EVALUATION OF ISLET NEOGENESIS FROM EXTRA-PANCREATIC CELL LINEAGES USING HERBAL PLANT

THESIS
SUBMITTED TO
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA, VADODARA, GUJARAT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

By

Nidheesh Dadheech

Under Guidance of
Prof. Sarita Gupta
Department Of Biochemistry
Faculty of Science
The Maharaja Sayajirao University of Baroda,
Vadodara- 390 002, Gujarat, INDIA
October, 2012

CERTIFICATE

DEPARTMENT OF BIOCHEMISTRY
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA,
FACULTY OF SCIENCE
VADODARA

STATEMENT UNDER O. Ph. D 8/ (iii) OF THE M.S. UNIVERSITY OF BARODA, VADODARA

This is to certify that the work presented in the form of a thesis entitled "Evaluation of Islet Neogenesis from Extra-Pancreatic Cell Lineages Using Herbal Plant" in fulfillment of the requirement for the award of the Ph.D. degree of The M.S. University of Baroda by Mr. Nidheesh Dadheech, is his original work. The entire research work and thesis have been built up under my supervision.

(Prof. Sarita Gupta)
Research supervisor,
Department of Biochemistry,
Faculty of Science,
M. S. University of Baroda,
Vadodara- 390 002.

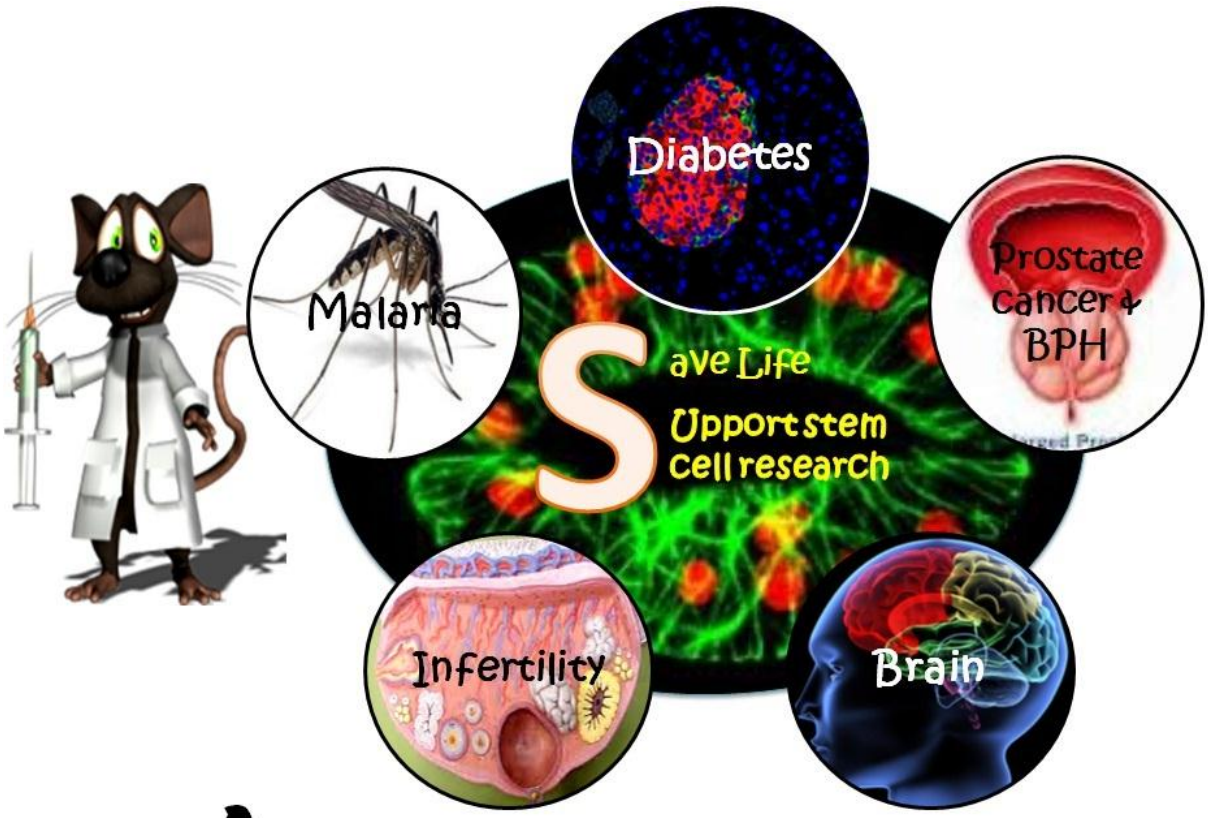
DECLARATION

This is to certify that the work presented in this thesis entitled "Evaluation of Islet Neogenesis from Extra-Pancreatic Cell Lineages Using Herbal Plant" for the degree of "Doctor of Philosophy "is original and was obtained from the studies undertaken by me under the supervision of **Dr. Sarita Gupta,** Professor, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara -390002, India.

I also declare that the contents in the thesis have not previously formed in any form for the award of any degree, diploma, fellowship, associateship or any other similar title or recognition.

Date: October 2, 2012 (Nidheesh Dadheech)
Place: Vadodara

"The deepest sin of the human mind is to believe things without evidence"



A Stem Cell cures for all...

MOLECULAR ENDOCRINOLOGY AND STEM CELL RESEARCH LAB

ACKNOWLEDGEMENT

Researcher is a traveler and his research is an endless journey. He keeps on moving with a continuous endeavor of exploring newer avenues with the torque of both failure and success. During this long journey, he needs to take halt and the completion of this thesis is the first halt of ***"my"*** *journey. Today when I finished this first phase, I go in a flash back and realize that I was a stone with no value, but I learned and opened my eyes with learning and blessings at a place called "Department of Biochemistry" and by the abundant grace and blessings of GOD and HIS everlasting light of guidance, I was able to accomplish this work. I would have never reached at this stage so far in my journey, if, l I was not fortunate to have support from ma well-wishers. I thank one and all for accompanying me, sharing my burdens and making this journey a happy and memorable experience, I should acknowledge all at this place, thought the word acknowledgement itself is a very small things in place of what I get.*

First a foremost, I should thanks and acknowledge ***Department of Biotechnology, Ministry of Science and Technology, Government of India,*** *for extending financial support for my Ph.D. tenure as JRF and SRF.*

I am grateful to ***Prof. Yogesh Singh****, Vice Chancellor, The Maharaja Sayajirao University of Baroda, Vadodara for giving me opportunity to work in this premier University of the nation and providing all necessary facilities and encouragement for the completion of my thesis work.*

An ostentatious use of words will not suffice to express my endeavoring sincere thanks and gratitude to my guide ***Dr. Sarita Gupta****, Professor, Department of Biochemistry, for her benevolent guidance, keen interest with critical and valuable suggestions without which this work could not have been possible to come to its fruition and worthy presentation. I am extremely grateful to her for giving me an opportunity to explore this field, even when I had no clue about this subject. She provided me full space and freedom to work and execute my ideas in research and prove them without any boundations. It is her qualities of respecting everyone's thinking and cultivating confidence in students that I admire the most. I must confess this here, that she had not been just a guide, but more than that a guardian. I always felt a motherly care, affection and offcourse anger when I needed but that all made me capable for all this and today I would like to say a million thanks to you madam for all what I have*

learned and got at this stage. I would certainly not have learnt cell biology so well, if you would have not shown trust on me. What all I can say is that she is indeed a best guide for any student. Thanks madam...for everything.

It gives me immense pleasure to express my deepest sense of gratitude and regards to ***Dr. Ramesh Bhonde****, Professor and Dean, Manipal Institute of stem cell and regenerative medicine, Manipal for his excellent guidance, incessant discussion and constant encouragement throughout my work. He has been the best mentor and teacher I could have wished for. It was his care and moral support that made this difficult task an easy one. His inspiration has been instrumental in shaping my future career.*

It is my privilege to express heartful thanks and sincere gratitude to ***Dr. Girish M Shah,*** *CRCHUL, Laval University, Quebec Canada, for offering me six months international Canadian Commonwealth Scholarship to pursue research work in his lab, and providing his valuable help in experimental planning, moral support and teaching in research aspect of my thesis work. I am immensely grateful to him. He has been constant source of encouragement, help and inspiration to me.*

One more agilent personality to whom I should pay respect and honor is ***Prof Nikhil Desai,*** *Dean Faculty of Science, for his support and guidance at time when I needed. Thank you sir….*

The Biochemistry department is not just an address where I conducted my research …. There's something more to it….a lot more…. For past five years, the department had been my second home. I call it a home because the freedom, love, respect, concern and warmth provided by people in the department makes you feel at home, with your family…. and that's the reason we proudly call it "Biochem Family". The temperament, spirit and enthusiasm prevailing in the air of this place energizes you to put in your best effort in whatever you do. ***Prof. G. Naresh Kumar****, Head of Department, whose humble and democratic nature allowed approaching him without slightest hesitation for any kind of problems or even suggestions. He always spared ear to it and readily acted upon when required. Those tea-time discussions with him on science-and-beyond, always encouraged to 'think big' and perform better.* ***Prof. S. D. Telang's*** *love and concern,* ***Prof. Rasheeda Begum's*** *continuous encouragement, lively discussions with* ***Dr. Acharya*** *that sharpens one's logic, sharing lighter moments with* ***Dr. C Ratnaprabha's****- a person with great sense of humor,* ***Dr. M D Jani's*** *appreciations & very healthy approach of* ***Dr. Jayshree Pohnerkar, Dr. Pushpa Robin, Dr. Laxmipriya*** *and* ***Dr.***

Devesh Suthar *played important role in carving my overall personality as a researcher. I am thankful to all of them for warmly accepting me and ensuring that I bring the best out of me.*

I am also thankful to few more teachers, ***Prof. AV Ramachandran****, Former, Dean,* ***Dr. A.N. Mishra (Dean, Pharmacy Dept)*** *and* ***Dr. T Bagchi,*** *Microbiology Dept., for being an examiner of research committee. Also many thanks to* ***Dr. Anandwardhan Hardikar****, Associate Professor, Melbourn University, for helping me in confocal studies at NCCS pune and providing guidance at various phases of my phd. I would also like to thank* ***Dr. H Krishnamurty****, Head C-Camp, NCBS-TIFR, Bangalore for giving me a wonderful training on flowcytometry, and here a very special thanks to* ***BD Biosciences*** *for helping financially and arranging FACS workshop. A warm heartdly acknowledge to* ***Dr. Aanad S Srivastava,*** *GIOSTAR,* ***Dr. Rangnath Mishra****, Univ. of California and* ***Dr. Rajarshi Pal****, MIRM, Bangalore for helping me in manuscript writing, correction, all your comments and suggestion made my science more valuable so thanks for that. At this place a very special thanks to* ***Dr. P.D. Gupta*** *who actually helped me in laying a strong foundation on which I was able to build my career, I looked upon him as my research idol and feel fortunate to have imbibed some of his great qualities in management of science.*

With a profound sense of gratitude I take this opportunity to express my thank to all past lab seniors ***Dr. Vihas Vasu, Dr. Jyoti, Dr. Anil Pillai, Dr Laxmipriya, Dr. Chirayu Pandya, Dr. Niraj Bhatt and Dr. Prakash Pillai*** *for not only helped me learning science and experimentation but also provided full support till the end of my phase. I would like to mention name of a very important person who actually contribution a lot in ma thesis and without this man it would have not been possible, ladies and gentleman, the one and only* ***Dr. Sanket Soni, A man of Phytochemistry and Ayurveda****. Sanket sir a special thanks for helping in isolating compounds and providing me as in when I required and whatever quantity. Thanks once again….*

Coming to my home place i.e. MESCRL…… it's a wonderful place, where anyone who comes observe ***" fell good factor".*** *With the grace of god and hardwork together with all lab member's, the lab is now standing on a comparable level with many institutional labs. I felt very genuine and homely environment in ma lab so I like to say thanks to all my lab members* ***Akhilesh*** *(Camel of MESCRL),* ***Tushar*** *(Jambojet and Voracious eater),* ***Muskaan****(a very simplified lady and a strong supporter of lab),* ***Regitha*** *(one and only sister of RAjni daDa),* ***Bhanvesh, Komal, Prateek, Abhay*** *(Scientist) and* ***Mittul*** *(Wakka wakka).*

Abhay, *a real fighter of my lab, is a man who can handel all the tension and work without any fold on a head, and that was spirit who helped me in last phase of my work. He is the one who promisingly behind and lookafter the work inspite I was not able to provide much time. Abhay a very sincere and deep thanks for our outstanding support in completion of this venture. I should not forget M**ittul** also a very new lab member, who also generously contributed in shaping this thesis. It was extremely difficult to complete this with help of both of you guys. I again thank you for being standing with me. At this place, I remember a very special friend and my colleague,* ***Anubha Singh.*** *I thanks you anubha for creating a start and supporting me whenever I required.*

One more chapter played by some contributing people are my dissertation student, who worked with me for their M. Sc. Dissertation and this was the hidden sources of energy that boosted my output. I thank ***Gauri Sanghvi, Sucheta Solanki*** *(Now my betterhalf),* ***Shivika Gupta, Veena Kinare, Arpita Dave****,* ***Neha Pranjape, Paresh Srimali, Mrudula Sane*** *and appreciate their sincerity, punctuality; obedience and dedication for work that helped me learn a few things from them as well.*

Moving beyond lab, the first names that come to my mind are my long time buddies ***Mrinal Sharma*** *(Bilal sahib),* ***Rushikesh*** *(Chotu),* ***Hemanta (****betu),* ***Prashant****, Thank you all of you for love and support. Many more names are there without whom I fee incomplete are* ***Hiren Bhai, Hemendra Bhai, Aditi Maa'm, Keyur Sir, Maulik sir, Shajil Sir, Vikas Sir, Iqbal, Vijay, Ansar Bhai, Swapnali, Purna,*** *thank them for being a continuous source of motivation and a sink for my frustrations. Mrinal and Rushi provided me lot of support and care, whenever I needed guidance, whenever I got confused. These angels provided me that needed comfort zone and accepted me the way I am. In my oddest times they stood by me whenever I wanted them…. the way I wanted them. Thanks ma Freindzzzzzzz……. I love u all……*

I would also like to say thanks to ***Dr. Sharad Gupta*** *for helping me in each and every phase of my life. Sir your constant support, jolly nature has inspired me a lot. A million thanks to you…*

I am highly grateful to the Non-teaching staff of my department ***Pethe Sir, Akshita Maa'm, Ramesh Bhai Nare, Ramesh Kamle, Shailesh Bhai, Manish Bhai,*** *a very special thanks to animal hose staff* ***Ramesh Bhai Parmar, Vijay Bhai, and Narayan Bhai*** *for helping in animal experimentation and being constant source of encouragement and help.*

This thesis would have been possible with hard work, devotion, dedication and concentration, which I was lessoned by my ever loving parents ***Smt. Santosh Dadheech & Shri. Om Prakash Dadheech****, my wife* ***Sucheta Dadheech*** *and my In-laws* ***Shri VC Solanki and Smt. Poonam Solanki.*** *Thanks for supporting. Since the very beginning of my childhood when I started withstand*

and running on the earth in support of their fingers. I would have been able to prove the perfection of their trainings and dream true. With gratitude and reverence, I acknowledge and admire the love, confidence and moral support bestowed on me. My wife, Sucheta, who was a constant source of inspiration to me. She is the one who understood me what I needed, when I needed and reacted upon. She never demanded and always provided a space and time in last phase where I really had to devote time in writing thesis. She was the one who shoed trsut and confidence that I will achieve all my goals and succeed in ma research and will do best out of it. If she could have not been there it would not been possible. Thanks u ma love for being standing with me in all phase of my life.

And finally, my heartfelt gratitude to ***all the people*** *within the MS University, Baroda who helped me to steer when sailing close to the wind of bureaucracy on the last leg of the race!. Last but not the least, I would like to acknowledge* ***all those whose name*** *do not figure here but helped me directly or indirectly in shaping this thesis.*

Date: 1st October, 2012 ***(Nidheesh Dadheech)***

AGE- advanced glycosylation end-product
ALP- Alkaline Phosphate
APC- Allophycocyanin
aPKC- Atypical protein kinase C
ATCC- American type culture collection
ATP- Adenosine triphosphate
BM- Bone marrow
BSA- Bovine serum albumin
cAMP- Cyclic Adenosine monophosphate
CCl_4- Carbon tetrachloride
CDK- Cyclin dependent kinase
CNS- Central Nervous System
CPCSEA- Committee for the purpose of control and supervision of experiments on animals
DAPI- 4'6'-diamidino-2-phenylindole dihydrochloride
DMEM- Delbecoo's Modified Eagle Media
DMSO- Dimethyl Sulphoxide
DTZ- Dithizone
EBs- Embryoid bodies
EDTA- Ethylenediaminetetraacetic acid
EG- Embryonic germ
EGFR- epidermal growth factor receptor
El- Enicostemma littorale
ES- Embryonic Stem
FACS- Fluorescent activated cell sorter
FBS- Fetal Bovine Serum
FGF- Fibroblast growth factor
FITC- Fluorescein isothiocyanate
GAD- Glutamate decarboxylase
GCK- Glucokinase
GFP- Green Fluorescent protein
GLP-1- Glucagon-like peptide-1
GLUT- Glucose Transporter
GPx- Glutathione peroxidase
GSH- Reduced glutathione
GSIS- Glucose-stimulated insulin secretion
GTT- Glucose tolerance test
H & E- Hematoxylin and Eosin
HDL- High density lipid
HNF- Hepatocyte nuclear factor
HP- Hydroperoxides
HPAP- Human Placental Alkaline Phosphatase
HPTLC-High Performance Thin Layer Chromatography
HSCs-Hematopoietic stem cells
I.P.- Intraperitoneal
I.V. Intravenous
ICC- Immunocytochemistry
Igt- Impaired Glucose Tolerance
IHC- Immunohistochemistry
IL- Interleukin
ILCC- Islet like cell clusters
INF- Interferon
IPC- Intra-Islet Precursor Cells
iPSC- Induced pluripotent stem cells
IR- Insulin receptor
IRS- Insulin receptor substrate
ISSCR- International Society for Stem cell Research
KD- Kilodaltons
KGF- Keratinocyte growth factor
KRBH- Krebs-Ringer bicarbonate hepes buffer
BMSC-Marrow Mesenchymal Stem Cells
Mesenchymal stem cells
MHC- Major histocompatibility complex
MSCs- Marrow stromal cells

MTT- (3-(4,5-Dimethylthiazol-2-yl)-2,5Diphenyltetrazolium Bromide)
NCCS- National center for cell sciences
NIDDM- Non insulin dependent diabetes mellitus
NO- Nitric Oxide
NOD- Non-obese diabetic
ONS- Oct4, Sox-2, Nanog
PARG- PAR glycohydrolase
PARP- Poly (ADP-ribose) Polymerase
PBMCs- Peripheral Blood Mononuclear Cells
PBS- Phosphate buffer saline
PCR- Polymerase chain reaction
PDL- Partial duct ligation
PDX-1- Pancreatic duodenal homeobox-1
PE- Phycoerythrin
PFA- Paraformaldehyde
PI3-K- phosphatidylinositol 3-kinase
PKB- Protein kinase B
PP-Pancreatic Polypeptide
PPx- Partial pancreatectomy
PSCs- Pancreatic stem cells
PTGS- Posttranscriptional gene silencing
q-PCR- Quantitative PCR
Reg- Regenerating islet-derived gene family
RISC- RNAi Silencing complex
ROS-Reactive Oxygen Species
rPSC- Rat Prostate Stromal Cells
RT-PCR- Reverse transcriptase PCR
SFM- Serum free media
SOD- superoxide dismutase
SSB- Single strand DNA breaks
STZ- Streptozotocin
T1DM- Type 1 diabetes mellitus
T2DM- Type 2 diabetes mellitus
TBARS- Thiobarbituric acid reactive substances
TGF-β- Transforming growth factor-β
TGS-Transcriptional gene silencing
TLC-Thin Layer Chromatography
TM- Tamoxifen
TNF- Tumor necrosis factor
TRITC-Tetramethyl Rhodamine Iso-Thiocyanate
UCB- Umbilical cord blood
VEGF-Vascular endothelial growth factor
XRCC1- X-ray repair complementing gene 1

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Chapter- 1

Introduction

&

Review Literature

1. INTRODUCTION AND REVIEW LITERATURE

1.1 The Pancreas

The pancreas is located next to the duodenum and performs two major functions: regulating blood glucose homeostasis and facilitating nutrient uptake. Pancreas consists of three distinct populations of cells: exocrine, endocrine and ductal cells. In the exocrine compartment, acinar cells are organized into small lobules and constitute maximum portion of the pancreas (90-95%) which synthesize digestive enzymes that are secreted through the highly branched system formed by the duct cells into the duodenum (Slack, 1995). The endocrine cells, which represent 2-5% of the adult pancreas, are clustered into small groups known as Islets of Langerhans. Isolated islets consist of approximately 70% β-cells, 25% α-cells, and <5% each of δ-cells, PP-cells as well as smaller numbers of grehelin-positive endocrine cells, microglial-like cells and vascular endothelium. In mouse, β-cells are located in the inner core of the islets and surrounded by a mantle of glucagon producing α-cells, somatostatin producing δ-cells and PP-cells that produce pancreatic polypeptides, however in humans, both α and β-cells are dispersed and surmounted by δ-cells and PP-cells on the periphery.

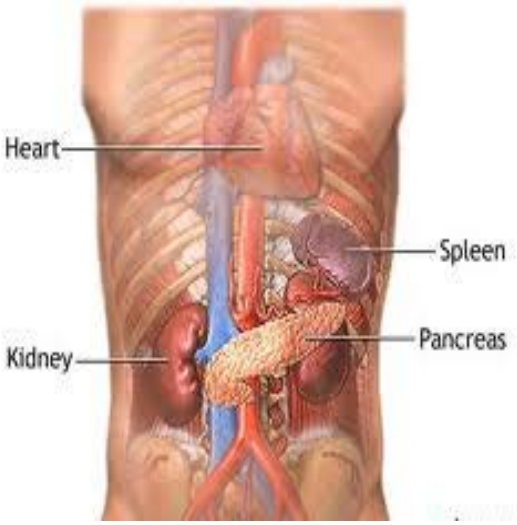


Figure 1.1 Human Alimentary Canal

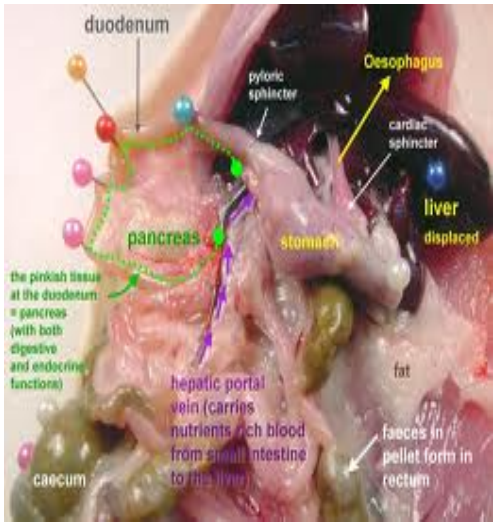


Figure 1.2 Rodent Alimentary Canal

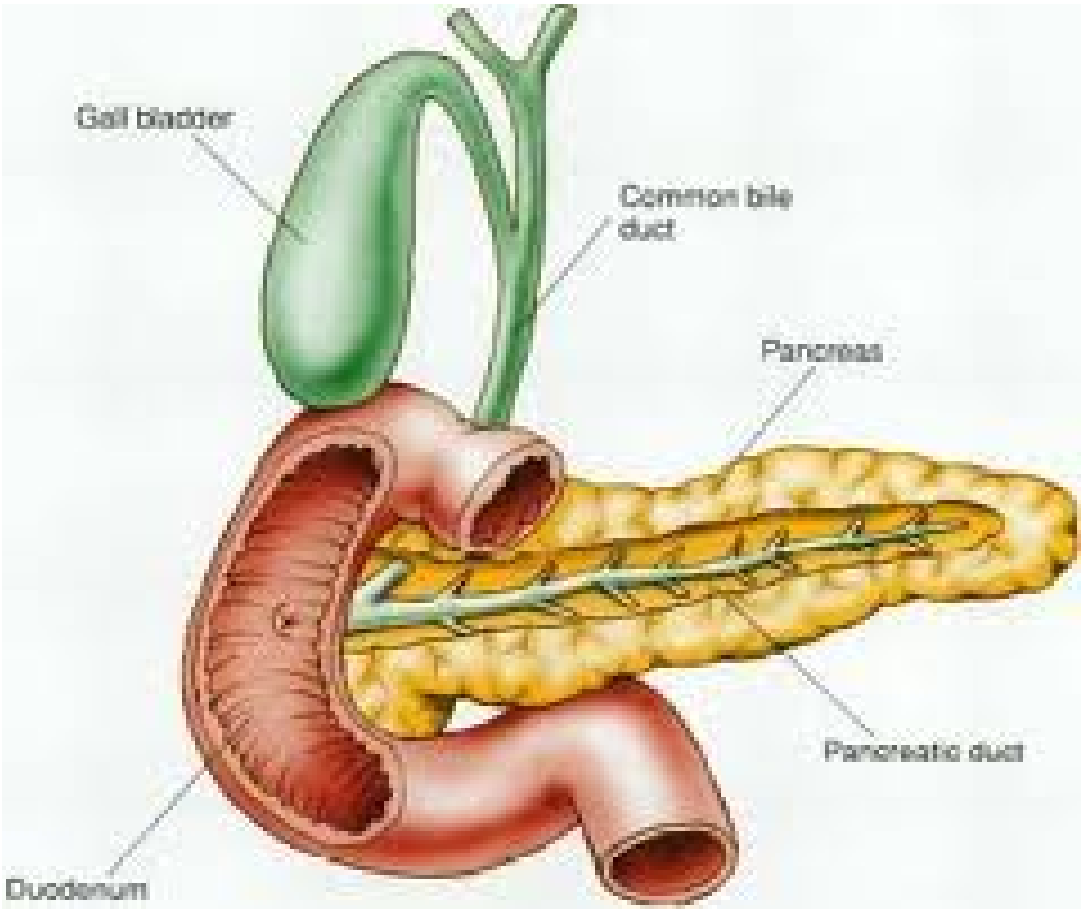


Figure 1.3 Pancreas located in the abdomen adjacent to the duodenum: Leaf Structure

1.1.1 Islet of Langerhans

Islet of Langerhans constitutes endocrine portion of pancreas. The β-cells are the most abundant cell types, within the islets. The β-cells are mainly responsible for the maintenance of the body's glucose levels within a very narrow range through secretion of appropriate quantity of insulin in response to the glucose levels,. (Edlund, 2001). The insulin producing β-cells develop from endodermal progenitor cells. The most significant increase in growth of β-cell mass occurs at a period of late fetal life. Low levels of β-cell replication (2–3% per day) seem to balance β-cell loss by apoptosis (Habener and Stoffers, 1998) throughout the lifespan and results in a slow increase in β-cell mass (Bonner-Weir, 2001).

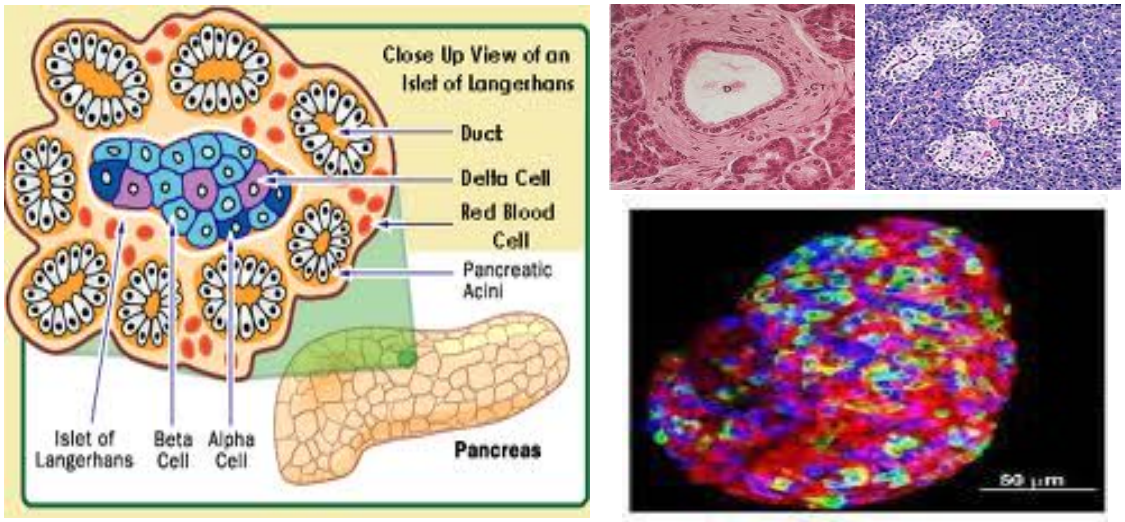


Fig. 1.4: Anatomy of endocrine Pancreas. (a) Pancreatic Duct with intact epithelial lining. (b) Islets of Langerhans surrounded by acinar tissue, secrete its hormones production into the bloodstream *via* pancreatic duct to control glucose homeostasis. (c) Confocal Image of Islet of langerhans showing all α-cells, β-cells, δ-cells and PP cells.

1.2 Physiology of Islet of Langerhans

1.2.1 Glucose response in islets and its homeostasis

Glucose regulation is the prime function of islet of Langerhans. Insulin producing β-cells are major entity which governs precise control over blood glucose concentration by releasing insulin required for glucose metabolism to facilitate glycolysis and ATP production. The glucose level become elevated and the β-cells are "turned on" to produce the hormone "Insulin". Glucose sensing is the key step leading to the release of insulin from the β- cells. Glucose transporter-GLUT2 with high Km and glucose phosphorylating enzyme glucokinase (GCK) play important function (Zalzman, Anker-Kitai et al. 2005) in glucose homeostasis. Therefore islets cells are known as "Built in Power Supply Mini-Organelle".

Normal glucose homeostasis in the human body represents a balance between glucose appearance and glucose uptake/ consumption at tissue level. The plasma glucose concentrations are maintained within a range of 60– 110 mg/dl for normal fasting state and <140 mg/dl for post 2-hour glucose influx (Phillips and Olson 2012). In fasting situation, glucose uptake at tissue level is predominantly insulin-independent and occurs in tissues of high priority for glucose such as the central nervous system (CNS). Following meal (absorptive state), plasma glucose

levels rise and promote hepatic glucose uptake. This elevated glucose level stimulates biogenesis and release of insulin by the β-cell, which acts as a glucose sensor. Three physiologic processes need to interact in a highly coordinated way to maintain glucose homeostasis *i.e.* β-cell insulin secretion, tissue glucose uptake, and hepatic glucose production.

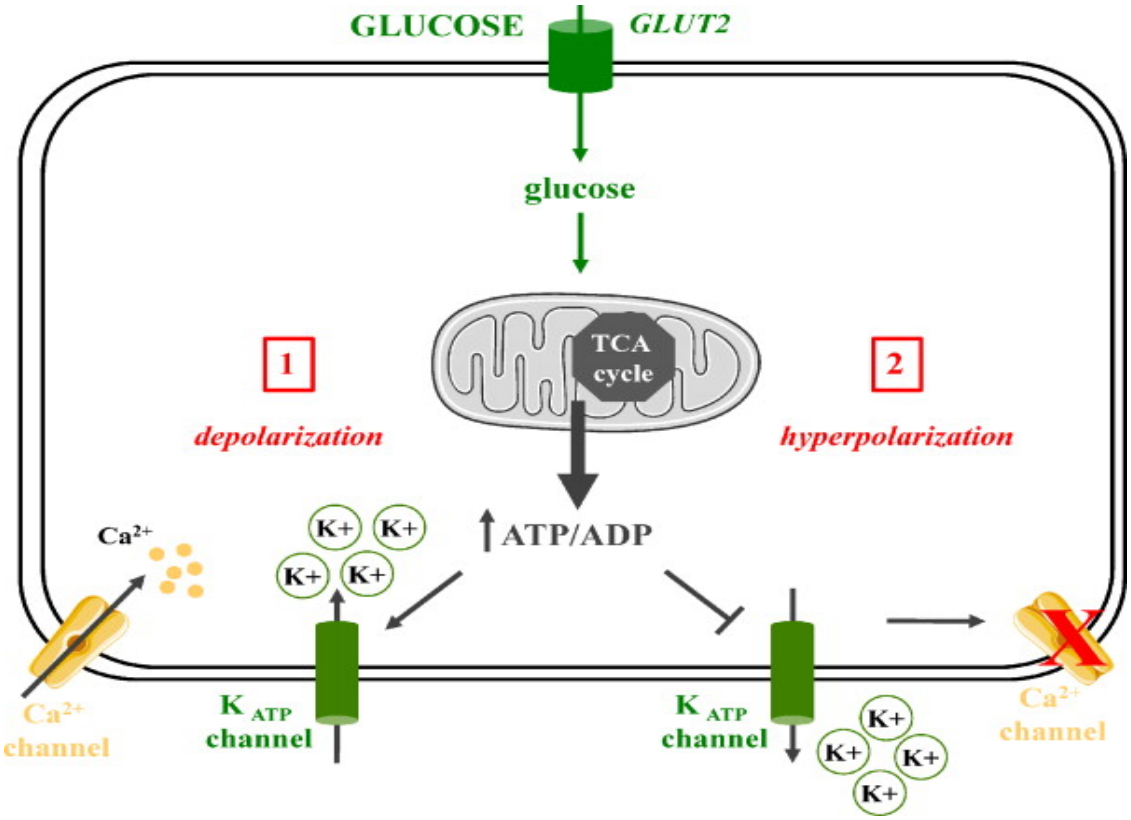


Figure 1.5: Glucose sensing in beta cells. In response to an increase of extracellular glucose leves, the ATP/ADP ratio raises, resuling in (1), the increased ATP/ADP ratio, which induces the closure of K+ATP channels and thus depolarises cell membrane leading to Ca2+ entry through voltage-gated channels. Finally, intracellular Ca2+ increases, (2) glucose induces hyperpolarization by several mechanisms (such as ATP-induced Na+/K+-ATPase, K+-ATPase and Cl– channel activation). (3) Opens GLUT, glucose transporter; KATP and mediate glucose internalization.

1.3 Development of Islet of Langerhans

1.3.1 Early patterning of the endoderm layer

The definitive endoderm forms the epithelium of the primitive gut tube from which the epithelial lining of pancreas (Wells and Melton 1999) develops. In mouse, at embryonic day 7.5 (e7.5), the endoderm layer is a cup of

approximately 500-1000 cells enveloping the mesoderm and ectoderm. These two layers send instructive signals to the endoderm making it competent to respond to subsequent permissive signals that will establish the organ domains along the anterior-posterior axis (A-P) (Wells and Melton 1999; Wells and Melton 2000). All three-cell types of the pancreas (ducts, acini, and islets) develop from a common pool of pancreatic duodenal homeobox 1-expressing (Pdx1/Ipf1) progenitor cells, by day (e8.5) governed from two mesoderm-derived structures: the adjacent notochord and the dorsal aorta.

1.3.2 Embryonic development of Islet of Langerhans

After e10.5 the newly formed pancreas expands rapidly from a compact structure of tightly packed cells which grows several folds in size, forming a branched structure into the surrounding mesenchyme (Guz, Montminy et al. 1995; Miralles, Battelino et al. 1998; Miralles, Czernichow et al. 1998). During the primary transition (e8.5), the only differentiated cell type present is glucagon producing α-cells which is Pdx1-independent. Mesenchymal cells accumulate around the primitive gut and form a blanket over the pancreatic diverticulum. The secondary transition starts at e13.5, peaks at e14.5 and ends at e15.5, during this period the first population of fully differentiated β-cells appears. After the secondary transition few progenitor cells are left (Fig.1.5). It has been observed that endocrine cell fate occurs temporally with glucagon expressing α-cells being the first to develop, thereafter δ-cells (e13.5) β-cells (15.5) and PP cells (e17). Once differentiated, the endocrine cells delaminate from the squamous epithelium and migrate into the extracellular matrix. This clusterization of endocrine cells and the subsequent islet formation begin right before the birth (e18.5 in mouse) and formation of capillary network occurs inside islets due to VEGF-a (vascular endothelial growth factor) that ensure that the islets are close to capillaries for the secretion of the hormones into the bloodstream (fig 1.6).

1.3.3 The mesenchyme controls Islet formation

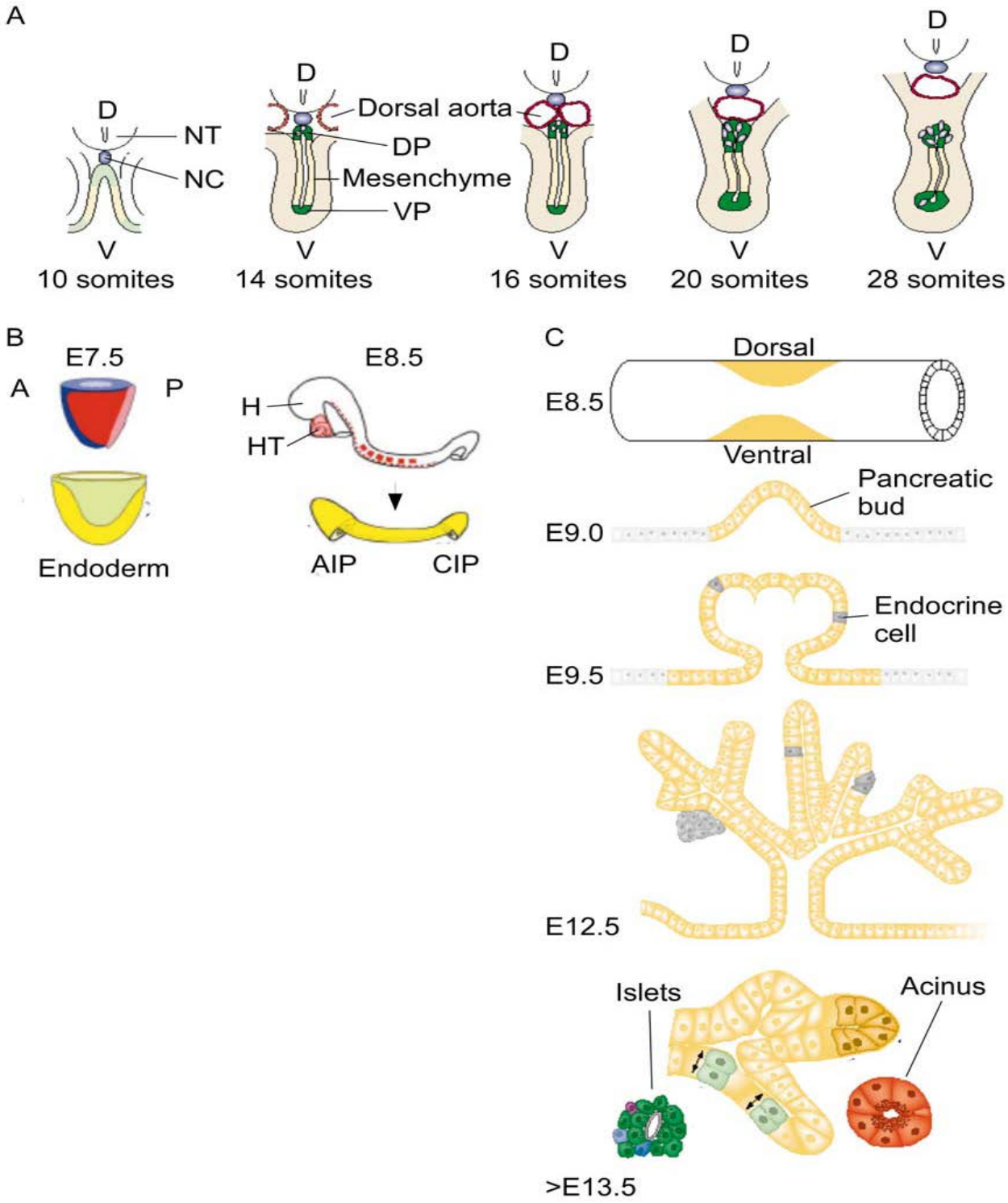
It was long suspected that the islets develop by interactions between the epithelium and its surrounding mesenchyme. Insulin expressing β-cells developed *in vitro* from embryonic pancreatic epithelium in the presence of

mesenchyme are immature, whereas those developed without mesenchyme become mature (Miralles, Czernichow et al. 1998; Miralles, Czernichow et al. 1999). The authors in these studies confirmed that pancreas grown *in-vitro* with rat pancreatic rudiments facilitated more of exocrine cell differentiation.

Later, few other studies have also contradicts such hypothesis showing that signals derived from the mesenchyme are also implicated in the control of islet cell differentiation. By now, it is clear that differentiation of the epithelium into acinar tissue is mesenchyme-dependent. However till today differentiation of endocrine tissue is still controversial (Pictet, Clark et al. 1972; Gittes, Galante et al. 1996; Miralles, Battelino et al. 1998; Miralles, Czernichow et al. 1999). Reports are there which suggest that in presence of fetal mesenchyme, both insulin and glucagon cells containing islet develops, whereas such islet fails to develop in absence of mesenchymal tissue, depicting importance of mesenchymal signals. These pilot experiments strongly suggested that the mesenchyme itslef is necessary for the development of endocrine tissue (islet of langerhans), at least during postnatal life (Dudek and Lawrence 1988; Dudek, Lawrence et al. 1991).

1.3.4 Notochord signals are required for pancreas development

The notochord belongs to mesodermal origin and considered as an axial structure of one of the earliest embryonic structures to be formed and functions as structural support. Some experiments have provided strong evidence that the notochord plays crucial role in development of the dorsal pancreas (Spooner, Walther et al. 1970). Interestingly, the notochord is only capable of inducing pancreas marker in anterior but not posterior endoderm, indicating that the endoderm is already pre-patterned at this early stage (Kim, Doudet et al. 2006). During early time of islet formation, the notochord secretes a variety of potent inducing molecules including the TGF-β family member activin-A and B and FGF2 (Fig. 1.7) crucial for differentiation signaling. Recent experiments showed that intercellular signaling of TGF-β, Notch and hedgehog pathway are important for appropriate specification of islet development.



<u>Fig 1.6 Schematic diagram of the pancreas development:</u> The pancreas develops at the foregut/midgut junction from a pre-patterned region of the primitive endoderm. The first morphological indications are around day e8.5 with the emergence of two evaginations, first the dorsal bud, and subsequently the ventral bud (first transition). The pancreatic epithelium proliferates invading the surrounding mesenchyme (second transition) and fuses at e18, after the rotation of the ventral portion and give rise to the mature organ (third transition). Hebner et al, 2005

TGF-β signaling is a major regulator of endocrine and exocrine pancreas development. TGF-β ligands activin and TGF-β1, its corresponding receptors, antagonist including follistatin, noggin and gremlin, and intracellular Smads proteins like Smad2 and 4 (Y. Eto and H. Shibai 1987; Furukawa, Eto et al. 1995; Manova, De Leon et al. 1995; Verschueren, Dewulf et al. 1995; Crisera, Maldonado et al. 2000; Tremblay, Hoodless et al. 2000). TGF-β1 also promotes the development of islet cells *in-vitro by* embryonic exposure of activin to β-cells and PP-cells (Sanvito, Herrera et al. 1994) but repress the epithelial branching and acinar formation (Ritvos, Tuuri et al. 1995).

