Synopsis
Synopsis of the thesis on

Evaluation of Islet Neogenesis from Extra Pancreatic Cell Lineages Using Herbal Plant

To be submitted to

The Maharaja Sayajirao University of Baroda

For the degree of

Doctor of Philosophy in Biochemistry

By

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Under Supervision of
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To
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Subject: Submission of synopsis of the Ph. D. work entitled – “Evaluation of Islet Neogenesis from Extra-Pancreatic Cell Lineages Using Herbal Plant”

Dear Sir,

Kindly accept the synopsis of my Ph. D work entitled – “Evaluation of Islet Neogenesis from Extra-Pancreatic Cell Lineages Using Herbal Plant”. My date of registration was 08/08/2008 and registration no. is 353.

Thanking you,

Sincerely yours,

(Nidheesh Dadheech)

(Prof. Sarita Gupta)
Guide

Head, Department of Biochemistry

Dean, Faculty of Science
Introduction

Diabetes mellitus is a debilitating metabolic disease caused by absence (juvenile or type 1) or insufficient (type 2) insulin production from β-cells. Type 1 (insulin-dependent) diabetes mellitus (IDDM) is an autoimmune disorder where destruction of insulin-producing pancreatic beta cells leads to insulin deficiency (Atkinson and Maclaren, 1994). India is now going to be first country in world to have highest number of diabetic patient till 2025 (Karvonen et al., 2000). Uncontrolled, diabetes is associated with serious long-term complications, such as cardiovascular disorders, kidney disease and blindness. The current treatment strategies for type 1 diabetes (5-10% of all diabetics) are based on insulin replacement therapy and dietary habits combined with meticulous blood glucose monitoring and lifestyle adaptations. This is not an appropriate treatment strategy for such patients as it involves only symptomatic management and lacks long term cure of the disease.

Pancreas (organ transplant) or Cadaveric islet transplantation treatment alternatives have their own limitations, such as lack of sufficient donor organs or islets from cadaveric tissue associated with long-term risk of chronic immunosuppression for graft rejection (Stratta and Alloway, 1998). Insulin secreting cells generated from either embryonic or adult stem cells might represent an attractive alternative approach to cure the disease (Vinik A.I. et al., 2009). Currently efforts are being made to generate insulin-producing cells from stem cells or tissue specific progenitor cells (Bonner-Weir et al., 2000; Lumelsky et al., 2001). Formation of insulin-producing beta cells can take place by two pathways: (1) insulin producing cell genesis from pancreatic resident islet precursors or (2) from stem/precursor cells other than pancreas. More pertinently isolation procedures of islet specific stem/precursor are rather controversial as diabetic pancreas itself is a very fragile and prone to inflammation therefore extra pancreatic cell types are better option. Limited availability of external factors, i.e. growth factors regulating beta cell neogenesis or differentiating agents which can enforce the fate of differentiation of beta cells even deteriorate the situation due to their high cost effectiveness. On the other hand novel strategies in diabetes therapy including transplanting genetically engineered β cells has its own problems due to slow turnover of β cell and uncontrolled regulation for insulin. Therefore today’s need is to focus research on increasing islet mass from stem cells other than pancreas using chemical, biological or herbal ingredients purified from medicinal plant as a differentiating agent for new islet cell generation.

The present study is focused to explore the possible sources of pluripotent/multipotent stem cells from extra pancreatic tissues/organs which can be utilized for differentiation into insulin producing cells using herbal plant extracts/compounds as differentiating agents, in in-vitro differentiated insulin producing cells when transplanted into streptozotocin (STZ) induced diabetic mice for reverting hyperglycemia is the aim of therapeutic interventions in diabetes. Limited sources for stem cells are available to clinician, hence such study is of utmost importance which
provides viable options to clinicians to identify stem cells and then transplant differentiated islets from these stem/precursor cells in diabetic patients who cannot undergo major surgery or invasive method for treatment except few minor ones. The second major hurdle which our study addressed is the accessibility of less cost effective islet cell differentiating agents. Not many differentiating agents are so far known and explored; those which are available are either chemical or synthetic agents or growth factors. None of these can be used for stem cell therapeutics in diabetic patients due to high cost effectiveness. So present research work highlight use of differentiating agents isolate from herbal plant *Enicostemma littorale*, which is very well reported from our lab for its antioxidant and anti-diabetic effects (Maroo et al., 2003a; Maroo et al., 2002; Maroo et al., 2003b; Vasu et al., 2003; Vasu et al., 2005).

