with GLUCAGON (red) were observed to focus on pancreatic resident endocrine progenitors and endocrine pancreas. Nuclei were stained with DAPI (blue) (n=3).**

5.4.Discussion

Type 1 Diabetes is associated with the degradation of β cells which leads to hyperglycaemia due to no insulin production (Wilcox et al., 2016). Various therapies have evolved till date for the amelioration of diabetic condition (Atkinson and Eisenbarth, 2001; Goodall and Halford, 1991; Rowan et al., 2008; Steil et al., 2006). Among these treatment strategies, regenerative therapy is the one associated with the transplantation of stem cells for islet replenishment(Aathira and Jain, 2014). There have been reports where mesenchymal stem cells have been used not only to manage diabetes but also for other diseases or injuries (Creusot et al., 2016; Froud et al., 2005; Hematti et al., 2013; Rekittke et al., 2016). Transplantation of mesenchymal stem cells has been used for the expression of lubricin in joint abnormalities(Nakagawa et al., 2016), managing cerebral ischemia(Feng et al., 2016), angiogenesis after ischemic limb injury(Zhu et al., 2016), cartilage repair(Mak et al., 2016), managing amyotrophic lateral sclerosis(Petrou et al., 2016), meniscus regeneration(Kondo et al., 2016), managing porcine myocardial infarction(Mu et al., 2016) and also to manage spinal cord injury(Chopp et al., 2000).

Among factors regulating the stem cells, classical role of PARP-1 in DNA repair and cell death has been extended to its role in proliferation, chromatin remodelling, differentiation etc. (Schreiber et al., 2002).More intriguingly, PARP-1 plays a role in islet differentiation by regulating the expression of *Reg*, which is the first transcription factor expressed in the Islet Neogenic pathway in pancreatic regeneration(Akiyama et al., 2001a). In addition to PARP-1, earlier data from our lab demonstrated that swertisin, a bioactive isolated from *Enicostemma littorale* had the virtue of islet neogenesis from various stem cell sources *invitro* as well as enhanced homing of mBMSC *in vivo* for amelioration of Type 1 diabetes (Dr.NidheeshDadheech Thesis, 2013),(Dadheech et al., 2013; Dadheech et al., 2015). Thus, the focus of the present study is the transplantation of PREPs with mesenchymal stem

cellcharacteristics to treat Type 1 Diabetic condition and to explore possible role of PARP-1 and swertisin in this process.

Streptozotocin(STZ), a potent oxidant is specifically used to insult β cells having poor anti-oxidant property for the induction of Type 1 Diabetes in murine models(Li et al., 2003; Szkudelski, 2001; Xin et al., 2016). Thus, mice were treated with 65 mg/kg bodyweight of STZ for 5 days to create Type 1 diabetic mouse model. These animals were monitored for the hyperglycaemic condition,where the animals with glycaemic status of more than 200mg/dL were included in the study.Since mesenchymal stem cells transplantation are widely used as a therapy to manage the diabetic condition, we attempted to ameliorate Type 1 Diabetic condition by intravenous transplantation of 2 x 10^6 mesenchymal stem cells i.e. PREPs into Type 1 Diabetic mouse model.

As per the literature it has been previously known that mesenchymal stem cells have a property of homing (Chamberlain et al., 2007; Henschler et al., 2008; Karp and Teo, 2009; Yagi et al., 2010). Homing is the trans-endothelial migration of the mesenchymal stem cells after getting localized into the vasculature of damaged/target tissue (Karp and Teo, 2009).Previous reports on transplantation of mesenchymal stem cells to manage diabetes also showed homing of these cells in the pancreas(Deans and Moseley, 2000; Ezquer et al., 2008; Hess et al., 2003; Lee et al., 2006). Reports suggest about ~3% homing of bone marrow mesenchymal stem cells when transplanted intravenously to ameliorate diabetes by repairing damaged pancreas(Ezquer et al., 2008; Hess et al., 2003). Previous study from our labwith mouse BMSC transplantation, we observed 1.6% homing(Dr.NidheeshDadheech Thesis, 2013) whereas in present study, we observed ~52% homing in the pancreas of the groups transplanted with PARP-1 positive PREPs. However, along with swertisin this number did not change much in contrast to our earlier report where swertisin induction improved homing from 1.6% to12% homing. This was possibly due to the fact that in present transplantation