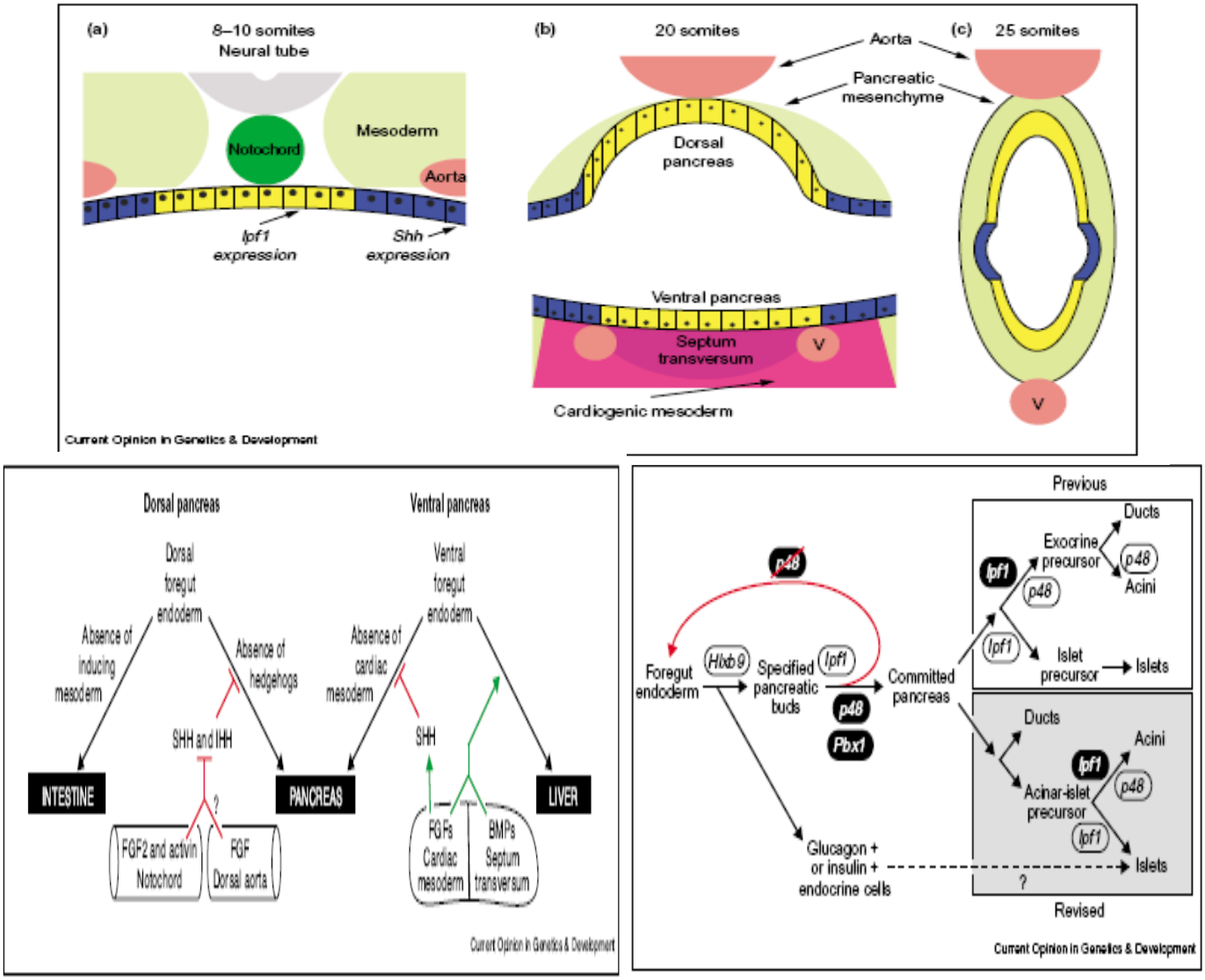


Fig 1.7: Signals from mesodermal derived structures such as nothocord, dorsal aorta, cardiac mesoderm and septum transversum regulating early dorsal and ventral pancreas development. Kim & McDonald, Current Opinion in Genetics & Development, 2002.

1.4 Transcription factors in Islet Development

1.4.1 Hierarchy of transcription factors in Islet development

Transcription factors involved in pancreas development belong to three different families: first, homeodomain family, second, basic helix-loop-helix (bHLH) family and third, windged helix family of proteins (Sander, Sussel et al. 2000). Recent reports on gene disruption in mice have helped to elucidate the hierarchy of transcription factors in pancreas development (fig.1.8).

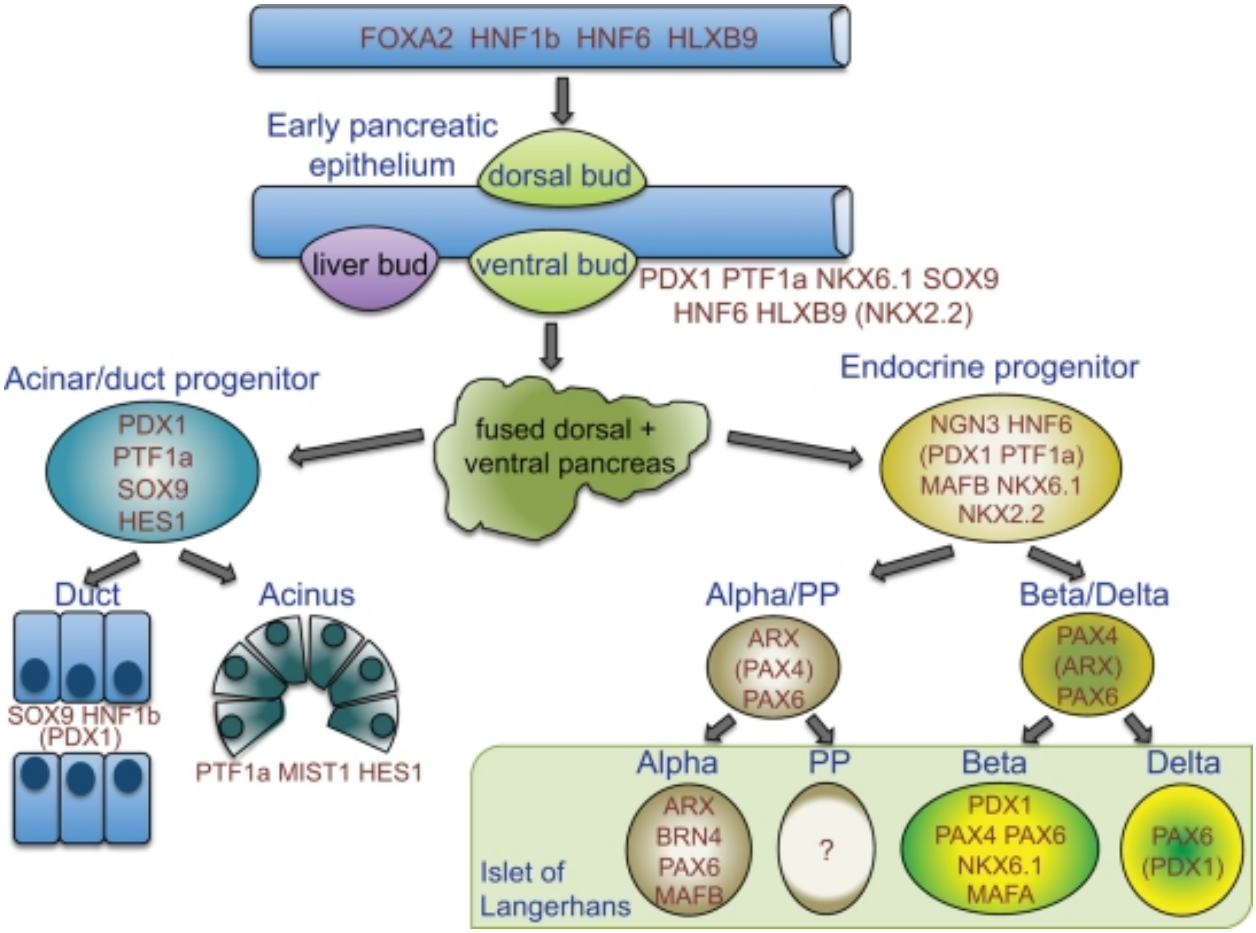


Figure-1.8. Model for the hierarchy of transcription factor expression in the developing pancreas. The proposed position for each transcription factor is based on its timing of expression or timing of predominant functional role (Edlund, 1998 and Kemp et al., 2003).

1.4.2 Hepatocyte nuclear factor (HNF)-3-beta/Forkhead box (Fox)-a2 & HNF-6

HNF-3β, also known as Foxa2, belongs to the winged helix family of transcription factors and is expressed at e5.5-e6.5 in the anterior part of the early primitive streak but later in the definitive endoderm (Monaghan, Kaestner et al. 1993;

Sasaki and Hogan 1993; Chiang and Melton 2003). Studies with specific knockout of Foxa2 in rodent model showed hyperinsulemia and hypoglycemia indicating that the gene is important for the maintenance of normal pancreatic histo-architecture and preservation of a normal physiological response to glucose. Furthermore it has been suggested for Foxa2 gene an essential role in glucagon cells development (Sund, Vatamaniuk et al. 2001; Itkin-Ansari, Marcora et al. 2005).

During pancreas development, HNF6 is detected at e9.5 in the epithelial cells and is later restricted to the exocrine and duct cells (Rausa, Samadani et al. 1997). Some investigation with HNF6 -/- knockout embryos suggest that the exocrine pancreas develops normally when HNF6 was completely knocked out, however the endocrine phenotype is severely inhibited.

1.4.3 Hlxb9

Hlxb9 gene is a homeobox gene encoding the HB9 protein. The early expression of Hlxb9 is detected at e8 in the notochord and gut endoderm (Johansson, Momma et al. 1999). Deficiency of Hlxb9 in mouse pancreas causes failure of the dorsal pancreas formation while the ventral portion forms at the expected time with reduced β-cell population (Johansson, Momma et al. 1999). It has also been observed that by e12.5, the expression level of Hlxb9 significantly do down but one day later the expression again upregulate in the dorsal and ventral portion of the pancreas (Harrison, Thaler et al. 1999).

1.4.4 Pancreatic duodenal homeobox 1 (Pdx1)/insulin promoter factor 1 (Ipf1)

The Pdx1 (Ipf1) gene belongs to the homeodomain family of proteins and it is the first gene shown to be inevitable for development of pancreas in mice (Ahlgren, Jonsson et al. 1996) and humans (Stoffers, Zinkin et al. 1997). Expression of Pdx1 begins in epithelial cells of the dorsal and ventral pancreatic somites stage (e8.5). Pdx1 expression persists in a homogenous way as the pancreatic epithelium branches. The early glucagon-producing cells are not Pdx1 positive. As the secondary transition starts, Pdx1 expression appears more evident. Insulin-positive cells up-regulate Pdx1 expression while differentiating exocrine cells

gradually lose the Pdx1 expression. Ductal-cells also down-regulate Pdx1 and only β-cells and a subset of β-cells express Pdx1 at levels readily detectable using standard histochemical techniques (Oster, Jensen et al. 1998). Pdx1-/- knockout mice show absence of pancreas, despite the initial formation of the pancreatic bud (Ahlgren, Jonsson et al. 1996; Johansson, Momma et al. 1999; Offield, Jetton et al. 1996).

1.4.5 Neurogenin3 (Ngn3)

Ngn3 is a transcription factor that belongs to the family of basic helix-loop-helix bHLH transcription factor. Its expression starts at e9.5 (in mice), peaks at e15.5 and diminishes to undetectable levels before birth (Apelqvist, Li et al. 1999). Experiments using knockout models have shown that ngn-3-/- animals lack the endocrine cell population before birth, while returning persistent expression of Ngn3 again drives the formation of additional endocrine cells (Apelqvist, Li et al. 1999; Gradwohl, Dierich et al. 2000).

1.4.6 Hairy Enhancer of Split-related protein (Hes1)

Hes1 also encodes a bHLH protein expressed at e9.5 in pancreas. The detection of pancreatic endocrine cells excess in Hes1 -/- animals has suggested that Hes1 may have a role in repressing endocrine cell development through the Notch signaling pathway (Jensen, Pedersen et al. 2000).

1.4.7 Pancreatic transcription factor 1 (PTF1-p48)

The bHLH transcription factor PTF1-p48 is described as a DNA-binding subunit of the hetero oligomeric transcription factor PTF-1 that governs the expression of genes in the exocrine pancreas (Krapp, Knofler et al. 1996). Recent results have revised the role of PTF1-p48 to include also the involvement in both exocrine and endocrine cell lineage development (Krapp, Knofler et al. 1998; Kawaguchi, Cooper et al. 2002; Lin, Biankin et al. 2004).

1.4.8 NeuroD1/Beta-2 cell E-Box trans-activator 2 (BETA2)

NeuroD1 or BETA2 (β-cell E-Box trans-activator-2), is a bHLH transcription factor and its expression start at e9.5 and goes throughout the development of β-cells (Naya, Stellrecht et al. 1995), induced by Ngn3 (Huang, Liu et al. 2000). The

essential role of NeuroD/BETA2 during pancreatic development has been shown by detection of severely reduced endocrine phenotypes in homozygous mice at birth. NeuroD/BETA2 null mice die after birth because of hyperglycemia due to impaired islet formation along with depletion in β-cell population (Naya, Huang et al. 1997; Chae, Stein et al. 2004). It has also been shown that NeuroD/BETA2 activates the insulin promoter in β-cells and represses the somatostatin promoter in human pancreatic β-cell line (Itkin-Ansari, Marcora et al. 2005).

1.4.9 Paired-box homeobox genes (Pax4/Pax6)

The paired homeobox genes encode key transcription factors involved in the organogenesis of many tissues (Mansouri, Goudreau et al. 1999). Pax4 and Pax6 are associated with pancreatic endocrine cell differentiation (Marsich, Vetere et al. 2003; (Smith, Gasa et al. 2003), and expression of Pax4 and Pax6 starts at e9.5 and e9.0, respectively (Turque, Plaza et al. 1994; Sosa-Pineda 2004). Knockdown of Pax4 in mice show complete absence of β- and δ-cells with little increase in α-cell population (Sosa-Pineda, Chowdhury et al. 1997). Insulin and glucagon expressing cells were detected in mutant mice at e10.5, although insulin is no longer detected by e13.5. Thus, Pax4 seems to play a role in maintenance of β-cell differentiation and maturation rather than to initiate it. On the other hand, Pax6 seems to be more crucial for the development of the α-cells (St-Onge, Sosa-Pineda et al. 1997; Sosa-Pineda 2004). Double knockout of Pax4 and Pax6 showed complete loss of endocrine cell types (St-Onge, Sosa-Pineda et al. 1997), so Pax4 and Pax6 probably act cooperatively to regulate islet cell identities.

1.4.10 NK class homeodomain protein Nkx2.2 and Nkx6.1

Nkx2.2 and 6.1 belong to the NK class of homeodomain proteins. The expression of the two proteins starts respectively at e9.5 and e10.5 (in mice). The expression of Nkx2.2 become restricted to β-, α- and PP cells in development time course while the Nkx6.1 expression become restricted to insulin producing cells in the adult pancreas (Oster, Jensen et al. 1998; Sussel, Kalamaras et al. 1998; Wang, Elghazi et al. 2004). Recent investigation by Prado et al. demonstrate that β-cells were replaced by ε-cells (ghrelin-producing cells) in

both Nkx2.2 -/- and Pax4 -/- animal models (Pugh-Bernard et al. 2004). Nkx6.1-/- animals display decrease in the β-cell population after e12.5 (Sander, Sussel et al. 2000), therefore seems to be required for the expansion and final differentiation of β-cell precursors.

1.4.11 Islet 1 (*Isl1*)

Isl1 encodes a transcription factor of the LIM (**L**in11, **I**sl-1 & **M**ec-3) homeodomain/LX3 protein family and is involved in the early formation of the pancreas (Ahlgren, Pfaff et al. 1997). The *Isl1* expression starts at e9.0 in the dorsal pancreatic epithelium and in the dorsal mesenchyme, but not in the ventral, invagination of the gut endoderm. *Isl1* express in fully differentiated cells and at e9.5 all the glucagon positive cells express *Isl1* then later it is detectable in all the islets cells. Mice mutant for *Isl1* are lethal and cannot develop after e9.0. The importance of the role of *Isl1* has been understood by co-culture of tissue from *Isl1* mutant mice with mesenchyme from wild type embryos. In these conditions the endocrine development can be partially restored but not the exocrine one. Also some more studies show *Isl1* to bind the promoter region of several genes such as glucagon, insulin and somatostatin, suggesting that the *Isl1* is also necessary for the maintenance of the islet function in the adult (Brink 2003).

1.5 Endocrine or Islet fate commitment: turning point for beta cell development

1.5.1 Ngn3 the master-switch of Islet cell development

Neurogenin-3 (Ngn3, atoh5) belongs to the bHLH (basic helix-loop-helix) transcription factor family and has the role of proendocrine factor in the development of the pancreas (Gradwohl, Dierich et al. 2000). Ngn3 is expressed in endocrine precursors of both the pancreas and gastrointestinal tract (Jenny, Uhl et al. 2002). Mice deficient for Ngn3 fail to develop any endocrine cells and die within 1-3 days after birth because of hyperglycemia, whereas exocrine cells develop normally (Gradwohl, Dierich et al. 2000). Ngn3 has been demonstrated as a very important and crucial transcription factor necessary to drive the formation of islet cells during pancreatic development (Apelqvist, Li et al. 1999).

Transcription factor	Onset and site of pancreatic expression	Mouse & human mutations	References
Homeodomain factors			
Pdx1	e8.5, early pancreatic epithelium, beta and delta cells of mature pancreas	Pancreatic agenesis, heterozygous human mutation: MODY4	Guz et al., 1995; Jonsson et al., 1994; Ohlsson et al., 1993; Ahlgren et al. 1998; Stoffers et al. 1997a, b
Hb9	e8, early prepancreatic endoderm, mature beta cells	Dorsal pancreatic agenesis	Harrison et al., 1999; Li et al., 1999; Li and Edlund 2001
Pbx1	e10.5, ubiquitous	Hypoplastic pancreas	Kim et al., 2002
HNF6	e9.5, throughout the pancreatic buds	Impaired endocrine differentiation	Jacquemin et al., 2000
Pax4	e9.5, in developing endocrine cells, not found in mature pancreas	Deficiency of beta and delta cells, increase in alpha cells	Dohrmann et al., 2000; Sosa-Pineda et al., 1997
Pax6	e9, in endocrine cells	No alpha cells, decrease in other endocrine cell types	St-Onge et al., 1997
Nkx2.2	e9.5, throughout pancreatic bud, later in endocrine cells	Lack of beta cells, decrese in alpha and PP cells	Sussel et. al, 1998
Nkx6.1	e9.5, in beta cells	Decrease in beta cells	Sander et al., 2000
Isl1	e9, in all islet cells	No differentiated islet cells	Ahlgren et al., 1997
HNF1α	e10.5, dorsal pancreas, mature beta cells	Heterozygous human mutation and homozygous mouse mutation: MODY3	Nammo et al., 2002; Pontoglio et al., 1998
HNF4α	e5.5, in visceral endoderm, e9.5 in pancreatic primordium	Early embryonic lethal Heterozygous human mutaion: MODY1	Ferrer, 2002; Duncan et al., 1994
Brn4	e9, in alpha cell progenitors	No phenotype	Hussain et al., 2002
Basic helix-loop-helix factors			
Ngn3	e9.5, endocrine precursors	No endocrine cells	Gradwohl et al., 2000; Gu et al., 2002
NeuroD	e9.5, endocrine precursors and adult islet cells	Impaired islet development	Jensen et al., 2000a; Naya et al., 1997
Hes1	e9.5, pancreatic precursors	Premature endocrine differentiation	Jensen et al., 2000b; Apelqvist et al., 1999
Ptf1-p48	e10, exocrine, endocrine and ductal progenitors	No exocrine cells	Kawaguchi et al., 2002; Krapp et al., 1996; Krapp et al., 1998
Forkhead / winged helix factors			
Foxa2 / HNF3β	e5.5-6.5, early foregut endoderm, adult acinar and islet tissue	Lack of foregut formation	Lee et al., 2002; Sund et al., 2001
Foxa1 / HNF3α	e7.5, notochord, later in all islet cell types	Reduced proglucagon expression	Kaestner et al., 1999

Fig- 1.9: Table summarizing the major pancreatic transcription factors, their expression and function in animals model and humans.

Ngn3 expressing cells in the pancreas are transient endocrine progenitor cells, committed to an endocrine fate. Various lineage tracing studies have shown that Ngn3 expressing cells do not give rise to exocrine cells at all and very rarely to duct cells but give rise to all endocrine cell types (Gu, Dubauskaite et al. 2002). Ngn3 protein can also be detected in very few cells in the early pancreas (e10.5-e12.5), while from e13.5 and onward, a major peak (between e14.5-e15.5) of expression is observed (Apelqvist, Li et al. 1999; Jensen, Heller et al. 2000).

The pattern of Ngn3 expression is observed for a transient period in the pancreas and gets completely turned off in postnatal mice, shown by immunohistochemical studies (Jensen, Heller et al. 2000). However, Gu et al. detected Ngn3 expression by RT-PCR in handpicked islets from 8-week old mice (Gu, Dubauskaite et al. 2002). In addition, using double transgenic Ngn3-Cre.ERTM;Z/AP mice they observed, upon Tamoxifen (TM) induction, three or eight-weeks old mice, Ngn3 expression in 15 out of 25 pancreata (identified by Human Placental Alkaline Phosphatase HPAP staining cells) and in islet cells insulin co-expression (Gu, Dubauskaite et al. 2002). These data suggest that Ngn3-expressing cells act as islet progenitor cells not only during embryogenesis but also in adults. This Ngn3 expression is transient and new Ngn3- positive cells are formed from Ngn3-negative cells during development. Once cells enter the endocrine pathway, they start expressing Ngn3 and stops proliferating (Jensen, Heller et al. 2000). Premature expression of Ngn3 in pancreas (Apelqvist, Li et al. 1999) and endoderm (Grapin-Botton, Majithia et al. 2001) gives rise to β-cells and it was suggested that β-cells are the default endocrine cell type to be formed. Insulin gene expression has been also achieved when pluripotent cells were transfected with an Ngn3- expressing vector (Vetere, Marsich et al. 2003) after induction of endoderm differentiation along with many other endocrine transcription factors (Vetere, Marsich et al. 2003).

It is now very well accepted that Ngn3-positive cells could adapt their responses to specific microenviromental inductive signals that changes during pancreatic development and different endocrine cell types adopt their fate at different time points. Further, It has also been reported that several other TF are required to

deviate from the adult β-cell fate. The Ngn3 gene expression is turned off before terminal differentiation and in the normal pancreas, Ngn3 protein has never been co-expressed with mature markers such as insulin. Interestingly, in a recent study by Lee et al., it has been demonstrated that pancreatic transcription factors that are seen to be expressed during the development are re-expressed also during the regeneration, with the only known exception of Ngn3 (Lee, De Leon et al. 2006). Although Ngn3 protein responsible to marks all the proendocrine cells during development, is not seen during mouse pancreas regeneration, functional neo-islets are generated by 4 weeks after 70% pancreatectomy (Lee, De Leon et al. 2006). The explanation for such phenomenon has been given only recently by Joglekar et al. (Joglekar, Parekh et al. 2007) where they carried some microRNA analysis during different stages of pancreatic development and regeneration and identified that, microRNAs that can potentially bind to Ngn3 transcript, are expressed at least 200-fold higher in the regenerating mouse pancreas.

1.5.2 Notch signaling pathway crucial for Islet differentiation

Notch signaling has been shown to play two major functions in the pancreas development: the early role is maintaining the progenitor cells in undifferentiated state while the later role is controlling the lateral inhibition, allowing selection of endocrine cells from non-endocrine cell population. As for as, the early role is concerned, notch signaling is known to controls ngn-3 expression directly and indirectly. In fact it is also known that Ngn3 expression appears to be dependent from positional signals that direct it to the gut and to the pancreatic endoderm and local signals that limit it to specific cells (Fig. 1.10). It has been observed that for lateral inhibition through notch signaling, the mechanism is more straightforward and has been recognized to be an almost "general" regulator for cell-fate decision in a variety of organisms and tissues (Kageyama and Ohtsuka 1999; Appel, Givan et al. 2001). Lateral inhibition, through the notch signaling pathway, prevents Ngn3 expression in few scattered cells within the pancreatic epithelium (Schroeter, Kisslinger et al. 1998; Apelqvist, Li et al. 1999; Gradwohl, Dierich et al. 2000; Jensen, Pedersen et al. 2000; Schwitzgebel, Scheel et al. 2000).

All the components of the Notch pathway have been shown to express in a developing pancreas (Jensen, Pedersen et al. 2000; Lammert, Brown et al. 2000). The Notch receptors Notch1 and 2 are expressed in pancreatic epithelial precursors, Notch3 in the surrounding mesenchyme, and Notch4 in the endothelial cells. The Notch ligands Jagged1, 2 and Delta1 (Dll1) are also expressed in pancreas. In addition the Notch in concert with the DNA-binding protein RBP-J activates the expression of the target gene Hes1 in the pancreatic bud and later in the pancreatic epithelial precursor cells (Apelqvist, Li et al. 1999) and specifically inhibits the Ngn3 promoter (Lee, Smith et al. 2001). Any defects in Notch signaling has been shown to accelerate the endocrine differentiation program leading to a depletion of the precursor cell population (Apelqvist, Li et al. 1999; Jensen, Pedersen et al. 2000; Hald, Hjorth et al. 2003; Hart, Papadopoulou et al. 2003). The maintenance of Notch activation was proposed to derive from the pancreatic mesenchyme and mediated by FGF10 (Gittes, Galante et al. 1996; Norgaard, Jensen et al. 2003; Miralles, Lamotte et al. 2006).

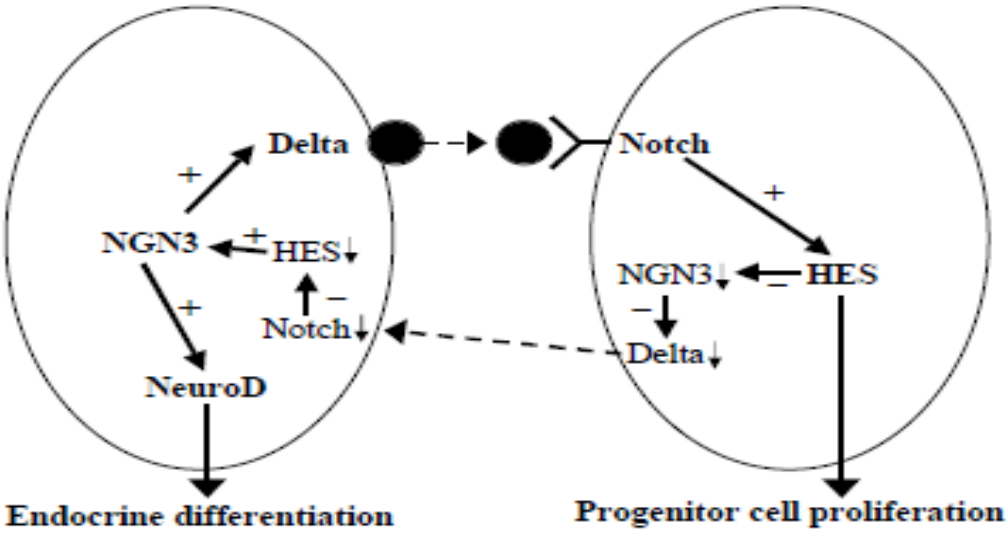


Fig. 1.10: Lateral inhibition is caused by cell-cell signaling between epithelial neighbors. Cells in which Notch1 is activated by the ligand Delts express high levels of Hes1, which in turn repress the proendocrine gene Ngn3. However, in ligand expressing cells, hes1 is not regulated, thus allowing robust Ngn3 expression and differentiation toward the endocrine lineage. Jensen J. et al., 2004

1.6 Diabetes Mellitus

Diabetes mellitus is one of the most common endocrine disorders today, with million people affected in the world in 2003 to approximately 220 million people in 2010 (Zimmet 2000; Zimmet, Alberti et al. 2001). Diabetes is a group of disorders of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (WHO 1999). The two most common types of diabetes mellitus are type 1 and type 2. About 90% of the patients with diabetes suffer from type 2 diabetes, which is characterized by insulin resistance and impaired insulin release.

Type-2 diabetes used to be called "adult onset diabetes", but today children are also being diagnosed. Type 2 diabetic patients have chronically increased levels of blood glucose, which is toxic and further impairs insulin sensitivity and β-cell function, leading to more severe type-2 diabetes. Chronic hyperglycemia is a major risk factor for the development of complications such as micro and macroangiopathy and neuropathy, which result in blindness, impotence, ischemic foot ulcers and amputations (Porte and Schwartz, 1996). One of the possible molecular mechanisms responsible for these complications is the increased oxidative stress induced by chronic hyperglycemia (King and Loeken, 2004). Briefly, continuous hyperglycemia activates insulin-independent tissues to consume glucose. The increased glucose inside of the cells will enter in the glycolysis pathway, Krebs cycle and oxidative pathways (King and Loeken, 2004). The increased flux will generate an excess of oxygen reactive species (ROS) and the accumulated ROS reduces the increased oxidative stress and may interfere with normal tissue functions. Another major factor in the development of the diabetic complications could be the increased glycosylation of proteins. This effect can lead to the accumulation of advanced glycosylation end-products and lipoxidation end-products (AGEs/ALEs) (Wada and Yagihashi, 2005). In addition to ROS and AGEs/ALEs, VEGF in diabetic retinopathy (Aiello, 2005) and microalbuminuria for cardiovascular and renal disorders in type-2 diabetes has also been shown (Lane, 2004). Patients with type-2 diabetes can often be treated with diet and exercise alone or in combination with insulin sensitizers,

which act to reduce the insulin resistance in target tissues, or sulphonylureas which stimulate the β-cells to secrete more insulin. Type 1 diabetes is caused by an autoimmune reaction or autoantibodies against insulin/ insulin receptor, which leads to destruction of β-cells and subsequent insulin deficiency. These patients are treated with insulin injections to compensate for the absence of natural insulin production. Whole organ pancreas transplants have been performed in type 1 diabetic patients. Now with the recent success of islet transplantation using the Edmonton protocol (Shapiro et al., 2000), type 1 diabetic patients have been shown to maintain normal blood glucose levels without daily insulin injections. This procedure unfortunately relies on islets available from pancreata from cadavers, a very limited supply. Each type 1 diabetic patient needs islets isolated from 2-3 pancreata and could need several transplants before achieving insulin independence. These results are promising, but a shortage of donors has led to an explosion in the number of investigations of methods to generate islets surrogates suitable for transplantation. Some of the roads taken so far have been expansion of existing β-cell lines, encapsulated islet xenografts, conversion of a stem cell population (either pancreatic or non-pancreatic adult stem cells) to β-cell, *in vitro* differentiation of embryonic stem cells to β-cells (Soria, 2001) or transdifferentiation from a different cell type (Nygaard Jensen and Jensen, 2004).

1.6.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) results from an absolute deficiency of insulin due to autoimmune attack and destruction of the β-cells. Type 1 diabetes mellitus usually appears suddenly in non-obese children or young adults, also known as juvenile-onset diabetes. This occurs as a result of direct attack of an aberrant β-specific auto-immune antibodies (Narendran et al., 2005) with involvement of cytokines and T-lymphocytes specific for β-cell antigens (Nerup et al., 1988), which only became symptomatic when the β-cell mass is reduced by approximately 90%. T1DM is a complex genetic trait, with multiple genetic loci contributing to susceptibility, but also with environmental factors playing a role in determining a risk.

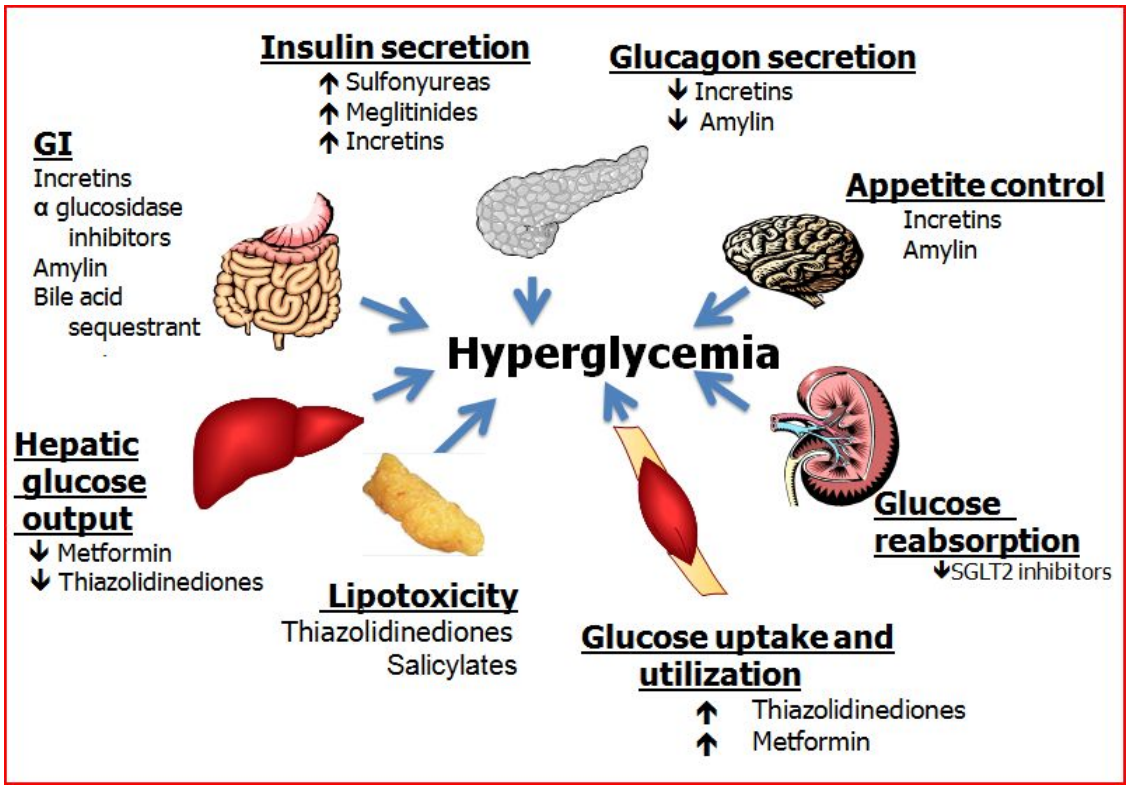


Fig-1.11 shows various factors leading to hyperglycemia, turning into diabetes. (Rushakoff et al., 2010)

It has been shown to exist a significant familial clustering of T1DM with an average risk in siblings of 6% compared to 0.4% in the general population (Karvonen et al., 1993; Karvonen et al., 2000). At least one locus that contributes strongly to this familiar clustering resides within the major histocompatibility (MHC) region on the chromosome 6p21.3. Other established T1DM risk genes include the insulin gene region on chromosome 11p15 and the cytotoxic T-lynphocyte associated 4 gene (CTLA4) region on the chromosome 2q33 (Pociot and McDermott, 2002). Low protein diet given to pregnant rats during gestation induces permanent changes in the offsprings like altered islet proliferation, islet size, pancreatic insulin, apoptotic rates and increased sensitivity to interleukin 1b (IL-1b) and nitric oxide (NO) (Merezak et al., 2001; Petrik et al., 1999; Snoeck et al., 1990). In the offspring of T1DM mothers, it has been suggested that the exposure to a diabetic environment in utero is associated with increased prevalence of Impaired Glucose Tolerance (IgT) and defective insulin secretory response in the adulthood (Sobngwi et al., 2003).

The present concept of the pathogenesis of T1DM is mainly based on evidence from studies on the animal models of human T1DM. In the non-obese diabetic mice model (NOD) autoimmune diabetes can be transferred from a diabetic to a non-diabetic animal via T lymphocytes. This confirms that the T lymphocytes are the mediators of autoimmune diabetes in NOD mice. It seems that both glutamate decarboxylase-(GAD) and insulin-reactive T cells are diabetogenic in the NOD mice. The major histocompatibility complex class II allele, HLA-DQ, is the major genetic element, conferring susceptibility to or protection from human T1DM. However, the mechanisms determining the contribution of the HLA-DQ allele remain to be elucidated (Moustakas and Papadopoulos, 2002). Accumulating evidence also suggests that the regulation of the gut immune system may be aberrant in T1DM, possibly due to environmental factors (Akerblom et al., 2002). Many autoimmune diseases are characterized by an overproduction of cytokines. In the case of T1DM, interferon (IFNγ), tumor necrosis factor-a (TNFα) and interleukin 1-β (IL-1β) have been shown to contribute to the pathology. In addition, the quality of the immune reactivity is an important factor in beta-cell destruction. According to the so-called "Copenhagen model" of T1DM , beta-cells are destroyed by cytokine-induced free radical formation before cytotoxic helper T cells and/or autoantibody-mediated cytolysis (Freiesleben De Blasio et al., 1999; Nerup et al., 1988). A consequence of this model is that proinflammatory cytokines induce both protective and deleterious mechanisms in all cells expressing cytokine receptors.

1.6.2 Type 2 Diabetes Mellitus

The glucose homeostasis dysregulation in type 2 diabetes mellitus (T2DM) is mainly due to impaired insulin secretion and reduced peripheral and hepatic insulin sensitivity (Bajaj and Defronzo, 2003; Beck-Nielsen and Groop, 1994). The development of T2DM is a gradual process. It is now agreed that overt diabetes arises when β-cells can no longer compensate for the insulin resistance. A prolonged exposure of pancreatic β-cells to glucose eventually leads to β-cells exhaustion and glucose toxicity. The effect of β-cell exhaustion is the early form of the disease and could be reversible whereas the glucose toxicity occurs later in

the disease and is less reversible (Rossetti et al., 1990). Detrimental effect of elevated levels of free fatty acids on β-cells function has also been studied more recently. The suggestion is that diabetes is not only the carbohydrate disease but also associated with disordered lipid metabolism (Unger, 1995). In addition, the deposits of islet amyloid present in T2DM diabetic pancreas may lower the functional β-cell mass (Kahn et al., 1999; Marzban et al., 2003). Several lines of evidence indicate that hyperglycemia induces β-cell apoptosis by inducing apoptotic molecules including glucose-induced NF-κB, apoptotic-associated protein caspase 3 and ROS. Once the apoptosis-related factors have accumulated, β-cell mass is reduced, insulin secretion is impaired and eventually diabetes occurs (Mandrup-Poulsen, 2003).

The working model for the β-cell dysfunction in T2DM can be discussed using animal model, 90% pancreatectomy (Px) rats. It has been found that insulin secretion is enhanced (hypersensivity) during the period when the blood glucose is as stable as the level in control animals (within 2 weeks post surgery) in Px rats (Porte, 2001). The occurrence of dysfunctional insulin receptor (IR) and downstream kinase provided another mechanism to explain why insulin resistance occurs. In the peripheral organ (e.g. muscle and liver) of insulin resistant subjects, a defect or IR and insulin receptor kinase have been observed (Froguel et al., 1993). Defects in IR function were originally determined by the detection of IR-specific autoimmune antibody, the mutation on IR and the decreased IR numbers (Horikawa et al., 1997; Stoffers et al., 1997). Insulin-IR binding activates insulin receptor substrate- 1 (IRS-1)-dependent phosphatidylinositol 3-kinase (PI3K) which activates atypical protein kinase C (aPKC) and protein kinase B (PKB/Akt) pathways and thereby regulates glucose transport (Malecki et al., 1999). The results exhibiting the reduced Akt kinase activity in the skeletal muscle tissue of T2DM patients (Busch and Hegele, 2001) and defective activation of aPKC in muscles and adipocytes of rats with T2DM (Guz et al., 1995) suggested that IR-related kinase plays a major role for the development of insulin resistance.

1.7 Current Therapies for Diabetes Mellitus

Individuals with type 1 diabetes require insulin daily to help maintain blood glucose values. Insulin supplementation is oral or by injection using either a syringe or an insulin- administering pump. Whole pancreas transplantation has been accepted as an alternative therapy for individuals with type 1 diabetes who are undergoing simultaneous kidney transplant (Meloche, 2007). Although there is still a significant cost and morbidity risk associated with this surgery, there is an 84-88% graft survival rate after one year (Halban, 2004; Lechner and Habener, 2003). Islet transplantation is a much less invasive procedure and may provide the best option for excellent glycemic control (Meloche, 2007). Early attempts to transplant islets into type 1 individuals resulted in only 8% patients independent of insulin after 1 year (Lechner and Habener, 2003). With advancements in immunosuppressive agents, the Edmonton Protocol was developed in the late 1990's with the aim to reduce toxicity and avoid the harmful effects of some immune-suppressants on the β-cells (Halban, 2004; Ryan et al., 2005). The Edmonton Protocol consisted of a glucocorticoid – free immune-suppressive protocol that included sirolimus, low dose tacrolimus and a monoclonal antibody against the interleukin-2 receptor (daclizumab) (Ryan et al., 2005). Insulin independence was achieved in ~30% and 10% of patients after 2 and 5 years, respectively, demonstrating a marked improvement in the effectiveness of islet transplantation (Lechner and Habener, 2003). However, there still remains a need to refine the islet isolation technique and the immunosuppressive regime to better maximize graft survival and function while limiting toxicity. Additionally, 2-3 cadaveric donors are required per islet transplant with some individuals requiring multiple transplants over time.

Lifestyle changes are recommended for individuals with type 2 diabetes, including diet and exercise, as well as weight loss for obese individuals. Additionally oral medications may be given, such as Metformin to reduce hepatic glucose production, Thiazolidinediones to improve peripheral tissue insulin sensitivity, or Sulfonylureas to stimulate insulin secretion. Glucagon-like peptide-1 (GLP-1) receptor agonists or inhibitors of the GLP-1 inactivating enzyme dipeptidyl peptidase-4 are used to enhance glucose-stimulated insulin

secretion (GSIS) (Sheehan, 2003). Insulin therapy may be required in addition to one of the above agents with progression of the disease.

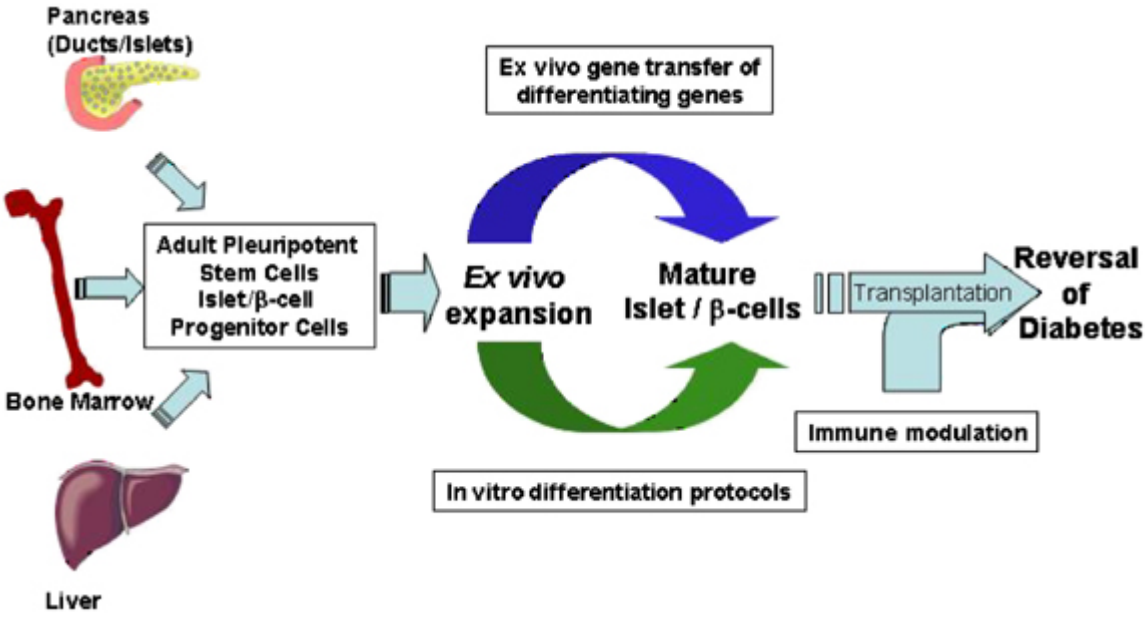


Fig-1.12 shows current therapy for diabetes using adult stem cells or expansion of pancreatic progenitor. (V. Yechoor and L Chan, 2005)

1.8 β-cell Regeneration and Islet Neogenesis

1.8.1 β-Cell Plasticity

An adequate source of proper functioning β-cells is necessary to improve the health of diabetic individuals. β-cell mass is a dynamic balance between β-cell growth and β-cell loss. β-cell growth occurs through replication of pre-existing β-cells, neogenesis of β-cells via stem/precursor cells and also through β-cell hypertrophy, while β-cell death occurs by apoptosis or necrosis (Juhl et al.). In rodents, there is an enhanced rate of replication and neogenesis that is interrupted by a brief episode of increased apoptosis during the neonatal period (Bonner-Weir et al., 2010; O'Brien et al., 2002).

These changes early in life lead to an increase in β- cell mass soon after weaning (Bonner-Weir et al., 2010). However, the rates of β-cell growth and death are dramatically decreased as the young rodent matures into adulthood. In adult rodents there is very slow turnover of β-cells, with about 0.5% of β-cells self-

replicating (Teta et al., 2005) and a corresponding rate of apoptosis of 0.5% of β-cells (Bernard et al., 1999; Pick et al., 1998). This would suggest a limited ability for β-cell growth in adult rodents under normal physiologic conditions.

1.8.2 Expansion of β-Cell Mass

Despite low replication rates only pregnancy and obesity are two physiologic situations in which β-cell expansion in the adult is possible. (Vasavada et al., 2000). A study showed that glucose homeostasis was maintained through endocrine tissue hyperplasia in non-diabetic obese subjects (Petrik et al., 1999). In animal models, there was a significant increase in β-cell mass in the obese non-diabetic neogenesis (Okamoto, 1999). Insulin resistance is a main feature of obesity, but two-thirds of obese individuals do not develop diabetes. Therefore plasticity of β-cell mass can exist in the adult under particular physiologic conditions. However, in the pathological setting of diabetes, the plasticity of β-cells from type 2 diabetic individuals was reduced when examined in vitro compared to non- diabetic β-cells (Ashcroft et al., 2004).

