CHAPTER 7

SUMMARY & CONCLUSION

7.1. Summary

The thesis highlights the development and optimization of pancreatic islet differentiation protocol from bone marrow-derived mesenchymal stem cells source using bioactive molecule cocktails along with augmentation of xeno-islet transplantation protocol by applying hollow fiber membrane. The main objectives of this thesis were to find three important aspects (1) Role of bioactive molecule cocktail in islet differentiation from hBMSCs (2) Evaluation of various transcription factors during four-stage differentiation protocol (3) Identification of key microRNAs expression in islet differentiation (4) Role HFM in "Rat to Mouse" xeno-islet-transplantation.

Objective 1 (Chapter :3) of the thesis focuses on the isolation, characterization, and islet differentiation potential of hBMSCs. hBMSCs were isolated and purified from six bone marrow of non-diabetic human subjects and were further characterized extensively for mesenchymal stem cell markers. Isolated hBMSCs were positive for stem cell markers like CD105, CD44, nestin, STRO-1, vimentin, α SMA, fibronectin. Further, we found basal level gene expression of unique pluripotent stemness markers like *SOX2, cMYC, KL4, NANOG, OCT-4*. After stem cell marker confirmation, the multipotency of hBMSCs was functionally evaluated by trilineage differentiation where they successfully differentiated into osteocytes, adipocytes, and chondrocytes. The major aim of this objective was to establish an effective islet differentiation protocol. Initially, we assessed the islet differentiation process from hBMSCs on 10^{th} day using two separate differentiating agents activin A and bioactive molecule cocktails. The color intensity of DTZ staining and insulin transcript of cell clusters on 10^{th} day was not remarkable enough to qualify for good islets. However, these results provided with the proof of concept for the possibility of pancreatic islet differentiation from hBMSCs using bioactive molecules. On that count, we extended our islet differentiation protocol from day 10 to day 18 four-stage protocol, with major modification like the addition of differentiating factors, growth factors along with manipulation of culture condition such as the use of ultra-low adherence plates with shaking culture condition. Thus, we obtained high yield 3-dimensional islets like cell clusters (ILCCs) with positive DTZ staining and high insulin transcript in both group activin A and bioactive molecules cocktail (BMC). Further, the functional characterization of these ILCCs was confirmed by important transcription factor's gene expressions such as NGN3, PDX-1, MAFA, insulin, glucagon, and protein expression such as insulin, human -C peptide, glucagon. These ILCCs obtained from hBMSCs shown

excellent human c-peptide release in response to the physiological concentration of glucose. Interestingly, bioactive molecules cocktail group including swertisin, curcumin, and genistein demonstrated a remarkably low ROS level and apoptosis in ILCCs as compared to activin A due to islet protective effects of curcumin and swertisin. The functional characterization of these ILCCs specified bioactive molecules cocktail to be more potent than activin A in forming islets like cell clusters. The results established the efficiency and reproducibility of our augmented multi-step islet differentiation protocol for the accomplishment of ILCCs from hBMSCs using bioactive molecules cocktails and activin A. Thus, our findings suggested that bioactive molecule cocktail containing small molecules "swertisin" could be one of the crucial inducers of hBMSCs differentiation towards a pancreatic islet phenotype.