**Specific objectives:** 

**Major objectives of the present study are:**

1. *In-vitro* Differentiation potential of Methanolic extract of *Enicostemma littorale* Blume for islet neogenesis from extra pancreatic stem/progenitor cell systems.

2. Differentiation of Mouse embryonic fibroblast (NIH-3T3) cells into insulin producing cells from extract/fractions/purified compounds isolated from *Enicostemma littorale*.
   (a). Characterization of NIH3T3 cells for stem/progenitor cell properties.
   (b). Molecular, structural and immunological characterization of differentiated islet Like Cell Cluster (ILCC) and *in-vivo* functionality of neoislets to mitigate hyperglycemia by transplantation into STZ induced diabetic mice as a model.

3. Differentiation of Mouse Bone Marrow Mesenchymal Stem cells into insulin producing cells using potential bioactive ingredient SGL-1 from *Enicostemma littorale* Blume.
   (a). *In-vitro* differentiation of mBMSC into insulin positive cells.
   (b). Endogenous *in-vivo* differentiation of mBMSC cells into insulin positive cells.

4. Assessment of molecular mechanism of SGL-1, a bioactive ingredient of *Enicostemma littorale* and role of Poly (ADP) Ribose Polymerase-1 in islet neogenesis.

**Obj-1: In-vitro Differentiation potential of Methanolic extract of *Enicostemma littorale* Blume for islet neogenesis from extra pancreatic stem/progenitor cell systems**

To study our hypothesis, we carried out a preliminary *in-vitro* experiment to evaluate the islet neogenic potential of methanolic extract of *Enicostemma littorale* (El). PANC-1 cells along with mouse embryonic fibroblast (NIH3T3) cells, mouse bone marrow mesenchymal stem cells (mBMSC) and rat prostatic mesenchymal stem cells (rPMSC) were subjected to differentiation into islet like cell clusters to examine the differentiating potential of methanolic extract of El. To test islet neogenic potential of El, a dose dependent study of Methanolic extract in various concentrations (5,10,15 and
20µg/ml) was used to show a quantitative increase in the number of ILCC on transition of stem/precursor cells from serum containing media to serum free media. The dose of methanolic extract for differentiating these cells in-vitro was determined from an in-vivo study of our lab, which indicated 2.5 g/kg bodyweight per day in alloxan induced diabetic animals (Maruo et al., 2003a), it was decided to start in-vitro assay with 10⁶-10⁷ cells and lowest dose of 5µg/ml to maximum of 20µg/ml in serum free differentiating media. Cells in serum containing medium proliferate into apparently homogenous adherent monolayer. After brief exposure to trypsin all cells form 3 dimensional clusters of compact mass of cells, further mature to hormone expressing islet-like budding structures within 8 days. Newly generated islets were stained with DTZ to give brick red color suggesting presence of insulin positive cells and differentiating potential of the methanolic extract of Enicostemma littorale.

**Obj-2: Differentiation of Mouse embryonic fibroblast (NIH-3T3) cells into insulin producing cells from extract/fractions/purified compounds isolated from Enicostemma littorale.**