experiment, pancreatic resident endocrine progenitors were used which have more affinity towards their own niche irrespective of any need of external stimulus. Further, pancreatic progenitor is a phenotypic state which is an intermediary state between any undifferentiated stem cell and a mature islet. As Swertisin is an excellent islet differentiating factor and. Swertisin's ability to bring mBMSCs into the pancreatic progenitor lineage could be the reason why we observed a more synergistic homing action with mBMSCs compared to pancreatic progenitors. Also, swertisin could not demonstrate any additive effect when treated with PARP 1 positive PREPS. Also, the ~52% homing in pancreas along with absence of any GFP+ve signal from any other major organ that we screened suggest that almost all the PREPs transplanted were localized into the pancreas of the diabetic mice. Hence, Swertisin was presented with no room to improve these statistics when administered alongsidePREPs.

The role of PARP-1 was elucidated by using RNA interference technology bysilencing PARP-1 in PREPs. In the mice transplanted with silenced PARP-1PREPs, nearly no homing was observedsuggesting that PARP-1 expression is essential for homing. This could be due to inability of PARP-1 KD cells to sustain themselves after transplantation and being cleared off from the host body. This can be further supported by the reports that suggest that PARP-1 negatively regulates FOXO-1 a transcription factor, which leads to inhibition of cell proliferation by playing role in cell cycle control, apoptosis, detoxification of reactive oxygen species, and gluconeogenesis through regulation of gene expression(Sakamaki et al., 2009).It is interesting to note that PARP-1 also affect the action of immune B cells by altering their homing to the target site(Ambrose et al., 2009). Since homing of B cells is controlled by chemokines or cytokines, we believe that PARP-1 might be playing a role in altering the homing of PREPs due to alterations in cytokines secretion from the damaged tissue. This might further result in reduction in cell survival in the PARP-1 deficient cells(Andreone et

al., 2012). Hence, cell death could be a major reason for no localization of the PARP-1 KD PREPs.

The differentiation of mesenchymal stem cells post transplantation into β cells is demonstrated in various reports. Mostly, the time required for the reversion of hyperglycaemic condition to normoglycaemic condition has been reported to be 7 days after transplantation of mesenchymal stem cells, this is monitored by the decrease in blood glucose levels(Deans and Moseley, 2000; Ezquer et al., 2008; Hess et al., 2003; Khorsandi et al., 2016; Rekittke et al., 2016; Xin et al., 2016). We observed normalization of the blood glucose levels on the 5^{th} day after transplantation of PREPs in STZ induced Type 1 Diabetic mice. This suggests that there was very rapid formation of functional neoislets in the damaged pancreas.

To understand the pattern of differentiation of the transplanted PARP-1 positive as well as PARP-1 KD undifferentiated PREPs flow cytometry and confocal imaging was performed. Nearly 46.3% of dual positive population for GFP and insulin was observed only in the groups transplanted with PARP-1 positive PREPsand PREPs with swertisin (~41%). Whereas, in previous reports the percentage of transplanted bone marrow mesenchymal stem cells which become terminally differentiated to β cells in the pancreas is only 1.5-3% (Deans and Moseley, 2000; Ezquer et al., 2008; Hess et al., 2003)(Akiyama et al., 2001a). Previous *in vitro* studies in the lab also demonstrated the role of PARP-1 in the islet neogenic pathway (Dr.NidheeshDadheech Thesis, 2013). This explains the presence of terminally differentiated β cells only in the groups transplanted with PARP-1 positive PREPs.

CD44 is reported to be a mesenchymal stem cell marker(Pittenger et al., 1999). Dual positive population of GFP and CD44 was monitored to determine the population of PREPs which got engrafted in the pancreas but did not terminally differentiate into β cells. Surprisingly, a large population was dual positive for GFP and CD44, which could mean that the terminally

differentiated β cells also express CD44. These results could be supported by reports that suggest the presence of CD44 on insulin producing β cells(Naor and Assayag-Asherie, 2013). There have been other reports where CD44 expression was observed in other parts of the pancreatic tissue including islets(Immervoll et al., 2011; Ringel et al., 2001). This could explain the slight increase in the CD44 population alone in the PARP-1 KD PREPs with Swertisin group. Further, we observe two distinct population in these graphs, it can be observed that the lower stream of population have reduced GFP intensity and moved to the left of CD44 scale as well suggesting that this population has moved from its original phenotype i.e. has differentiated. We believe that the graph has captured the population in between a transition state of differentiation.