After establishing the islet differentiation protocol, we explored the transcription factors in the paradigm of islet differentiation from hBMSCs. In chapter 4, we examined to recapitulate all-important transcription factors involved in pancreatic islet development. We generated islets by the progressive differentiation of hBMSCs through the known stages of embryonic pancreatic development. This progression begins with the formation of definitive endoderm from mesoderm origin stem cell, followed by a transition to pancreatic progenitors, endocrine progenitors, and finally, hormone-expressing mature pancreatic islets. We examined temporal (0 Day,5 Day,10 Day,15 Day, and 18 Day) genes and proteins expression of all essential transcription factors involved in islet differentiation. First, we investigated definitive endocrine progenitor markers expression such as GATA4/6, SOX 9/17, FOXA2, and HNF-1β during islet differentiation in both groups. Secondly, we demonstrated pancreatic endocrine progenitor markers expression like PDX-1, NKX2.2, NKX6.1 during islet differentiation in both groups. In order to figure out the islet differentiation progression, we found pancreatic endocrine progenitor key markers expression such as NGN-3, NEUROD1, PAX4/6, MAFA/B, RFX-6, and ARX during islet differentiation in both groups. Interestingly, in the current study, some important transcription factors such as FOXA2, PDX-1, and NGN-3 expression were early detected in the bioactive molecules cocktail group as compared to activin A group, indicating rapid initiation of islet differentiation because of the presence of "swertisin" as a leading bioactive molecule in bioactive molecules cocktail group. Finally, we examined the gradual increase in the expression of mature functional pancreatic islet markers like *INS, GCG, SST, PPY*, and *GLUT2* in the final stage of islet differentiation in both groups. To the best of our understanding, to date, this is the foremost report for addressing a multi-step islet

differentiation event triggered by the ectopic expression of crucial transcription factors during hBMSCs-derived ILCCs.

Many reports suggest that microRNAs are associated with the regulation of pancreatic transcription factors networks. After identifying the job of transcription factors in islet differentiation, we explored the role of microRNAs in the paradigm of islet differentiation from hBMSCs. In chapter 5, the 28 candidate microRNAs expression was profiled at different stages during the process of islet differentiation using activin A and bioactive molecules cocktail from hBMSCs (isolated three different non-diabetic human subjects). We found dynamic expression of candidate microRNAs such as miR-375, miR-146a, miR-124a, miR-182, miR-187, miR-95, miR-204, miR-342, miR-34a, miR-7, miR-382 during islet differentiation in both groups. Further, we also validated our candidate microRNA expression in an early stage of islet differentiation & confirmed miR-124a and miR-195 highly upregulated in the initial stage of islet differentiation that eventually reduced protein expression of FOXA2 (a key regulator of definitive endoderm progenitors) and NGN3 (master regulator of pancreatic endocrine progenitor) respectively in both groups. The goal of the current objective was to identify key microRNA and manipulate them in order to accelerate the islet differentiation process from hBMSCs. Thus, we inhibited miR-124a using power inhibitor lock nucleic acid (LNA) i.e. LNA-has-miR-124a at the initial stage of the islet differentiation process in both groups. Our immunocytochemistry data revealed that cell clusters treated with LNA-has-miR-124a demonstrated increased expression of FOXA2 and NGN-3 protein as compared to non-treated in both groups, indicating that inhibitor strategy leads to promote the early stage of islet differentiation. Thus, to the best of our knowledge, we established for the first time the dynamics of candidate miRNAs that will provide crucial insight in islet differentiation from hBMSCs. Hence, our present study has established a novel islet differentiation protocol using both approaches bioactive molecules cocktail as well as microRNA inhibitor that induced the differentiation of hBMSCs into islet-like cell clusters and leads to enhancement of islet quality in term of functionality, yield & viability for creating efficient new therapeutic avenues in diabetes treatments.

After clearly defining the role of bioactive molecules cocktail and microRNAs in islet differentiation (Chapter:3,4 & 5), we moved towards another important aspect of the thesis, which was to improve islet encapsulation and islet transplantation using bioactive molecules and hollow fiber membrane (HFM). First, we differentiated hBMSCs into ILCCs using 18th

day islet differentiation protocol in the presence of a bioactive molecule cocktail as a differentiating agent and we performed *in vitro* islet encapsulation experiments using calcium alginate techniques along with HFM. We confirmed that the treatment of bioactive molecules during islet encapsulation increased islet viability in the presence of diabetic serum as compared to the non-bioactive molecule's treatment group. Secondly, we executed similar experiments with isolated rat pancreatic islets in order to validate islet encapsulation techniques and we found identical results, suggesting that bioactive molecules like curcumin, swertisin, and genistein play an important role in increasing islet viability during islet encapsulation. Thus, based on our *in vitro* study, we confirmed that our islet encapsulation strategy using a combination of bioactive molecules and HFM is superior to the conventional method of islet encapsulation in terms of reinforces encapsulated islet viability.