a. **Characterization of NIH3T3 cells for stem/progenitor cell properties.**

Mouse Embryonic Fibroblast NIH3T3 has been considered as differentiated dermal fibroblast cells and not been observed for its pluripotency or multipotency till 2005. Many groups have used NIH3T3 as control cells which fails to differentiate (Russo et al., 1998; Wu et al., 2010; Zuo et al., 2001). In 2006, Yamanaka and his group compared pluripotent ES cells and untransformed NIH3T3 cells by microarray analysis and found that NIH3T3 do not possess pluripotency genes esp. the four crucial ones oct3/4, Nanog, sox-2, and Klf4 but they express c-Myc (Takahashi and Yamanaka, 2006). But on the contrary this group did not examine or comment upon the multipotential nature of these cells which is still a lacuna to consider NIH3T3 as inert cells. Three years down the line group of Win Ping Deng had published two reports stating that untransformed NIH3T3 cells differentiates into osteocytes and neuronal cell types in bone and spinal cord injury mouse models (Lo et al., 2009; Lo et al., 2008). Another two groups of Wang et al. and Abdallah reported NIH3T3 differentiation into neural cells and oesteoblast respectively (Abdallah, 2006; Wu et al., 2010). In parallel, we also cited islet cell differentiation property of NIH3T3 cells with appropriate induction (Gupta, 2010). To test whether NIH3T3 cells do possess stem/progenitor cell potential, we carried out a comparative study of NIH3T3 with pluripotent Bone Marrow Mesenchymal Stem Cells (mBMSC). Systematic screening of NIH3T3 cells for pluripotent and multipotent stem cell markers using RT-PCR, immunochemistry and in-vivo transplantation analyses revealed the presence of pluripotent stem cell like characteristics in undifferentiated NIH3T3 cells compared to bone marrow mesenchymal stem cells. Morphological and histological observations are in accordance with earlier studies (Conrad et al., 2008; Kanatsu-Shinohara et al., 2004; Li et al., 2003; Lowry et al., 2008; Reisi et al., 2010). Also remarkable ability to form spheroid bodies
which stained positive for high alkaline phosphatase staining confirmed characteristic feature of pluripotent stem cells. The presence of stem cell like markers at both mRNA and protein levels by RT-PCR, Q-PCR, immunocytochemistry and immunoblotting showed most of stem cell relevant genes and proteins like Oct3/4, Nanog, Sox-2, Nestin Vimentin, SMA, Fibronectin, E-cadherin and PDX-1 confirming its mesenchymal and fibroblastic state with pluripotent nature. In-vivo study in direction for pluripotency of NIH3T3 cells for teratoma formation also resulted interesting results, which explains pluripotent nature of NIH3T3 cells and strong reason to differentiate into various cell lineages.

(b). Molecular, structural and immunological characterization of differentiated Islet Like Cell Cluster (ILCC) and in-vivo functionality of neo-islets to mitigate hyperglycemia by transplantation into STZ induced diabetic mice as a model.

Under this objective we aimed to prove the differentiation potential of various extracts/ fractions and series of purified compounds which we isolated from Enicostemma littorale and screened with NIH3T3 cell system. The major emphasis was laid on three facts. First, which of these compounds/fractions provides highest yield of ILCC generation, as this is a solemn obstacle to clinical professionals and patient which are limiting with inadequate yield of islets for transplantation? Second, out of all herbal ingredients, which of these effectively differentiate and form functional islet cells other than beta cells, esp. glucagon positive cells which are indispensable for proper beta cell functioning and regulated glucose responsive insulin release? Third and last, are these herbal ingredient induced pseudo islets generated, able to functionally integrate in-vivo to mitigate the STZ induced hyperglycemic condition into diabetic mouse model? Based on all above facts most potent and active herbal ingredient was selected for islet differentiation with subsequent stable or primary cultured cell systems hereafter.