1.8.3 Alternative Sources of β-Cells

Given the demand for β-cell replacement therapy in diabetes and the current limitations to pancreas and islet transplantation, alternative approaches to generate β- cells are being pursued. Three areas of focus include differentiation of stem cells, expansion of primary β-cells ex vivo and transdifferentiation. The embryonic stem (ES) cells are pluripotent cells capable of differentiation into β-like cells (fig 1.12). Several groups have demonstrated the ability to produce β-cells from mouse ES cells or human ES cell lines (Creutzfeldt, 1979; Drucker, 2003a).

These studies provided evidence that the ES-derived cells expressed key markers of β-cells such as PDX-1, insulin, GLUT2, GCK, NKX2.2, NKX6.1; they released insulin in response to glucose and improved glycaemia following transplants into diabetic mice (Creutzfeldt, 1979; Drucker, 2003a). Following transplantation into immune compromised mice, measurements of serum C-peptide levels, an indication of insulin production. While advancements are being made, ES-derived

β-cells must express the full complement of mature β-cell markers and be capable of tightly regulating blood glucose levels.

Isolation of β-cells and their subsequent expansion and transplantation back into the individual is a promising approach. Isolated islets placed into culture will dedifferentiate into a mesenchymal-like phenotype, losing expression of islet hormones and key β-cell transcription factors (Zhou et al., 1999). These more primitive cells can be expanded and then directed to redifferentiate back to a β-cell phenotype, however, these newly formed β-cells have very low levels of insulin mRNA (Drucker, 2003b). There are many social and ethical issues associated with ES cell use and limited success with the expansion of β-cells *ex vivo.* This has forced researchers to look at other cell types as possible sources for new β-cells. The process of converting one cell type into another cell type is called transdifferentiation.

A study in mice showed that liver cells transfected *in vivo* with an adenovirus expressing PDX-1 led to the expression of β- cell markers and considerable amounts of serum insulin, as well as the reversal of chemically induced diabetes (Farilla et al., 2002). Acinar cells can be converted into β-cells through adenoviral transfection of three key transcription factors: PDX-1, NGN3 and MafA (Buteau et al., 1999). Recently α-cells were also converted into β-cells through the ectopic expression of Pax4 (Buteau et al., 2003), while another group observed the spontaneous conversion of α-cells into β-cells following near-total β-cell ablation using a lineage tracing model (Perfetti et al., 2000).

1.8.4 β-Cell Replication

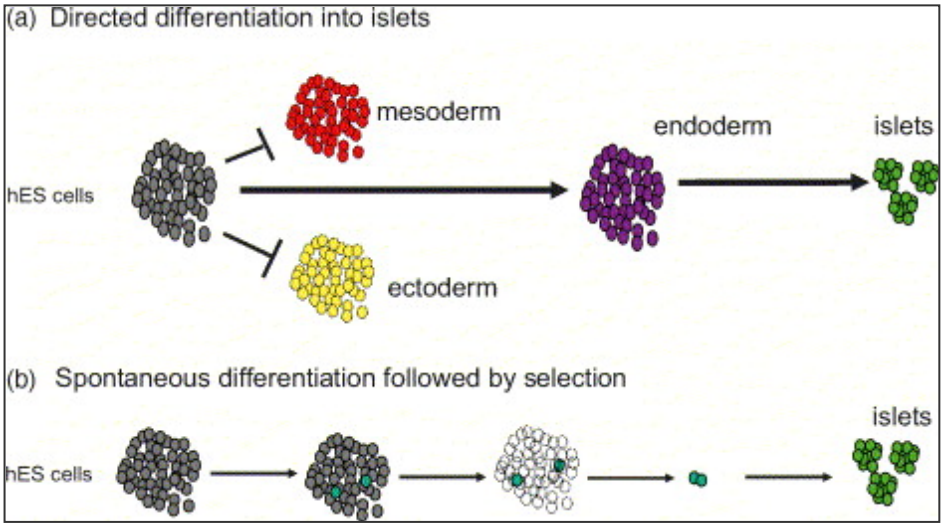
The first source for increasing β-cell numbers is through cell replication. Cell cycle involves the initiation and completion of the DNA replication (S) phase, cell division or mitosis and a number of gaps (G) between these phases (De Leon et al., 2003). Adenoviral overexpression of CDK4 and cyclin D1 in rodent and human islets resulted in marked increase pRb phosphorylation and increased β-cell replication (Kim et al., 2006).

1.8.5 Islet Neogenesis

During embryonic development, the formation of new islets, or islet neogenesis, is believed to originate from ductal epithelial cells. The growing epithelium forms proliferating ductules that branch and ultimately differentiate giving rise to mature ducts, acinar cells and islets (Butler et al., 2003). A recapitulation of this process has been observed during regeneration in duct ligation model or after following 90% partial pancreatectomy (Px) in the rat. Increased proliferation of ductal epithelium is followed by expression of PDX-1 during the differentiation stage, leading to the formation of new islets and acinar cells (Butler et al., 2003; Meier et al., 2006). In less rigorous models of regeneration, evidence of islet neogenesis was observed by islet hormone positive cells within ducts or as small clusters budding from ducts (Bonner-Weir et al., 2011; Joglekar and Hardikar, 2012; Kopp et al., 2011).

Few other reports suggest generation of islet cells from islet precursor within the islets itself (Joglekar and Hardikar, 2012). These cells are now considered as "Intra-Islet Precursor Cells" (IPC). Reports are there which suggest that even under *in-vivo* conditions, these IPC's are responsible for the replenishment of the lost beta cells in diabetic animal model (Smukler et al., 2012). New β-cells may also arise from the differentiation of stem or precursor cells from both pancreatic and extra-pancreatic source such as liver. A colony forming assay using adult mouse pancreatic cells identified multipotent precursor cells capable of producing pancreatic endocrine cells *in vitro* (Herold et al., 2002). In addition to mature duct cells, another study suggests that the ductal structures contain Ngn-3 expressing endocrine precursor cells within the ductal lining and are responsible for giving rise to new β-cells (Ryan et al., 2005). Other studies provided evidence of an early endocrine precursor cell marked by PDX-1 expression in the absence of insulin expression (Zhang et al., 2006). Co-hormone expression may represent differentiation of precursor cells. Expression of somatostatin and insulin or glucagon and insulin has been observed by immunohistochemistry during regeneration (Nir et al., 2007). In contrast, strong evidence using lineage cell tracking has shown that

adult β-cells arise from replication rather than stem cell differentiation, however, this does not exclude the contribution of stem cells to β-cell formation during the neonatal period and during regeneration (Ashcroft et al., 2004).



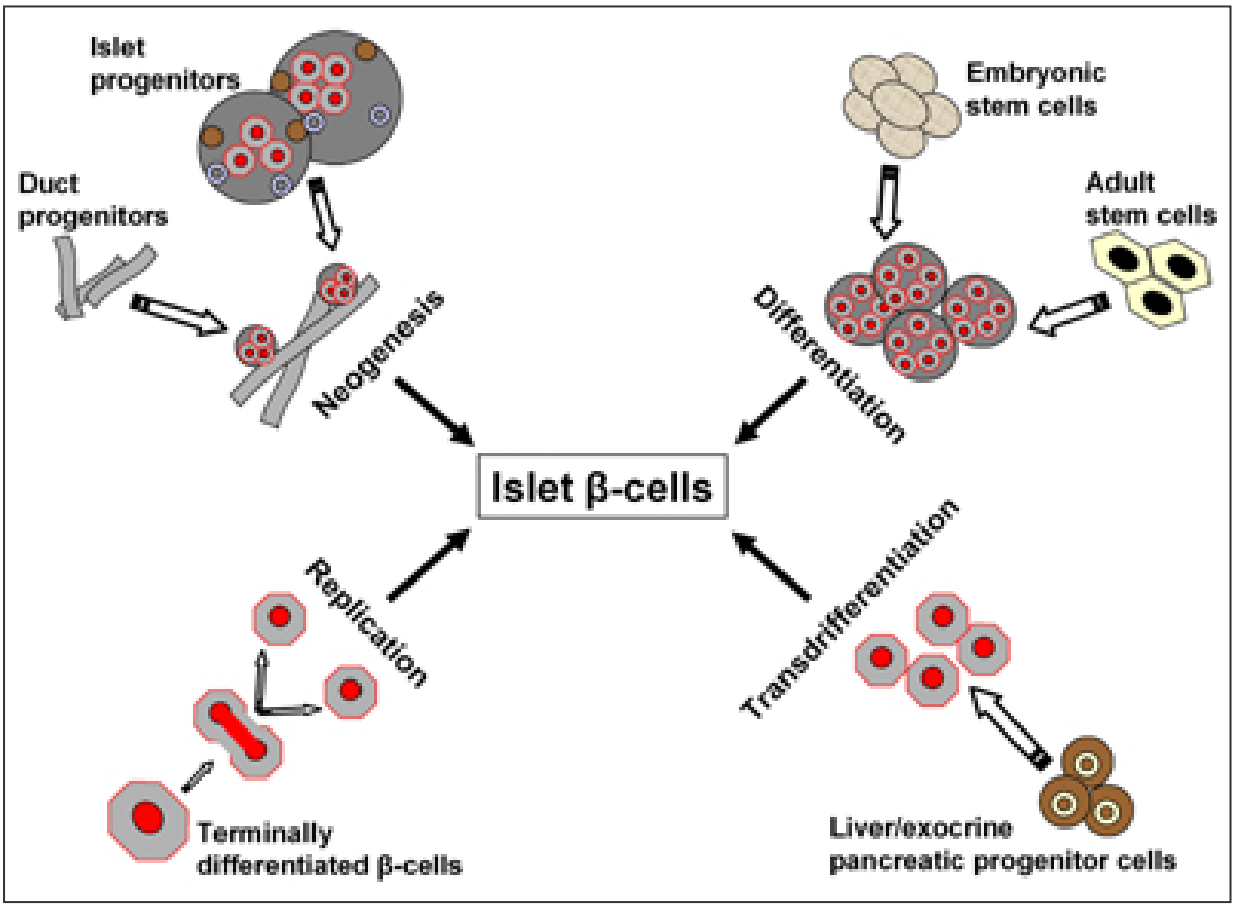


Fig-1.13 demonstrating various sources for islet neogenesis from both ES and cult stem cells. (Alan Colman, 2004; Bhonde et al., 2005).

1.9 Stem cells

Stem cells identified as a group of cells, with capacity to self-renew and differentiate into various cell types, which can construct tissues and organs. Stem cells are biological identity found in all multicellular organisms. Being small portion in body mass, they can divide through mitosis and differentiate into diverse specialized cell types and can self-renew to produce more stem cells. There are many types of stem cells known, differing only in their degree of differentiation and ability of self-renewal. Embryonic stem cells are derived from the part of a human embryo or fetus, are the stem cells having full potential for differentiation into any cell types including extra embryonic cell types like placenta, amniotic membrane, amniotic fluid, placental cord etc. Adult stem cells are partially differentiated cells which are found amongst specialized (differentiated) cells in almost all tissues or organs. Recent research shows that, adult stem cells appear to possess more restricted ability of producing different cell types and self-renewal capacity compared with that of embryonic stem cells.

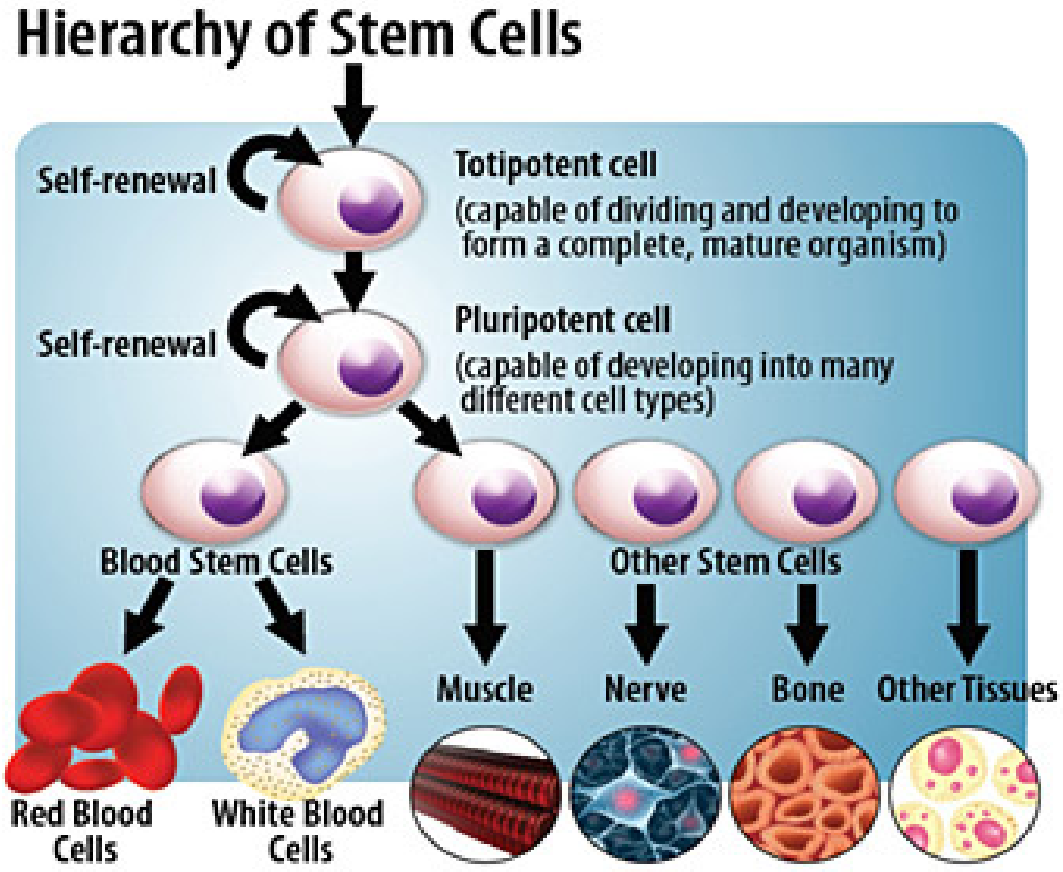


Fig-1.14 demonstrating Hierarchy of stem cells leading to various cell tyoes on differentiation.

1.9.1 Stem cells classification:

Stem cells are being classified as below according to their plasticity and sources--

Classification		Characteristics
Sources/types	Embryonic stem cells	are pluripotent stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo.
	Adult stem cells	Endodermal Origin: Pulmonary Epithelial SCs, Gastrointestinal Tract SCs, Pancreatic SCs, Hepatic Oval Cells, Mammary and Prostatic Gland SCs, Ovarian and Testicular SCs. Mesodermal Origin: Hematopoietic SCs, Mesenchymal Stroma SCs, Mesenchymal SCs, mesenchymal precursor SCs, multipotent adult progenitor cells, bone marrow SCs, Fetal somatic SCs, Unrestricted Somatic SCs, Cardiac SCs, Satellite cells of muscle Ectodermal Origin: Neural SCs, Skin SCs, Ocular SCs
	Cancer stem cells	have been identified in almost all caner/tumor, such as Acute Myeloid leukemic SCs (CD34+/CD38-), Brain tumor SCs (CD133+), Breast cancer SCs (CD44+/CD24-), Multiple Myeloma SCs (CD138+), Colon cancer SCs (CD133+), Liver cancer SCs (CD133+), Pancreatic cancer SCs (CD44+/CD24+), Lung cancer SCs (CD133+), Ovary cancer SCs (CD44+/CD117+), Prostate cancer SCs (CD133+/CD44+), Melanoma SCs (CD4+/CD25+/FoxP3+), Gastric cancer SCs (CD44+).

	Induced pluripotent stem cells	a type of pluripotent stem cells artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a "forced" expression of specific genes.
Cell potency	Totipotent cells	Zygote, Spore, Morula; It has the potential to give rise to any and all human cells, such as brain, liver, blood or heart cells. It can even give rise to an entire functional organism.
	Pluripotent cells	Embryonic stem cell, Callus; They can give rise to all tissue types, but cannot give rise to an entire organism.
	Multipotent cells	Progenitor cell, such as hematopoietic stem cell and mesenchymal stem cell; They give rise to a limited range of cells within a tissue type.
	Unipotent cells	Precursor cells.

(Source: Xiaoning Zhao et. Al., 2011)

1.9.2 Embryonic stem cells

Embryo in humans is consisting of 50–150 cells at blastocyst stage, 4-5 days after fertilization termed as Embryonic stem cells (ES cells) and are derived from the inner cell mass of the blastocyst. These cells represent two distinctive properties. First, they are able to differentiate into all derivatives of three primary germ layers (pluripotency), and second, they are capable of propagating themselves indefinitely, under defined media conditions (Ying et al., 2003). These studies of gene expression in ES cells have demonstrated many proteins associated with the "stemness" phenotype which can serve as markers such as 5T4, Nanog, ABCG2, Oct-3/4, Alkaline Phosphatase/ALPL, Oct-4A, E-Cadherin, Podocalyxin, CXCR4, SCF R/ckit, CD30/TNFRSF8, sFRP-2, Rex-1/ZFP42, CD9, CDX2, Smad2,

Chorionic Gonadotropin, lpha Chain (alpha HCG), Smad2/3, SSEA-1, ESGP, SSEA-3, FGF-4, Cripto, SOX2, DPPA4, SPARC/Osteonectin, DPPA5/ESG1, SSEA-4, GCNF/NR6A1, STAT3, GDF-3, SUZ12, Integrin alpha 6/CD49f, TBX2, Integrin alpha 6 beta 4, TBX3, Integrin beta 1/CD29, TBX5, KLF5, TEX19, Lefty, THAP11, Lefty-1, TRA-1-60(R), Lefty-A, TROP-2, LIN-28, UTF1, LIN-41, ZIC3, c-Myc etc. (Zhao et al., 2011)

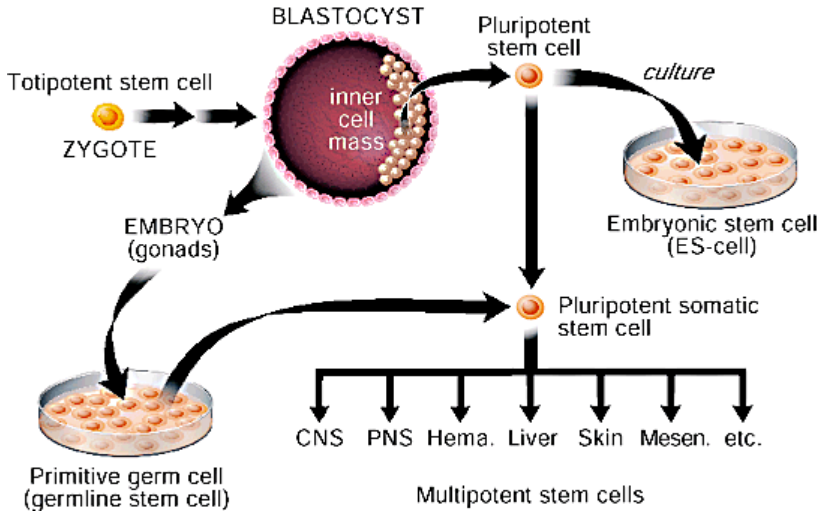


Fig- 1.15 Embryonic stem cells isolated from inner mass of blastocyst.

1.9.3 Embryonic germ (EG) stem cells

EG cells are derived from primordial germline cells (PGCs) during early development. EG cells share most of the characteristics of human ES cells, however differ significantly. Cells in these colonies expressed SSEA-1, SSEA-3, SSEA-4, TRA1–60, TRA-1–81, and alkaline phosphatase markers. Media for growth for EB cultures lacked LIF, bFGF, and forskolin (Roach et al., 1993).

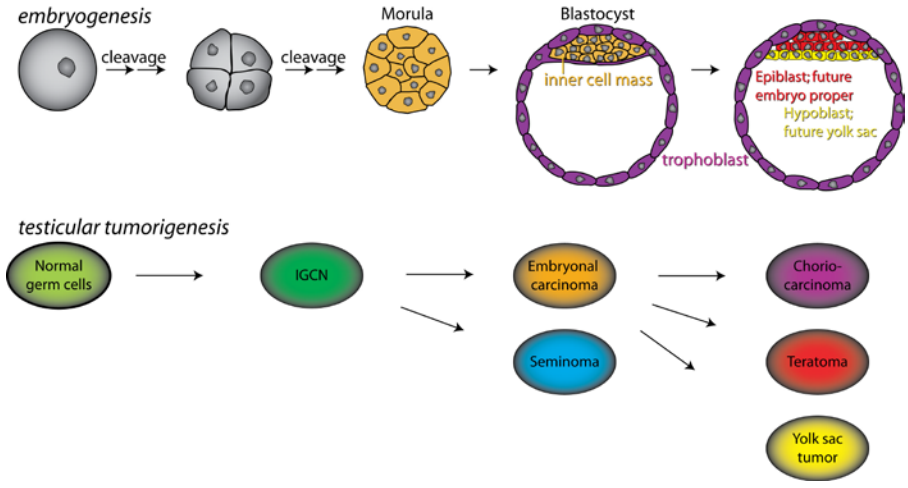


Fig 1.16 showing embryonic germ stem cells.

1.9.4 Fetal stem cells

Fetal stem cells are also primitive cell types which are found in fetus organs. Fetal stem cells potent to differentiate into two types of stem cells: pluripotent stem cells and hematopoietic stem cells. pancreatic islet progenitors, Neural crest stem cells and fetal hematopoietic stem cells have been isolated from fetuses (Beattie et al., 1997). Fetal blood, placenta and have been used by many people including children and adults suffering from various devastating diseases (Alvarez et al., 2009). Fetal neural stem cells found in the fetal brain has been shown to differentiate into both neurons and glial cells (Villa et al., 2000). While human fetal liver progenitor cells have also been shown for enormous proliferation and differentiation capacity to generate mature hepatocytes after transplantation in immuno-deficient animal model (Soto-Gutierrez et al., 2009). Suzuki et al. showed that even a single cell in the c-Met+CD49f- lowc-Kit-CD45-Ter119- fraction isolated from mid gestational fetal liver has the capacity for self-renewal in-vitro and bipotential differentiation, indicating that this subset of population does contains hepatic stem cells (Suzuki et al., 2002).

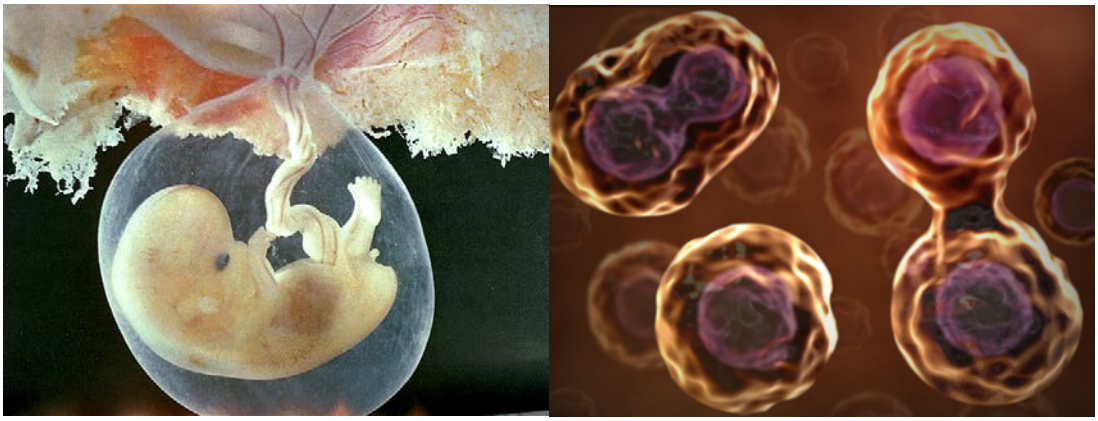


Fig-1.17 demonstrating fetal stem cells can be isolated from developing fetus

1.9.5 Induced pluripotent stem cells

Induced pluripotent stem cells (Thomson et al., 1998), commonly abbreviated as iPS cells or iPSCs are type of pluripotent stem cell which are artificially derived from a non-pluripotent cell or adult somatic cell, by inducing a "forced" expression of specific subset of genes. Induced Pluripotent Stem Cells are similar to naturally occurring pluripotent stem cells, such as embryonic stem (ES) cells.

in many respects, such as the expression of certain stem cell genes and proteins, doubling time, embryoid body formation, teratoma formation, chromatin methylation patterns, viable chimera formation, and potency and differentiability (Ying et al., 2003). iPSCs were first introduced in 2006 from mouse cells and in 2007 from human cells by Japanese group of Nobel laureate Yamanaka et al.

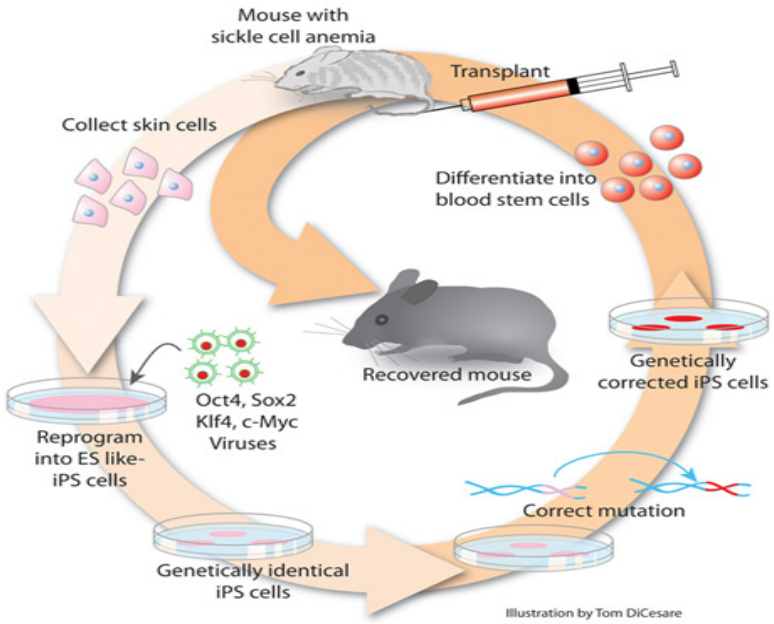


Fig-1.18 depicts generation of Induced Pluripotent Stem from somatic cells

1.9.6 Bone Marrow (BM) stem cells

Adult BM mainly consist of two populations of progenitor cells, *hematopoietic stem cells* (HSCs) and marrow stromal cells (MSCs) (Lagasse et al., 2000). HSC and MSC are both multipotent stem cells. HSCs are present in circulating blood and umbilical cord blood (UCB) which are able to sustain production of all blood cells throughout life. Whereas MSCs can be isolated from several other tissues sources, including adipose tissue, placenta, amniotic fluid, UCB and fetal tissues and are able to differentiate into osteocytes, adipocytes, chondrocytes, smooth muscle cells and haematopoietic supportive stroma etc. (Herzog et al., 2003; Yagi et al., 2010). Human HSCs have been defined with respect to staining for Lin, CD34, CD38, CD43, CD45RO, CD45RA, CD59, CD90, CD109, CD117, CD133, CD166, and HLA DR (human). The positive markers useful for MSC identification are CD106, CD105, CD73, CD29, CD44, and Sca-1 (Domen, et al., 2006).

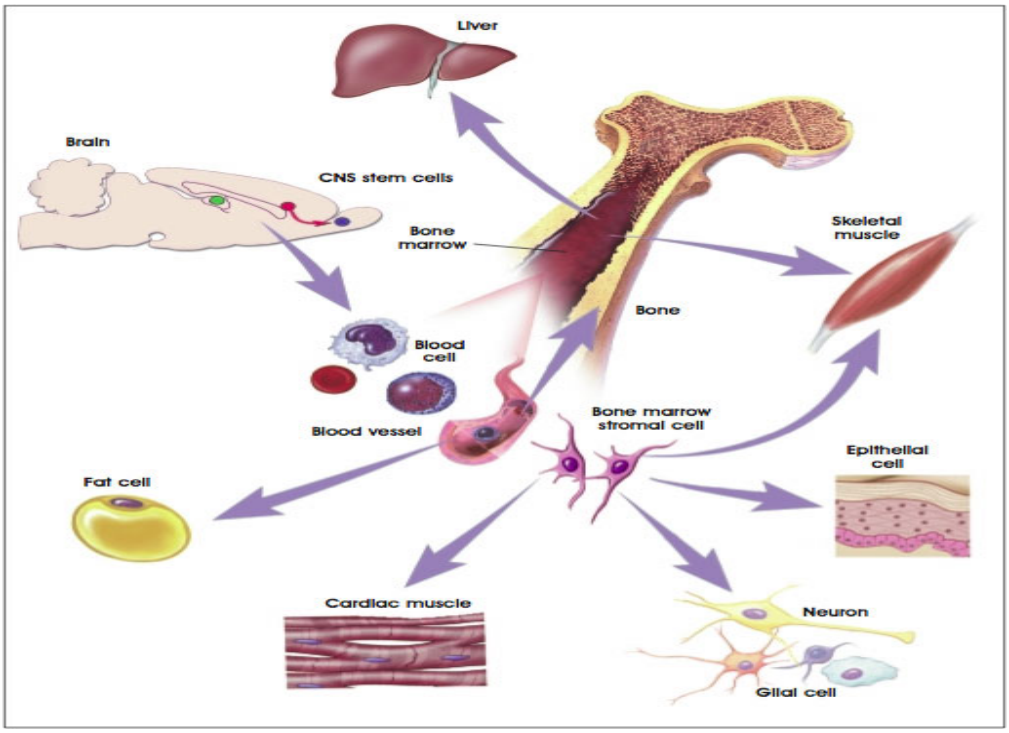


Fig-1.19 shows bone marrow stem cells potential to form cell of multiple lineages.

1.9.7 Adult stem cells

Adult stem cells are stem cells isolated from mature tissues. Because of the stage of development of these cell types, they have limited potential compared to that of stem cells derived from embryos and fetuses etc (Robinson, 2001). Most adult stem cells are lineage-restricted (multipotent) and are generally referred from their tissue origin (mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, dental pulp stem cell, etc.) (Barrilleaux et al., 2006; Gimble et al., 2007). These cells play important roles in local tissue repair and regeneration.

1.9.8 Pancreatic stem cells

Pancreatic islet transplantation has been demonstrated in an very efficient way to achieve the long-term insulin independence for the patients suffering from type-1 diabetes mellitus. However, because of limited availability of islet mass, new sources of insulin producing beta cells that are responsive to glucose are required. Development of pancreatic or non-pancreatic beta-cell lines from rodent or human origin had been progressed quite slowly in recent years. Till

date, the best candidate sources for adult pancreatic stem or progenitor cells are: ductal cells, exocrine portion of pancreas, nestin-positive islet derived progenitor cells, neurogenin-3-positive cells, pancreas-derived multi-potent precursor cells. The very first report to describe in-vitro generated insulin-producing islet-like clusters was based on the expansion of mouse pancreatic duct cells. Thereafter, Bonner-Weir et al generated the same insulin-producing islet-like clusters from cultivated islet cells developed from human pancreatic duct cells in-vitro (Bonner-Weir et al., 2000). One such other study also provided evidences that GLP-1 is able to induce pancreatic ductal cells with the elevated expression of PDX-1 to differentiate into insulin producing beta cells (Hui et al., 2001) and is also able to stimulate glucose-derived de-novo fatty acid synthesis and chain elongation during cell differentiation and insulin release (Bulotta et al., 2003). These sets of data indicated pancreatic ductal cells are potential source for generation of insulin-producing islet cell types. A recent genetic lineage tracing study (Dor et al., 2004) claimed the replication success of pre-existing β-cells turned to be the dominant pathway for the formation of new β-cells in adult mice. Another similar study I these lines (Seaberg et al., 2004) also showed a clonal isolation of multi-potential precursor cells from mouse adult pancreas called pancreas-derived multi-potent precursor cells. These precursor cells arose from single islet or ductal cell. The generation of insulin-producing cells from pancreatic exocrine tissue had also been recently reported (Baeyens et al., 2005). Both exocrine and endocrine pancreas originates from a domain of the foregut endoderm, which expresses master or key-step gene pancreatic duodenal homeobox factor (Pdx-1) at early developmental stages. Silencing of this gene, lead to non-pancreatic phenotype, demonstrating its major impact on both exocrine and endocrine pancreatic development. Baeyens et al., indicated, the existence of acinar-islet transitional cells *in-vivo* and the "spontaneous" trans-differentiation of acinar cells to insulin-expressing beta cells. Altogether, these evidences may suggest that a population of acinar cells, in the presence of certain soluble factors, is competent to adopt an endocrine fate. Some other reports suggest that pancreatic precursor cells express nestin (Zulewski et al., 2001), an intermediate filament protein which is a marker of neural stem cells. These

nestin-positive islet-derived progenitor cells also express insulin, glucagon, and Pdx-1 as well as low with levels of insulin secretion too. However, some other studies also suggest that nestin expression is not related to pancreatic precursor identity only. Recent data in this direction indicate that Ngn-3-positive cells are endocrine progenitors present in the adult pancreas and in the embryo both and Ngn-3 expression is not seen outside the pancreatic islets (Gu et al., 2002). Nevertheless, low levels of Ngn-3 expression within a population of duct cells are not excluded by these studies. Pancreatic stem cells (PSCs) have identified with a potential to differentiate into all three germ layers. Major markers present on the surface of PSCs include Oct-4, Nestin, and c-kit. DCAMKL-1 is a novel putative stem/progenitor marker, these sets of markers can be used to isolate normal pancreatic stem/progenitors, and potentially regenerate pancreatic tissues.

1.10 Mechanisms of β-Cell Regeneration

Stimulation of endogenous tissue regeneration is another avenue that is being examined for β-cell replacement therapy. The capacity to repair tissue following an insult has been demonstrated in many tissues, including the skin, liver and heart. This mechanistic tissue regeneration has also been demonstrated in many animal models of pancreas injury such as partial duct ligation and partial pancreatectomy or by β-cell specific injury with alloxan and Streptozotocin. These studies have attempted to identify the source of new β-cells following regeneration as a means to generate an increased supply of β-cells. There are three main sources of new β-cells: replication, islet ductal neogenesis and stem/precursor cell differentiation.

1.10.1 Genes involved in β-cell Regeneration

Understanding the mechanism of β-cell regeneration will also require the elucidation of the genes involved. Following β-cell loss, upregulation of some genes are expected to initiate the process of regeneration, while other genes may play a role in cell proliferation and/or precursor differentiation.

1.10.2 The Regenerating gene family

The regenerating islet-derived gene family (Reg) belongs to the calcium dependent C-type lectin gene superfamily (Okamoto, 1999). The Reg gene

family encodes for group of small secreted proteins, which can function as acute phase reactants, lectins, anti-apoptotic factors or growth factors for β-cells, neural cells and epithelial cells in the digestive system. Additionally, Reg genes have been shown to act on the ducts to stimulate β-cell neogenesis (Zenilman et al., 1996).

	Species				
Type	Human	Rat	Mouse	Hamster	Cow
I	Reg1α (PSP/Lithostathine/PTP) Reg1β	Reg1	Reg1		
II			Reg2		
III	HIP/PAP	Reg3/PAP2 PAP1/Peptide 23 PAP3	Reg3α Reg3β Reg3γ Reg3δ	INGAP	PTP

Fig-1.20 representing family of reg proteins in various species.

1.10.3 PDX-1 and β-cell Regeneration

In addition to its role in development, PDX-1 has also been shown to play a role in β- cell maturation and differentiation in the adult. A population of PDX-1+ cells expressing little or no insulin (low) were tracked *in vitro* and over time acquired insulin expression (insulin+) without cell division (McKinnon and Docherty, 2001). This suggested the existence of a precursor pool of immature β-cells in the adult. These immature β-cells had increased expression of MAFB and NKX2.2, markers of early β-cell development, and also had higher replication rates than mature β-cells.

1.11 Poly (ADP) Ribose polymerase (PARP)-1

PARP-1 is the foremost and important enzyme which is oldest and best characterized member of the PARP family, accounting for 80% of cellular PAR formation and targeting the negatively charged PAR polymers to numerous nuclear proteins, affecting their functioning through steric hindrance and electrostatic repulsion. Out of 17 different PARP proteins (Han et al., 2011). PARP-1 have been extensively studied so far because of its highly participating role in many cellular events like proliferation, DNA repair, cell cycle, including

cell differentiation and gene transcriptional regulation. Poly(ADPr) in turn is very rapidly degraded by PAR glycohydrolase, thereby allowing fast and dynamic signaling. Although originally thought to be only involved in DNA repair, mounting evidence now points to PARP-1 as a key regulator of gene transcription, cell activation, migration, proliferation and differentiation (Kim et al., 2005).

1.11.1 PARP and its structure:

Poly (ADP-ribose) (PAR) polymerase (PARP)-1 is a nuclear enzyme which catalyses the polarization of PAR from cleaved NAD+ moieties. PARP is composed of four domains of interest namely, N terminal DNA-binding domain, central auto-modification domain (a caspase-cleaved domain domain) and C terminal catalytic domain. The DNA-binding domain (44kD) is composed of two zinc finger motifs F1 and F11. F 1 is known to interact with double stranded nick whereas F11 recognize and binds single stranded nicks. It also contains a NLS sequence which is required for the nuclear translocation of PARP protein and is cleaved off at DEVD site upon activation of caspase 3 and 7. The central auto-modification domain (16kD) is responsible for releasing the protein from the DNA after catalysis this region is rich in glutamine, serine and leucine residues. Also, it plays an integral role in cleavage-induced inactivation. The last C terminal catalytic domain (55kD) is required for the catalysis of ADPr from reduced NAD+ species and its polymerization into linear or branch chain polymers (Kim et al., 2005).

1.11.2 How does PARP functions

PARP is found in the cell's nucleus, the main role is to detect and signal single strand DNA breaks (SSB) to the enzymatic machinery involved in the SSB repair. Once PARP detects a SSB it binds to the DNA, and, after a structural change, begin the synthesis of a poly(ADP-ribose)chain (PAR)as a signal for the other DNA repairing enzymes such as DNA ligase III (LigIII), DNA polymerase beta (polβ) and scaffolding proteins such as x-ray repair complementing gene 1 (XRCC1). After repairing, the PAR chains are degraded via PAR glycohydrolase (PARG). Interestingly, NAD+ is required as substrate for generating ADP-ribose

monomers. The overactivation of PARP may deplete the stores of cellular NAD+ and induce a progressive ATP depletion, since glucose oxidation is inhibited leading to necrotic cell death.

1.11.3 Evidence for PARP role in Cell/Stem cell Differentiation

Cellular Differentiation Programs. PARP-1, as well as PARP-2, has been implicated in the differentiation of other cell types as well. For example, in a model of neuronal differentiation, PARP-1 is required for the exchange of corepressors for coactivators at the promoters of genes regulated by the transcriptiofactor HES1 (Ju et al., 2004). PARP-1 is also required for T cell dependent immunoglobulin class switching in B cells (Ambrose et al., 2009; Morrison et al., 1997), and PARP-1 deficiency promotes the differentiation of regulatory (CD4+/CD25+/Foxp3+) T cells (Nasta et al., 2010). In a model of endodermal differentiation, PARP-1 and PARP-2 play distinct roles in a pathway involving physical and functional interactions with the heterochromatin-associated proteins HP1 and TIF1b: PARP-2 is required for differentiation of mouse embryonal carcinoma cells into primitive endoderm-like cells in response to retinoic acid, while PARP-1 is required for subsequent differentiation into parietal endoderm-like cells in response to retinoic acid and dibutyryl cAMP (Quenet et al., 2008). PARP-2 is required for adipogenesis (Bai et al., 2007), spermiogenesis (Dantzer et al., 2006), and T cell survival during thymopoiesis (Yelamos et al., 2006). During adipogenesis, PARP-2 functions as a coactivator of the adipogenic transcription factor PPARγ (Bai et al., 2007).

1.11.4 Stem Cell Differentiation and Functions

In embryonic stem cells (ESCs) fromParp-$1^{-/-}$ mice, about 10% of genes analyzed showed altered expression compared to about 3% of genes in livers from the same animals (Ogino et al., 2007). The number of genes downregulated by PARP-1 knockout was about 2-fold more than the number of upregulated genes in both cases, indicating a major role for PARP-1 in keeping genes active in ESCs and liver cells (Ogino et al., 2007). The large panel of genes whose expression is dependent on PARP-1 in ESCs suggests a role for PARP-1 in the developmental programming of these cells. A recent study has revealed some of the molecular

mechanisms whereby PARP-1 might help to promote the differentiation of stem cells (Gao et al., 2009). Specifically, PARP-1 antagonizes the DNA binding transcription factor Sox2 to stimulate expression of the gene encoding fibroblast growth factor 4 (FGF4), a growth factor that promotes differentiation. In response to appropriate cellular signals, PARP-1 PARylates Sox2 at the FGF4 enhancer, this promotes the dissociation and degradation of Sox2 and leads to enhanced expression of FGF4. These results indicate that PARP-1 can regulate the pluripotent state of ESCs by controlling the activity of key stem cell transcription factors

1.12 Models of β-cell Regeneration

Several different experimental models have been developed to study β-cell regeneration. These models range from pancreas injury to β-cell specific insults and describe observations of β-cell replication, ductal islet neogenesis or stem/precursor cell differentiation during regeneration.

1.12.1 Partial Duct Ligation

The partial pancreatic duct obstruction model was developed by Rosenberg and colleagues by wrapping a narrow piece of cellophane tape around the head of the pancreas of the adult Syrian golden hamster (Rosenberg et al., 1983). This led to the formation of new islets through migration of cells out from the epithelium of small intralobular ductules reiterating normal fetal ontogeny (Rosenberg, 1998; Rosenberg et al., 1983). There was a significant increase in ductal cell proliferation 2 weeks following surgery and at 8 weeks an increase in islet cell proliferation culminating in a 2.5 fold increase in islet mass (Rosenberg, 1998; Rosenberg et al., 1983) . This phenomenon of islet neogenesis from duct cells was also observed in the adult rat following pancreatic partial duct ligation (PDL) (Wang et al., 1995). These findings were supported by lineage tracing of the ductal marker CA II in the mouse (Inada et al., 2008). The results of this study showed that β-cells were marked by the tracer, β-galactosidase, following PDL suggesting they were derived from CA II expressing duct cells (Inada et al., 2008). However, a study by Solar et al. found contrasting results by cell

lineage tracking of the duct cell marker HNF1β . No evidence that duct cells gave rise to new endocrine cells following PDL was observed. Previous work from this group suggests that NGN3+ endocrine precursor cells are responsible for giving rise to new β-cells following PDL (Solar et al., 2009).

1.12.2 Partial Pancreatectomy

Partial pancreatectomy (Px) is another method that has been used to study β-cell regeneration and is typically performed in adult animals. Following 90% resection of the rat pancreas there is substantial regeneration of the exocrine and endocrine pancreas (Bonner-Weir et al., 1983; Brockenbrough et al., 1988). In this model, two pathways of regeneration were observed: 1) replication of pre-existing, differentiated exocrine and endocrine cells, and 2) the proliferation and differentiation of ductal epithelium to form new pancreatic lobules (Bonner-Weir et al., 1993; Brockenbrough et al., 1988). More recently, lineage tracing was performed using RIP-CreER;Z/AP adult mice to label the β-cells and look at regeneration after 70% Px . They determined that β-cell replication as the major source of new β-cells and not stem cell differentiation as previously suggested (Dor et al., 2004).

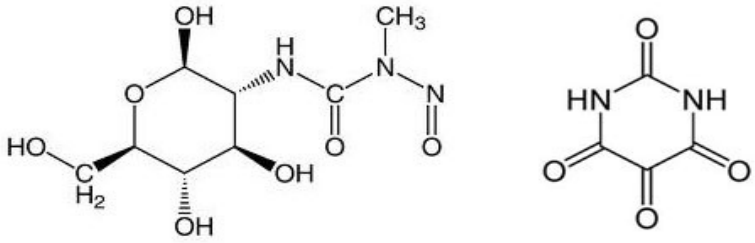
1.12.3 Alloxan

In 1943, it was reported that alloxan could induce diabetes in the rat due to specific necrosis of the β-cells. Alloxan is a very unstable compound that is structurally similar to glucose (Fig 1.21) allowing for its uptake into the β-cell through the GLUT2 transporter (Lenzen and Munday, 1991; Weaver et al., 1978). Once in the β-cell, alloxan selectively inhibits GSIS through specific inhibition of the glucose sensor, GCK, and causes necrosis of β-cells through its ability to induce reactive oxygen species (ROS) formation. Regeneration of mouse β-cells was observed following selective perfusion of alloxan after clamping the superior mesenteric artery. β-cell destruction occurred in perfused regions of the pancreas, while non-perfused regions were spared . Histological evidence suggested islet neogenesis from differentiation of duct cells in the alloxan perfused region and a significantly increased islet area due to β-cell replication in the non- perfused region (Waguri et al., 1997). In rats, a

single intraperitoneal (i.p.) injection of alloxan led to increased serum glucose levels followed by β-cell regeneration 12 days later. β- cell replication rates were increased and neogenesis was observed as transdifferentiation of acinar and duct cells to β-cells (De Haro-Hernandez et al., 2004).

