Synopsis of the Thesis

"Exploring And Potentiating Human Bone Marrow Derived Mesenchymal Stem Cells [hBMSCs] And Differentiated Islets For Effective Diabetes Therapy"

To be submitted to

The Maharaja Sayajirao University of Baroda

For the degree of

Doctor of Philosophy in Biochemistry

By

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Diabetes Mellitus (DM) describes a group of metabolic disorder depicted by chronic hyperglycemia, leading to life-threatening health complications resulting in expensive medical care, reduced quality of life and increased mortality. In recent decades incidence of diabetes and impaired glucose tolerance in adults has been increasing globally. International Diabetes Foundation (IDF) estimated 451 million (age group: 18–99 years) diabetics globally in the year of 2017.These numbers are expected to increase to 693 million by 2045 (Cho, Shaw et al. 2018).

Although there are numerous therapeutics for management of diabetes viz. insulin injectables and/or oral hypoglycemic drugs, a cure for diabetes awaits an insight and naïve phase. Ever since the Edmonton protocol in 2000, islet transplantation is perceived as a viable treatment for diabetes as compared to insulin in term of perfectly timed regulated glucose sensor & insulin release (Shapiro, Ricordi et al. 2006). However, the major limitation is the availability and number of functional islets. Hence, researchers have been exploring different sources and types of stem cells for islet neogenesis. Cell therapy treatment using autologous adult stem cells has been identified for its potential to progress the field of regenerative medicine. Of the numerous sources of adult stem cells, human bone marrow has been the chosen source and it is broadly used for clinical transplantation in the treatment of a varied range of diseases (Potdar and Deshpande 2013). Human bone marrow-derived mesenchymal stem cells [hBMSCs] have demonstrated one of the most clinically acceptable in the context of easy availability, less ethical issue and promising potential in term of multilineage for generating new islets for diabetic therapy. Further, experiments with human bone marrow mesenchymal stem cells demonstrated that they are able to differentiate into insulin-expressing cells *in vitro* by a mechanism involving several transcription factors of the beta-cell developmental pathway, when cultured in an appropriate microenvironment (Moriscot, De Fraipont et al. 2005) (Zanini, Bruno et al. 2011). The bone marrow-derived MSCs isolated from both type 1 and type 2 diabetic patients have the potential to differentiate into insulin producing cells (IPCs) *in-vitro* (Sun, Chen et al. 2007). The therapeutic outcome of transplantation of IPCs differentiated from adult hBMSCs proved that they were able to control streptozotocin (STZ) induced diabetes in nude mice(Gabr, Zakaria et al. 2013) (Xin, Jiang et al. 2016). Thus, taken together, all these experimental findings support the potential of adult human bone marrow-derived MSCs to differentiate into islet-like clusters. However, the challenge is to demonstrate that enough IPCs with adequate functional capacity can be produced in a culture which then can be used for transplantation therapy.

A number of transcription factors play a crucial role during pancreatic development as well as in Islet differentiation. Further, these transcription factors are also regulated by microRNA. microRNAs are small non-coding RNAs that play significant roles in the posttranscriptional regulation of gene expression by interaction with specific sites located in the 3′ untranslated region of target mRNAs(Ha and Kim 2014). Currently, researchers have identified various microRNAs that have crucial roles in pancreas development, insulin secretion, islet function and diabetic related complications. Apart from this, microRNAs are involved in stem cell maintenance and differentiation (Wei, Yang et al. 2013) (Chen, Yu et al. 2011).The researchers demonstrated that microRNA-375 is expressed in pancreatic β-cells and regulates insulin secretion and may constitute a novel pharmacological target for the treatment of diabetes (Lahmy, Soleimani et al. 2014).

Islets from cadaveric donors are one of the conceivable cures for Type 1 diabetes. However, this practice has limitations such as the shortage of transplantable islets, graft rejection, use of immune suppressants (Rother and Harlan 2004). The main motive of cell encapsulation methodology is to overcome the existing problem of graft rejection and decrease the requirement for long-term use of immunosuppressive drugs. Use of an encapsulation device is to provide a physical barrier between transplanted β-cells and their recipients to overcome some of these challenges by eliminating the need for immunosuppression and promote islets survival post-transplantation (Ludwig, Ludwig et al. 2017). To overcome the issues of immune-barrier biocompatibility, the researcher designed a hollow fiber membrane (HFM) (Teotia, Kadam et al. 2017).