We first carried out screening experiment for islet differentiating property of methanolic extract, aqueous extract and series of compounds namely swertiamerin, SGL-1, SGL-2 and SGL-3 (all purified from methanolic extract of El) and compared the yield and differentiation stages with known islet cell differentiating agent keratinocyte growth factor (KGF). In this initial screening setup, we observed methanolic extract, swertiamerin, SGL-1 and SGL-2 are able to form ILCC’s whereas aqueous extract and SGL-3 fails to make them effectively with very few clusters even after 8 day. DTZ staining was used to assess the insulin positivity in clusters differentiated with all extracts/fractions and compounds. Out of all SGL-1 showed highest number of clusters positive for DTZ stain-brick red color with higher yield and more average area. Further immunochemical characterization of SGL-1 induced clusters showed intense C-peptide, Insulin and glucagon positive staining similar to KGF induced cluster compared with normal islets. Gene expression analysis of stem cells, islet differentiation pathway and islet cell specific genes after 8 day differentiation protocol, revealed absence of stem cell
relevant gene expression (*Nestin, Vimentin, SMA*) over differentiation time period 0-8 day, while islet cell genesis pathway gene ike PDX-1, Ngn-3, Pax-4, Nkx 6.1 etc starts expressing from 2-3 days of differentiation window onwards. Functional maturity of ILCC showed significant high release of insulin in response to 20mM glucose challenge represented mature nature of newly generated clusters. Differentiated ILCC from SGL-1 Compound in in-vivo study mitigated STZ induced hyperglycemia in a week upon transplantation in balb/c mice and remained functionally integrated in renal capsule.

**Obj-3: Differentiation of Mouse Bone Marrow Mesenchymal Stem cells into insulin producing cells using potential bioactive ingredient SGL-1 from *Enicostemma littorale* Blume.**

(a). *In-vitro* differentiation of mBMSC into insulin positive cells.

This part of the study involves differentiation of primary pluripotent bone marrow mesenchymal stem cells with SGL-1 compound to show its islet differentiating property. Herein, we used properly characterized mouse bone marrow mesenchymal stem cells for differentiation using SGL-1 as a differentiating agent. We observed similar ILCC generation from undifferentiated mBMSC upon 8 day differentiation protocol confirmed by positive DTZ staining. Immunocytochemical and molecular analysis again confirmed the presence of islet hormones in differentiated clusters. Functional assessment for glucose and arginine challenge for insulin release assay and transplantation of newly generated clusters into renal capsule of diabetic mice are under progress to test reversal of hyperglycemia.

(b). Endogenous *in-vivo* differentiation of mBMSC cells into insulin positive cells.

An approach for *in-vivo* endogenous differentiation of any stem cell type is the actual therapeutic implementation for any diabetic patient in treatment. Drugs or agents which can be capable of enforcing organ resident stem cells or circulating stem cells like bone marrow mesenchymal stem cells to form functional beta cells into diabetic individual endogenously will promise a attractive and synergistic appeal for diabetic medication. Aiming this, efforts were done to understand whether El can potentiate endogenous differentiation. For this we successfully created GFP labeled mouse bone marrow mesenchymal stem cells for monitoring the *in-vivo* differentiation into insulin positive cells and functional integrity upon induction with SGL-1. GFP labeled BMS cells were injected into STZ induced diabetic mice and monitored constantly for lowering of blood glucose levels over a period of time until animal become normoglycemic. The analysis part for the identification of dual positive population of GFP+/Insulin + cells upon differentiation with SGL-1 induction is under progress.
**Objective:** Assessment of molecular mechanism of SGL-1, a bioactive ingredient of *Enicostemma littorale* and role of Poly (ADP) Ribose Polymerase-1 in islet cell genesis.

*Enicostemma littorale* has been examined for the capacity of anti-diabetic herbal medicinal plant in promoting islet neogenesis. More pertinently, we observed in-vitro formation of functional islet-like cell clusters containing both β and α cells starting from mouse embryonic fibroblast NIH3T3 cells and human pancreatic ductal epithelioid carcinoma cell PANC-1 when treated with herbal bioactive ingredient SGL-1 (Gupta, 2010). This led us to investigate the molecular mechanism of this potent bioactive ingredient SGL-1 for its effective islet cell differentiation property. In light of this, a molecular study to unravel the mechanistic pathway of islet differentiation was carried out. Only few reports which show pancreatic regeneration using herbal extract or compounds are there. One of such study done by Kojima et al., who isolated a vincra alkaloid called Conophyllin from a herbal plant *Evertimia microphylla* showed insulin positive cell differentiation from AR42J cells (pancreatic acinar cells) which cause over expression of Neurogenein-3 protein in response to stimulated phosphorylation of p38 MAPKinase protein identical to Activin-A mediated differentiation pathway (Ogihara et al., 2003). We also hypothesized and tested the same pathway for understanding the mode of action of SGL-1 mediated differentiation of stem cells. For this we used PANC-1 cells to investigate the mechanism of action of SGL-1 induced differentiation.