1.12.4 Streptozotocin

Streptozotocin (STZ) is an antimicrobial agent and has also been used as a chemotherapeutic alkylating agent (Schein et al., 1974; White, 1963). In 1963, Rakieten et al. reported that STZ was diabetogenic. STZ has become the main choice for the chemical induction of diabetes in animal models and for the study of β-cell regeneration (Rakieten et al., 1963). STZ is a glucose analogue known as 2-dexoymethyl-nitrosurea-glycopyranose (Fig 1.21). Similar to alloxan, GLUT2 on the β-cell plasma membrane selectively recognizes STZ due to its similar structure to glucose and transports it into the cell (Karunanayake et al., 1976; Tjalve et al., 1976). The toxic effects of STZ mainly arise from the transfer of the methyl group to a DNA molecule leading to DNA fragmentation. Overstimulation of poly(ADP-ribose) polymerase occurs in an attempt to repair DNA, which leads to depletion of cellular NAD+ and ATP stores (Uchigata et al., 1982; Yamamoto et al., 1981). Accordingly, diabetes develops due to necrosis of β-cells leading to hypoinsulinemia and hyperglycemia (Portha et al., 1974; Wang et al., 1994).



Streptozotocin Alloxan

Figure-1.21: Comparison of the molecular structures of Streptozotocin and Alloxan.

1.12.5 Single Dose STZ Model

STZ is conventionally administered as a single injection. It has a serum half-life of 15 minutes (Schein et al., 1973). β-cell necrosis is apparent within 24 hours by ultrastructural examination (Junod et al., 1967). Blood glucose values peak 1-

2 days after STZ and remain elevated depending on the dose given (Junod et al., 1969). In neonatal rats, there are a few different protocols used. First, the Na-STZ model is generated by injecting 100 mg/kg STZ i.p. or intravenously at birth. Rats exhibit insulin deficient acute diabetes 3-5 days after birth. At 3-4 weeks of age, body weight and basal plasma glucose values cannot be distinguished from control values. However, by 8 weeks of age, rats show a mild basal hyperglycemia, an abnormal response to an i.p. glucose tolerance test (IPGTT) and 50% decrease in pancreatic insulin stores. Other neonatal models involve the injection of STZ on day 2, 4, or 5 (Bonner-Weir et al., 1981; Thyssen et al., 2006) and the severity of diabetes and the degree of recovery that follows depends on the timing of STZ, as rats injected on day 5 lacked re-accumulation of insulin in the pancreas 2 weeks following STZ . In the n0-STZ model, the recovery of plasma glucose to normal values 1 week after birth was related to recovery of pancreatic insulin content and β-cell mass . From day 4 onward, signs of regeneration became apparent in the numerous insulin positive cells found throughout the acinar parenchyma and within the ductal epithelium (Cantenys et al., 1981). The appearance of islets budding from ducts was a common feature. Examining the mitotic rate suggested that most β-cells were formed by mitosis of undifferentiated cells (Dutrillaux et al., 1982). However, the long term impairment of the plasma glucose homeostasis and persistence of a reduced β-cell mass in 4 month old animals are proof that the regeneration process was incomplete. It has also been suggested that there may be strain differences in the regeneration potential in rats (Portha et al., 1989).

1.12.6 Multiple Low Dose STZ Model

The multiple low dose model, first described by Like and Rossini , used five daily injections of a sub-diabetogenic dose of STZ in mice, as compared to one large diabetes-inducing dose of STZ as used in the rat model. CD-1 mice were injected with 40 mg/kg STZ daily for 5 days. Plasma glucose levels were significantly elevated after the 4th injection and increased further in the following days . Large numbers of lymphocytes and moderate numbers of macrophages surrounded or permeated the islets . β-cell necrosis was observed and remaining β-cells showed degranulation . Five daily injections of STZ (40 mg/kg) in rats

failed to produce similar islet inflammatory lesions, despite the fact that an equivalent single large injection of STZ (160 mg/kg) produced marked hyperglycemia . With the one injection technique, necrosis occurs within 4 hours, hyperglycemia is achieved rapidly and all islets are free of inflammatory lesions . In contrast, multiple sub-diabetogenic injections induced gradual elevation of plasma glucose, with mononuclear inflammatory cells in and around the islets (Like and Rossini, 1976). Multiple low doses of alloxan similarly altered islet morphology and increased blood glucose levels, however, no inflammatory infiltration was observed (Rossini et al., 1977).

1.12.7 STZ-Induced Regeneration

It has been shown that young rodents have a large capacity to regenerate β-cell mass after STZ-mediated destruction . Subtotal destruction of β-cells with STZ is followed by regeneration of β-cells and remission of hyperglycemia in young rodents (Wang et al., 1994). The capacity to regenerate decreases with age, as observed when STZ was administered to adult rats, showing incomplete regeneration leading to diabetes later in life (Portha et al., 1989; Wang et al., 1996). However, it was found that transplantation of bone marrow-derived stem cells were able to improve regeneration and survival rates, suggesting that regeneration is possible even in the adult.

The limitation of regeneration in the adult may be a paucity of inductive signals from endothelial progenitor cells or a lack of β-cell precursors. In the PDL and pancreatectomy models, there has been much debate about the source of regenerated β-cells. There is strong evidence to suggest that new β-cells mainly arise from replication of pre-existing β-cells (Dor et al., 2004; Teta et al., 2007) while others contest that differentiation of progenitor cells is the main source of β-cells during regeneration (Inada et al., 2008; Xu et al., 2008). However, there is less known about the source of new β-cells in STZ induced regeneration. A recent study using lineage tracing technology demonstrated an increase in Pdx-1+/MafB+/insulin— cells following STZ treatment suggesting an increase in differentiation of β-cell progenitors leading to regeneration of mature insulin expressing cells (Liu et al.). While endogenous (Chamson-Reig et al.) or

transplanted (Hess et al., 2003) bone marrow cells have been shown to home to the regenerating pancreas, they differentiate into supportive vascular endothelium. Moreover, vascular growth in parallel to that of β-cell replication appears to be important for regeneration and involves growth factor signalling and extracellular matrix interactions (Nicholson et al.).

Together these results suggest that the intra-islet niche may facilitate β-cell replication, in addition to the differentiation of precursor cells for regeneration of β-cells following STZ. From fig 1.22 there is evidence to support the generation of new β-cells from all three main sources (replication, neogenesis, and differentiation), depending on the model. A key question is which mechanism can best be utilized to generate a functional supply of new β-cells and how can we stimulate this process?

1.13 Agents facilitating Islet Neogenesis

1.13.1 Various chemical or biological agents used for the generation of insulin-producing cells

Bioorganic molecules have provided efficient chemical tools to control cellular processes by modulation of metabolism, signal transduction pathways and gene regulation. Some of the compounds have been instrumental in islet differentiation protocols, such as HGF, IGF, Activin-A, Exendin-4, GLP-1, INGAP, Betacellulin etc. In this sense, numerous molecules have been used by various investigators in order to drive the differentiation of stem cells towards β-cell-like phenotype, as well as to promote the proliferation and differentiation of β-cell precursors isolated from adult tissues.

1.13.1.1 Nicotinamide

Nicotinamide is also called niacinamide or nicotinic acid amide. It is the amide of nicotinic acid or vitamin B3. Nicotinamide represents another commonly used extrinsic induction factor. As early as the 1990s Otonkoski et al. had used nicotinamide as an inducer of endocrine differentiation in cultured human foetal pancreatic cells . It was shown that treatment of human foetal pancreatic cells with 10 nM nicotinamide resulted in a twofold increase in DNA content and a threefold increase in insulin content associated with development of beta cell

outgrowths from undifferentiated epithelial cell clusters. There was also an increase in the expression of the insulin, glucagon, and somatostatin genes (Otonkoski et al., 1993). More recently, many studies almost inevitably used nicotinamide in their induction protocols in one or more stages together with other extrinsic induction factors (Chao et al., 2008; Chen et al., 2004; Segev et al., 2004).

Model	Regimen	Species	Mechanism
STZ	multiple low dose (50 mg/kg)	Mouse	differentiation
	n0-STZ (100 mg/kg)	Rat	replication, neogenesis
	n4-STZ (70 mg/kg)	Rat	replication, differentiation
	multiple low dose (40 mg/kg)	Rat	ND
Alloxan	perfusion; adult	Mouse	replication, differentiation, neogenesis
	multiple dose; adult	Mouse	ND
	single injection; adult	Rat	replication, neogenesis
Partial Pancreatectomy	70, 90% resection; adult	Rat	replication, neogenesis
Partial Duct Obstruction	cellophane wrapping; adult	Hamster	neogenesis
Partial Duct Ligation	adult	Mouse	neogenesis, differentiation
	adult	Rat	neogenesis
Glucose infusion	adult	Rat	replication
Pregnancy		Human	neogenesis
		Rat	replication, hypertrophy
		Mouse	proliferation
Obesity	adult	Human	replication, neogenesis
	adult	Mouse	replication, neogenesis
	adult	Rat	replication, neogenesis

Fig 1.22 showing summarized table on various animal model to study beta cells regeneration in various species with their regimen for induction of regeneration.

1.13.1.2 Epidermal Growth Factor (EGF) and Basic Fibroblast Growth Factor (bFGF)

EGF is a growth factor belonging to the EGF family of proteins. It plays an important role in cellular proliferation, differentiation, and survival (Herbst, 2004). EGF acts by binding to epidermal growth factor receptor (EGFR) on the cell surface which stimulates the intrinsic protein-tyrosine kinase activity of the receptor leading to the initiation of a signal transduction cascade. This in turn leads to a series of biochemical changes in the cell such as an increase in intracellular calcium levels, increased glycolysis and protein synthesis, and expression of certain genes, all of which lead to DNA synthesis and cell proliferation (Fallon et al., 1984). Fibroblast growth factor (FGF) was first discovered as an activity in extracts of pituitary and brain which had a stimulatory effect on the growth of mouse fibroblast cells. It was later shown that the activity was due to two proteins, namely, acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) (Rifkin and Moscatelli, 1989). bFGF is also known as FGF2 or FGF-β, which is a member of the fibroblast growth factor family of proteins. bFGF demonstrates a broad spectrum of biological activities ranging from increased growth and cell migration on many cell types in vitro to involvement in neovascularization and wound repair *in vivo* (Rifkin and Moscatelli, 1989). Low concentrations of EGF and bFGF are used in the culture of stem cells, especially the MSCs. Interestingly, high concentrations of EGF and bFGF, either used alone, or in combination, have been shown to be useful in IPC differentiation (Lumelsky et al., 2001; Sun et al., 2007). On the contrary, Dalvi et al. demonstrated that when human pancreatic islets were exposed to medium containing a high concentration of EGF (50 ng/mL), such populations demonstrated a high degree of proliferation (Dalvi et al., 2009). Removal of EGF from the medium resulted in the formation of islet-like cell aggregates. This phenomenon was also supported by Cras-M´eneur et al. who demonstrated that EGF increased undifferentiated pancreatic embryonic cells *in vitro*. Therefore, a careful use of EGF may be important in successful IPC differentiation (Cras-Meneur et al., 2001). It is also important to note that bFGF may play a role in the clustering of IPCs. In a study performed by Hardikar et al.,

it was shown that out of several growth and differentiation factors tested on pancreatic duct progenitor cells, bFGF secreted by endocrine precursor cells was found to be the most effective chemo attractant in the clustering of these pancreatic precursor cells (Hardikar et al., 2003).

1.13.1.3 Activin A and Betacellulin

Activin proteins are members of the transforming growth factor-beta (TGF-β) superfamily. Activin A has a wide range of biological activities including regulation of cellular proliferation and differentiation and promotion of neuronal survival. Like other members of the TGF-β superfamily, activin A has been reported to affect embryogenesis, haematopoiesis and angiogenesis (Breit et al., 2000; Y. Eto et al., 1987). Activin A is found in abundance in bone matrix (Hirotani et al., 2002), and bone marrow-derived fibroblasts are a major source of activin A (Gaddy-Kurten et al., 2002). Betacellulin is a member of the EGF family isolated as a 32 kDa glycoprotein from the conditioned medium of a mouse pancreatic insulinoma cell line (Sasada et al., 1993). It is widely expressed in many organs and highly expressed in the pancreas and intestine (Yamamoto et al., 2000). It is also expressed in human foetal pancreas (Silver et al., 2005). Much of the knowledge about the biological function of this protein mainly came from studies of its effect on cultured cell line *in vitro*. In a study carried out by Demeterco et al. to analyze the effect of activin A and betacellulin on islet development and growth, it was found that betacellulin acted as a mitogen for undifferentiated pancreatic epithelial cells with an increase in the number of islet-like clusters. On the other hand, activin A was responsible for increased insulin contents. Interestingly, combined use of both factors led to weaker effects when compared to the use of either of the factor alone (Demeterco et al., 2000). It has also been shown that intraperitoneal injection of betacellulin enhanced beta cell regeneration in 90% pancreaticised rats (Li et al., 2001). The use of betacellulin and activin A, either alone or in combination, has resulted in differentiation of stem cells into IPCs (Mashima et al., 1996; Sun et al., 2007; Zalzman et al., 2005). For example, Sun et al. showed that combined use of betacellulin and activin A was involved in the differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into IPCs . On the other

hand, Thowfeequ et al. demonstrated that betacellulin inhibited amylase and glucagon production and promoted beta cell differentiation in mouse embryonic pancreas (Thowfeequ et al., 2007). Using betacellulin and/or activin A, other studies have successfully differentiated stem cells of liver and pancreas.

1.13.1.4 Exendin-4

Exendin-4 is a 39 amino acid protein isolated from the venom of the lizard Heloderma suspectum (Eng et al., 1992). Several studies have been carried out to elucidate the effect of exendin-4 *in vivo*. Xu et al. demonstrated that exendin-4 increased beta cell mass by stimulating beta cell proliferation and neogenesis in diabetic rats (Xu et al., 1999)while Yang et al. demonstrated that when combined with lisofylline, treatment with exendin-4 reversed pancreatic beta cell destruction in diabetic rats (Yang et al., 2006). Exendin-4 has been shown to differentiate stem cells of various origins into IPCs (Gabr et al., 2008; Gao et al., 2008; Tang et al., 2004) . It has also been reported that high levels of the protein *in vitro* resulted in an increase in insulin release by IPCs derived from mouse embryonic stem cells (Wang et al., 1997).

1.13.2 Herbal Differentiating agents

1.13.2.1 Conophylline from *Ervatamia Microphylla*

Conophylline is a vinca alkaloid extracted from the tropical plant Ervatamia microphylla. This compound acts as well in the *Shh* pathway, mimicking the effect of activins repressing *Shh* expression. In addition, activin A, when bound to its receptor, can activate p38 mitogen-activated protein kinase inducing the expression of neurogenin3 (Kojima and Umezawa, 2006). Conophylline addition to pancreatic rudiments in culture as well as to AR42J cells (a model of pancreatic progenitors) increased the number of insulin, and PDX-1-positive cells. Treatment with conophylline of neonatal streptozotocin diabetic rats increased as well the number of insulin-positive clusters budding from ductal structures. The data support the role of conophylline in β-cell regeneration, which has also been described in the same animal model treated with glucagon-like peptide and exendin-4 (Ogata et al., 2004).

1.13.2.2 *Enicostemma littorale* a new target for islet neogenesis

Enicostemma littorale (Gentianaceae) belonging to the family Gentianaceae is a glabrous perennial herb. It grows throughout India up to 1.5 feet height and more frequently near the sea. It is called as Chota-kirayat or Chota chirayata in Hindi, Mamejavo in Gujarati, Nagajivha in Bengal and Vellarugu or Vallari in Tamil.

The taxonomic position of *Enicostemma littorale* is as follows

Subdivision Angiospermae
Class Dicotyledonae
Subclass Gamopetalae
Serius Bicarpellatae
Order Gentianales
Genus Enicostemma
Species littorale

Phyto-chemistry

Many compounds have been isolated and reported from this plant. The aerial part of the plant gave 34% of dry alcoholic extract and 15.7% of ash. Five alkaloids, two sterols and volatile oil, have been reported by Natarajan and Prasad (Natarajan PN, 1972). Monoterpene alkaloids like-enicoflavin and gentiocrucine were also isolated (Chaudhuri, 1975). The presence of catechins, saponins, steroids and triterpenoids were reported by earlier workers (Retnam, 1988a). A new flavone C-glucoside named as Verticilliside was isolated recently. Swertiamarin was isolated from the green viscous mass obtained from an alcoholic extract of the drug treated with ether followed by ethylacetate (Desai et al., 1966).

Flavonoids and xanthones are also found to be present in this plant. Six phenolic acids viz: vanillic acid, syringic acid, p-hydroxy benzoic acid, protocatechuic acid, p-coumaric acid and ferulic acid were reported (Daniel, 1978), whereas seven flavonoids were also isolated from alcoholic extract and their structures were identified as apigenin, genkwanin, isovitexin, swertisin, saponarin, 5-Oglucosylswertisin and 5-Oglucosylisoswertisin (Ghosal, 1980). Methanol extract of *E. littorale* was found to contain different aminoacids like L-glutamic acid, tryptophane, alanine, serine, aspartic acid, L-proline, L-tyrosine, threonine, phenyl alanine, L-histidine monohydrochloride, methionine, iso leucine, L-

arginine monohydrochloride, DOPA, LGlycine, 2-amino butyric acid and valine (Retnam, 1988b).

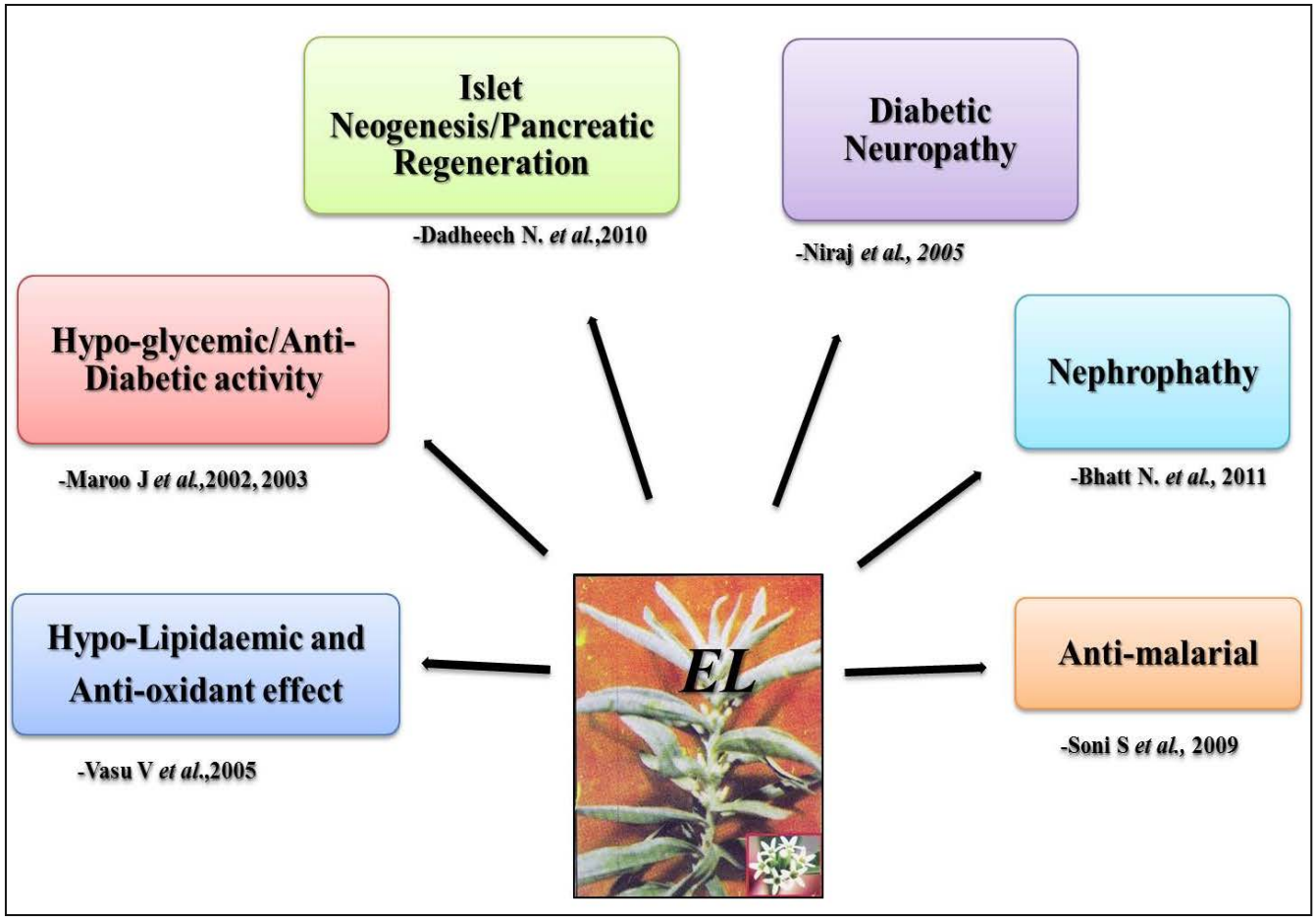


Fig-1.23 shows picture of Enicostemma littorale plant and various activities reported from our lab.

Pharmacological Activities

Hypoglycemic activity

Our lab have been published few reports which demonstrated hypoglycemic and anti-diabetic effects of *E. littorale*. A single dose of aqueous extract of *E. littorale* (15 g dry plant equivalent extract per kg) had shown significant increase in the serum insulin levels in alloxan-induced diabetic rats along with insulinotropic action in isolated rat pancreatic islets(Maroo J. et al., 2000; Maroo J. et al., 2003; Vasu et al., 2003; Vijayvargia et al., 2000). The dose dependent effect of three weeks treatment with hot and cold aqueous extract of *E. littorale* (0.5, 1 and 2 g/kg) in streptozotocin induced type 1 diabetic rats (45 mg/kg,iv single dose) showed improved normoglycemia and possess potential antidiabetic activity and improves lipid profile at a small dose of 0.5 g/kg (Vishwakarma Santosh L. et al., 2004). Gupta et al. has also reported antidiabetic effect of *E. littorale* in newly diagnosed NIDDM patients showing hypoglycemic, antioxidant and hypolipidaemic actions with aqueous extract (Vasu et al., 2003)

Hypolipidaemic activity

Aqueous extract of *E. littorale* (1.5 g/100g body weight/day) has also shown by our lab having hypolipidaemic and antioxidant effect within 6 weeks in hypercholesterolemia induced rats (Vasu et al., 2005). Rats treated in this study showed improved serum TG, LDL, HDL levels with normalized antioxidant enzyme activity. One more report from thirumalai et al states that rats treated with leaf extract of *E. littorale* showed hypolipidaemic and antioxidant effect with restoring serum and tissue triglyceride, cholesterol, and free fatty acids to normal level with antioxidant enzymes (Thirumalai T et al., 2011).

Antioxidant activity

Potent antioxidant activity was observed for all extracts of *E. littorale*. The crude powder form of the whole dried plant, prepared in form of extract showed enzymatic and non-enzymatic antioxidant activity after treatment with a decrease in activities of erythrocyte catalase, superoxide dismutase and lipid peroxidation levels and increase in reduced glutathione levels as compared to diabetic and cholesterol fed untreated rats(Maroo J. et al., 2003; Vasu et al.,

2005). The antioxidant activity of *Enicostemma littorale* showed a strong free radical scavenging activity and ferric reducing property indicating it to be a good source of natural antioxidants to prevent free. The *in-vitro* antioxidant activity of aqueous, hydro alcoholic, methanolic, chloroform and ethyl acetate extract of leaves of this plant has also been evaluated (Sharata L Derore, 2008). A significant increase in blood glucose and increased concentration of thiobarbituric acid reactive substances (TBARS) and hydroperoxides (HP) in liver, kidney and pancreas were observed in alloxan induced diabetic rats with decreased concentration of reduced glutathione (GSH) and decreased activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were also observed in diabetic rats (Thirumalai T et al., 2011)

Islet neogenic activity

Very recently stem/ progenitor differentiation activity of E. littorale has also been showed where human pancreatic carcinoma cells Panc-1 and mouse embryonic fibroblast cells NIH3T3 were converted to functional insulin producing islet like clusters (Gupta S et al., 2010).

Anti-inflammatory activity

The alcohol extract at a concentration of 300 and 600 mg kg-1bwt and its ethyl acetate fractions at 25 and 50 mg kg-1bwt showed a significant dose dependent anti-inflammatory activity in carrageen induced rat hind paw edema as well as formalin induced rat hind paw edema chronic model in rats. The study showed that the alcohol extract of *Enicostemma littorale*, and its ethyl acetate fractions, exhibited significant anti-inflammatory activity (Arivukkarasu .R, 2009).

Anti-tumor activity

The methanolic extract of *Enicostemma littorale* indirectly inhibited tumor cell growth and it was examined on the peritoneal exudates cells of the normal mice. Methanolic extract of *Enicostemma littorale* found to enhance potential cell counts. These results demonstrated the indirect effect on the cells, probably mediated through enhancement and activation of macrophages or through some cytokine product inside the peritoneal cavity produced by methanolic extract of *Enicostemma littorale* treatment (Kavimani, 2000).

Effect on diabetic complications

Neuro-protective activity

A very recent report from Niraj et al., states that rats treated extract of E. littorale showed significant reduction in nociception within 45 days treatment. A significant increase in MDA levels and reduction in antioxidant enzymes activity and improved Na-K-ATPase activity in diabetic rats was observed (Bhatt et al., 2009). In another study in-vivo antinociceptive activity of swertiamarin isolated from E. axillare was carried out using three different methods in mice. In the hot plate method, a significant increase in the latency period was observed for the treatment with swertiamarin at 100 and 200 mg/kg after 30 and 45 min. A significant increase in the tail withdrawal reflex was observed for the swertiamarin treatment (Jaishree Vaijanathappa, 2008).

Nephro-protective activity

One more study on nephroprotective effect of EL was done which showed increase in insulin sensitivity, normalizing dyslipidaemia and provides nephroprotection in diabetic rats upon el extract treatment. Also the diabetic rats treated with plant extract for 45 days showed reduction in blood glucose levels (Niraj Mukundray Bhatt, 2009).

Hepato-protective activity

Swertiamarin isolated from *Enicostemma axillare* possesses significant antioxidant and hepatoprotective properties against D-GalN induced hepatotoxicity given at 100 and 200 mg/kg body weight orally for 8 days, which might be due to its *in vitro* antioxidant activity. (Jaishree Vaijanathappa, 2008). The present investigation indicate the ethanolic extract of *E. littorale*, extract exhibited significant hepatomodulation against oxidative stress induced liver injury by CCl4 in rats through antioxidant potential and free radical scavenging activities along with reduction of fat metabolism (Gupta R.S. and Singh, 2007).

Cardio-protective activity

Protective effects of E. littorale Blume (EL) extract on hypertension and insulin resistance along with its associated cardiovascular complications in high fructose

(HF) fed rats was also observed. The study showed that HF-fed rats treated with EL showed improved insulin resistance, along with reduced hypertriglyceridemia, hypertension, platelet aggregability, blood coagulation, serum enzymes (CK-MB, SGOT, LDH and SGPT), and vascular reactivity (Bhatt et al.). Another recent property of swertiamarin as a potent lipid lowering agent and comparable to atorvastatin which may contribute to its cardioprotective and antiatherosclerotic role (Hitesh Vaidya and Goyal, 2009).

Anti-microbial

The *in-vitro* antimicrobial activity of aqueous, hydro alcoholic, methanolic, chloroform and ethyl acetate extract of leaves of this plant has been evaluated. The antimicrobial activity against *Bacillus subtilis, Staphyloccous aureus, Escheichia coli, Shigella sonni, Pseudomaonas aeruginosa, Proteus vulgaris, Aspergillus niger and Candida albicans* by well diffusion method. It was observed that chloroform, ethyl acetate and hydrochloric extract showed prominent antimicrobial against all microorganisms (Sharata L Derore, 2008).

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Chapter-2

Aims

&

Objectives

2.1 Aim and Rational for study:

Stem cells open up new avenues for the novel therapies for treating various diseases. Studies of embryonic stem cell greatly fuel the incredible use but they are still clouded with controversial ethical issues. However adult stem cells solve this obstacle and provide better option to develop as novel therapeutic. Strategies in diabetes therapy including transplanting genetically engineered β-cells and the use of β-cell progenitors has its own problems due to slow turn-over of β cell and insufficient amount required for transplantation at therapeutic level. Therefore today research is focused on increasing islet mass from pancreatic progenitors using chemical or biological differentiating agents. Although isolating pancreatic progenitor and supplying them to the verge of demand has yet not achieved so far. So now a day's use of adult stem cells other than pancreas provides the light to solve this hurdle.

Plants with medicinal properties are gift of nature to mankind, providing effective, safe and better therapy for various diseases including diabetes. *Enicostemma littorale* Blume belonging to family Gentianaceae is being used by rural folk. Studies carried out on *E. littorale (EL)* in our lab had earlier demonstrated hypoglycemic and anti-oxidant effect of aqueous and methanolic extract in dose dependent manner with no alteration in toxicity parameters at therapeutic dose. Also few indications of pancreatic regeneration have been predicted from previous work which suggests its importance in islet neogenesis as a differentiating agent. Hence, in the present study we made an attempt to explore various tissue specific adult stem cells for screening compounds isolated from EL as potent islet differentiating agent and use these newly generated islets for therapeutic in diabetic animal model. Thus we proposed following objective for the thesis.

2.2. Objectives

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

2.2.2 Differentiation of Mouse embryonic fibroblast (NIH3T3) 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.

2.2.3 Differentiation of Mouse Bone Marrow Mesenchymal Stem cells into insulin producing cells using potential bioactive ingredient Swertisin (SGL-1) from *Enicostemma littorale* Blume.

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

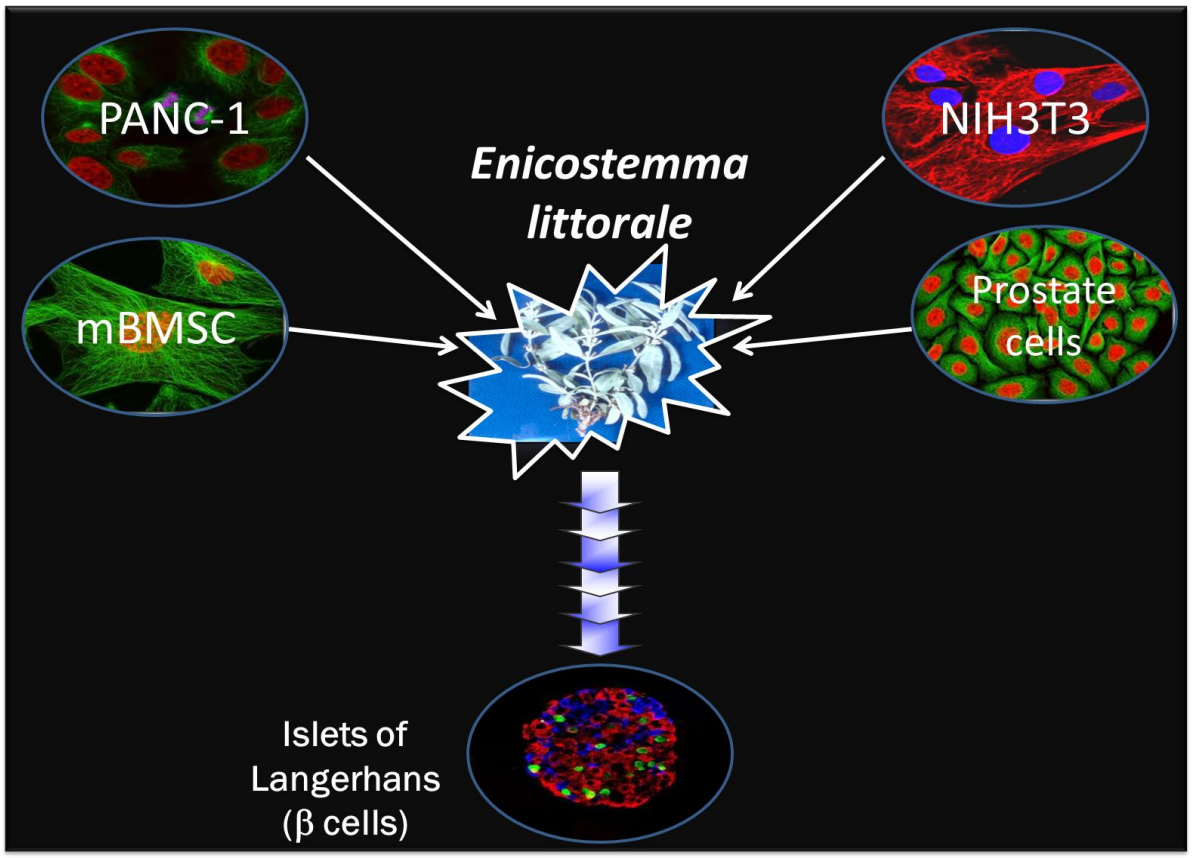
(b).Endogenous *in-vivo* differentiation of mBMSC cells into insulin positive cells.

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

Chapter 3

Title

"*In-vitro* Differentiation potential of Methanolic Extract of *Enicostemma littorale* Blume for Islet Neogenesis from Extra Pancreatic Stem/ Progenitor Cell Systems"



Enicostemma Littorale: A new therapeutic target for islet neogenesis

Short Communication

International Journal of Integrative Biology
A journal for biology beyond borders

IJIB
ISSN 0973-8363

***Enicostemma Littorale*: A new therapeutic target for islet neogenesis**

Sarita Gupta [1,*], Nidheesh Dadheech [1], Anubha Singh [1], Sanket Soni [1], R R Bhonde [2]

[1] *Molecular Endocrinology and Stem Cell Research Lab, Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Gujarat, India*
[2] *Stempeutics Research Pvt. Ltd, Bangalore, Karnataka, India*

Submitted: 18 Jan. 2010; Accepted: 16 Feb. 2010

Publications: Gupta, S., **Dadheech, N.,** Singh, A., Soni, S. and Bhonde, R.R. (2010). Enicostemma littorale: a new therapeutic target for islet Neogenesis. IJIB, 9(1), 49-53.

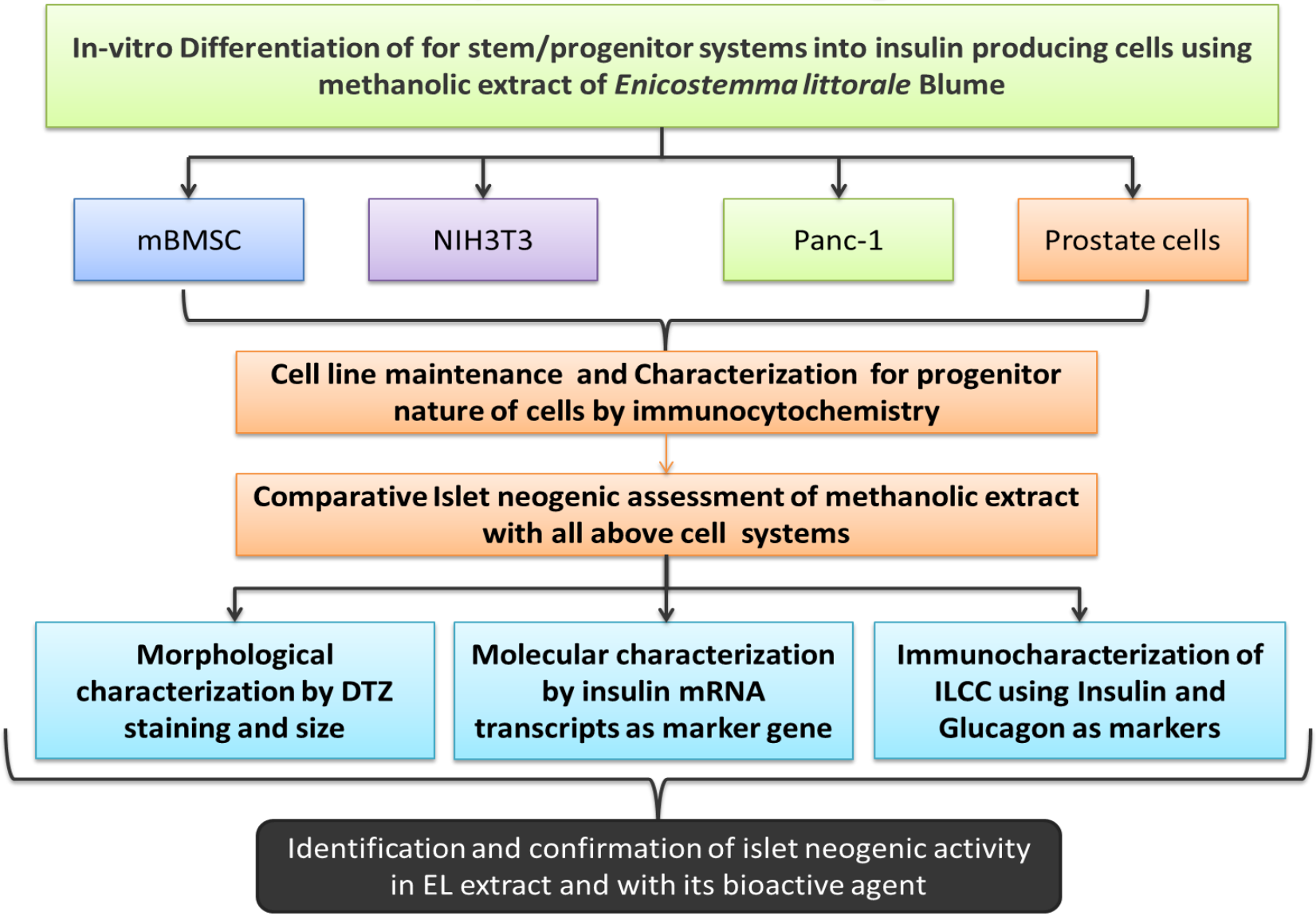
3.1 Introduction

Diabetes mellitus is a metabolic disorder that affects millions of people and number of patients suffering from diabetes continues to increase all over the world. Both type 1 and type 2 diabetes results from an inadequate mass of functioning beta cells. The prevalence of both type 1 and type 2 diabetes mellitus is increasing throughout the world along with the ensuing morbidity and early mortality because of premature microvascular and macrovascular disease. Type-I diabetes is a consequence of self-destruction of insulin producing pancreatic β cells which creates a chaos state for β-cells, for which there is presently no cure. Clinical trials indicate that, in some instances, control of blood glucose can be restored by transplantation of cadaveric derived islets [Korsgen O *et al.,* 2005]. Stem cells both embryonic and adult open new vistas in islet therapy. Recently researchers look for fibroblast-like cells emerging from islets in culture due to gradual loss of endocrine cells as an alternative and potential source of beta cells [Scharfmann R *et al.,* 2008; Schmied BM *et al.,* 2001; Zulewski H *et al.,* 2001]. Proper lineage, nature and origin of these fibroblast-like cells are still a matter of conflict. Various groups now started characterizing these cells as endocrine precursors [Dorisetty RK *et al.,* 2008; Zulewski H *et al.,* 2001; Lars S and Helena E, 2002] but not many evidences have been generated so far to value them as islet progenitor cells. In fact queries still persist about their origin and whether these cells have proliferative potencies or not. These cell based therapies may eventually provide new rays of hopes for a curative treatment for diabetes. The availability of this treatment option is limited due to (1) dearth of cadaveric islet donors [Holland AM *et al.,* 2009], (2) generation of new islets from stem cell pool, and (3) availability of islet neogenic or differentiating agents [You-Qing Zhang *et al.,* 2005]. However, hurdles of islet transplantation can be significantly improved. (Medical practitioners have started using islet transplantation therapy to treat diabetes in year 2000 [Bretzel RG *et al.,* 2004].) Stem cell pool is prodigious target that is being exploited to generate islets for clinical intention. Various differentiating agents for this rationale are now being rummage around. Biological growth factor like KGF, FGF, GLP-1, and betacellulin [Abraham EL *et al.,* 2002; Katdare M.R. *et al.,* 2004; Meenal Banerjee *et al.,* 2005] and chemical

agents like Nicotinamide, Activin-A, Exendin-4 [Chandra V *et al.,* 2009; Meenal Banerjee and Bhonde R.R, 2003; Xu G *et al.,* 1999] are conveniently used by researchers. But the yield of islets after differentiation is not sufficient to overcome the verge of demand and the high cost of therapy. Hence scientists are now shifting towards the use of dramatic medicinal properties of herbal plants that may possess islet neogenic activity. This is less explored area except for a report from a Japanese group [Itaru K and Kazuo U, 2006]. One compound Conophylline isolated from plant *Ervatamia microphylla* do show islet regeneration [Itaru K and Kazuo U, 2006]. Kojima's idea was that the generation of new islets was from Activin-A signaling pathway, and Conophylline may act as a ligand for Activin-A which was later proven by performing differentiation in presence of Activin-A antagonist. The antagonist blocked islet generation when incubated with Conophylline confirming the mode of differentiation [Itaru K and Kazuo U, 2006]. This is first evidence which provided some clue that herbal compounds may possess neogenic property and thus play crucial role in islet differentiation. *Enicostemma littorale* (El) is a plant which is well characterized by our lab. Antidiabetic, hypolipidaemic, and antioxidant properties of EL are very well reported by us in recent years [Jyoti Maroo *et al.,* 2003a and 20003b; Ravi Vijayvargia *et al.,* 2000; Vihas T. Vasu *et al.,* 2005]. We have also demonstrated earlier that EL has insulin secretory activity in isolated islets [Jyoti Maroo *et al.,* 2002]. Preliminary experiment with methanolic extract treated alloxan induced diabetic rats not only showed improved glucose status but also enlighted some blueprints for new islet generation histologically [Jyoti Maroo, 2003]. Considering all above results, this led us to hypothesize that there might be some potential agents in methanolic extracts of the plant which could be responsible for islet neogenic activity, apart from those responsible for antioxidant, hypoglycemic and hypolipidaemic actions, due to which we are seeing its anti-diabetic action. To test this we performed a comparative study for evaluating islet neogenic activity of methanolic extract using stem/progenitor cells from various sources.

The Experimental design of work done under this chapter is described in figure below.

Experimental Design



3.2 Materials and methods

3.2.1 Chemicals and media

All chemicals, media and antibodies used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA. Molecular biology reagents and cDNA and PCR kits were procured from Fermentas Inc USA. All plastic wares were purchased from NUNC, USA (Naperville, IL, USA).

3.2.2 Plant material

Plant material was procured from Saurastra region of Gujarat after proper identification with voucher specimen [Oza 51, 51 (a)] and kept in the herbarium of the Department of Botany, MS University of Baroda, Vadodara.

3.2.3 Methanolic Extract Preparation from EL

Whole plant material was dried and cut into small pieces. Methanolic extract from EL plant was prepared according to Maroo et al., [Jyoti Maroo, 2003]. Breifly, the plant was chopped into small pieces and soaked with petroleum ether for 12 h in a Soxhlet apparatus. Residues were again extracted with methanol for 24 h. After the extraction, methanol was recovered by distillation and remaining traces of methanol were completely removed by keeping extract at 60°C for 4 days.

3.2.4 Cell culture and Induction of Differentiation

All four different cell systems (two cell lines: PANC-1 and NIH3T3 cells and two primary culture cells: mBMSC and rPSC) were maintained and cultured in DMEM high glucose medium with 10% Fetal Bovine Serum. Cells were passaged and split into 1:1 ratio in new tissue culture flask upon reaching 90% confluency. Once confluent, the cells were then washed twice with DMEM medium and replaced by neogenic differentiating medium prepared with DMEM Ham's F-12 (1:1, 8mM Glucose) medium with no serum, containing cocktail supplements of Insulin 5mg/L, Transferrin 5ug/L and Selenite 5 ug/L (Sigma Aldrich, USA) and BSA 1.5g/L with antibiotics Penicillin 25ug/ml, Streptomycin 25ug/ml, Gentamycin 25ug/ml, Oxy-tetracyclin 25ug/ml and Amphotericin B 25ug/ml. The differentiation was carried out with control serum free media (SFM),

positive control keratinocyte growth factor (KGF) (10ng/ml), EL methanolic extract (15ug/ml). The medium was changed every alternate day till 6 days. Finally on 8th day cells were observed for islet like cell clusters (ILCC) in phase contrast inverted microscope (Nikon TE200, Japan) and confirmed with Dithizone (DTZ) staining.