The primary aim of the present study is to empower efficient islet differentiation of Human bone marrow derived mesenchymal stem cells using bioactive molecules cocktail. Several parameters for differentiation proficiency of hBMSCs were assessed, comprises expressional studies of several transcriptional factors essential for pancreatic endocrine differentiation, islet functionality assays, and microRNA profiling of neogenic pancreatic islets at different intervals of the differentiation protocol. Translation of this project *in vivo* requires a transplant of approximately 0.5 million islets per adult human being. To attain such a generous yield several strategies can be implemented, one being microRNA manipulation. Inhibition (LNA-Lock Nucleic Acid) of certain overexpressed and non-favorable microRNA, can abrogate translational repression, in turn enhancing protein expression which, is crucial for islet yield, functionality and longevity (Stenvang, Petri et al. 2012). For the islets to

achieve greater survival *in vivo,* the work further focuses on islet encapsulation within a hollow fiber membrane (HFM).

Significance: Optimized islets can be translated for a clinical purpose for the treatment of diabetes. These islets can be used for autologous transplantation in diabetic patients. We expect these optimized islets to be more efficient in terms of functionality and longevity, thus, providing better treatment and an extended relief from successive insulin administration.

Specific objectives:

1.Systematic evaluation of combinatorial activity of bio-active molecules for islet neogensis of hBMSCs into functional islets.

2. Assessment of molecular mechanism in islet differentiation pathway from hBMSCs using best combination of bioactive molecules.

3. Assessment of microRNA profile & microRNA modulation by silencing /inhibition (LNA)for augmentation of islet differentiation from hBMSCs.

4. Encapsulation & transplantation of islets into a diabetic mouse model for effective therapy.

Objective-1: Systematic evaluation of combinatorial activity of bio-active molecules for islet neogensis of hBMSCs into functional islets

Isolation and characterization of Human bone marrow-derived MSCs(hBMSCs)

Under the aseptic condition, 5-10 ml of heparinized bone marrow samples were obtained from patients undergoing orthopedic surgical process using standard protocol (Sun, Chen et al. 2007). hBMSCs were isolated and characterized for mesenchymal stem cell markers using flow cytometry. At the passage 3-5 these cells were positive for CD105(71.6 %), CD44(56.1%) and expressed low levels of CD34(18.2%), CD31(2.7%) with high vimentin expression. Further, hBMSCs were differentiated to form adipocytes, chondrocytes, and osteocytes with appropriate growth factors assessing for their trilineage potential (Multipotent).

Standardization of Islet differentiation & potentiation of islet differentiation with bioactive molecules cocktail

Present research work focused on the use of bioactive molecule "swertisin" as differentiating agents isolated & characterized from herbal plant *Enicostemma littorale*, which is very well reported from our lab for its anti-diabetic and anti-oxidant properties (Gupta, Dadheech et al. 2010) (Dadheech, Soni et al. 2013) (Dadheech, Srivastava et al. 2015) (Srivastava, Bhatt et al. 2016) (Srivastava, Dadheech et al. 2018). Initially, hBMSCs were differentiated into islet like cell clusters within 10 days using swertisin (15 µg/ml) as a bioactive differentiating molecule. At the end of the 10^{th} day, clusters were harvested and subjected to Dithizone Staining (DTZ). C-peptide & glucagon expression by Immunocytochemistry (ICC) were done. 10^{th} day clusters demonstrated bi-hormonal (Insulin, Glucagon) phenotype. Hence, based on our 10^{th} day results and scientific reports(Sun, Chen et al. 2007) (Jafarian, Taghikhani et al. 2014) for improvement, protocol was extended till 18^{th}day which revealed a maturation in the clusters. The revised protocol of 18^{th} day was thus selected for further experiments.

To combat the oxidative stress (protect islets), we decided to use curcumin(Meghana, Sanjeev et al. 2007) and genistein(Fu, Zhang et al. 2010) in a sequential manner during islet differentiation protocol (four stages) from hBMSCs. Apart from differentiating agents, culture condition here optimized for enhanced islets yield by using ultra-low adherence plates.

Differentiated islet functionality was assessed by three methods: (1) Immunocytochemistry (2) Flow cytometry (3) Human c-peptide release assay. In Immunocytochemistry experiments, we observed the high intensity of fluorescence for human c-peptide, Glucagon, Glut2, Nkx6.1protein expression in islet-like cell clusters. In flow cytometry, results demonstrated that bioactive molecules cocktail expressed 77.8 % & 16.7 % c-peptide and glucagon respectively. I*n vitro* functionality of islet like clusters was finally assessed by the amount of C-peptide release assay by islet like cell clusters with glucose challenge. A significant increase of c-peptide release was observed in bioactive molecules cocktail group as compare to undifferenatiated hBMSCs, confirming excellent functionality.