During neogenesis of islets from PANC-1 cells by treatment with Activin A and SGL-1, we noted that Nestin, subsequently declined by 7-8 days, with strong upregulation of Ngn-3 in both Activin-A and SGL-1 induced differentiated clusters. In parallel, phospho-p38 MAP kinase expression also increased dramatically prior to Ngn-3 expression. These results provide us the basis to conclude that SGL-1 also differentiates stem/precursor cell via same ACTII- MAPK -TKK pathway.

Oxidative stress is a prime reason for beta cell damage in diabetic condition and Poly (ADP)Ribose Polymerase-1 (PARP-1) protein is fundamental factor for repair of DNA in such stress condition. Moreover, PARP-1 also been reported to play crucial role in pancreas regeneration and beta cell replication in mouse model (Okamoto et al., 2001). Earlier observations in a simpler model of only β-cell lines revealed that PARP-inhibitor could promote transcription of Reg-1 (Akiyama et al., 2001) and MafA (Wu et al., 2010) genes, which are implicated in β-cell proliferation or insulin gene expression, respectively. Therefore it becomes more evident to understand the role of PARP-1 in islet cell differentiation from stem/precursor cells. We examined the impact of PARP inhibition with a very specific inhibitor PJ-34 influencing islet differentiating property. Our results from inhibitor study indicated that PARP activity is playing a negative role in islet neogenesis from precursor cells while presence of free enzyme facilitates islet cell genesis. Further, to understand the effect of absolute depletion of PARP we carried out RNAi mediated knockdown of PARP-1 in PANC-1 cells. Cells treated with Activin A
and SGL-1 fails to differentiate into insulin producing cells in PARP-1 deplete SiP cells whereas PARP-1-replete U6 cells formed large islet-like clusters identical to normal untrasfected PANC-1 cells with strong positive staining for insulin, as measured by DTZ and immunofluorescence. The results with PARP-1-depleted SiP cells clearly show that PARP-1 protein is required for proper formation of islet-like clusters from PANC-1 cells.

Conclusion

From this study we conclude stem cell differentiating potential of Eunicostemma littorale for islet cell differentiation which can be exciting therapeutic tool for effective treatment of diabetes. SGL1, a compound isolated from Eunicostemma littorale has been successfully demonstrated as a novel differentiating agent for new islet like cell cluster formation and thus it is a promising candidate to increase islet cell mass. Further we also confirmed the pluripotent state of NIH3T3 cells capable of multilineage cell differentiation esp. in insulin producing cells. SGL1 compound differentiate stem/progenitor cells via ACT-MAPK-TPK pathway, and Poly (ADP)Ribose polymerase-1 has a crucial role and is inevitable for islet cell neogenesis.

References


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Publications:

Manuscript in communication:

Abstract Published:

Poster presented:
1. Presented poster entitled “Evaluation of differentiation potential of Indian herb Enicostemma littorale into neoislet formation from human pancreatic stem cell line PANC-1 and extra pancreatic mouse embryonic fibroblast cell line NIH3T3” in 7th annual meeting, ISSCR, Barcelona, Spain, 7th to 11th July, 2009.
Achievement:

1. Awarded with Canadian Commonwealth Fellowship (10,000 CAD) for 6 month to work at Dr. Girish Shah Lab, Laval University, Quebec Canada in 2010-2011.
2. Awarded with Young Investigator International Travel award for attending 7th annual meeting, ISSCR, Barcelona, Spain in 2009.

International Conference attended in Ph.D. duration:

1. Attended 7th Annual meeting on Stem Cell Research organised by International Society for stem cell Research held at Barcelona Spain, 7-11 July, 2009.

Date: October 2, 2011

Signature of the candidate

Nidheesh Dadheech

(Prof. Sarita Gupta)
Guide

Head
Biochemistry Department

Dean
Faculty of science
This is not just an end but it’s a
“Beginning.....”