3.2.5 Morphometric analysis

To carry out primary screening for ILCC generation and morphological examination, ILCC were resuspended in 1ml PBS and was stained with 10ul of DTZ and brick red or crimson red stained islet like clusters were counted in bright field inverted microscope in three independent experiments.

3.2.6 Molecular characterization

ILCC were spun at 1500rpm (Eppendorf 5415R) and total RNA was isolated using TRizol (Sigma Aldrich). 1µg RNA was reverse transcribed using Mulv Reverse transcriptase, oligo dT, dNTPs, and ribolock inhibitor using manufacturer's instructions (Fermentas). Gene specific forward and reverse primer for insulin was used to amplify the desired cDNA product at 56^0C annealing temperature. Amplified product was confirmed on 15% polyacrylamide gel.

3.2.7 Immuno-characterization

ILCC were fixed on glass coverslip with DMEM and 10% FBS for 30 min. Upon attachment on cover slip clusters were immediately fixed with 3.7% paraformaldehyde for immunocytochemistry. Fixed cells were incubated for 1 hour at room temperature in blocking solution (1% BSA and 1% horse serum in PBS). After incubation clusters were stained with anti-Insulin and C-peptide primary antibodies (dilutions used were 1:100, 1:4000 and 1:100 respectively) overnight at 4^0C. Coverslips were then washed for three times with washing buffer and further incubated with anti-mouse FITC (1:400), anti-guinea pig alexafluor-688 conjugated secondary antibodies for 1 hour at room temperature and finally stained with DAPI and mounted with DABCO and glycerol mountant.

3.3 Results

3.3.1 Cell line maintenance and immunocytochemistry

For islet neogenic evaluation, we had chosen four different cell types- Panc-1 and NIH3T3 as cell lines and two primary culture cell systems i.e. Mouse Bone Marrow Mesenchymal Stem Cells (mBMSC) and Rat Prostate Stromal Cells (rPSC). All cell systems were screened for ILCC formation using methanolic extract of EL along with KGF as positive control. A Panc-1 cell show origin from human pancreatic ductal carcinoma, apart from this is has also been observed to possess stem/ progenitor like property. Therefore Panc-1 system provided us pancreatic precursor population for islet differentiation screening. The other cell system like NIH3T3- a dermal fibroblast cells, mBMSC- potential stem cell source and rPSC- a representative of adult progenitor cells provided cells of extra-pancreatic origin. When observed all cell types microscopically, panc-1 showed typically epithelial cell morphology with tight junctions and no intracellular spaces. It also showed positive staining for pan-cytokeratin (CK)-19. While all other cell types showed elongated fibroblastic morphology with intense positive vimentin staining (Fig 3.1).

3.3.2 Islet neogenic assessment of methanolic extract:

For investigating islet like cluster formation activity of El methanolic extract, all the four cell types were allowed to differentiate with differentiating medium alone (SFM), positive control-KGF and methanolic extract (fig 3.2a). A four stage protocol for islet formation provided reasonably good ILCC generation with methanolic extract suggesting its islet differentiation potency. An evident zone of activation for cluster formation started by day 2 (stage-2 in Fig 3.2b), followed by mature clustering and tight aggregation of cells. Differentiated clusters showed intense crimson/brick red colored DTZ staining with mature islet architecture (Fig 3.2a and b).

Efficient and rapid ILCC formation by methanolic extract similar to KGF (positive control) suggests presence of some strong differentiating agent which is facilitating ILCC generation and enforcing stem/progenitors to differentiate into insulin positive cells.

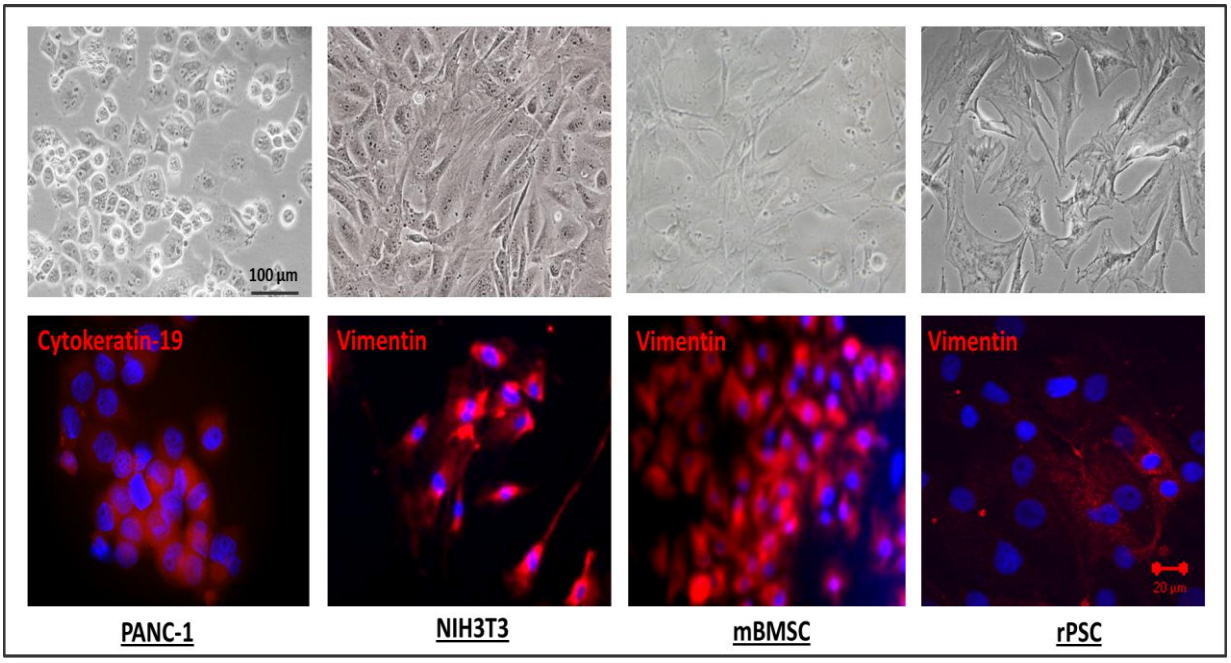


Figure 3.1 show phase contrast/ bright field images of PANC-1, NIH3T3 cell lines, mBMSC and rPS cells during differentiation. Undifferentiated Panc-1 cells depicts pan-cytokeratin-19 staining (red color) while NIH3T3, mBMSC and rPS cells do show deep vimentin staining (red color) in normal culture conditions.

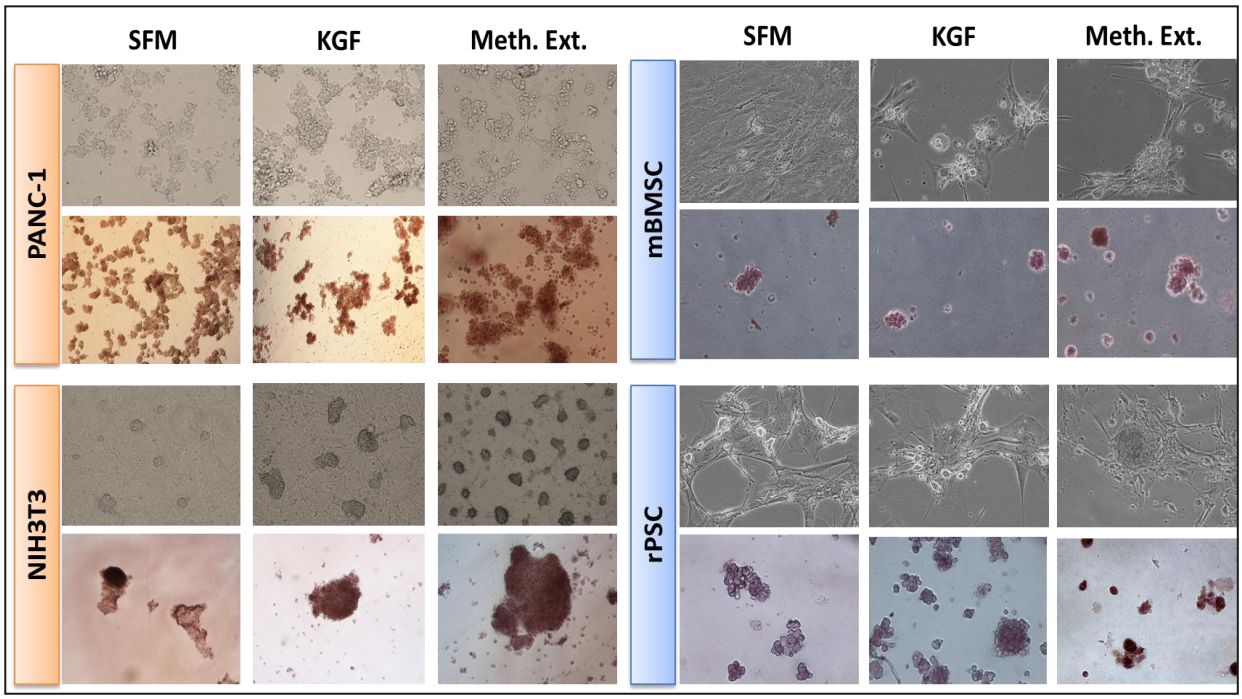


Figure 3.2a shows differentiation of PANC-1, NIH3T3, mBMSC and rPS cells upon induction with SFM; KGF and methanolic extract. Upper picture show cluster formation (phase contrast) and lower show DTZ staining.

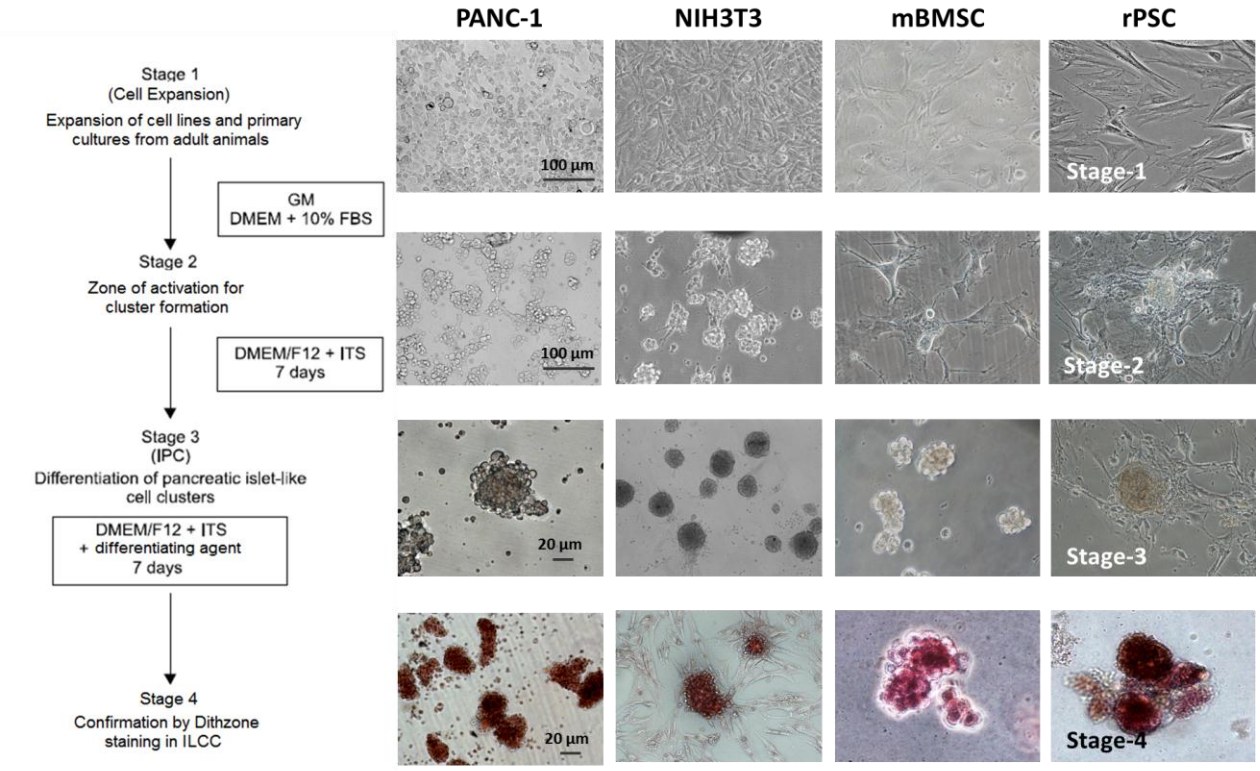


Figure 3.2b shows four stage differentiation protocol for islet cluster generation from PANC-1, NIH3T3, mBMSC and rPS cells upon induction with methanolic extract.

3.3.3 Molecular characterization of generated ILCC

We then examined insulin expression at mRNA level in newly differentiated clusters from all four stem/progenitor cell sources. Comparative assessment of insulin gene expression with control SFM, KGF (positive control) and methanolic extract showed insulin biogenesis in all ILCC with EL methanolic extract induction from all four cell systems. Panc-1 being a pancreatic progenitor showed insulin mRNA in control cells as well, while it was not been observed in NIH3T3 ILCC cDNA.

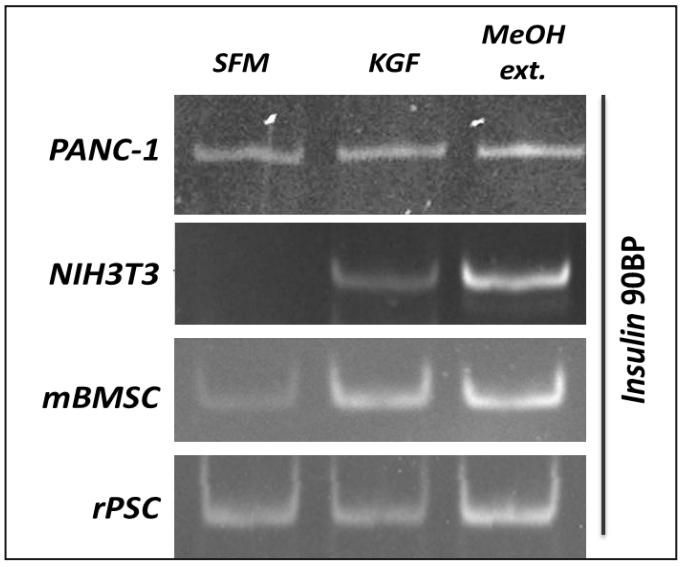


Figure 3.3 shows insulin gene expression by semi-quantitative RT-PCR in SFM control, KGF (positive control, and methanolic extract in ILCC generated with all four cell systems over 8 day induction period.

Due to presence of nestin and mesodermal in origin mBMSC could show little insulin expression and same is case with rPSC which are endocrine in nature and also highly nestin positive.

3.3.4 Immuno characterization of generated ILCC

We further examined the generated ILCC from each cell type for the presence of islet hormones esp. insulin and glucagon. Insulin biogenesis is the most significant and gold standard evidence to show differentiation with methanolic extract induction. Clusters generated from human pancreatic progenitor - Panc-1 cells showed intense insulin staining. Few glucagon positive cells at periphery also supported mature islet like cluster formation (Figure 3.4a).

Mouse embryonic fibroblast NIH3T3 cells of non-pancreatic origin upon differentiation with EL methanolic extract also revealed similar features. ILCC with these cells show insulin and glucagon staining in newly generated aggregates with sufficient 8^{th} day induction (Figure-3.4b). Presence of insulin in whole cluster surmounted with few glucagon positive cells scattered confirmed rodent islet morphology, unlike human islets where glucagon cells are present only at periphery (as seen with PANC-1 cells).

It is indeed more important to further evaluate potential of EL extract for differentiation of primary cultures adult stem/progenitor cells like mBMSC and rPSC. When analyzed, we observed that methanolic extract was equally potent to differentiate adult primary cultured stem/progenitor cells

Both mBMSC and rPSC showed presence of insulin and glucagon in newly generated clusters (Figure 3.4c and d). The potency to differentiate adult stem cells isolated from animals further signified and strongly indicated the presence of some crucial and most potent agent which is differentiating cell of multiple lineages more evenly without changing much of medium condition similar to KGF a known differentiating agent.

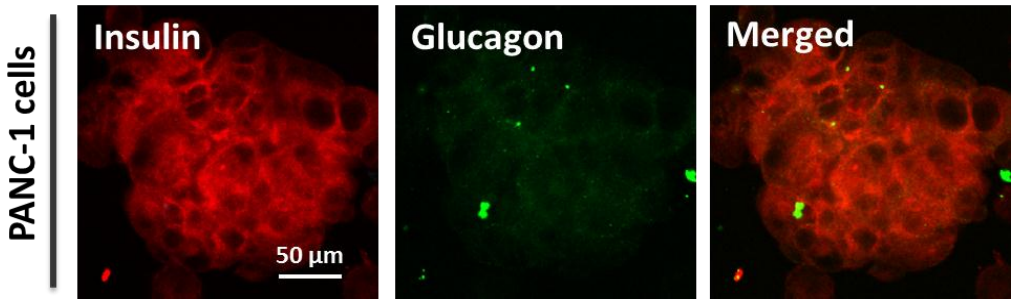


Figure 3.4a represents insulin (red) and glucagon (green) staining in human pancreatic progenitor- PANC-1 cells generated ILCC on 8th day upon methanolic extract induction.

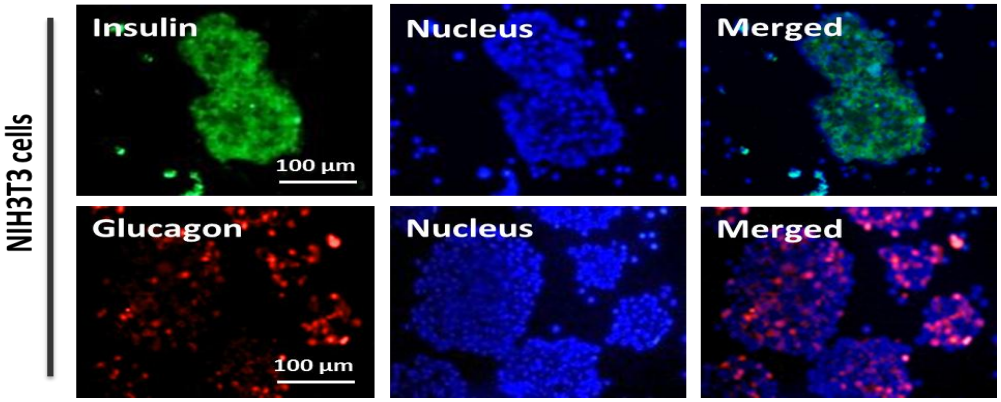


Figure 3.4b represents insulin (green) and glucagon (red) staining in mouse embryonic fibroblast NIH3T3 cells generated ILCC on 8th day upon methanolic extract induction.

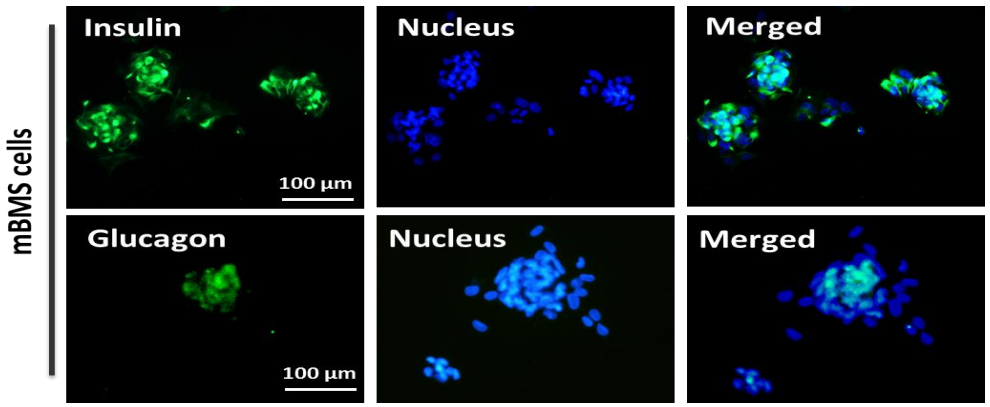


Figure 3.4c represents insulin (green) and glucagon (green) staining in mouse bone marrow mesenchymal stem cells generated ILCC on 8th day upon methanolic extract induction.

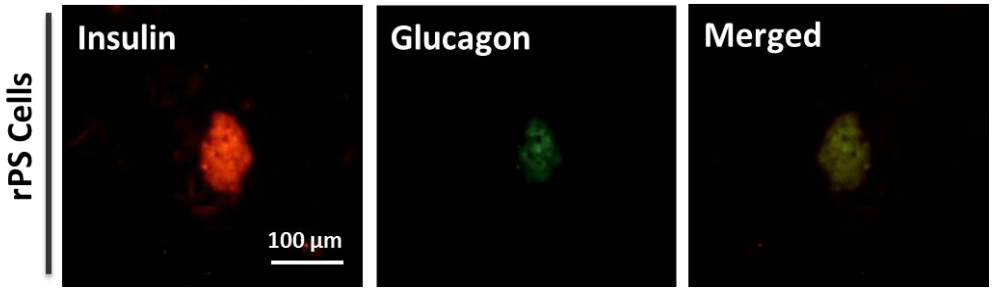


Figure 3.4d represents insulin (red) and glucagon (green) staining in rat prostatic mesenchymal stem cells generated ILCC on 8th day upon methanolic extract induction.

3.4 Discussion

The present investigation relates to an *in vitro* method to identify agents capable of inducing islet neogenesis. Besides, subcutaneous insulin injection, total endocrine replacement therapy in the form of whole pancreas islets transplantation has been considered a better treatment option for diabetes. In order to achieve this, in current study we tested *Enicostemma littorale* methanolic extract, a known anti-diabetic plant, for its capacity to induce islet neogenesis and supply islet required for transplantation. To achieve this we attempted to investigate cell lines like PANC-1, of pancreatic nature and NIH3T3, of extra-pancreatic nature along with mBSMC and rPSC having stem cell like property which was chosen to screen islet neogenic activity of methanolic extract of El. DTZ is an zinc chelating agent which in turn binds with insulin and therefore provides way to detect insulin presence [Chausmer, A. B., 1998; Shiroi, A., et al., 2002]. DTZ staining serves as a very preliminary method to test presence of insulin and monitor differentiation property of any compound [Banerjee M and Bhonde RR, 2005]. We observed that the ILCC generated from all sources with methanolic extract induction, were deeply stained crimson red with DTZ, showing presence of insulin (Figure 3.2). Morphometric features of these ILCC in all groups (Control, Methanolic and KGF as positive control) found similar to normal islets. It was clearly observed with the architecture of new ILCC that methanolic extract differentiate all cell types to form a tight aggregate of clusters with compact mass and 3D structure, cells formed an active zone of cluster right away by day 2 which eventually resulted in floated 3D islet of langerhans in good number.

Earlier reports have reasonably shown islet cell differentiation from many stem cell sources using various categories of differentiating agents [Wong, et al., 2011]. Insulin biogenesis by mRNA expression demonstrated new insulin synthesis from differentiated cells. In our study also, molecular characterization of ILCC by Reverse Transcriptase PCR for Insulin mRNA expression profile confirmed the expression of endocrine markers upon differentiation. Insulin expression on 8^{th} day was found in PANC-1, NIH3T3, mBMSc and rPSC differentiated ILCC respectively with methanolic extract similar to KGF. The

results were in accordance with many groups showing insulin transcript after differentiation [Gershengorn et al., 2007; Lie et al., 2007; Ghoneim et. al., 2011].

Immuno-positivity of ILCC for islet hormones proved them differentiated. Prominent insulin staining was seen in whole clusters whereas stain for few glucagon positive cells was also observed either scattered or at periphery. This characterization implies that newly generated islets are more or less identical to that of normal islets [Banerjee M and Bhonde RR, 2005].

Hence methanolic extract from *Enicostemma littorale* does contain certain factors/ agents which could be of potential capacity for differentiation of stem/ progenitor cells of both pancreatic and extra-pancreatic lineages into insulin producing cells or islets. This requires a new venture to screen, isolate and identify the most potent compound in methanolic extract that enhance huge islet mass with mature architecture.

4.5 References

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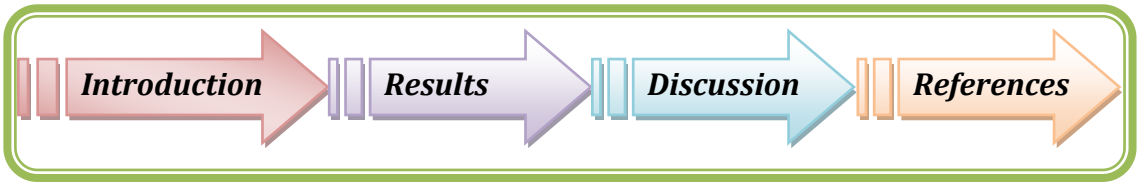
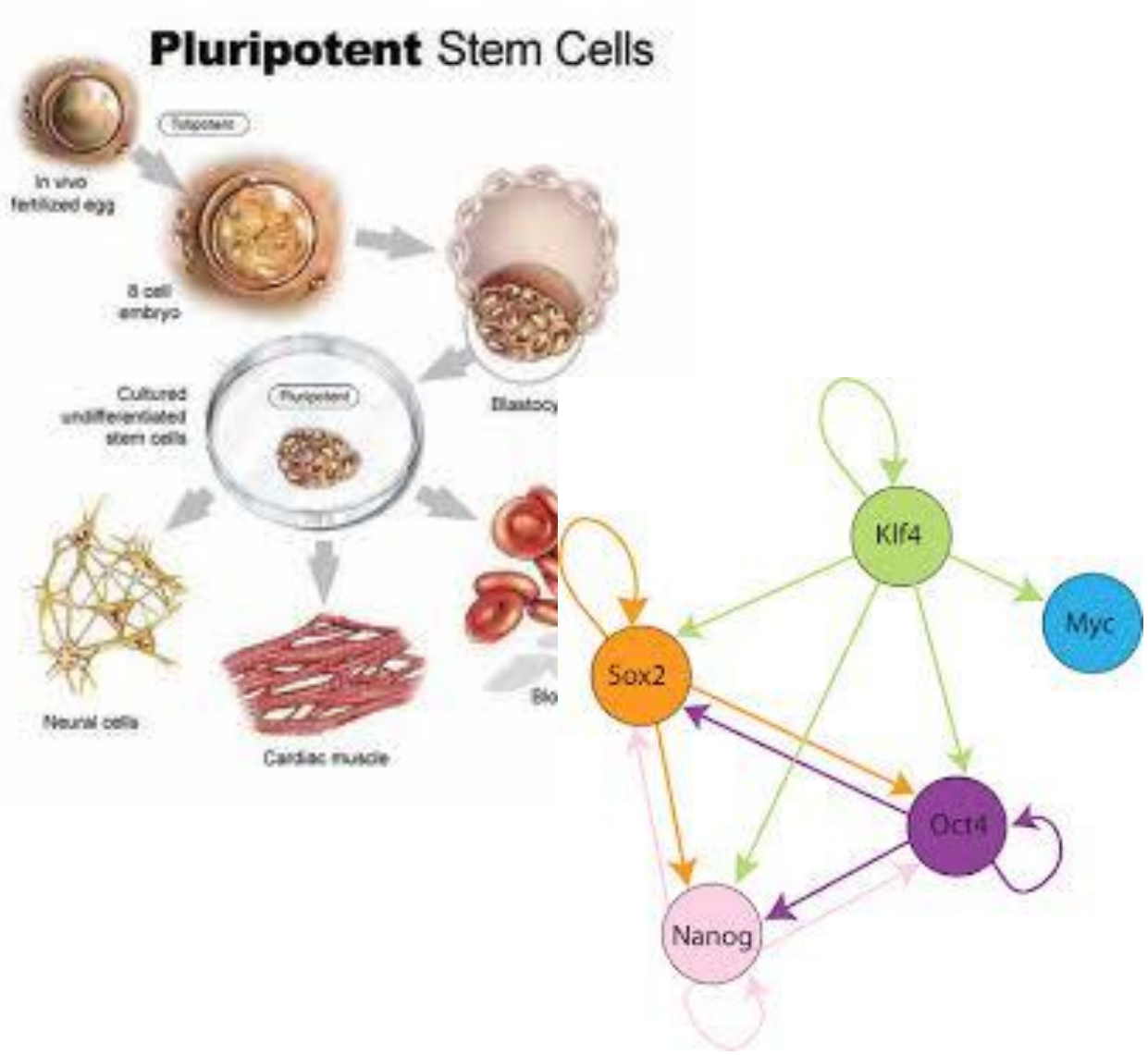
Chapter 4

Title

"Differentiation of Mouse embryonic fibroblast (NIH3T3) cells into insulin producing cells from extract/fractions/purified compounds isolated from *Enicostemma littorale*"

Part-A:- Characterization of NIH3T3 cells for stem/progenitor cell properties.





4a.1 Introduction:

Ability to isolate and establish pluripotent or multipotent stem/precursor population is a major objective for the biotechnological industry and clinical translation of regenerative medicine. A major impediment for using stem cells in clinical setup is poor availability of cells, especially those obtained with non-invasive procedures without raising much ethical issues. These limitations greatly restrict the usage of stem cells in clinics disabling treatments of many degenerative diseases. This lacuna can be filled if any tissue specific cells can be verifiably demonstrated to possess pluripotent or multipotent capacity. This may elevate the hope to find a well suited stem cell-like cell line which can serve as autologous, noncontroversial and renewable source for the cell therapy without ethical and immunological concerns, usually associated with ES cells.

Numerous gene and protein expression criteria have been set for recognizing a cell as pluripotent. Microarray analyses have demonstrated a set of various transcripts that are associated with stemness, as in case of ES cells (Conrad et al., 2008). Notably, it has been demonstrated by Yamanaka et.al. that the combinations of four major transcription factors, *Oct-3/4, Nanog, Sox-2,* and *Klf-4* is indispensable for the cells to maintain pluripotency as in case of ES cells (Riekstina et al., 2009; Takahashi and Yamanaka, 2006). However, there are some other reports which indicate that expressions of not all the four genes are essential to maintain the stemness. According to NIH and ISSCR guidelines teratoma formation is one of the major criteria for classifying a cell to be pluripotent (Hyun et al., 2008). Apart from this, presence of *alkaline phosphatase* is another reliable property shown for pluripotent cells like ES cells (Conrad et al., 2008; Li et al., 2003).

In principle a cell which is able to differentiate into cell types of all three germinal layers, is considered to be pluripotent. On the other hand, a multipotent cell can give rise to cells originating from the same germ layer (Gardner, 2002). A classic example of stem cell with pluripotent and multipotent potential is mesenchymal stem cells (MSC). MSCs can differentiate into various cell types. Depending upon isolation procedure and tissue source, both pluripotent or

multipotent type of stem cells can be isolated (Medici et al., 2010). Mesenchymal cells display fibroblastic cell morphology and express vimentin protein as a characteristic marker along with high ONS expression (Phinney and Prockop, 2007). BMSC isolated from crude bone marrow are reported to possess pluripotent gene expression and also show tri-lineage differentiation with efficient teratoma formation (D'Ippolito et al., 2004; Jiang et al., 2002; Mezey et al., 2003; Zhu et al., 2010). Therefore it appears that BMSC can serve as a model to screen various cell types for their degree of stemness. Hence a cell which portrays specific features that is in accordance to above mentioned parameters can be designated as pluripotent cells.

Wang et al. in very recent report exemplify that ONS genes are the core regulators of pluripotency. This group showed that *Oct4* regulates and interacts with the BMP4 pathway to specify four developmental fates. High levels of *Oct4* enable self-renewal in the absence of BMP4; *Nanog* represses embryonic ectoderm differentiation but has little effect on other lineages whereas *Sox-2* is redundant and represses mesendoderm differentiation. Thus, instead of being panrepressors of differentiation, each factor controls specific cell fates (Wang et al., 2012). Numerous cell types isolated from tissues and organs like heart, kidney, liver, lungs, brain, pancreas, spleen, muscles, adipose tissue, dental pulp, placenta, and amnion are now being tested on these above parameters with an aim to generate patient specific pluripotent stem cell lines for treating various incurable degenerative diseases.

With numerous published reports, it becomes a general belief that pluripotency, stemness and differentiation potential into trigerminal lineages are associated with high levels of ONS genes, but this does not explain the fact that many low ONS expressing cells can also demonstrate multilineage differentiation potential, high ALP staining and teratoma like structure formation and hence generate valid questions. (1) What should be the threshold of ONS gene expression for any cell to be pluripotent? (2) Whether pluripotency is really a function of high ONS gene expression or basal level expressing cells can also show lower but sustained pluripotency? Attempts are made in the present study to answer these questions

scientifically using NIH3T3 as a model and compared with high ONS expressing cells.

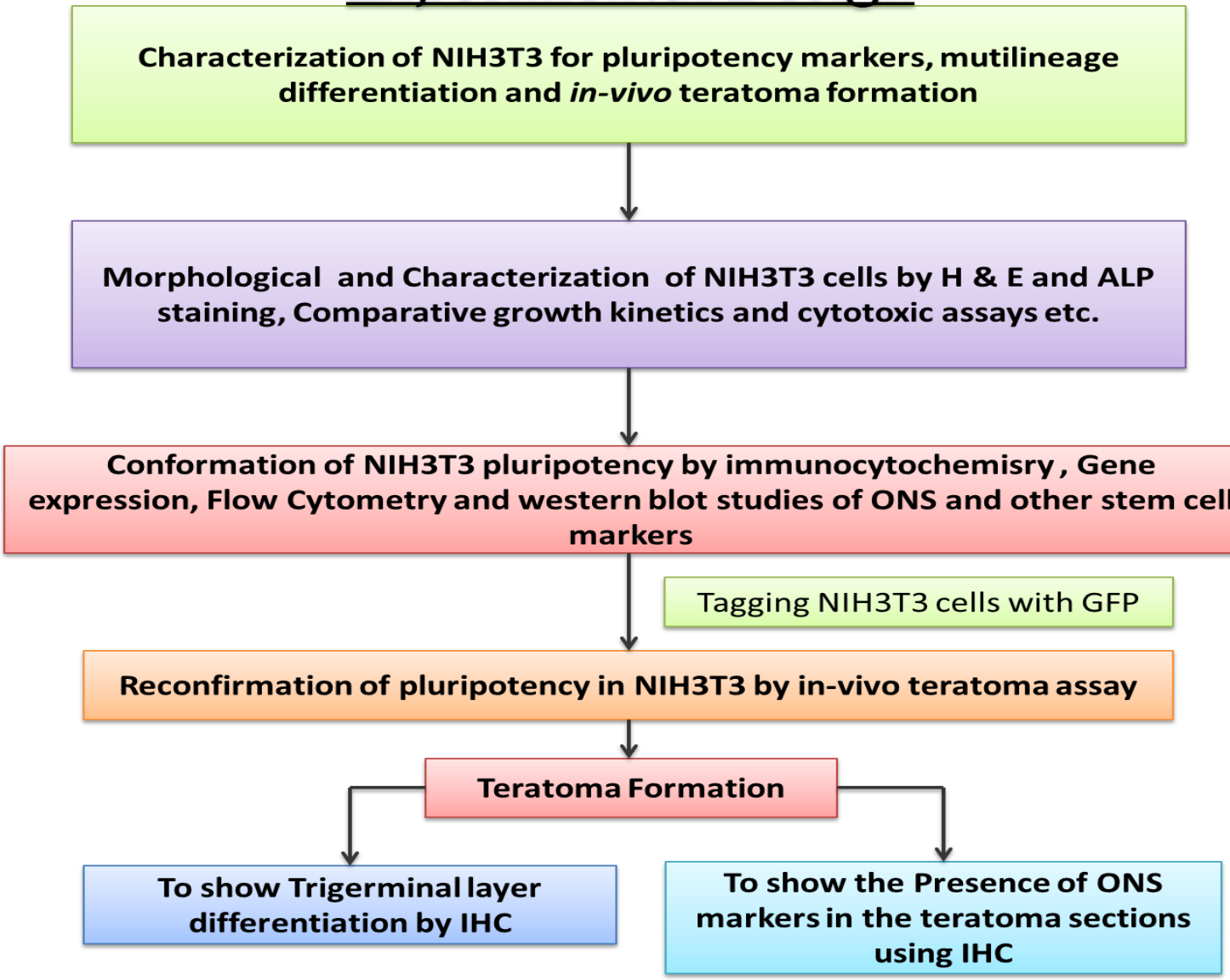
NIH3T3 cells, isolated from Swiss albino mice (Todaro and Green, 1963) are not known as stem cells due to the reduced expression of pluripotency associated genes despite being embryonic in nature. These cells have been extensively used as a model system for various molecular, cellular and toxicological studies (De Groene et al., 1995; Dhar et al., 2010; Patki, 2011; Yu et al., 2007) and are considered to be differentiated dermal fibroblast cells. Many groups used NIH3T3 as control cells that fail to differentiate (Russo et al., 1998; Wang et al., 2011; Zuo et al., 2001). However, their differentiation into adipocytes, myocytes and neural cell types has been reported either after transforming genetically or if differentiated under defined media conditions (Russo et al., 1998; Wang et al., 2011; Zuo et al., 2001). Piestun et al. reported that NIH3T3 cells after being transfected with *Nanog* show induced pluripotency and expressed markers specific to various differentiating cell types (Piestun et al., 2006). In 2006, Yamanaka and his group also compared pluripotent ES cells and untransformed NIH3T3 by microarray analysis and concluded that although these cells did not express pluripotency genes especially the four crucial ones i.e. *Oct3/4, Nanog, Sox-2,* and *Klf4;* they did express *c-Myc*(Takahashi and Yamanaka, 2006). However, this group neither examined nor commented on the multipotent nature of these cells. Later Win Ping Deng et al in 2008 and 2009 published two reports demonstrating differentiation of untransformed NIH3T3 cells into oesteocytes and neuronal cell types in bone and spinal cord injury mouse models (Conrad et al., 2008; Lo et al., 2009; Lo et al., 2008). Two other groups also reported that NIH3T3 can give rise to neural cells and oesteoblasts respectively (Abdallah, 2006; Wang et al., 2011). Recently our group also reported pancreatic islet cell differentiation of untransformed NIH3T3 with appropriate induction (Gupta, 2010).

To answer these questions and to understand whether pluripotency can also be associated with basal ONS gene expression, we carried out a comparative study of basal ONS expressing NIH3T3 cells with pluripotent mBMSC and mESC. We carefully examined NIH3T3 cells for pluripotent and multipotent stem cell

specific markers and compared them with those of mBMSC and mESC with respect to morphological features, expression profile of stemness related genes, ability to undergo multilineage differentiation and *in-vivo* teratoma formation.

The Experimental design of work done under this chapter is described in figure below.

Experimental Design



4a.2 Material and Methods:

4a.2.1 Animals: Male *Balb/c* mice, 3-4 weeks old, weighing around 25-30 grams, were used for transplantation and bone marrow cell isolation experiments. All animal experiments were performed in accordance with our institutional ''Ethical Committee for Animal Experiments'' and CPCSEA guidelines and regulations.

4a.2.2 Chemicals and cell culture media: All chemicals, media and antibodies used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA. Molecular biology reagents and cDNA and PCR kits were procured from Fermentas Inc. All plasticwares were purchased from Nunc Inc. (Naperville, IL, USA).

4a.2.3 Cell culture and maintenance: NIH3T3 cells were obtained from three different sources (Cell Repository, National Centre for Cell Science, Pune, ATCC procured cells from Zydus Pharmaceuticals, Ahmadabad, Gujarat and a generous gift from Dr. Girish M Shah's Lab. Universite Laval, Quebec, Canada) and were maintained in high glucose DMEM supplemented with 10% Fetal Bovine Serum. mBMSC and mES cells were also cultured in same culture conditions. Cells were split into 1:1 ratio in new tissue culture flasks upon being about 90% confluent. Culture media was changed after every two days.

4a.2.4 Cell count and Growth Curve: Fully confluent mBMSC and NIH3T3 cells were trypsinized with 0.1% Trypsin EDTA solution and counted under an inverted phase contrast microscope (Nikon TE2000, Japan). One hundred thousand cells were seeded into each well of 24-well plates for growth curve studies. Cells were eventually trypsinized and counted at different time points (0h, 24h, 48h, 72h, 96h, and 120h). Cell counts were then plotted versus time to establish the growth curve of cells. Growth curve of NIH3T3 cells was compared with that of mBMSC. Doubling time of both the cells were also determined using the algorithm ln (Nt-N0) ln (t), where Nt and N0 were number of cells at final time point and at initial seeding point respectively, and t was time period in hours for which cell counts were recorded.

4a.2.5 MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5Diphenyltetrazolium Bromide) Assay: Cell proliferation rate and effect of cytotoxic agent (DMSO) were determined by MTT assay (Roche). NIH3T3 cells were seeded into 96-well plates at a density of fifty thousand cells per well in complete medium. MTT reagent was added in culture in each well after 24 hours incubation with different concentrations of DMSO. The optical density values were analyzed 4 h after the MTT reaction using Multiskan PC (Thermo Lab), and cell survival curves were then plotted against time in culture.

4a.2.6 H & E staining: Cells were trypsinized and fixed in 4% Paraformaldehyde (PFA) in PBS solution. Histological examination was carried out by standard histological techniques. Fixed cells were stained with H&E stain and observations were recorded under a light microscope.

4a.2.7 Transfection and GFP labeling: Before carrying out *in-vivo* teratoma assay, we transfected NIH3T3 cells with GFP harboring plasmid p-eGFP-N1 (Clontech, USA), so that the cells could be differentiated from those of the host. One hundred thousand cells were transfected with 1µg p-eGFP-N1 plasmid DNA using lipofectamine 2000 (Invitrogen) as a transfecting agent in 1:3 volume ratio in 3.5 cm2 dish, according to manufacturer's protocol. Following the transfection, stably GFP expressing clones were selected by growing them in media containing G418 at 300µg/ml concentration for first 2 days and thereafter in 900µg/ml G418 for following 7 days. Clones were purified from isolated colonies using clonal discs and scaled up for subcutaneous transplant with agarose plugs in *Balb/c* mice.

4a.2.8 Teratoma formation: For each graft, approximately 0.2 million GFP expressing NIH3T3 cells, washed and resuspended in 300 µl DMEM complete medium, were injected subcutaneously into *Balb/c* mice (maintained in MSU in-house animal house facility) in 1.5% melted agarose (Ando et al., 2007; Awad et al., 2004; Chen et al., 2007). Visible tumors, after 2 weeks of transplantation, were dissected out and fixed overnight with 4% PFA solution. The tissues were then paraffin embedded, sectioned, stained with H&E, and, sections were

examined for the presence of cells representatives of all three germ layers produced by NIH3T3-eGFP cells (Byrne et al., 2009) .

4a.2.9 Alkaline phosphatase staining: Alkaline phosphatase staining of spheroids of both NIH3T3 and mBMSC origin was carried out with BCIP/NBT stain. Cell clusters were mechanically isolated and cells were dissociated with trypsin EDTA digestion for 5 min at 37^{o}C. After washing with 10% FBS and then with PBS, the cells were fixed with 2% PFA solution for 5 min and stained with NBT/BCIP (Conrad et al., 2008). 3T3L-1 cells served as controls.

4a.2.10 Immunocytochemistry: Immunocytochemical analysis was performed for various stem/progenitor markers in both cell types as previously described (Das et al., 2006; Sugano et al., 2005). Cells were seeded on glass coverslips in high glucose DMEM complete medium and allowed to adhere for 3-4 hours. Following attachment, cells were fixed with 4% PFA solution for 10 min at room temperature. After permeabilizing them with 0.1% Triton X-100 in PBS for 5 min, the cells were incubated in blocking buffer (1% bovine serum albumin and 4% FBS in PBS) for 1 hour at room temperature. Primary antibodies were added in blocking solution and coverslips were incubated overnight at 4^{o}C. A list of the antibodies used and their dilutions are given in Table-1. Cells were then washed thrice with washing buffer (1:10 dilution of blocking buffer in PBS) and incubated with secondary antibodies conjugated to FITC and TRITC flurophores (Sigma Aldrich, USA) in dark for 30 min at room temperature. For negative control cells were incubated with normal IgG. Cells were mounted with Vectashield mounting medium containing 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Inc.). Fluorescence imaging was carried out by using a Nikon TES2000 microscope. All images were analyzed with help of NIH element software supplied by Nikon Japan.