In this present study it has been observed that the cells after homing have differentiated into functional β cells as observed by the confocal imaging which show the co-localization of GFP with Nkx 6.1 and GFP with C-peptide. Also, the elevated serum insulin levels indicate increased pancreatic function leading to amelioration of the diabetic condition in the PREPs transplanted groups. Although we saw an increased serum insulin level in the PREPs + Swertisin group it lacked statistical significance because of high SEM within the group.

In our present study, we observed no synergistic effect of swertisin treatment along with transplanted PREPs in the amelioration of type I diabetes, which is different from our previous study with BMSCs (Dr.NidheeshDadheech Thesis, 2013) where an increased homing and differentiation was observed. It's interesting to observe that the group in which PARP-1 KD cells were transplanted along with swertisin treatment showed glucose lowering and upregulation of islet neogenic markers suggestingan inherent potential of swertisin in Islet neogenesis and glucose homeostasis. To prove the efficacy of swertisin alone in the amelioration of diabetic condition, we performed an experiment for extended period of fifteen days (Experimental plan 2). In this study, we tested whether exogenous administration

of swertisin in STZ treated diabetic BALB/c mice can enhance islet regeneration by triggering pancreatic resident endocrine progenitors toamend islet function and thusreverting them to normoglycemia. Here, the BALB/c mice were treated with STZ for a period of five days followed by confirmation of hyperglycaemia. Swertisin was then intravenously administered in these diabetic mice starting from 14th day till 30 days for total period of 15 days. At the end of Swertisin dose, mice were sacrificed and analysed for amelioration in the diabetic condition. In Swertisin administered group of the mice fasting blood glucose levels came back to normoglycaemic values by tenth day of treatment along with recovery in fasting serum insulin levels. Our results are in accordance with other studies where herbal compounds like Pterosin A and Conophyllin, can effectively reverse pancreatic endocrine injury and dysfunction in STZ induced diabetic mice model (Hsu et al., 2013; Kojima and Umezawa, 2006).

At molecular level, we observed a significant increase in the protein expression of GLUT2 upon Swertisin treatment in pancreas of diabetic mice. It has been previously reported that in the regenerating mice pancreas, post STZ treatment, GLUT2 positive progenitor population have been observed. Also, pancreatic progenitor population expressing GLUT2 were not as susceptible to STZ exposure as beta cells and were able to survive and retain their potential of islet neogenesis (Banerjee and Bhonde, 2003b; Guz et al., 2001). In another study also, GLUT2 was identified for enriching pancreatic progenitor population (Segev et al., 2012). Hence, a significant rapid rise in the GLUT2 levels could be an indication of increased pool of endogenous pancreatic progenitor population triggered by Swertisin treatment in diabetic mice. Upregulation of other key transcription factors such as PDX1, NEUROG3, MAFA and NKX6.1 were also observed suggesting an increase in pancreatic endocrine progenitor population and increased endocrine function which also reflected in fasting blood glucose and insulin levels. In pancreatic development, these transcription factors are also expressed after

E9.5 followed by expression of GLUT2 at E10.5 (Murtaugh, 2007; Zhang and Sarvetnick, 2003). In the immunohistochemistry sections of Swertisin treated diabetic mice pancreas, NESTIN and NEUROG3 dual positive cells were observed along with increased expression of GLUT2 and NESTIN indicating increased progenitor population and islet formation. Correspondingly, there was an increased c-peptide expression within the islets indicative of enhanced insulin biosynthesis. Thus, comparing results of both short and long-term treatment of swertisin, it can be noted that swertisin not only plays a role in differentiation but also stimulate insulin secretion by targeting other signaling pathways.

Hence,PREPs when transplanted can not only home in towards the damaged pancreas without trapping in any other major organ but also differentiated into insulin producing cells without any exogenous stimuli. On the other hand, PARP-1 KD PREPs could not home to the pancreas which emphasises PARP-1's expression for functional homing of stem/progenitors. Also, Swertisin showed highly encouraging effects, which indicate that its administration may be a promising strategy for diabetes therapeutics. Furthermore, phytochemical-based therapies may be established in concert with stem cells as unique pharmacological methodologies for the treatment of diabetes. This study thus opens up a window of hope for a plausible diabetic cure, by triggering PREPs towards islet neogenesis withregulatedinsulin release in diabetic patients.

Special appreciation to My Loving "Mice"

I express my humblest gratitude to you my friends, without you this would not have been possible….