Objective 2: Assessments of molecular mechanism in islet differentiation pathway from hBMSC using best combination of bioactive molecules

From objective 1, we have optimized islet differentiation protocol. In this objective, expression of various transcription factors involved in islet differentiation were assessed using Taqman Low-Density Array (TLDA). We screened day 18th islet-like clusters from the test plate as well for the positive control (Activin A) in comparison to undifferentiated hBMSCs for 11 different genes involved in pancreatic islet development. *NGN3* and *MAFA's* (Marker for Beta-cell specification) expression was seen to an increase in 18th-day clusters. In western blot analysis REG-1, NGN-3, and PDX-1 proteins expressions peaked on the fourth day, followed by a reduction in the former with increase in the latter's expression indicating differentiation into an islet-like lineage. This was further strengthened by GLUT2's expression in day 18 clusters. Additionally, during islet differentiation, a 10thday cluster were harvested and immunocytochemistry for protein expression of FOXA2, REG-1, PDX-1, NGN-3 was performed. Thus, bioactive molecules cocktail demonstrated efficient differentiation by expressing gene and protein in differentiated islets compared to normal undifferentiation cells.

Objective: 3 Assessments of microRNA profile & microRNA modulation by silencing /inhibition (LNA) for augmentation of islet differentiation from hBMSCs.

We evaluated microRNA profile during islet differentiation from human bone marrow-derived mesenchymal stem cells. We harvested clusters on Day 10 and Day 18, along with undifferentiated hBMSCs during differentiation. Based on the scientific reports from in vitro differentiation of embryonic stem cells, 28 candidate microRNAs were selected whose targets have been identified and role in islet differentiation has been studied. Our results showed prominent alteration in the expression of 11 microRNAs known to target β-cell differentiation, function, and homeostasis. However, we will be using microRNA inhibitor (Lock Nucleic acid LNA) to increase the efficiency of islet differentiation and increase islet longevity.

Objective: 4 Encapsulation & transplantation of islets into diabetic mouse model for effective therapy.

In the present objective, the HFM (Hollow Fiber Membrane) were used along with bioactive cocktail to increase islet longevity for effective islet encapsulation.

Rat islets from young rat pancreas were successfully isolated & cultured overnight before encapsulating them in HFM, islets viability was checked after encapsulating islets & bioactive molecules cocktail in HFM & placing them in media containing normal & diabetic mice serum for 48-hour incubation (*In Vitro*). Annexin V/PI staining result suggested islets were viable & bioactive cocktail prevented necrosis due to their islets protective & antioxidant properties. A similar study will be performed in differentiated islets from hBMSCs in the presence and absence of bioactive molecule cocktails for efficient islet transplantation.

Physiological competence of isolated rat islets to maintain glucose homeostasis was assessed *in vivo* by transplantation of rat islets (≈ 1000 IEQ islets) encapsulated in 3 HFM (3 cm length) & transplanted in STZ mice. Xeno-transplantation was successfully performed using rat islets & Hollow Fiber Membrane (HFM) in STZ diabetic mice, which resulted in normoglycemia of diabetic mice in a month.

Conclusion:

From this study, we conclude that bioactive molecules cocktail potentially differentiates hBMSCs to pancreatic islets having higher longevity. Therefore, this cocktail could be a promising therapeutic tool for effective diabetes therapy. To our knowledge, the expression of candidate microRNAs in islet differentiation, using hBMSCs as the source has been reported for the first time. In nutshell, use of bioactive molecules cocktail & novel encapsulating device are breakthrough in an exploration of hBMSCs for treating diabetes mellitus.

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4. Dadheech, N., et al. (2015). "Swertisin an anti-diabetic compound facilitate islet neogenesis from pancreatic stem/progenitor cells via p-38 MAP kinase-SMAD pathway: an in-vitro and in-vivo study." PloS one **10**(6): e0128244.
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Publication:

1. "Potentiating of Islet differentiation from hBMSCs using bioactive molecules cocktail" **Mitul Vakani**, Abhay Srivastava, Shubham Rathore, Sarita Gupta (Manuscript under preparation)
2. "Swertisin ameliorates diabetes by triggering pancreatic progenitors for islet neogenesis in Streptozotocin treated BALB/c mice". Biomedicine & Pharmacotherapy April 2018. [Abhay Srivastava, Nidheesh Dadheech, **Mitul Vakani,** Gupta Sarita]
3. "Pancreatic resident endocrine progenitors demonstrate high islet neogenic fidelity with significant homing towards its niche in diabetic mice" 2018. [Abhay Srivastava, Nidheesh Dadheech, **Mitul Vakani**, Sarita Gupta] Journal of Cellular Physiology (Under Revision)
4. "Herbs to Stem cells: A perspective in diabetes therapeutics: Review", (2018); [Abhay Srivastava, **Mitul Vakani**, Gurpreet Bharadwaj and Sarita Gupta] **(Communicated)**

Abstracts Published through Poster Presentations in International Conference:

1. **Mitul Vakani**, Abhay Srivastava, Shubham Rathore, Deep Maheshwari and Sarita Gupta "Dynamic expression of microRNAs in Islet Differentiation from Human Bone Marrow Derived Mesenchymal Stem Cells" Frontiers in Islet Biology and Diabetes" Keystone, Colorado, USA. February 2018
2. **Mitul Vakani**, Abhay Srivastava, NidheeshDadheech, Sarita Gupta. "Generation of functional islet-like insulin-producing clusters from human BMSCs using novel bioactive molecules: An in vitro study". Till & McCulloch meeting, Whistler, BC, Canada October 2016.
3. Abhay Srivastava, **Mitul Vakani**, NidheeshDadheech, Sarita Gupta. "Novel Bioactive in potentiating Islet Neogenesis from mouse Intra-Islet Mesenchymal Stem Cells". Till & McCulloch Meetings, The Sheraton Center, Toronto, Canada. October 2015.
4. Abhay Srivastava, Tushar Patel, **Mitul Vakani**, Sarita Gupta. "PARP-1 protein regulates Islet differentiation in mouse intra-islet progenitors through Smad signaling". 3rd Stem Cell Biology meeting at Cold Spring Harbor Laboratory, New York, USA. October 2015.

Award:

1. Graduated Aptitude Test in Engineering (**GATE-2014**) (Life Science)
2. Council of scientific &Industrial Research-National Eligibility Test (**CSIR-NET-LS - 2014**) (Life Science)

3. **First prize awarded** for best poster presentation in DBT-MSUB-ILSPARE-JRF's poster competition organized on 3rd October 2013 at Dr.vikram Sarabhai Institute of cells and molecular biology, faculty of science, The M.S.University of Baroda.
4. Received **Travel Grant** from "Till and McCulloch" for attending & presenting poster in international conference held in Whistler, British Colombia, Canada, October 2016.
5. Received **Travel Grant** from "Department of Biotechnology (DBT)-CTEP" for attending & presenting poster in international conference "Keystone Symposia Conference-Frontiers in Islet Biology and Diabetes" held in Keystone, Colorado, USA, February 2018.

Poster/Oral Presentation

1. Poster Presentation entitle "Studying gene expression kinetics of various transcription factors involved in islet differentiation from pancreatic progenitor /stem cells using bio-active compound isolated from EL" on Poster Presentation competition organized by Dr,Vikramsarabhai Institute of Cells and Molecular Biology DBT-MSUB-ILSPARE,Facutly of Science, The M.S.University of Baroda, Vadodara on 3rd October 2013.
2. Oral Presentation entitle "Assessing the effect of PARP inhibitor & EL on ROS mediated mitochondrial and islet dysfunction" on oral Presentation competition organized by Dr,Vikram sarabhai Institute of Cells and Molecular Biology DBT-MSUB-ILSPARE,Facutly of Science, The M.S.University of Baroda, Vadodara on 30th September 2014.
3. Oral Presentation entitle "Optimizing differentiation of human Bone Marrow Mesenchymal Stem Cell (hBMSC) to Islets of langerhans using novel bio-active molecule" on oral Presentation competition organized by Dr,Vikram sarabhai Institute of Cells and Molecular Biology DBT-MSUB-ILSPARE,Facutly of Science, The M.S.University of Baroda, Vadodara on 28th Septamber2015.
4. Poster presentation entitled "Mesenchymal stem cells in Islet differentiation: A transition from mouse to human" in Two Day National Symposium on "Omics….to structural Basis of Diseases" organized by Department of Biochemistry, Faculty of Science,The M.S. University of Baroda, Vadodara on 30th -01st October 2016.

Workshop:

1. Attended Three Days Hands on training on "Advanced microscopy & Imaging Techniques" jointly organized by DSS Imagetech Pvt Ltd,Olympus medical Systems India

Pvt Ltd and Photometric(USA) along with The M.S.University of Baroda,Vadodara,during 1st -3rd December 2015.

2.Actively Participated in Organizing the Four Day Workshop on "Advanced Techniques in Stem cell Research" Jointly Organized by DBT-MSUB-ILSPARE and Department of Biochemistry, Faculty of Science, The maharaja Sayajirao University of Baroda, Vadodara held on 31st December 2014 to 3rd January 2015.

3.Participated in the Technical Education Quality Improvement Programme (TEQIP) workshop on "Tissue Engineering: Biomaterial & Stem cells for Manufacturing of Biological Tissue" organized at IIT-Hyderabad, during 18th -23rd July 2016.

4. Participated in Three Day hand on workshop in "In Vivo Preclinical Imaging and Drug Discovery" by Advanced Centre for Treatment, Research and Education in Cancer (ACTREC)Tata Memorial Centre, Navi-Mumbai, Mumbai held on 20th -22nd September 2017.

Patent (Filed):

Sarita Gupta, **Mitul Vakani**, Abhay Srivastava, Nidheesh Dadheech. "Swertisin as potent and novel molecule for islet differentiation from human bone marrow derived mesenchymal stem cells". Patent filed to intellectual property India, application no. 201621012988(Filed)

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