4a.2.11 RNA extraction and semi quantitative Reverse Transcriptase PCR (RT-PCR): Total RNA was isolated from cells using TRizol Reagent (Sigma Aldrich, USA) and 5µg of total RNA was reverse transcribed into first strand

cDNA and subjected to PCR amplification for various stem cell genes according to conditions mentioned in Table 2. Optimal conditions for PCR were determined by running gradient PCR performed with a range of annealing temperature from 51-60 ^{0}C. One µl of cDNA products was used to amplify genes using Fermentas 2X master mix containing 1.5 µl Taq Polymerase, 2mM dNTP, 10X Tris, glycerol reaction Buffer, 25mM MgCl2, and 20pM appropriate forward and reverse primers for each gene. PCR products were then resolved on a 1.5% Agarose gel (Sigma Aldrich, USA) and visualized with ethidium bromide staining. Images were analyzed using Alpha Imager software (UVP image analysis software systems, USA).

4a.2.12 Real time quantitative PCR: cDNA, synthesized by using Maxima First Strand Reverse Transcription System Kit (Fermentas INC, USA), was used for real time analysis (Applied- Biosystem Step OneTM Real-Time PCR Sequence detection System) of pluripotent stem cell gene expression. The primer sequences used for quantitative real-time polymerase chain reactions (qRTPCR) are listed in Table 2 (Appendices). Quantitative RT-PCRs were performed in two independent experiments in triplicates. Five µl of total reaction volume containing 2.5µl Maxima SYBR-Green Master-mix (Fermentas INC, USA), 20pM of each forward and reverse primers using 100ng cDNA (1/20th of total cDNA preparation) was used. PCR amplification programme was 2 minutes at 50^{o}C, 10 minutes at 95^{o}C, and 40 cycles of 15 seconds at 95^{o}C and 1 minute at 54-57^{o}C). All qRT-PCR results were normalized to the level of beta-actin determined in parallel reaction mixtures to correct for differences in RNA input. Fold changes by qRT-PCR gene expression was analyzed using Step OneTM software V.2.2.2 (Applied Biosystems Inc.) which led to a possible estimation of the actual fold change (downlegulation after setting mBMSC expression to base in log10). The qRT-PCR results are mean±S.E.M of RQ values vs target gene.

4a.2.13 FACS Analysis for ONS markers: Cultures of fixed mBMSC and NIH3T3 cells were examined for presence of ONS markers in their normal cell culture condition. Cells were stained in solution using a mixture of *OCT-3/4 -PE*, *Nanog* (unlabeled) and *Sox-2-APC*labelled antibodies. Unstained cell samples included

unlabeled cells, cells labeled with secondary FITC conjugated antibody alone (for *Nanog* only) and single fluorochrome labeled cells. Cells were analyzed using a FACS Aria III (BD Biosciences) 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 *OCT-3/4* and *Sox-2* labeling. Cells stained with Nanog were compared to secondary antibody control cells at FITC channel. All the calculations and statistics were calculated using FlowJo software (Treestar INC, USA).

4a.2.14 Protein extraction and Western blotting: Western blotting analysis of both NIH3T3 cells and mBMSC at two different passages was performed as previously described (Takahashi and Yamanaka, 2006). Both NIH3T3 cells and mBMSC were lysed with urea containing lysis buffer (1mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) supplemented with protease inhibitor cocktail (Fermentas INC.). Protein estimation in all samples was carried out using Bradford reagent according to manufacturer's suggestions (BIO-RAD). Cell lysates (50μg) were separated on Polyacrylamide gel using Mini-tetrapod electrophoresis system (BIO-RAD) and transferred onto nitrocellulose blotting membrane (Thermo Inc.). Blots were then incubated with blocking milk buffer (5% fat free skimmed milk with 0.1% Tween-20 in PBS). Dilutions of primary antibodies used, against various stem cell genes, is Table 5 (Appendices). Primary antibodies were added to blots and incubated overnight at 4^{o}C. Anti-rabbit and Anti-mouse IgG conjugated with HRP were used to develop the blots using Ultra-sensitive enhanced chemiluminiscence reagent (Millipore, USA) and imprinted on X-ray films.

4a.3 Results: To determine whether pluripotency is a function of ONS gene expression and if basal ONS gene expression can contribute to multilineage differentiation potential, a comparative study of NIH3T3 cells was done with pluripotent mBMSC and mESC. Stemness in NIH3T3 was monitored in cells procured from three independent sources (as mentioned earlier) after which presence of stemness related genes were screened separately with cells obtained from each individual sources at both mRNA and protein levels. In this study we

have monitored and compared growth curve, doubling time and growth kinetics of NIH3T3 cells with that of mBMSC. Presence of various proteins and gene expression profile of pluripotent, multipotent, self-renewal and differentiation related genes were also carried out. Here-in we also examined *in-vivo* differentiation potential and phenotypic features for pluripotent stem cells with specific reference to teratoma formation and trigerminal layer representatives.

4a.3.1 Morphological Studies and H&E Staining Showed Stem cell like features in NIH3T3 cells:

Both NIH3T3 and mBMSC were cultured in high glucose DMEM with 10% FBS in absence of any growth factor or growth supplements and sub-cultured when they became about 70% confluent to ensure continued availability of log phase culture conditions for both the cell types. All the characterizations for comparative studies were performed in their native and untransformed state. Morphologically NIH3T3 cells attain basic fibroblastic morphology, as explained by Todaro and Green (Todaro and Green, 1963), similar to that of fibroblastic mBMSC (Fig. 4.1a and 4.1d).

In confluent cultures NIH3T3 cells progressively formed a monolayer and got interlaced with each other, same as in case of mBMSC. Since these cells showed neoplastic behavior, we observed an absence of contact inhibition and cells continued to grow to form multiple layers when cultured for longer period of time. H&E staining of NIH3T3 cells showed rapidly dividing nuclei and cells with high chromatin content (Fig. 4.1c). All these observations are in congruence with the previous report by Todaro and Green (Todaro and Green, 1963). Comparison of morphological behavior of NIH3T3 cells with mBMSC demonstrated many similarities between the two cell types in cell shape, pattern of growth, cell contacts and proliferative nature.

Phase contrast and Hematoxylin and eosin staining

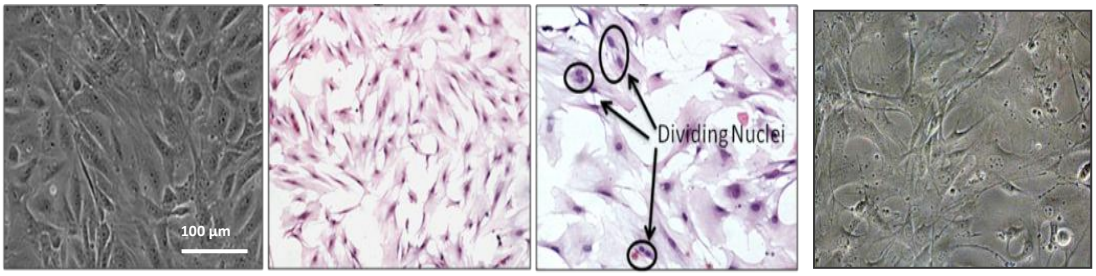


Fig 4.1a Fig 4.1b Fig 4.1c Fig 4.1d

Fig 4.1 Phase contrast and H&E stain photomicrographs of NIH3T3 cells and mBMSC: Fig. 4.1a represents cells in high glucose DMEM complete medium at 37°C and 5% CO_2. Fig. 4.1b and 4.1c represent H&E stained NIH3T3 cells at 200 X magnifications. Images represent fibroblastic cell morphology. Encircled arrow marked cells show mitotically dividing nuclei with high chromatin content. Fig. 4.1d represents Phase contrast photomicrograph of mBMSC at Passage # 10.

Alkaline Phosphatase Staining (ALP) In Clusters of mBMSC and NIH3T3, mESC and 3T3-L1.

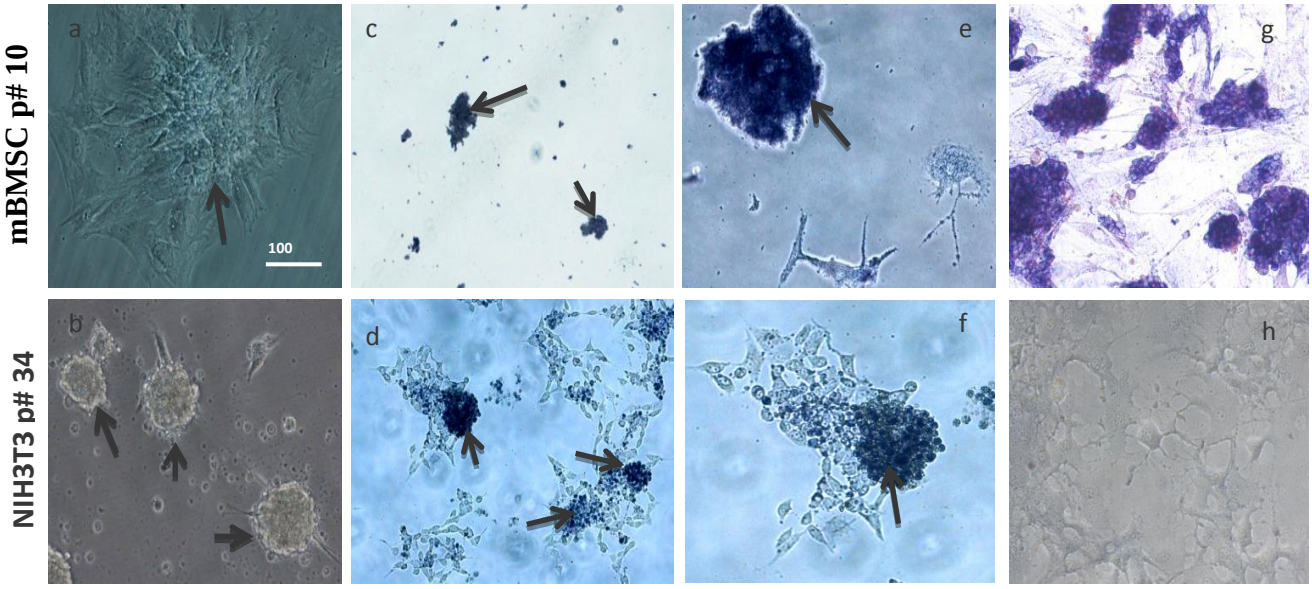


Fig 4.2 Alkaline Phosphatase staining in NIH3T3 and mBMSC: Fig. 4.2a and Fig. 4.2b represents typical spheroid formation after 8 day culture in absence of any growth factor supplement. Fig. 4.2c and Fig. 4.2d represent positive alkaline phosphatase staining in cell clusters generated in both cell types. Fig. 4.2e and Fig. 4.2f represent magnified view of clusters. Fig. 4.2g represents stained EB's from mESC and Fig. 4.2h represents 3T3-L1 cells as negative control.

4a.3.2 Spheroid body formation with Alkaline Phosphatase (ALP) staining indicated in vitro differentiation potential in NIH3T3 cells:

Next, in order to determine the *in vitro* differentiation potential of NIH3T3 cells, we carried out ALP staining of spheroid body from two cell types, as it is more important to characterize these cells based on recent ISSCR guidelines and other reports available from current literature. Spheroid bodies were generated by seeding high cell density in minimum surface area in plates without any coating with growth factors. As expected, most of the cells adhered and some of them died which were excluded from the culture upon medium replenishment. Varying sizes of small to large spheroid bodies were formed in each cell type by day 3-5 very similar to EBs formed from ES cells. Upon comparing, NIH3T3 cells showed similar features to mBMSC which are in accordance to Yamanaka and Riekstina group (Riekstina et al., 2009). The floating spheroids from both cell types were collected and stained for ALP. We noticed NIH3T3 cells formed spheroids and remained intensely positive for ALP similar to mBMSC and mESC, confirming the pluripotent nature of these cells (Riekstina et al., 2009). Normal 3T3-L1 cells served as a control which was negative for ALP (Fig. 4.2).

4a.3.3 Growth curve; Cumulative duplication number and doubling measurement of NIH3T3 demonstrated growth pattern identical to mBMSCs:

Healthy cells from sub-confluent cultures of both cell types were plated in 24-well plates and used for determining the population doubling potential, progression and proliferative activity. Cumulative population doublings were calculated by considering initial number of NIH3T3 and mBMSC cells seeded at 0 hrs and number of cells harvested at each destined time point respectively without passaging (Figure 4.3a and 4.3b). These observations provide a theoretical growth curve that is directly proportional to the cell number. With the help of the curve generated, doubling time was found to be 27.53±3.4 hrs and 21.87±2.39 hrs for NIH3T3 and mBMSC respectively, suggesting that both the cells have more or less similar population doublings time. A small but insignificant difference of 1.2 fold was found in their doubling time (Figure 4.3c),

which is in accordance with Todaro and Green (Todaro and Green, 1963). Correlation analysis curve of both cell system, from log scale graph gave a trendline that agreed with linear curve equation i.e. y=mx+c. The regression value (R^2 value) of both curves was found to be 8.9 and 8.2 respectively suggesting no significant difference in growth patterns of the two cell systems.

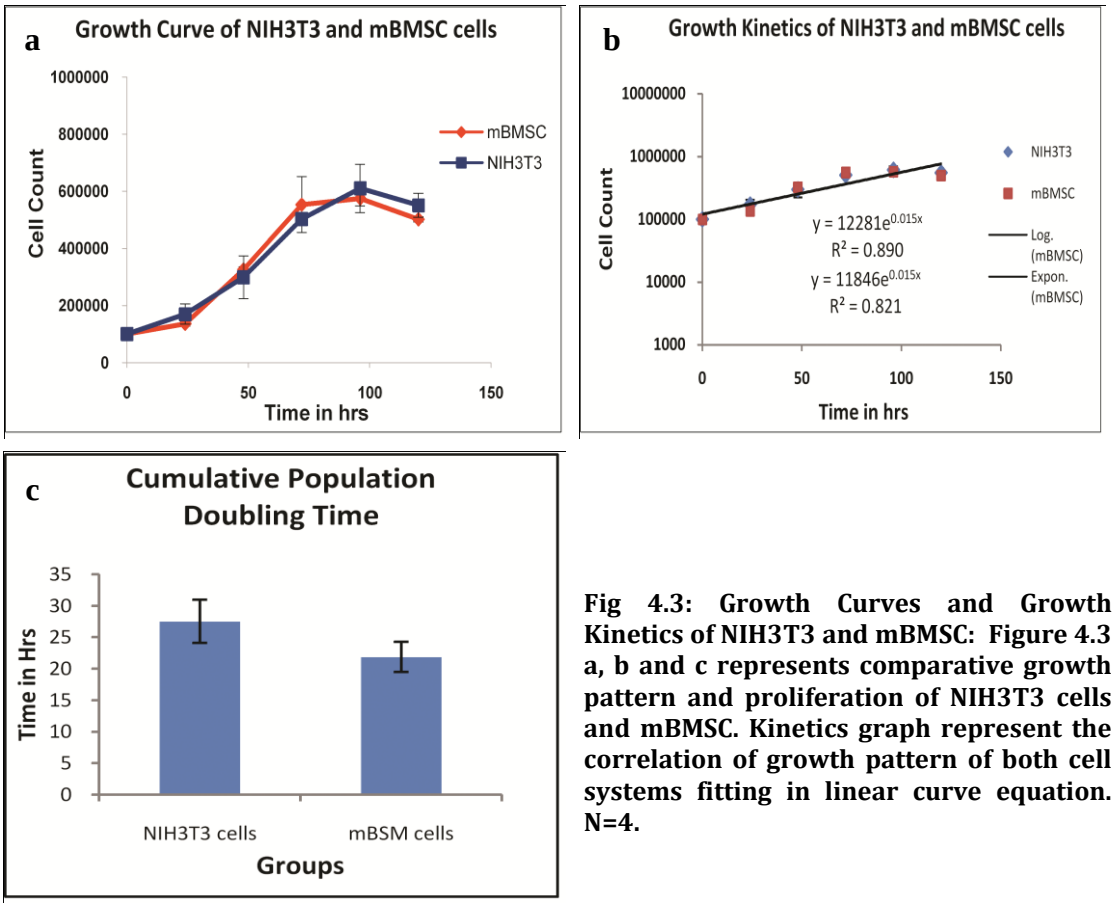


Fig 4.3: Growth Curves and Growth Kinetics of NIH3T3 and mBMSC: Figure 4.3 a, b and c represents comparative growth pattern and proliferation of NIH3T3 cells and mBMSC. Kinetics graph represent the correlation of growth pattern of both cell systems fitting in linear curve equation. N=4.

4a.3.4 Cell Proliferation and cytotoxicity assays revealed stemness behavior of NIH3T3 cells:

For determining the proliferative nature of NIH3T3 cells and their tolerance to a cytotoxic material like DMSO, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay was employed. We found that starting from 24 hours to 72 hours NIH3T3 cells proliferated aggressively with a fold change of 2.8 from 0 to 24 hour and 2.78 from 24 to 72 hours respectively (Figure 4.4a). It has been shown that cytotoxicity tolerance of DMSO is evidently much higher in stem cells rather than normal differentiated ones. It is worth

mentioning that differentiated cells can tolerate optimal DMSO concentration up to 0.5% however Peripheral Blood Mononuclear Cells (PBMC's) and MSC's are shown to tolerate DMSO even till 5% (Liseth et al., 2005; Qi et al., 2008). When carefully observed, these cells were found to have enormous tolerance for potent cytotoxic agents DMSO. Unexpectedly, NIH3T3 cells showed tolerance to DMSO even at 5% with approximately 50% viability. The cells were growing normally in media containing 5% of DMSO. Almost 20% cells even survived in very high dose (upto 10%) of DMSO (Figure 4.4b).

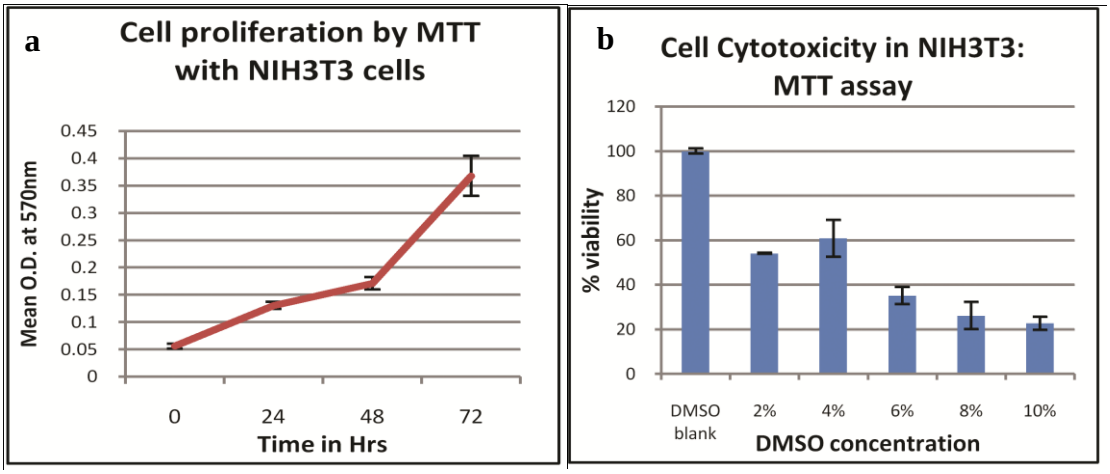


Fig 4.4: MTT cell proliferation and cytotoxicity of NIH3T3: Figure 4.4a represents proliferation of NIH3T3 cells by MTT assay and 4.4b represents percentage survival of NIH3T3 cells with varying concentration of DMSO. N=4.

4a.3.5 Immunocytochemistry confirms Pluripotency in NIH3T3 cells:

To determine whether NIH3T3 cells do express pluripotent or multipotent markers, cells were cultured on glass coverslips and immunostained after 24 hours for ES cell (ONS) and various other markers associated with stemness and progenitor state. We observed intense positive staining in both mBMSC and NIH3T3 for *Oct3/4, Nanog* and *Sox-2* similar to mESC (Fig-4.5a). Moreover we also found that NIH3T3 cells were positive for *Nestin, Vimentin, Smooth Muscle Actin (SMA), Ki-67, Fibronectin, E-cadherin* and *Pancreatic Duodenal Homeobox Gene-1 (PDX-1)* (Fig-4.5b). *Nestin* and *Vimentin* are markers of neural and MSC respectively where as *Fibronectin* and *SMA* confirm mesenchymal stem cell like features. Also, we noticed that NIH3T3 cells were strongly positive for *PDX-1* which is not only a pancreatic progenitor marker but it is also expressed by pluripotent stem cells (Riekstina et al., 2009).

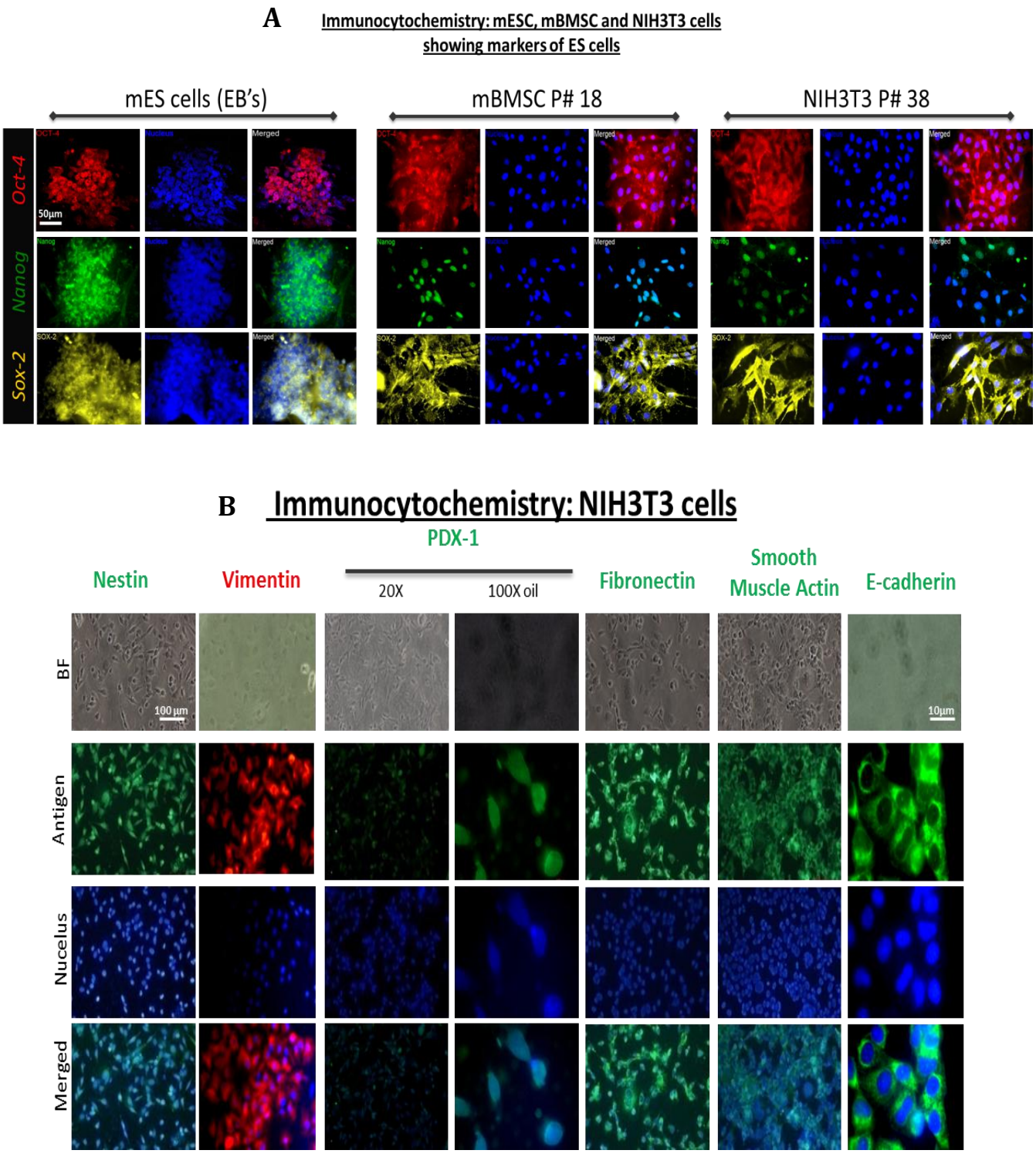


Fig 4.5 Immunocytochemistry of mESC, mBMSC and NIH3T3: Fig 4.5a represents pluripotency marker staining OCT3/4 (red), Nanog (green) and Sox-2 (yellow) in mESC, mBMSC and NIH3T3 cells respectively. Fig 4.5b represents comparative immunophenotyping of other stem cell markers in NIH3T3 cells.

4a.3.6 Gene expression analysis indicates Pluripotent nature of NIH3T3 cells:

Subsequently, when we found NIH3T3 cells positive for stemness markers by immunocytochemistry, we tested them for the presence of these pluripotent, multipotent and differentiation markers at transcript (mRNA) levels by semiquantative reverse transcriptase and Quantitative real time PCR on NIH3T3 cells procured from second source Zydus Pharmaceuticals, Ahmedabad, India. Expression profiles of all stem cell markers like *Oct3/4, Nanog, Sox-2, Nestin, Vimentin, SMA, PDX-1, and Neurogenin-3 (Ngn-3)* were carried out. mBMSC at passage number 10 were found to be positive for all *Oct3/4*, *Nanog, Sox-2* (by q-PCR), *Nestin, Vimentin, SMA, and PDX-1* and *Ngn-3* (by RT-PCR) (Fig-4.6a and 4.6b). These marker profile, determined by q-PCR, clearly demonstrated the pluripotency of mBMSC as *Oct3/4*, *Nanog* and *Sox-2* expression was upregulated in them. Expression profiles of the same markers were screened in NIH3T3 cells of passage number 15. The cells were found to be positive for *Oct3/4, Nanog, Sox-2*,*Vimentin, SMA, Nestin, PDX-1 and Ngn-3* by RT-PCR whereas contradictorily downregulation of *Oct3/4, Nanog* (100 times) and *Sox-2* (1000 times) was also observed in these cells by q-PCR when compared to mBMSC expression (Fig-4.6c). To further validate and confirm that NIH3T3 cells do retain pluripotent nature, we compared NIH3T3 with mESC by RT PCR; interestingly we found these cells to express all three ONS genes similar to mESC along with *ABCG2,* an important transporter molecule in ES cells. We also examined the expression of tri-lineage germ layer markers like *β-III tubulin, Otx-2* for Neuro-ectoderm *GATA-2, Brachyury (T)* for Mesoderm, and *GATA-4 and Pdx-1* for Endoderm in NIH3T3 aggregated clusters similar to EBs generated from mESC (Fig-4.6a).

4a.3.7 Flow cytometery analysis supports pluripotent nature of NIH3T3 cells:

FACS analysis of ONS markers in both mBMSC and NIH3T3 cells was carried out. We clearly observed that both mBMSC and NIH3T3 cells showed ONS positivity. On comparing the percentage population of mBMSC, we found 7.91% cells were positive for *Oct-3/4*, 70.87% cells for *Nanog* and 75.37% cells for *Sox-2 (Fig-4.7)*.

Likewise in case of NIH3T3, 6.13% cells were positive for *Oct-3/4*, 70% cells for *Nanog* and 7.96% cells for *Sox-2 (Fig-4.7)*.

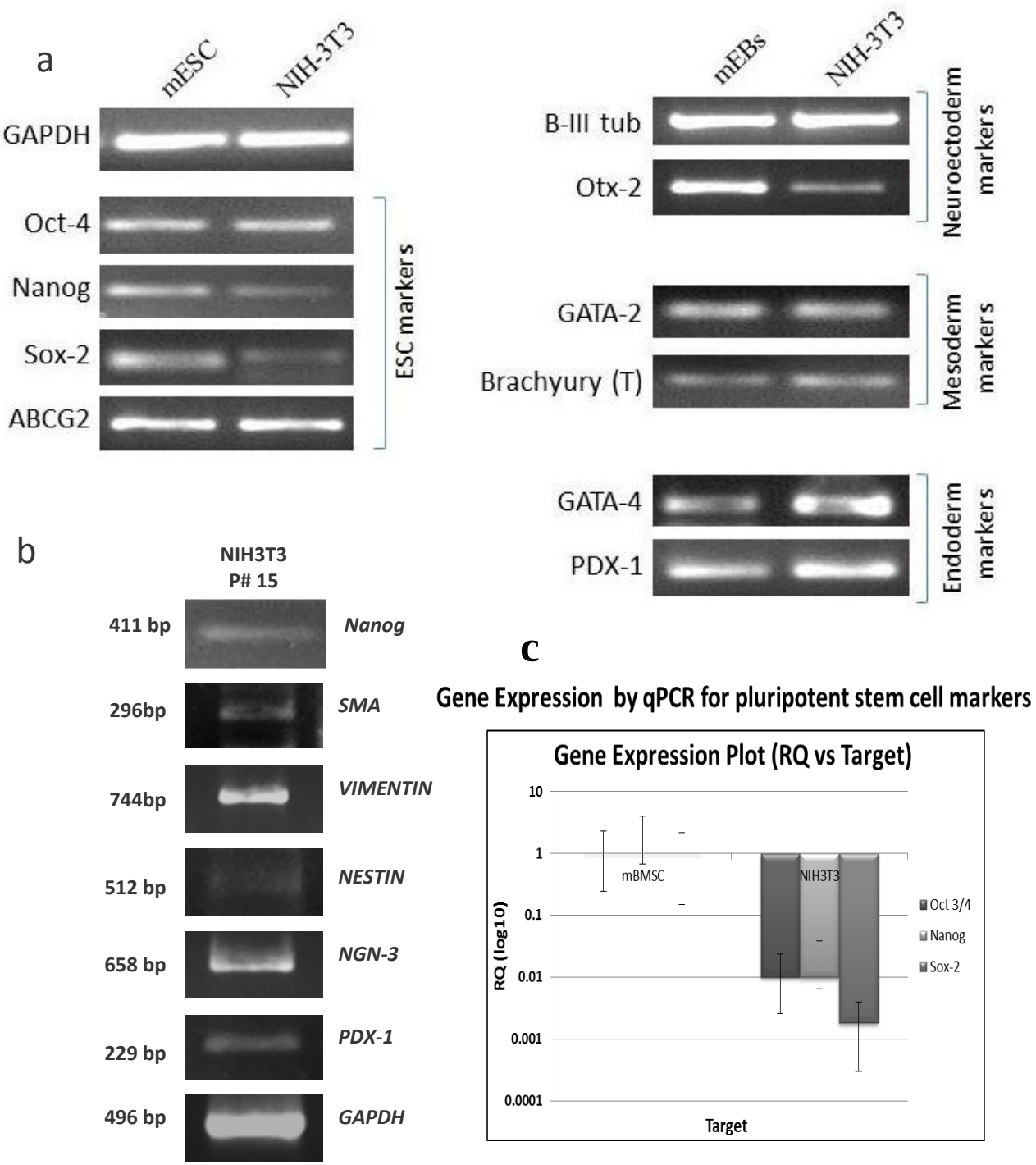


Fig 4.6 Gene Expression analysis of mESC, mBMSC and NIH3T3: Fig- 4.6a represents comparative gene expression profile of pluripotency markers and trigerminal layer markers in mESC and NIH3T3 cells. Fig-4.6b represents mRNA expression of other stem cell genes in mBMSC and NIH3T3 cells. Fig 4.6c represents comparative study of gene expression profile of 3 crucial stem cell markers- Oct3/4, Nanog and Sox-2 in NIH3T3 and mBMSC by real time PCR. Results are mean of triplicates performed independently in two sets of experiments using cells at different passage number.

FACS histograms of ONS markers in mBMSC and NIH3T3 cells

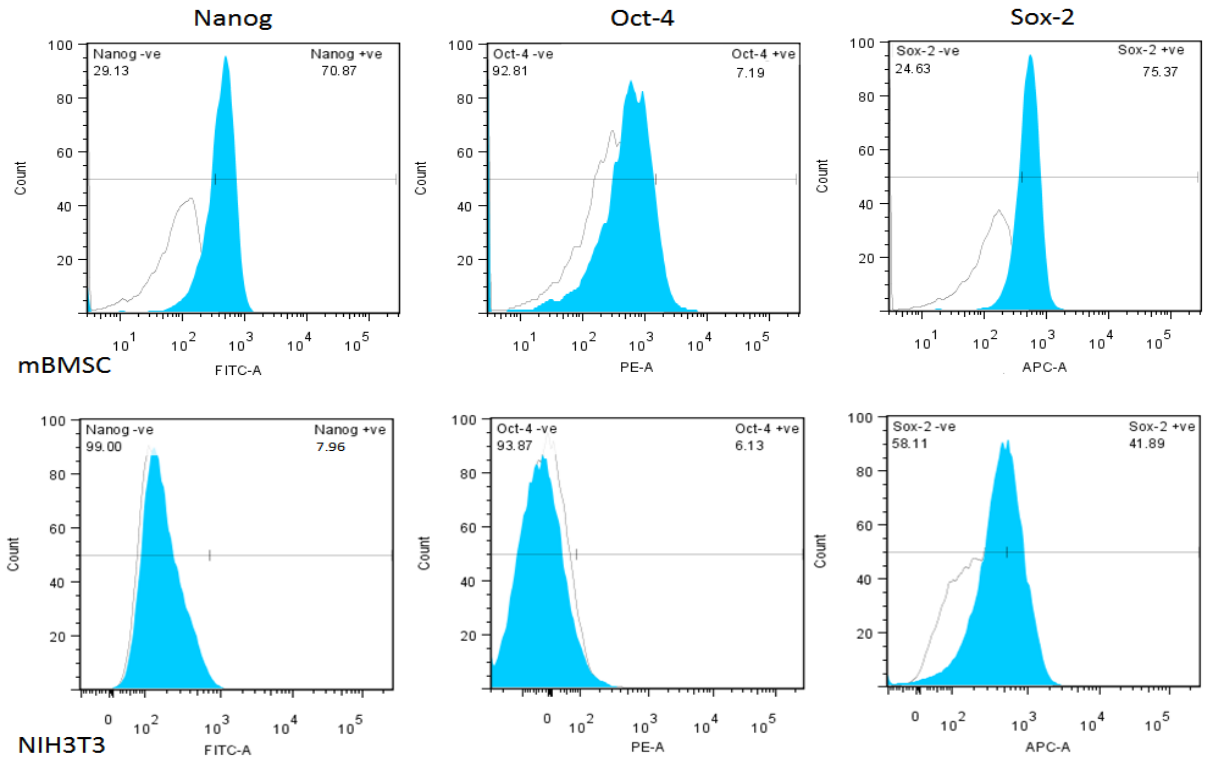


Fig 4.7 FACS histograms of ONS markers in mBMSC and NIH3T3 cells: Fig 4.7 represents comparative histograms of pluripotency marker profile from mBMSC and NIH3T3 cells.

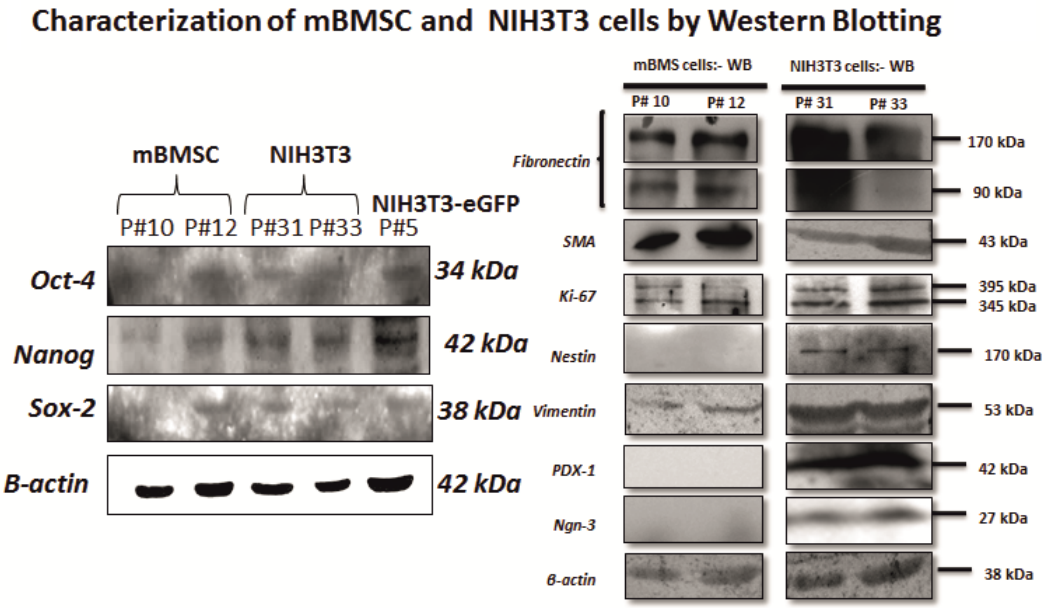


Fig 4.8: Western blotting profile of NIH3T3 and mBMSC: Fig: 4.8 represents comparative protein expression profile of pluripotency and other stem cell markers from mBMSC (p# 10 and 12) and NIH3T3 (p# 31 and 33). Figure also represents ONS expression in NIH3T3-eGFP cells post transfection at p# 5.

4a.3.8 Western blot profile validates pluripotent stem cell-like nature of NIH3T3 cells:

For confirming the presence of ONS genes at the protein level, western blot was carried out with the cell lysate of mBMSC and NIH3T3. As expected, both the cell types from two different passage numbers (P# 10 and 12 in mBMSC and P# 31 and 33 in NIH3T3; see Fig. 4.8) were positive for *Vimentin, Fibronectin, SMA, Ki-67, PDX-1* and *Ngn-3*. Both mBMSC and NIH3T3 cells showed high expression levels of both precursor and mature isoforms of Fibronectin protein which were 170 and 90kDa respectively. Level of *SMA* expression in NIH3T3, however, was a little lower than that in mBMSC where as that of *Ki67* isoforms of 395 and 365 kDa were higher in NIH3T3 cells. Further, the expression of *Nestin* was seen in NIH3T3 cells only but not in mBMSC. In fact the expression levels of *Vimentin, PDX-1* and *Ngn*-3 were also very high in NIH3T3 compared to those in mBMSC (Fig-4.8). More interestingly, we further found that mBMSC cells demonstrated increased protein expression of ONS markers from passage number 10 to 12; nonetheless NIH3T3 cells showed bands for *Oct-3/4* of 34kDa, *Nanog* of 42 kDa and *Sox-2* of 38 kDa at both 31 and 33 passage number. We also checked expression of ONS in GFP labeled NIH3T3 cells before teratoma formation during in vivo studies to ensure pluripotency. NIH3T3-eGFP cells as per our expectation showed ONS protein bands even after GFP labeling (Fig-4.8).

4a.3.9 *In-vivo* Teratoma reconfirms pluripotency in NIH3T3 cells:

To further validate the phenotypic properties of NIH3T3 cells in terms of pluripotency, *in-vivo* experiment for teratoma formation in *balb/c* mice was carried out. NIH3T3-eGFP cells in a small aliquot of medium with 1.5% warm agarose plugs were transplanted subcutaneously on left side of ten *balb/c* mice (Ando et al., 2007; Awad et al., 2004; Blum et al., 2009; Chen et al., 2007; Prokhorova et al., 2009). Right side of the same animals was used for control or placebo i.e. only agarose plugs were injected in that site. After 15 days of transplantation, mice were sacrificed after teratoma formation from NIH3T3-eGFP cells was observed in these animals. All of the 10 mice developed evident teratomas (left side) wherein skin bulges were bigger in size than that of agarose plugs alone (right side). Also we noted that teratomas formed by NIH3T3-eGFP

cells injected in agarose plugs were significantly larger (average weight of 0.999±0.1 grams and volume 2.70±0.48 mm^3) than those formed by agarose plugs alone (average weight 0.70±0.03 grams and volume 1.5±0.16 mm^3). GFP imaging of teratoma sections demonstrated that major mass of tumor plugs were mainly derived from NIH3T3-eGFP cells only and not from host dermal cells (Fig-4.10). Immunohistochemistry of tri-lineage markers clearly depicted the presence of pluripotency markers ONS in developed tumor tissues. Representative regions of slides showed co-labeling of *Oct-3/4, Nanog* and *Sox-2* with GFP staining (Fig-4.12). Histological studies confirmed the *in-vivo* functionality and representation of tri-germinal layers in developed teratomas (Fig-4.11). The infused cells were found to be confined to the transplanted areas only as all other organs in the alimentary canal were normal and no significant metastasis was observed. Since the cells were encapsulated in agarose and transplanted subcutaneously beneath the forearms and not directly in vasculature; hence the chances of spreading were minimal to begin with. Apart from this, in order to effectively validate the signatures of all three germ layers in the excised teratomas, we confirmed the markers of ectoderm, mesoderm and endoderm using immunohistochemistry with GFP co-labeling. NIH3T3-eGFP teratoma slices stained positive for *Nestin,* neurofilament markers for ectodermal germ layer, *CD44* specific marker for Mesodermal cell types and *PDX-1*, for endodermal germ cells (Fig-4.13).

4a.4 Discussion

NIH3T3 cells, till 2005, were not known to possess stemness properties. Recent studies have shown stemness in NIH3T3 as the cells are capable of differentiating into various cells types like neural cells, oesteocytes, adipocytes including insulin producing beta cells (Abdallah, 2006; Conrad et al., 2008; Gupta, 2010; Lo et al., 2009; Russo et al., 1998; Wang et al., 2011; Zuo et al., 2001). The present study is an attempt to demonstrate, whether pluripotency and stemness is associated with basal levels of ONS genes.

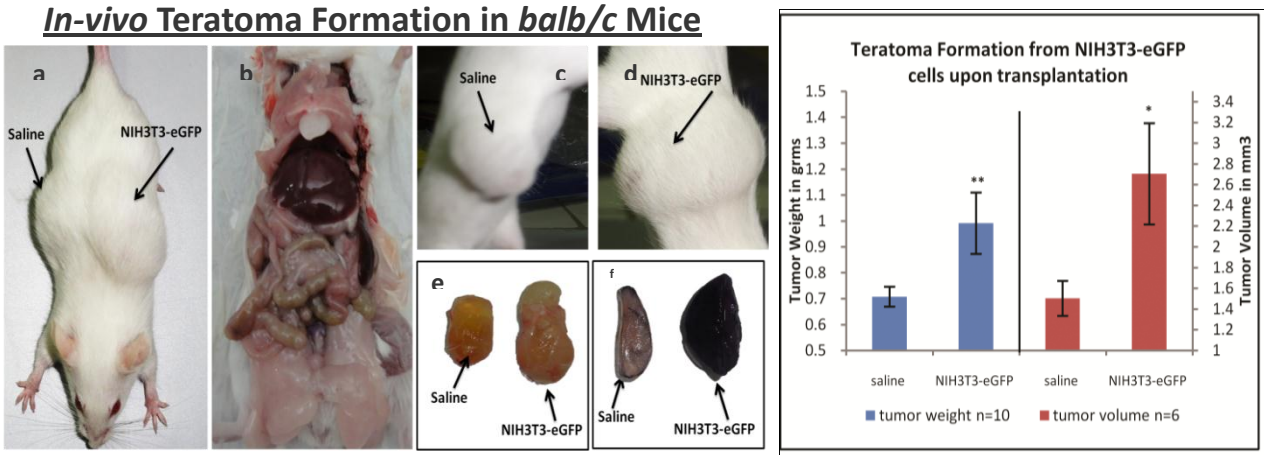


Fig 4.9 *In-vivo* study for teratoma formation in balb/c mice: Fig 4.9a shows NIH3T3-eGFP produced teratomas in balb/c mice on left side of mice while saline showed no outgrowth on right side of body. Fig. 4.9b represents that no other organ was affected as transplanted cells did not metastasize. Fig. 4.9e indicates difference in size of teratomas between transplanted cells in saline and those in agarose plugs. Fig. 4.9f represents alkaline phosphatase staining of cells transplanted in agarose plug after 15 day teratoma formation. Fig. 4.10 shows comparative graph for weight and volume of NIH3T3-eGFP induced teratoma and agarose plug alone after 15 days. Results are expressed as Mean±SEM with N=6-10 animals. * and ** represents p<0.01 and p<0.001 Vs control plugs (Saline) respectively.