After the achievement of our *in vitro* islet encapsulation study, we further extended the study at *in vivo* islet encapsulation level. Here, we attempted to solve islet longevity problems during islet transplantation by conducting a systematic study on encapsulated rat islets transplantation into STZ diabetic mouse using HFM as an immune-isolating device and "Rat to Mouse" xeno-islet transplantation model system. We found that transplantation of encapsulated rat islets successfully brings hyperglycaemia to normoglycemia and the graft was stable for up to 3 months in the intraperitoneal cavity of the STZ mouse model, suggesting that reversal of diabetes in the HFM transplanted group. We further noticed that the transplanted group-maintained body weight throughout the post-transplantation period. Interestingly, our graft retrieval study revealed the absence of the immune cell infiltrations inside HFM and ample new vascularization outside HFM, indicating graft acceptance with islet longevity. In the broader context, the current investigation exhibited that islet xeno-graft can survive in the HFM capsule with enhanced biocompatibility without the usage of immunosuppression, henceforth offered "proof of concept" in the field of islet encapsulation and islet transplantation for successful diabetes therapy.

In nutshell, in this thesis, we have successfully resolved some outstanding challenges in the field of pancreatic islet differentiation, microRNA biology, and islet transplantation to design better therapeutic strategies for treating diabetes.

7.2. Overall conclusions:

The conclusions of the present study can be understood as following:

- In the present study, we are able to isolate and characterize a human bone marrow-derived mesenchymal stem cells from the bone marrow of six non-diabetic human subjects.
- We developed a scalable and easily reproducible novel 18-day islet differentiation protocol for generating 3-dimensional 3D- ILCCs from hBMSCs using a combination of bioactive molecule cocktail and manipulating culture condition. Day 18 ILCCs released human c-peptide in a glucose-dependent manner, demonstrating *in vitro* functionality. The fact that these novel protocol permit creation of ILCCs in the context of the cellular complement of stem cell-based islets further improves their utility for research and, ultimately, therapeutics.
- We demonstrated that hBMSCs have tremendous *in vitro* expansion potential and can be differentiated into the highly efficient stepwise multistep lineage, including definitive endocrine, pancreatic progenitor, endocrine progenitor, and mature pancreatic islets, by evaluating temporal gene and protein expression. Thus, recapitulating in *vivo* pancreatic islet development.
- Our study is the first to report dynamic expression profile of candidate miRNAs of islet differentiation from hBMSCs and further suggested the role of anti-miR-124a to accelerate *in vitro* islet differentiation by immense expression of key transcription factors such as FOXA2 and NGN-3 in both bioactive molecule cocktail and activin A. Our results strongly indicate a positive effect of power inhibitor LNA -miR-124a on islet differentiation from hBMSCs, which could be a promising therapeutic strategy for diabetes.
- We augmented *in vitro* rat islets/ differentiated ILCCs islet encapsulation process by reinforcing the islet viability using bioactive molecules cocktail and HFM scaffold for effective islet transplantation.
- Further, we developed an HFM encapsulation protocol to increased islet longevity and effectively combating diabetes in the “Rat to Mouse” xeno-islet-transplantation model without the use of immunosuppression, bringing us one step closer to applying this islet transplantation therapy in humans.

Thus, in this study, we used multiple approaches, such as the use of hBMSCs, bioactive molecules cocktail, microRNA inhibitor along with hollow fiber membrane which should be extrapolated to a human subject in the near future to achieve better therapeutic strategies from bench to bedside for the treatment of diabetes mellitus.

7.3. Thesis summary