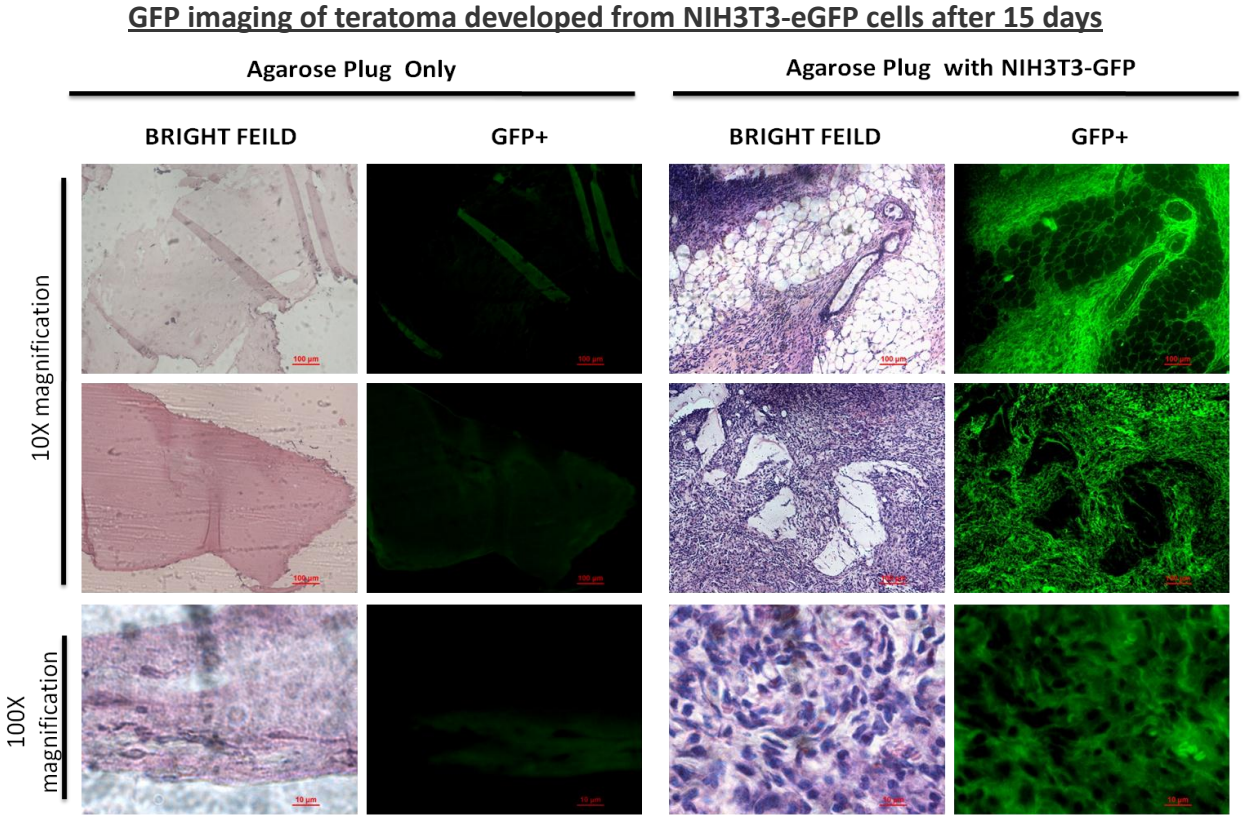


Fig 4.10 Histological GFP images of teratoma : Fig 4.10 represent histological and GFP fluorescence in panel (a) agarose plug alone and (b) NIH3T3-GFP developed teratoma. Teratoma sections of agarose plugs with NIH3T3-eGFP cells showing GFP fluorescence with representation of three germinal layers.

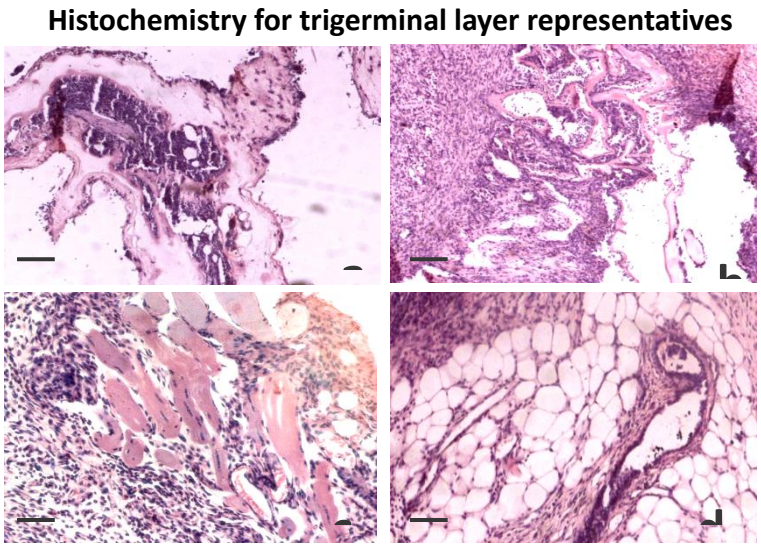


Fig 4.11 Histological images of teratoma: Fig 4.11 represent histological representatives for trigerminal lineages, panel (a) Ectoderm (b) Endoderm (c) Mesoderm (Skeleton Muscles) and (d) Mesoderm (Adipose tissue).

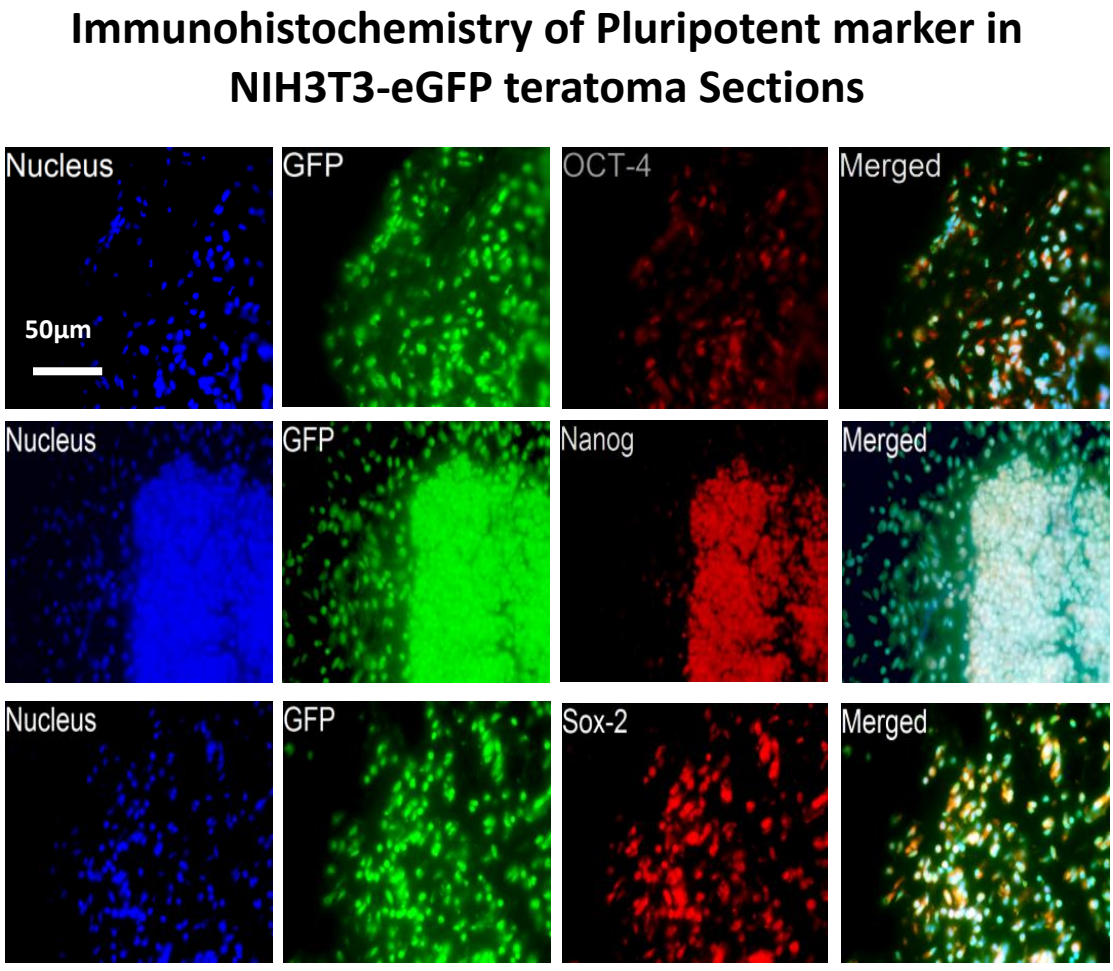


Fig 4.12 Immunohistochemistry of (ONS) NIH3T3-eGFP teratoma Sections: Fig 4.12 represents pluripotency markers OCT-3/4, Nanog and Sox-2 staining in NIH3T3-eGFP teratoma sections with GFP Co-localization. Panel represents ONS staining in red, GFP staining in green and DAPI nuclear staining in blue with merged image.

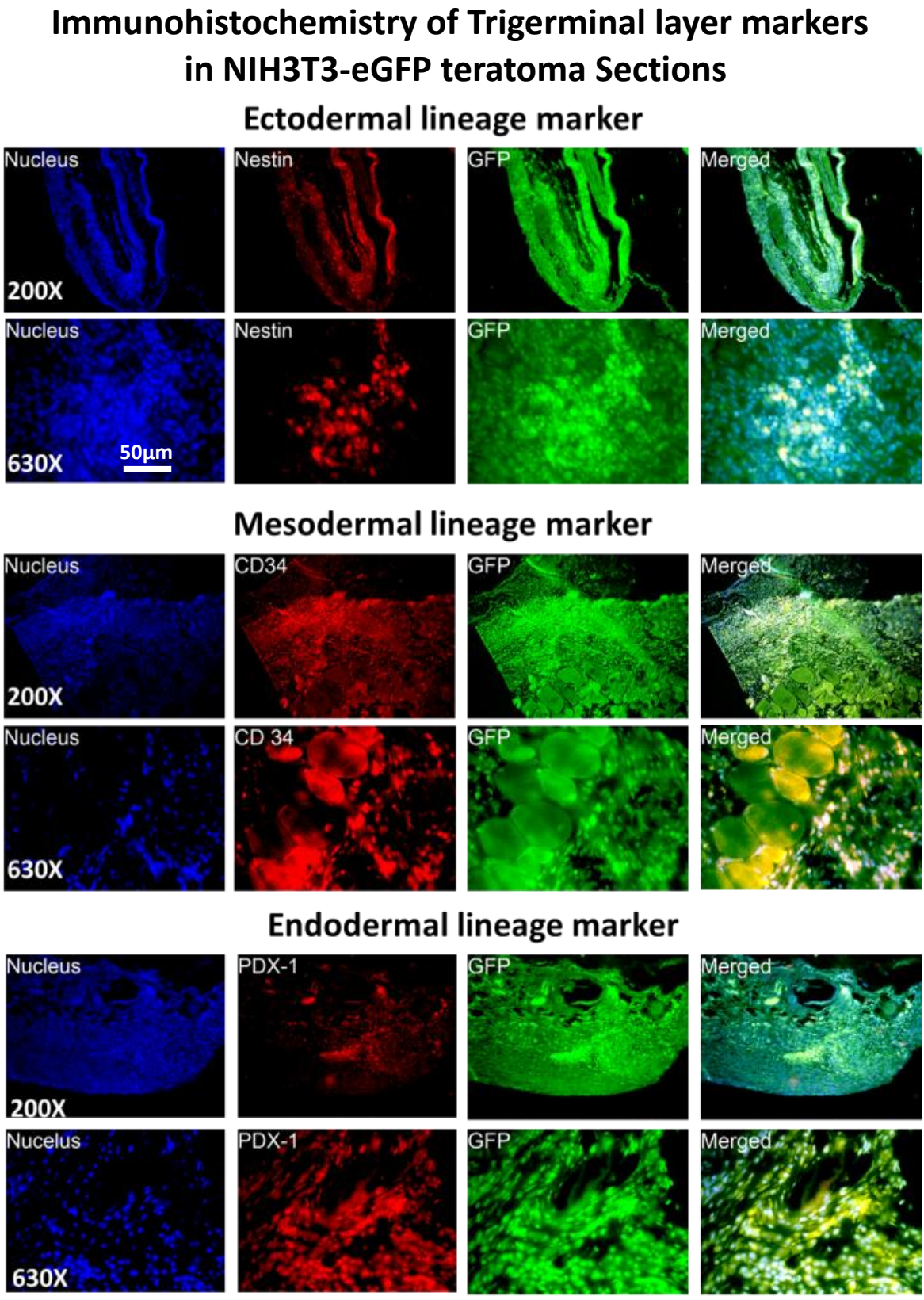


Fig 4.13 Immunohistochemistry of (Trigerminal layer) NIH3T3-eGFP teratoma Sections: Figure Shows ectodermal; Nestin (red) expression in few GFP (green) positive cell representing neuro-ectodermal cell lineage, Mesoderm; CD34 (red) positive nuclei marker of chondrocyte cells, and Endoderm; PDX-1 positive (red) nuclei depicting presence of pancreatic endodermal layer. DAPI (blue) was used for nuclear staining.

Efforts were made to unravel these facts by demonstrating characteristic features of NIH3T3 in terms of stemness by undertaking comparative analysis of NIH3T3 with mBMSC employing cellular and molecular techniques. Morphologically NIH3T3 showed more or less similar shape like fibroblastic mBMSC with the ability to survive in culture conditions for numerous passages. We also found that NIH3T3 became progressively hyperplastic and get interlaced with one over other with increase in the number of days in culture. Multiple layers of cells are produced since the cells growth is not inhibited by contact inhibition as described earlier (Todaro and Green, 1963). Histological observation also supported the proliferative rate and plasticity of NIH3T3 cells. High chromatin content and frequent appearance of dividing nuclei indicated progenitor like features which are in accordance with earlier studies done with pluripotent stem cells (Conrad et al., 2008; Kanatsu-Shinohara et al., 2004; Li et al., 2003; Lowry et al., 2008; Reiisi et al., 2010).

ISSCR guidelines suggest ALP activity as one of the critical criteria of pluripotency which has also been emphasized by Riekstina et al. (Riekstina et al., 2009). On this ground we demonstrated that NIH3T3 cells formed spheroid bodies which stained positive for ALP staining. The purple color in NIH3T3 spheroids indicated characteristic feature of embryoid bodies (EBs) derived from pluripotent ES cells reinforcing their enhanced stemness. These findings are in concurrence with those of Li and Riekstina et. al. (Li et al., 2003; Riekstina et al., 2009).

Molecular and immunological characterization is important to investigate the presence of stem cell like markers at both mRNA and protein levels. To achieve this, we characterized NIH3T3 cells from all the three different sources as mentioned earlier at gene and protein levels by RT-PCR, Q-PCR, immunocytochemistry, flow-cytometery and western blotting. Based on the immunocytochemistry results, we conclude that NIH3T3 cells obtained from the first source i.e. NCCS Pune, India at passage no-4 showed presence of all characteristic markers relevant to pluripotency. They express ES cell markers like *Oct3/4, Nanog* and *Sox-2.* NIH3T3 cells were highly positive for *Nestin*, a

neuroendocrine filament protein, expressed in pluripotent neural stem cell (Dahlstrand J et al., 1992; Lendahl U et al., 1990). It is also reported that *Nestin* is widely expressed in pluripotent stem cells from various other tissues like ear (Li et al., 2003), dermal fibroblast (Byrne et al., 2009) and pancreatic lineage cells (Ling Zhang et al., 2005). NIH3T3 cells were also found to be positive for mesenchymal lineage markers like *Vimentin, SMA,* and *Fibronectin* confirming their mesenchymal or multipotent status. Various pluripotent or multipotent MSCs are known to express *Vimentin, Fibronectin*(Conrad et al., 2008; Kawase et al., 2004) and *SMA* (Medici et al., 2010). *PDX-1*, a pancreatic progenitor marker present in pluripotent MSC (Riekstina et al., 2009), was also found to be expressed by NIH3T3 indicating their predisposition to give rise to definitive endoderm. Pluripotent stem cells are also known to have high *E-cadherin* content (Conrad et al., 2008), which represents higher self-renewal capacity and undifferentiated status. When we examined mBMSC and NIH3T3 cells, both strongly expressed *E-cadherin,* a finding in good agreement with that of Conrad and group (Conrad et al., 2008). These finding clearly suggested that NIH3T3 cells do harbor pluripotent characteristics.

We further tested NIH3T3 stemness at molecular level at P#15 obtained from the second source i.e. Zydus Pharmaceuticals, Ahmedabad, India. The idea was to demonstrate that the results and observations we had recorded so far in early passage of NIH3T3 by immunocytochemistry held true in cells of higher passage numbers obtained from another source. The presence of *OCT-3/4*, *Nanog* and *Sox-2* represented ES cell-like state as reported by Yamanaka and group (Takahashi and Yamanaka, 2006), *Nanog* is known to be responsible for self-renewal, pluripotency and for maintaining undifferentiated state of cells (Lei Lei et al., 2008). As per our observations evident by RT-PCR, NIH3T3 cells express *Oct3/4*, *Nanog* and *Sox-2* albeit in slightly lower levels thus indicating their pluripotent nature. Further NIH3T3 cells also expressed *ABCG2,* an important transporter molecule abundant in ES cells. In order to reconfirm our semi-quantitative PCR data, we decided to quantify the levels of the genes by a more sensitive technique like quantitative real-time PCR. Results showed NIH3T3 exhibited 100 fold lesser level of *Oct3/4* and *Nanog* expression, while

that of *Sox-2* was 1000 fold less than that in mBMSC. We hypothesize that NIH3T3 cells do sustain their pluripotency, through sub basal levels of *Oct3/4, Nanog* and *Sox-2 unlike ES cells*. However the presence of ONS can perhaps explain the differentiation potential of NIH3T3 cells into neural cells, oesteocytes, adipocytes as reported by Win Ping Deng group (Lo et al., 2008; Wang et al., 2010). In one of the recent reports we also demonstrated successful differentiation of NIH3T3 cells into islet like cell clusters (Gupta, 2010), further validating the pluripotent nature of these cells. Even further, flow cytometery and western blotting confirming the presence of ONS proteins in NIH3T3 cells similar to mBMSC provides additional support to the hypothesis and authenticates the fact that NIH3T3 may represent a connecting link to ES cells with respect to pluripotency.

Pluripotent cells have the ability to differentiate into cell types of all three germinal layers namely ectoderm, mesoderm and endoderm under suitable culture conditions. *In-vitro* pluripotency of cells can be assessed by their ability to form three dimensional structures called EBs or aggregates or spheroids; on the other hand *in-vivo* pluripotency can be characterized by the ability to form putative tumors when implanted into immunocompromised animals (Prokhorova et al., 2009). Noncancerous benign tumors formed by ES cells are called teratomas (Blum et al., 2009; Prokhorova et al., 2009). Our studies demonstrated interesting results in terms of spheroid formation *in-vitro* and teratoma formation *in-vivo* with NIH3T3 cells. GFP expressing NIH3T3 cells, when engrafted with agarose plugs without any growth factors, showed high incidence of teratoma formation in immune compromised mice like *balb/c*. NIH3T3eGFP cells, not only demonstrated the ability to proliferate under *in-vivo* conditions to form noncancerous cell mass, but they also stimulated angiogenesis which was evident by new blood vessel formation within 15 days. The behavior of NIH3T3 cells *in-vivo* was fairly identical to ES cell-induced teratoma formation. With immunological confirmation of three lineage markers like Nestin, CD44 and PDX-1 for ecto-, meso- and endoderm layers further verified *in-vivo* pluripotency of NIH3T3 cells. All these results demonstrate

pluripotent nature of NIH3T3 cells representing connecting link between mBMSC and mESC.

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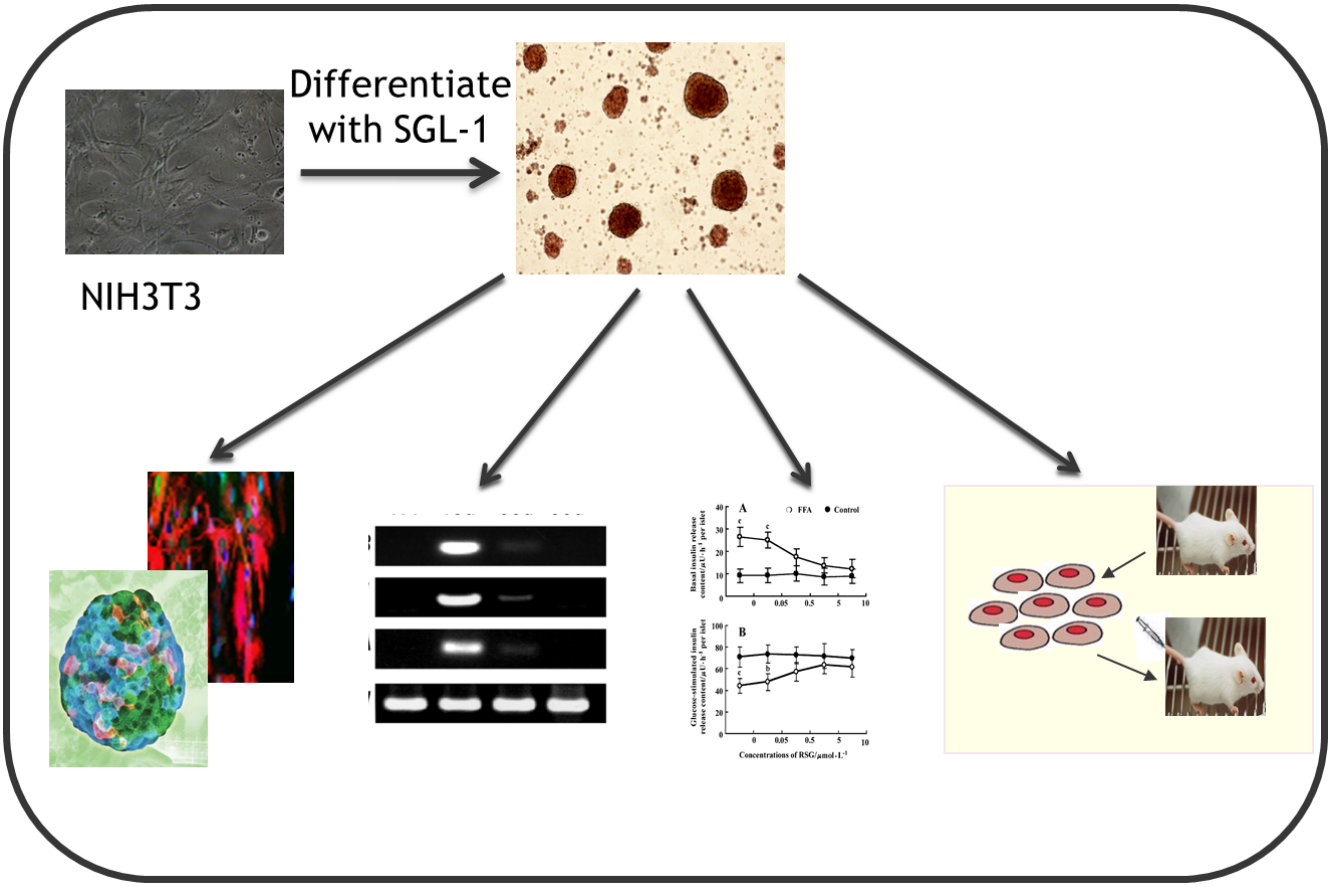
Title

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

Part-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.



4b.1 Introduction

Islet transplantation is modern approach and became more prevalent in clinics now a day for the treatment of diabetes. It offers internal glucose homeostasis with low surgery risk and reduces complications in diabetic patients. However, islets derived from multiple donors require immunosuppressors. Also inadequate islet supply from cadaveric pancreas has limited, the wide utilization of this approach (Shapiro et al., 2000).

Cell based therapy, principally new islets derived from stem cell differentiation, is new area of research in diabetes. Recent studies have shown that embryonic stem cells, induced pluripotent stem cells, adult bone marrow mesenchymal stem cells and many other tissue specific progenitors have the ability to convert into cell of multiple lineages like blood, liver, lung, skin, cardiac, muscles, neurons including insulin producing β cells upon appropriate induction (Jiang, Jahagirdar et al. 2002; Mezey, Key et al. 2003). Although few reports have been shown to generate islet mass from various pluripotent and multipotent stem cells, the progress has been hampered in increasing differentiated islet yield due to lack of potent and economical islet differentiating agent. Success in exploring various adult tissues to isolate stem cell population has been recorded for autologous transplant. However, not much work has been done for identification of potent differentiating agents that not only accelerate the rate of differentiation but produces functional cell types in large numbers. If this can be achieved, the utilization of huge islet mass seems to be feasible to withstand the shortage of autologous islet transplant in near future and prevent diabetes and its complications. In first part of this chapter it has been clearly demonstrated that NIH3T3 cell line possess basal level of pluripotency and is capable of differentiating in tri-lineage germ layers when transplanted *in vivo, s*uggesting it as ideal model system for evaluating islet differentiation and further exploring efficacy of novel differentiating agents.

There are many growth factors and differentiating agents known to promote differentiation or regeneration of pancreatic β cells (Nielsen, 1999). These include nicotinamide, glucagon-like peptide, gastrin, activin A, betacellulin, Reg

protein, INGAP and hepatocyte growth factor (HGF). Practically none of them were translated as therapeutic for islet generation and transplant in clinics, as all of them are associated with high cost and low yield of islet clusters.

Plants are exemplary sources of medicinal values, an important thing is to properly identify and screen for their miraculous properties. From a practical point of view, low molecular weight compounds are favorable for such cellular interaction studies, because such agents are not immunogenic and may be effective even when administered orally. As mentioned earlier our group has reported anti-diabetic activity of *Enicostemma littorale Blume* (E. littorale) which is a perennial herb, belonging to Gentianaceae family and distributed throughout India. Aqueous and methanolic extract of *E. littorale* demonstrated hypoglycemic, hypolipidaemic and antioxidant potential in alloxan-induced diabetic rats and newly diagnosed NIDDM patients (Vijayvargia, R. et al., 2000; Maroo, J. et al., 2002, 2003a, 2003b, Vasu, V. T., et al., 2003). The antidiabetic effect of this plant has been reported by other workers too (Murali, B. et al., 2002; Vishwakarma, S. L. et. al, 2003, Upadhyay, U. M. and Goyal, R. K. 2004; Srinivasan, M. et al., 2005). Apart from these properties, various fractions of *E. littorale* also demonstrated cyto-protective effect in isolated islets and pancreatic regeneration in both T1D and T2D animal models (Data unpublished; PhD Thesis, N Bhatt, 2010). Major chemical constitutes of the plant are Swertiamarin and gentianine (Fig 4.14) (Rai, J. and Thakar, K. A., 1966, Govindachari, T. R. et al., 1956, 1957, 1966). While, apigenin, genkwanin, isovitexin, swertisin, saponarin, and gentiocrucine (Ghosal, S. et al., 1974) are also reported to be present in minor amount.

Based on these observations a preliminary study was conducted for islet differentiation property with methanolic extract of *E.littorale* (chapter II). Further to identify potent islet differentiating agent, in present study we screened various biomolecules isolated from E. littorale and monitored for effective stem cell differentiation with NIH3T3 cells. These molecules were assessed at morphological, molecular, immunological and functional levels for confirming proper differentiated islets (*in-vitro*). The most potent compound had been identified and further characterized. ILCC generated with most potent

compound were evaluated for Glucose lowering effect after transplantation in Streptozotocin (STZ) induced diabetic *balb/c* mice.

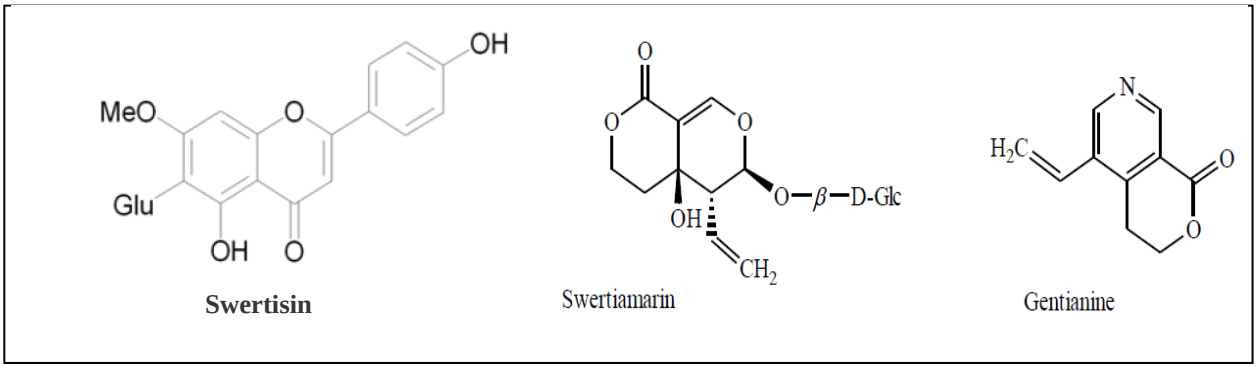
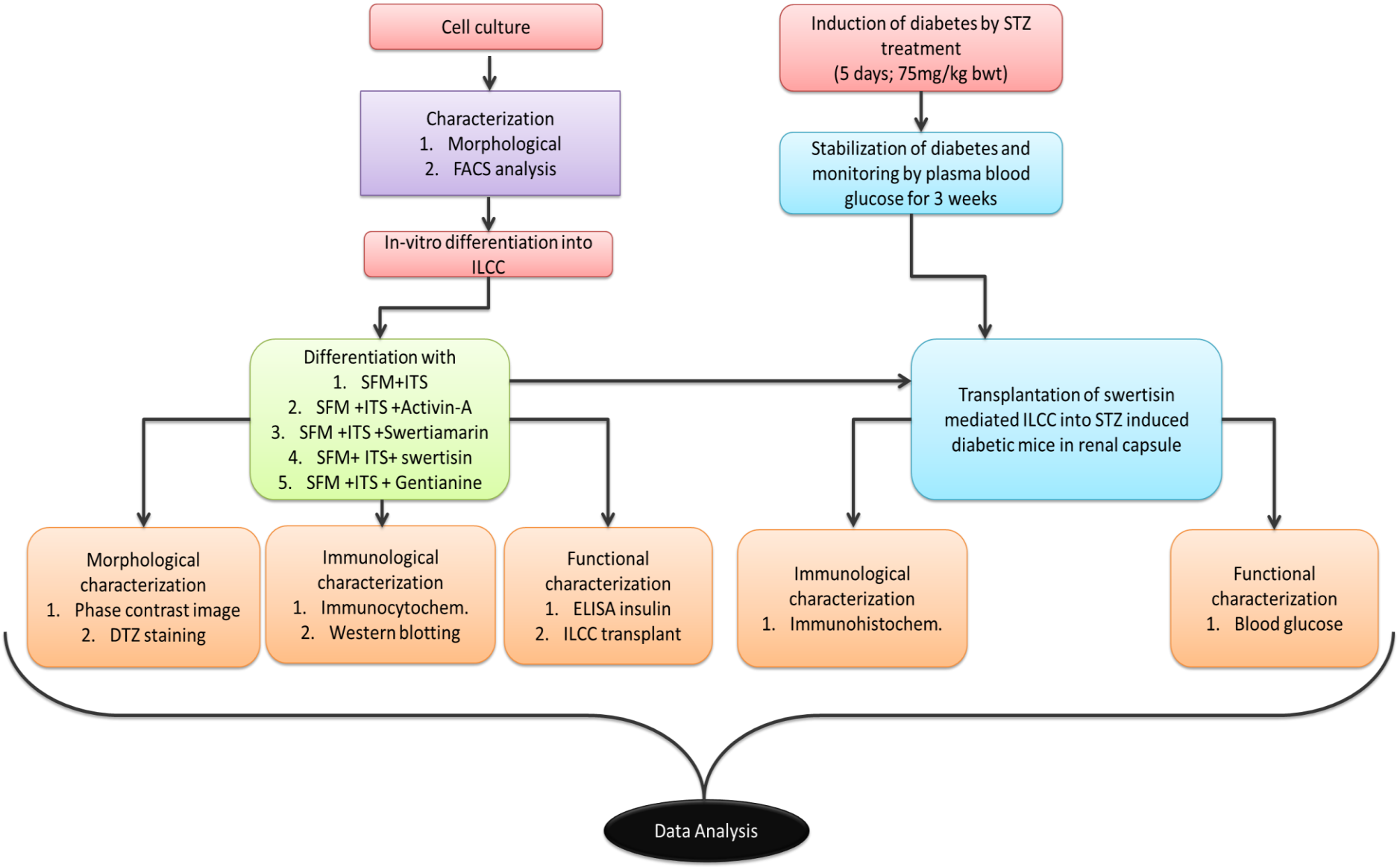


Fig 4.14 representing molecular structure of compounds present in E. littorale blume

The Experimental design of work done under this chapter is described in figure below.

Experimental Design



4b.2 Materials and methods:

4b.2.1 Plant material

Whole dried plant was procured from Bhavnagar district in Gujarat state, India, in month of august after authentication from taxonomist with voucher specimen no. [Oza 51, 51 (a)] present in the Herbarium, Department of Botany, The M.S University of Baroda, Vadodara, Gujarat, India.

4b.2.2 Isolation and characterization of compounds from *E. littorale*:

Methanolic extract of E. littorale was prepared as discussed in earlier reports (Maroo J. et al., 2003 and 2005). In brief, the extract (50 ml) was dissolved in distilled water (100 ml) and transferred to a separating funnel. Successive fractionation was carried out with solvents like butanol, chloroform and ethyl acetate (250 ml each) which lay in descending manner of polarity index. Ethyl acetate fraction yielded a, pale white precipitate which was further purified by washing with acetone whereas other compounds obtained from different fractions were confirmed as swertiamarin and gentianine. This pale yellow compound was further confirmed by UV spectrum, Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) (M.B. Patel and Mishra., 2012), and Mass fragmentation pattern using ESI-MS/MS under positive and negative ionization mode.

4b.2.3 Cell culture and preparation of differentiation medium

NIH3T3 (ATCC procured and gifted from Zydus Research Centre, Ahmedabad, Gujarat) was maintained as explained earlier in chapter 4a, section 4a.2.3.

4a.2.4 FACS analysis

The flow cytometric analysis of NIH3T3 cells for determining the undifferentiated state, cells were stained and analyzed in same procedure as explained in chapter 4a; section 4a.2.13.

4b.2.5 Comparison assessment of islet neogenic property of various fractions / compounds with NIH3T3 cells (*In-vitro*).

ILCC's were generated from NIH3T3cells in four step protocol as described earlier in previous chapter 3; section 3.2.4. Cells were seeded with differentiating

medium (described in section 3.2.4) supplemented with various fractions and compounds isolated from *E. littorale* with dose of 15µg/ml (*in-vitro*), whereas control cells received only differentiation medium without any growth factor. Medium was replenished every alternate day till 8 days. On 8th day, clusters thus formed were collected; comparative assessment was undertaken for morphological changes. Differentiated NIH3T3 ILCC were washed three times with ice cold Phosphate buffered saline and stained with 10ul of DTZ stain. Bright crimson red colored clusters were observed in light microscope and photographed.

4b.2.6 Characterization of most potent compound by TLC and HPTLC:

Based on morphometric analysis of ILCC generated above, most potent compound in producing high yield of islets was identified. The yellow pale compound, which yielded highest ILCC number with proper islet architecture was screened with TLC, using one mg and dissolved in 25 ml of methanol and applied on a plate pre coated with Silica gel GF254 (E. Merck). The test solution was loaded on the plate by fine glass capillary. The plate was then developed in the solvent system of Ethyl acetate: Methanol (8:2) up to 8 cm. After development, the solvent on the plate was evaporated and visualized at UV 254 nm. For HPTLC spectrum, twenty five mg of compound was dissolved in small aliquot of methanol and TLC plates were developed in the solvent system mentioned above and scanned densitometrically at 243 nm at which the peak area was recorded using CAMAG TLC Scanner 3 system.

4b.2.7 Characterization of most potent compound by UV and ESI MS/MS:

One milligram of most potent compound was dissolved in 1ml of methanol and scanned for the wavelength ranging between 200–800 nm on Helios UV spectrophotometer using quartz cuvette. The wavelength at maxima peak was recorded and compared with previous reports. For mass spectral identification by ESI-MS/MS, one mg of this compound was dissolved in methanol and was introduced for direct fragmentation in ionization chamber in positive and negative ionization mode. The base peak was recorded and compared with the molecular weight as reported earlier (M.B. Patel and Mishra., 2012).

4b.2.8 Immunocytochemistry and Confocal Microscopy

For immunocytochemical analysis of various islet specific markers, ILCC were collected and spun at 1200 rpm (Eppendorf 5415R) and kept for adherence on glass coverslip with high glucose DMEM complete medium for 3-4 hours. Upon attachment, clusters were immediately fixed with 3.7% Paraformaldehyde solution for 20 min and then stained for various islet markers with method as explained earlier in chapter 4a; section 4a.2.10.

4b.2.9 RNA extraction and semi quantitative Reverse Transcriptase PCR

Total RNA from differentiated ILCC's was isolated and 5ug of total RNA was reverse transcribed into first strand cDNA, which thereafter subjected to PCR amplification for monitoring various stem cell and islet specific genes as explained in methods under chapter 4a; section 4a.2.11.

4b.2.10 Immunoblotting for islet differentiation proteins

Again in order to understand the differentiation process for new islet formation by swertisin compound, ILCC protein was probed for immunoblotting of various islet markers and transcription factors as mentioned earlier in detail under chapet4a; section 4a.2.14.

4b.2.11 Glucose induced insulin release assay

Differentiated swertisin mediated ILCC were initially incubated for 3 hours in glucose-free Krebs-Ringer bicarbonate hepes buffer (KRBH) containing 0.5% bovine serum albumin and then induced with 5.5 and 25 mM glucose for additional 2 hours. After brief centrifugation the supernatant was collected and frozen at –70°C until further analysis. Insulin assay was performed using mouse-insulin ELISA (Mercodia Inc, USA).

4b.2.12 Animal selection and induction of diabetes

Male balb/c mice; age 3-4 weeks, weighing 15-20 grams were used for transplantation experiments. All animal experiments were performed in accordance with our institutional 'Ethical Committee for Animal Experiments' and CPCSEA guidelines and regulations. Animals were kept with 12 hours light and 12 hours dark cycle and allowed to have water and pellet diet ad-libitum.

Diabetes was induced with Streptozotocin (STZ;65 mg/kg bwt) intraperitonealy for 5 days upon overnight fasting. Diabetic status of animals was confirmed by monitoring FBS using Accu check glucometer (Accu check, Roche, USA) till 3 weeks for stabilization of hyperglycemia.

4b.2.13 Transplantation of ILCC and reversal of hyperglycemia

Transplantation was performed under anesthesia with ketamine and Xylazine. Briefly, the abdomen was incised to expose kidney capsule (n=5-6 per group). Small incision was given to open capsule membrane for implanting the ILCC. The abdominal cavity and overlying skin were sutured back, and animals were allowed to recover for 2 weeks. An additional group of mice (n 5-6) underwent a sham operation, in which the same surgical procedure was performed without implanting ILCC. Fasting blood glucose was monitored weekly till four weeks in both the group of animals.

4b.2.14 Histological and Immuno-histo-chemical assessment of ILCC

Transplanted ILCC from kidney graft were excised after 4 weeks and histologically examined under light microscope with H&E staining. For immunohistochemical analysis section were deparafinized and rehydrated using varying alcohol grades (1 min each) followed by rehydration in distilled water (1 min). Each section was then incubated in blocking solution at room temperature for 1-2 hr. Primary antibodies for Insulin, C-peptide, somatostatin and glucagon (for details, see table-4) were incubated overnight at 4^0C and washed three times with 0.1 M PBS. FITC conjugated secondary antibodies were incubated for 1h in 0.1 M PBS at RT (details table-4). Cell nuclei were visualized with 4-9-6-9 diamindino-2-phenylindole (DAPI). Sections were fixed with antifade mountant and images were recorded on confocal microscope (LSM710, Zeiss, Germany).

4b.2.15 Statistical Analysis

Data given as mean±SEM. significant difference was evaluated by the paired t-test. If more than one group was compared with control, significance was evaluated according to one-way ANOVA. Statistical difference was indicated by $P< 0.05$.

4b.3 Results

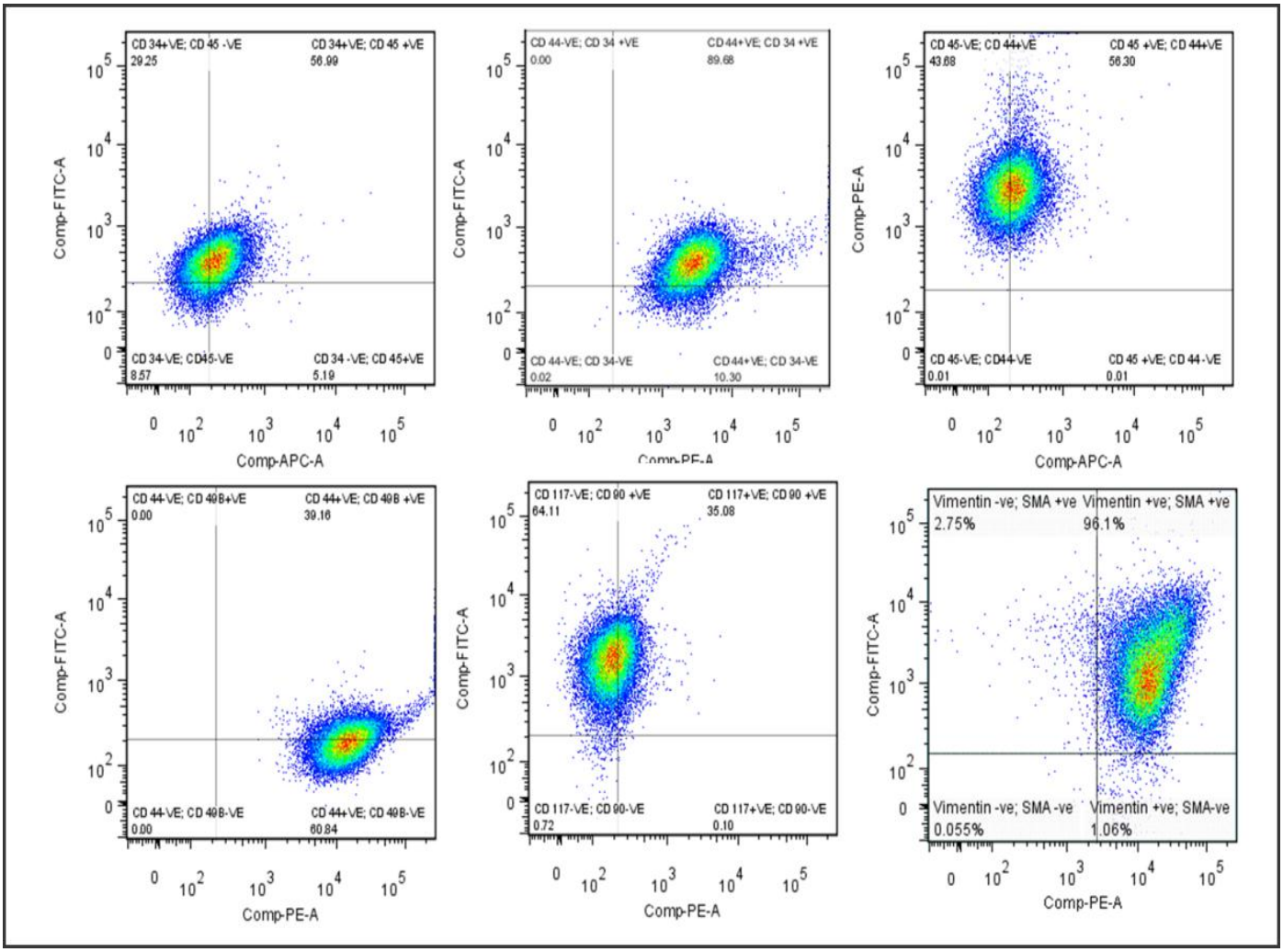
4b.3.1 Morphologic characteristics of undifferentiated NIH3T3 cells:

At full confluence, NIH3T3 cells became homogenous and attain spindle shape, fibroblast-like morphology and arranged in monolayer. Flow-cytometric data showed positive peaks for mesenchymal stem cell markers CD34, CD44, c-kit (CD117), CD90, Vimentin, SMA and negative staining for CD45, a hematopoietic stem cell marker (Fig.-4.16). This result indicated stem/progenitor like features in NIH3T3 cells similar to multipotent stem cells (Chateauvieux et al., 2007; Gang et al., 2007; Kagami et al., 2011; Sung et al., 2008).

4b.3.2 Comparative assessment for islet-cell differentiation potency of various fractions/ compounds

In order to identify most potent islet differentiating agent among various isolated compounds from *E. littorale*, a comparative islet differentiation was carried out with various compounds using NIH3T3 cells as extra pancreatic progenitor cells for 8 days along with positive islet differentiation inducer agent Activin-A. After 8 day induction, cluster formation was observed microscopically where a flavonoid extracted from ethyl acetate fraction induced group showed maximum zone of activation and cell aggregation, followed by other compound gentianine (Fig-4.16). ILCC yield and morphological features like average size and area were quantified. The flavonoid induced NIH3T3 cells showed highest yield of ILCC with significant increase of 3.9 fold compared to SFM alone and activin-A induced differentiation group (Fig-4.17a). On calculating average area of ILCC generated among all groups, no significant difference was observed in all tested compounds compared to control indicating clusters generated were more or less similar (Fig-4.17b). More pertinently, both flavonoid and gentianine showed significantly higher number of ILCC categorized in two groups based on size, first ranging from 150-300 μm and second ranging from 300-3000 μm. On the other hand, activin-A generated more of ILCC ranging between 150-300 μm size and less between 300-3000 μm. An increase of approximately 9.3 fold bigger size ILCC from isolated flavonoid compared to activin-A, eventually demonstrated generation of mature ILCC (Fig-4.17c and d). These results represent the mean of three independent

observations and differences were found statistically significant ($P < 0.01$ and 0.001).



<u>Fig 4.15 FACS dot plots of various stem cell markers in NIH3T3 cells:</u> Fig 4.15 depict dot plots of flow cytometery data analyzed for (1) CD34 + CD44, (2) CD 34 + CD44, (3) CD44 + CD45, (4) CD 44 + CD49b, (5) CD 117 + CD90 and (6) Vimentin + SMA.

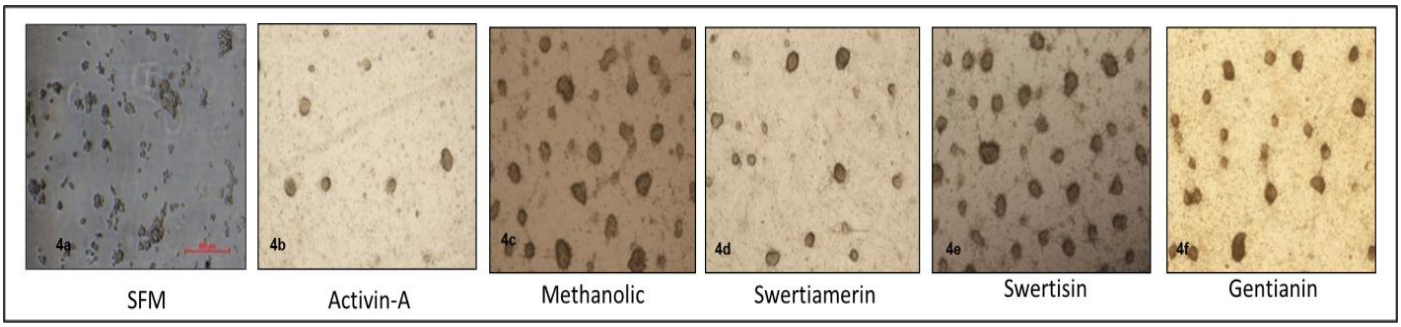
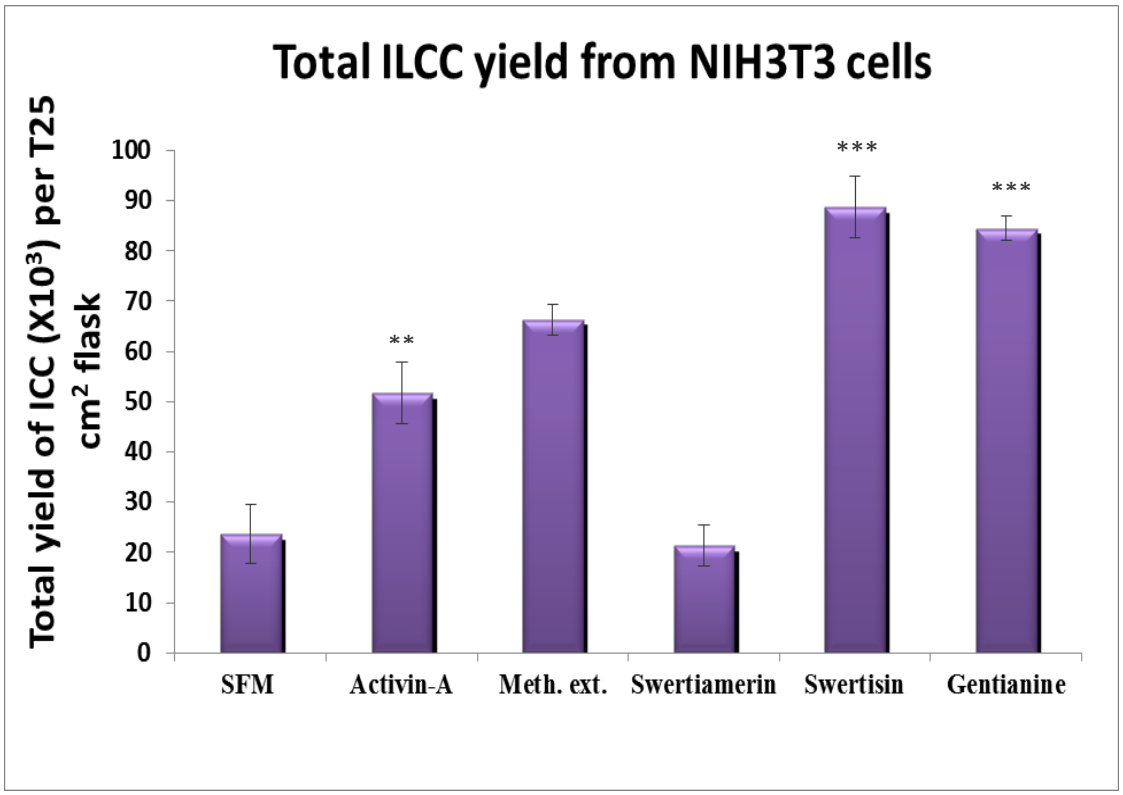
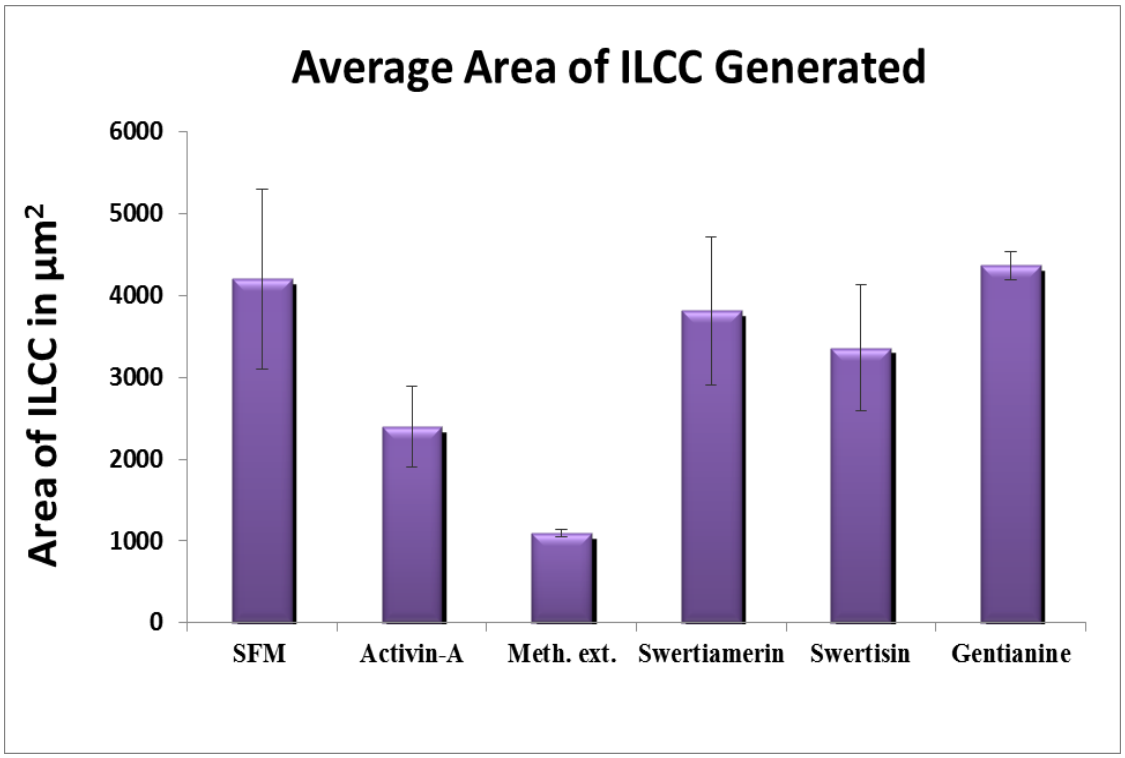
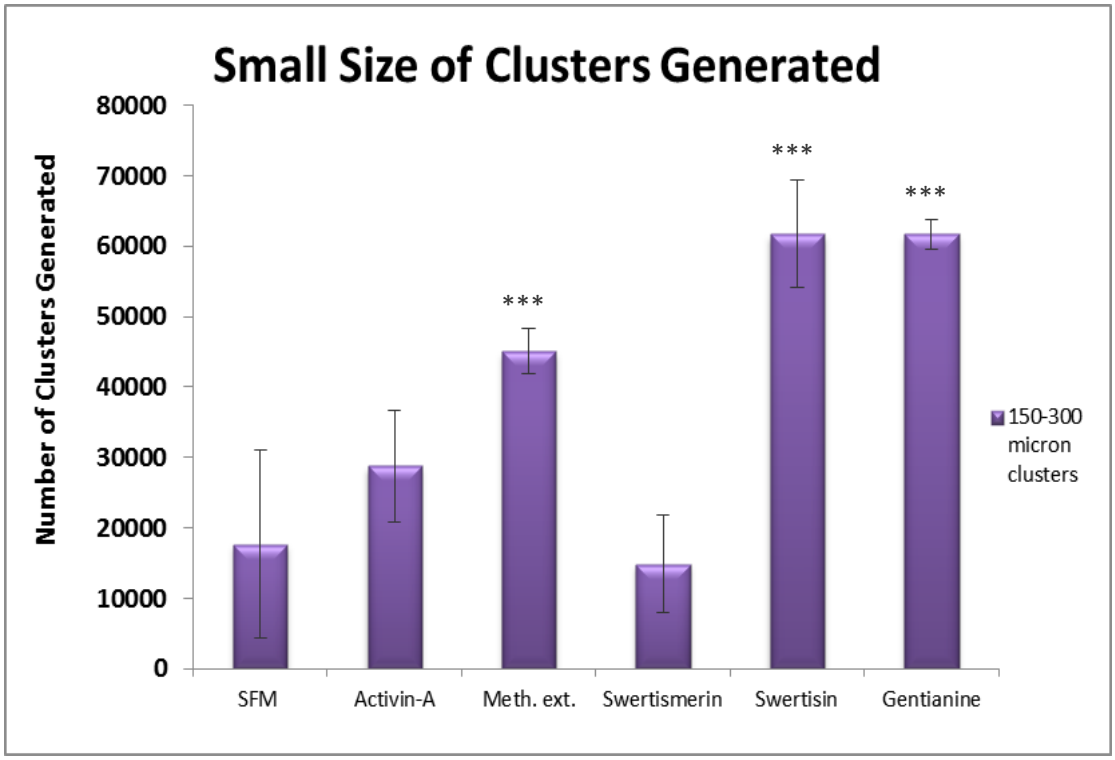


Fig 4.16 Pictorial representation of ILCC generation from control, activin-A and various other E. littorale compounds: Fig 4.16a-f demonstrate comparative pictorial representation of ILCC generation from NIH3T3 cells using SFM control, Activin-A and various EL compounds. Out of all fig 4.16e indicating maximum ILCC generation with swertisin induction.


Total ILCC yield from NIH3T3 cells
Total yield of ICC (X10³) per T25 cm² flask
100
90
80
70
60
50
40
30
20
10
0
**
***
***
SFM
Activin-A
Meth. ext.
Swertiamerin
Swertisin
Gentianine


Average Area of ILCC Generated
Area of ILCC in µm²
6000
5000
4000
3000
2000
1000
0
SFM
Activin-A
Meth. ext.
Swertiamerin
Swertisin
Gentianine



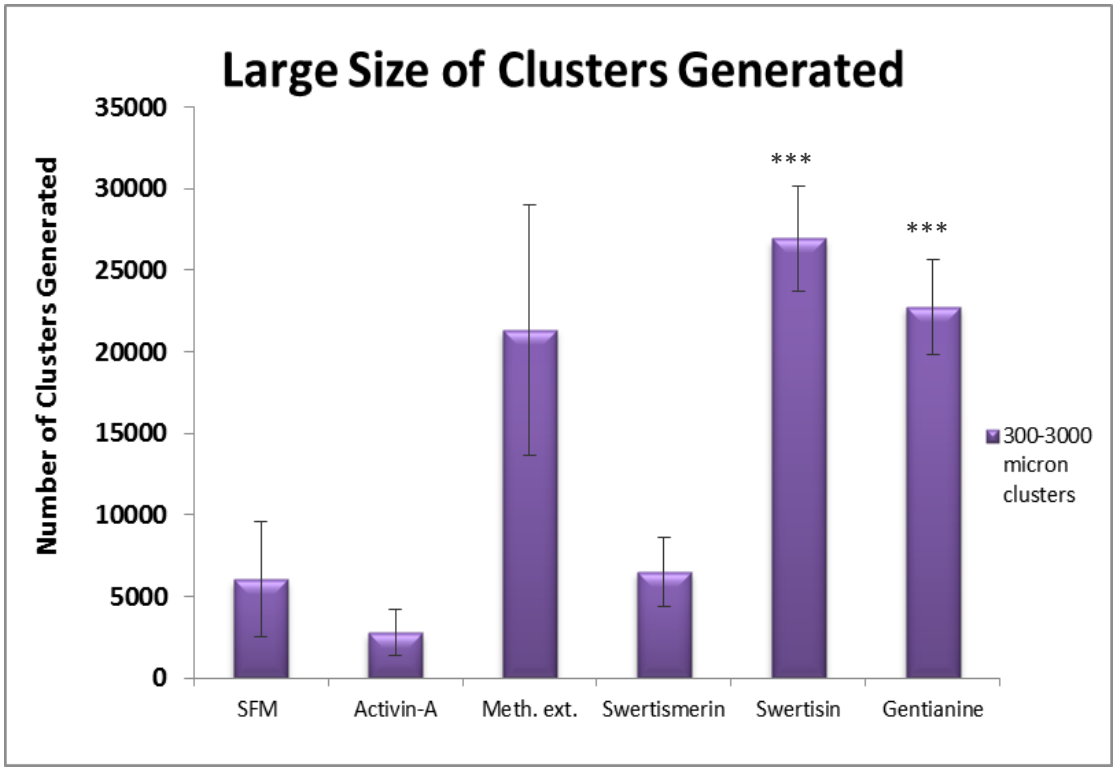


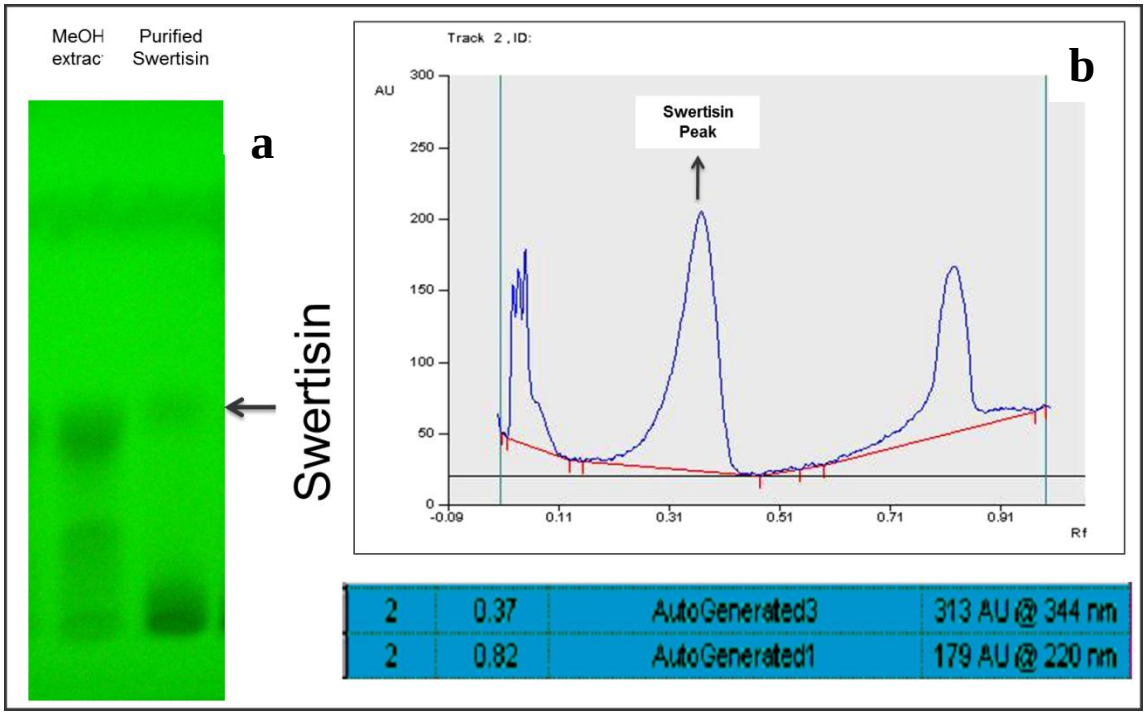
Fig 4.17 Morphometric analysis of NIH3T3 generated ILCC: 4.17a depicts total yield of ILCC. Swertisin showed significantly higher yield compared to SFM and Activin-A group. Fig 4.17b shows the comparative data of average area of ILCC generated with various compounds. Fig 4.17 c and d represents small and large size of ILCC clusters generated between range of 150-300 (small) and 300-3000 µm (large). Results are expressed as mean ± SEM of three independent observations. *** p< 0.001 vs SFM control and activin-A.

To further test the mode of islet differentiation between control-SFM, Activin-A, and most potent compound, a day wise microscopic observations were recorded and confirmed for insulin biogenesis. After induction with this flavonoid, the morphology of NIH3T3 cell gradually changed from a fibroblast-like to round cells within 2 days and large number of cells formed tight clusters (Fig.-4.16). The differentiated NIH3T3 cells showed intense zone of activity and clustering in just 4 days induction, while untreated cells did not (Fig.-4.16). These clusters turned up to mature ILCC within 8 days and float into the medium resembling to islet like mini-organelles.

4b.3.3 Compound from ethyl acetate fraction identified as Swertisin

The compound isolated from ethyl acetate fraction of E. littorale was confirmed as a single peak at 344 nm in UV-Vis spectrum analysis (Fig.-4.18a) and a single spot at Rf 0.72 of HPTLC plate again at λmax of 344 nm when scanned on CAMAG TLC Scanner-3 system (Fig.-4.18a and 4.18b). These results indicated the presence of Swertisin compound as reported earlier by Renata and Mishra et al . (ColomboI; et al., 2009; M.B. Patel and Mishra., 2012).

Mass fragmentation pattern of this compound was also compared with earlier references and found to be identical to Swertisin (ColomboI; et al., 2009). Our results showed a base peak of m/z 447 (M+H) which corresponded to molecular weight of Swertisin in positive ionization mode. Further-more, a peak of M+H – 120 was also obtained which indicated a loss of 120u a characteristic peak of C – glucosyl flavonoids as mentioned by Renata et al. (Fig.-4.18b) (ColomboI; et al., 2009). The compound was found to be soluble in solvents like DMSO and dioxane while, sparingly soluble in methanol and insoluble in water. All the above results shown by us are in accordance of swertisin reported data with structure shown in Fig-4.18c.



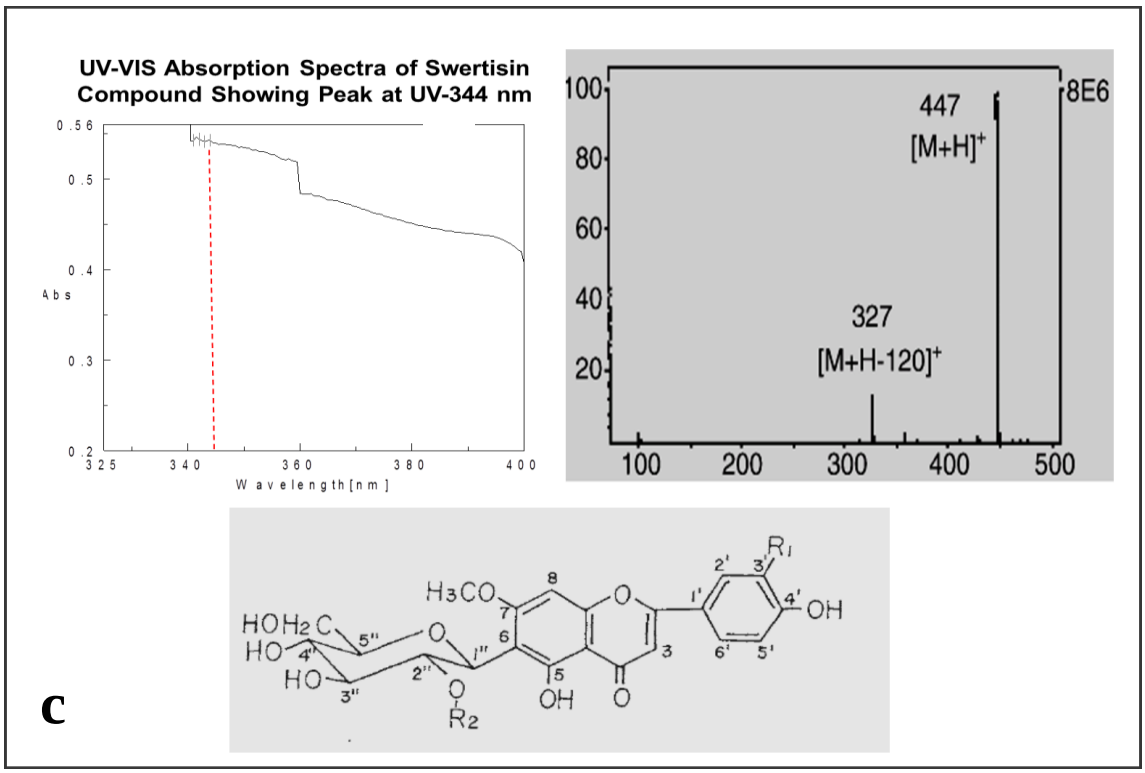


Fig 4.18 TLC and HPTLC profile of Swertisin compound: Fig- 4.18a represents TLC plate scan at UV-265 nm showing methanolic extract and purified Swertisin compound profile as a single band at Rf 0.32 (arrow marked), Fig 4.18b represents HPTLC curve showing single peak of Swertisin at Rf 0.32 and UV-344nm wavelength. Fig 4.18c UV-Vis and Mass Fragmentation pattern of Swertisin compound: Fig 4.18a represents UV-Vis absorption spectra of swertisin compound. A base peak at height of 0.54 at 344 nm was seen purified Swertisin (Red line marked), Fig 4.18b represents Mass Fragmentation curve showing two single peak 447 $[M+H]^+$ representing Swertisin compound and 327 [M+H -120u] release of Glyucosyl ring. Fig 4.18c shows Structure of Swertisin based on Mass fragmentation pattern.

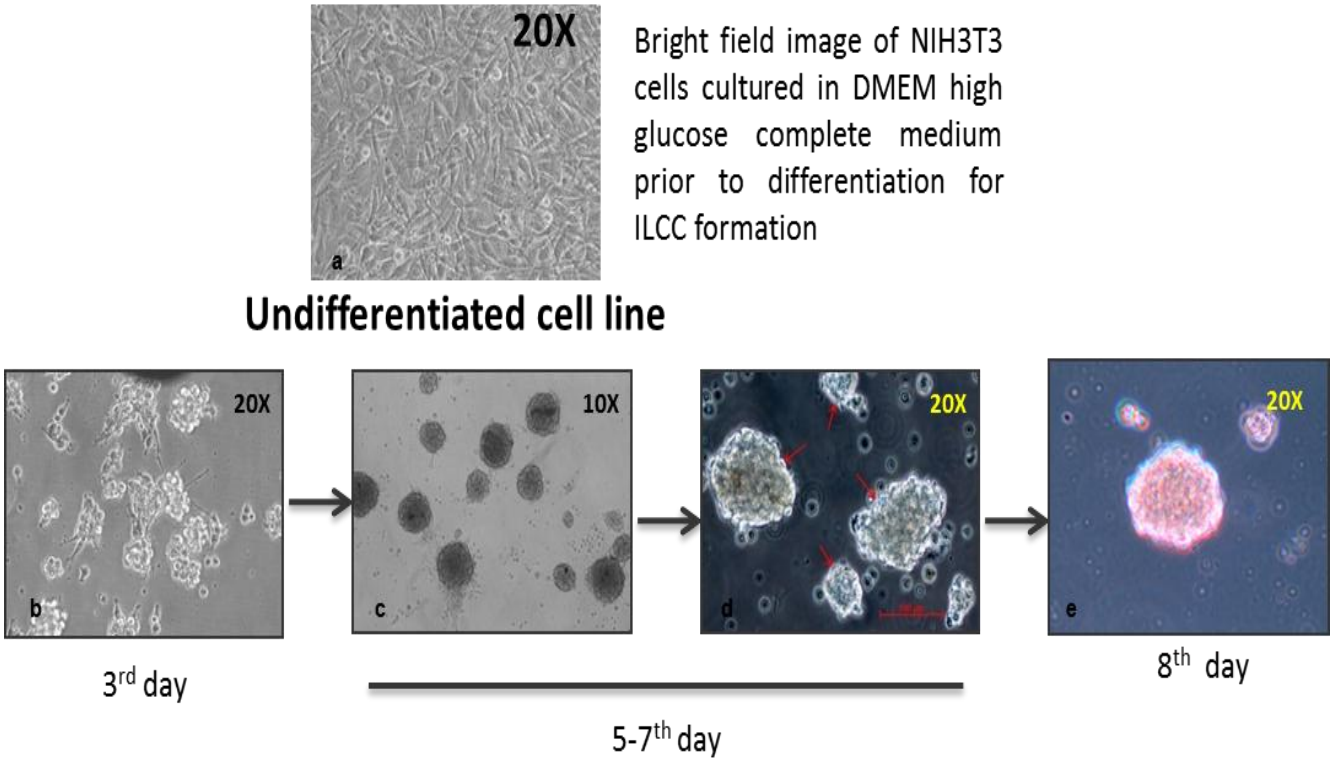


Fig 4.19: Schematic representation of event occurring in Swertisin induced ILCC formation. Fig 4.19a represents basic fibroblastic morphology of NIH3T3 cells cultured in complete medium. Fig 4.19b-d depicts event occurring during swertisin induced differentiation process from day 3rd to day 8th. Fig 4.19e represents DTZ staining in differentiated ILCC for primary confirmation of beta cell Differentiation.

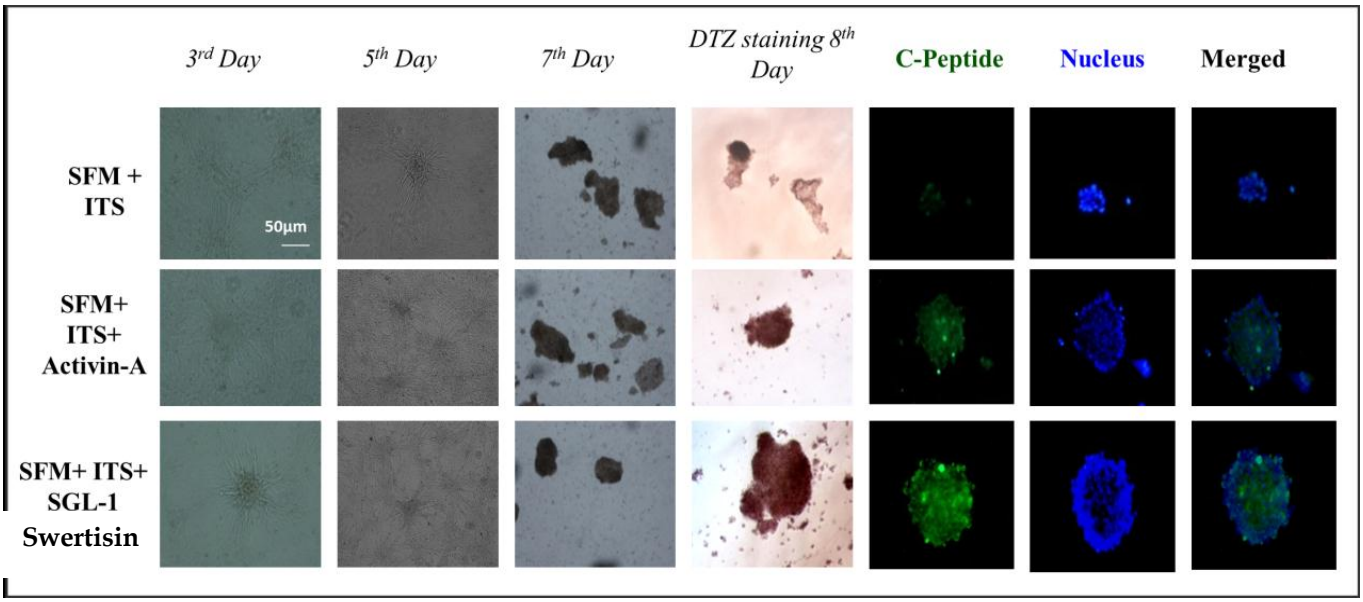


Fig 4.20: Comparative montage of morphological events occurring in 3-8 day time duration: Figure represents day wise representation of morphological changes and differentiation event occurring with SFM, activin-A and swertisin mediated ILC. Figure also show DTZ staining in differentiated ILCC for primary confirmation of beta cell differentiation and C-peptide staining representing presence of insulin biogenesis and insulin granules.

4b.3.4 DTZ and C-peptide staining depicted presence of new insulin synthesis in NIH3T3 ILCC.

We examined the clusters thus generated in each group, for insulin-producing cells and stained them with zinc chelating agent DTZ which is a zinc-binding substance. Pancreatic islets are known to stain crimson red in color after DTZ incubation. Swertisin induced NIH3T3-ILCC were distinctly stained crimson red by DTZ indicating a sequestration of zinc along with insulin granules (Fig.-4.20). To further examine the new insulin biogenesis, we carried out c-peptide staining. C-peptide is a 3 kDa peptide released from insulin molecules within the beta cells when beta cells carry out new insulin biogenesis. In accordance to our expectations, when swertisin induced differentiated clusters were tested for C-peptide, along with SFM and activin-A group, we found immense positive cytoplasmic staining of mouse c-peptide in swertisin generated ILCC whereas activin-A mediated ILCC showed less staining with no staining in control SFM clusters (Fig.-4.20).

4b.3.5 Confocal imaging show differentiation in swertisin mediated ILCC:

To monitor the presence of various islet hormones and differentiation markers, immunocytochemical staining was performed in cell clusters generated with swertisin induction and stained for PDX-1, insulin, c-peptide and glucagon proteins. PDX-1 is considered as an important marker for initiation of islet differentiation. Pancreatic progenitor cells are known to be highly PDX-1 expressing, many groups have also shown that ectopic expression of PDX-1 leads to islet cell differentiation (Kajiyama et al., 2010; Mauda-Havakuk et al., 2011; Oliver-Krasinski et al., 2009), moreover it is also reported that even mature beta cells do express basal level of PDX-1 protein (Li et al., 2005; McKinnon and Docherty, 2001). In our study, we observed that undifferentiated NIH3T3 cells were slightly PDX-1 positive (Fig.-4.21) but negative for c-peptide staining, depicting potential to form ILCC similar to pancreatic progenitors. Upon differentiation with activin-A, we observed D-NIH3T3 ILCC showed both insulin and c-peptide staining. Similarly, swertisin induced ILCC also intensely stained for c-peptide, insulin, somatostatin and glucagon proteins (Fig.-4.21).

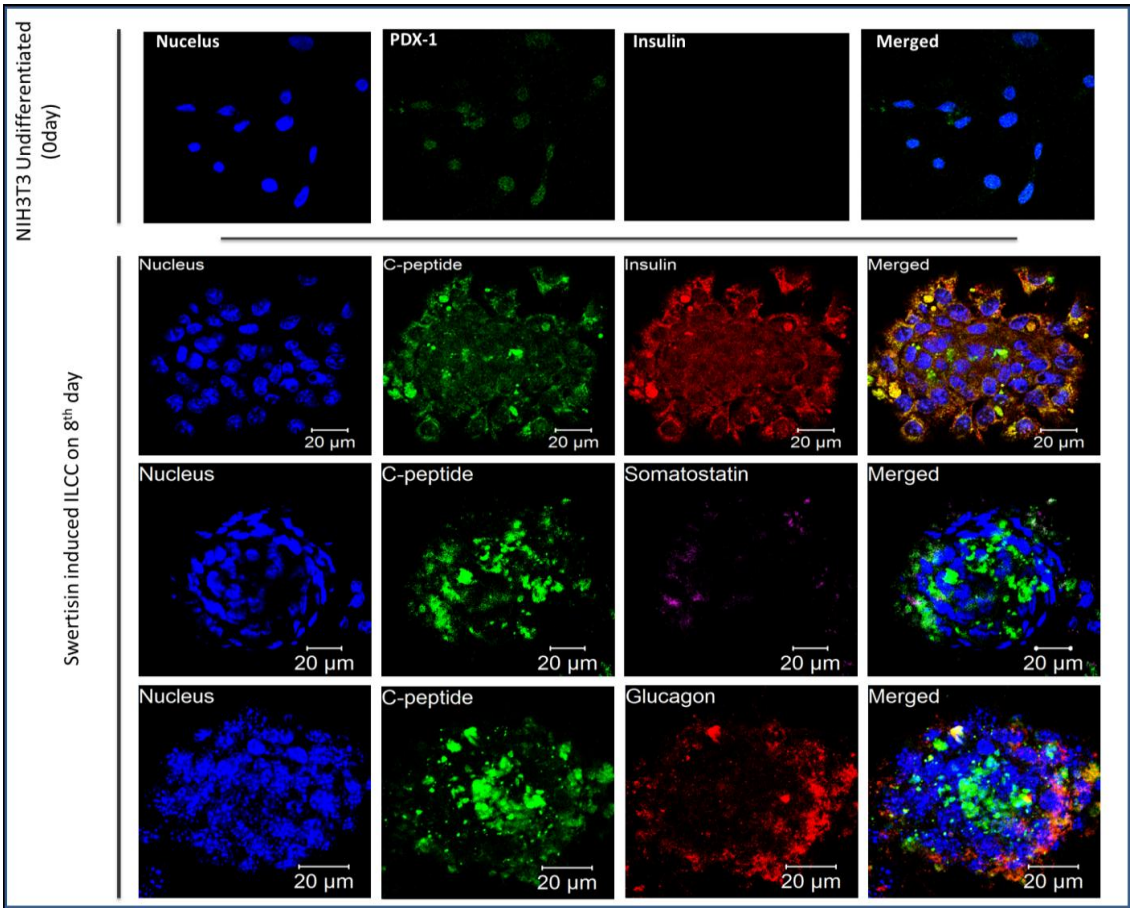


Fig 4.21 Confocal images of various islet markers in swertisin induced ILCC: Fig 4.21 depicts intense positive staining for Insulin (red color), C-peptide (Green color), Glucagon (Red color) and Somatostatin (pink color). Nucleus of all the clusters was stained with DAPI shown in blue color.

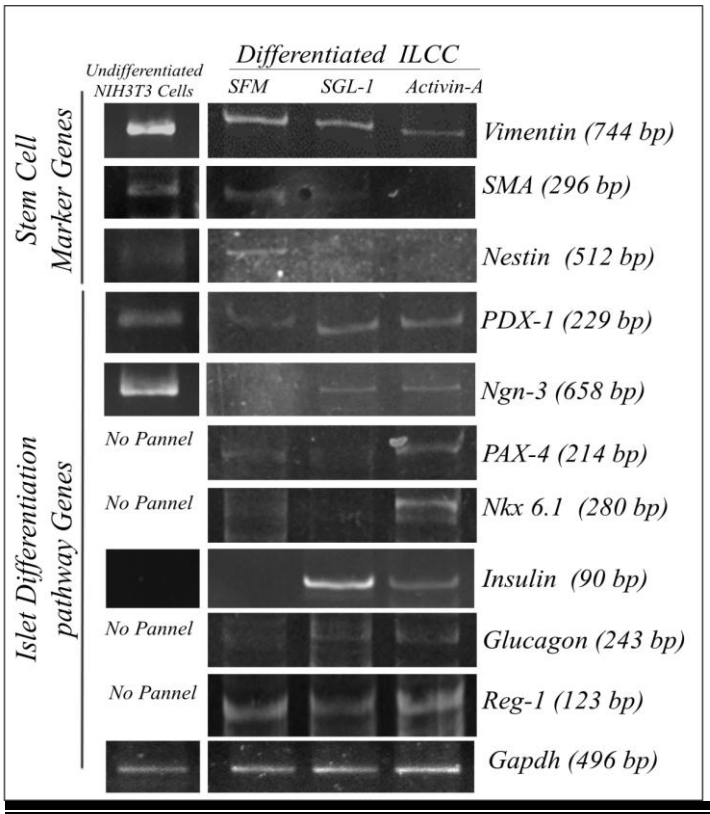


Fig 4.22 RT-PCR expression data of various stem/progenitor and islet differentiation pathway genes Fig-10 shows mRNA expression of various stem/progenitor genes and key islet differentiation pathway transcription factor genes harvested at 10 day of differentiation protocol in SFM alone, activin-A and swertisin mediated ILCC.

4b.3.6 Gene expression by RT PCR:

Using reverse transcriptase PCR, mRNA expression of islet differentiation pathway genes and mature islets were quantified. In our study, mRNA transcripts were not detected in the undifferentiated NIH3T3 cells neither for pancreatic endocrine development genes nor for islet specific genes. However, undifferentiated NIH3T3 cells found to be strongly expressing transcripts of stem/progenitor related genes like Nestin, Vimentin and Smooth Muscle Actin (SMA). Expression of pluripotent stem cell markers like Oct-4 and Nanog was not observed in any case. Upon differentiation, we found significant elevated expression of both differentiation pathway transcription factors like Nestin, PDX-1, Ngn-3, Pax-4, Nkx 6.1, Reg-1 and endocrine genes like insulin and glucagon at the end in Swertisin induced ILCC with significant reduction in expression for stem cell markers like Nestin, Vimentin, and SMA genes was also noted (Fig.-4.22). The most peculiar observation about swertisin induction is that, the flavonoid, not only potentiate islet differentiation pathway, but it also accelerate it with faster pace. This phenomenon is more obvious with significant reduction in Nestin, Vimentin, and SMA transcript and absence of Pax-4 and Nkx 6.1 expression in swertisin induced clusters while the same is elevated in SFM alone and activin-A induced ILCC even at 8th day. Expression of the reference gene Gapdh confirmed the uniformity of the experimental system and acted as input template control.

4b.3.7 Immunoblot analysis of Swertisin mediated islet differentiation pathway

Protein immunoblotting was performed to monitor the initiation and fate of differentiation signaling pathway operated with SFM alone, activin-A, and swertisin induction. Major transcription factors like Nestin, PDX-1, Ngn-3 (a master regulator protein for islets), phopho-Smad-2 and Smad-7 (key signaling modulator for endocrine fate) were analyzed in a time dependent manner from day zero to day eight. In this kinetic study, we observed that SFM alone ILCC fails to down regulate stem/progenitor marker vimentin, resulting no differentiation, whereas both activin-A and swertisin showed decrease in

vimentin from 4th day onwards. Compared to activin-A, swertisin clusters showed more steep decrease in vimentin protein, which got completely lost by 8th day. Further, SFM alone clusters failed to up-regulate Nestin, Ngn-3 and PDX-1 protein, which are crucial for differentiation signaling to initiate (Fig.-4.23), but same was found to be elevated in both activin-A and swertisin, but more prominently in swertisin induced ILCC. Activin-A induced ILCC showed little shoot in Nestin protein expression (cleaved-form 90kDa) starting from day 2 which remain high even till day 8 (Fig.-4.23) suggesting slower pace in differentiation process. On the contrary, swertisin generated ILCC showed upregulated form of both 170 kDa and 90 kDa which peaked at 4th day, decreased significantly by 6th day and completely lost by 8th day, representing complete and proper differentiation of NIH3T3 cells with swertisin induction.

Apart from this swertisin mediated clusters also showed high expression of PDX-1 protein within 2 day and persisted even till 8th day, whereas both SFM alone and activin-A did'nt demonstrate same (Fig.-4.23). All these results were found to be in accordance with earlier reports by numerous groups(Jiang et al., 2007; Kim and Hebrok, 2001; Shi et al., 2005; Sulzbacher et al., 2009). Smad protiens are known to play role in islet differentiation mediated by activin-A. Abundant expression for Smad2 and Smad4 with low expression of Smad1, Smad3 and Smad7 was earlier reported by Zhang et al. (Zhang et al., 1999) in developing pancreas.

To purely understand the fate of islet differentiation by swertisin we also targeted Smad signaling by monitoring Smad 2 and 7 proteins. In day wise study, we found that swertisin clusters showed dramatic decrease in Smad7 expression by day 2 with continuous increase in smad2 phosphorylation till 8 day whereas activin-A showed less Smad2 phosphorylation inspite of steep decrease in Smad 7 expression (Fig.-4.23). Same is the case with SFM alone group too, which failed to phosphorylate Smad2. It is pertinent to note that Smad protein phosphorylation in swertisin clusters clearly demonstrate immense potential for islet differentiation than activin-A.

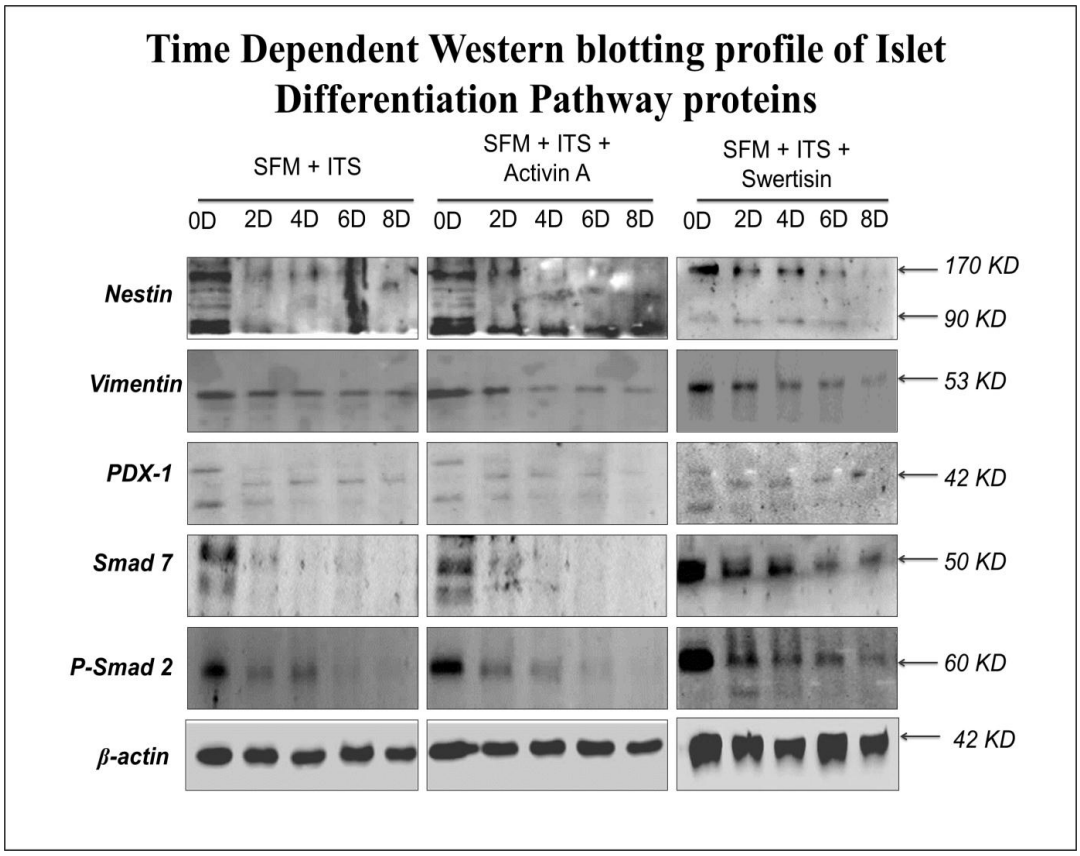


Fig 4.23: Time dependent Western blot study of various stem/progenitor and islet differentiation pathway protein: Fig-11 shows time dependent expression of various stem/progenitor marker and key islet differentiation pathway transcription factor during differentiation protocol ranging from day 0 to day 8.

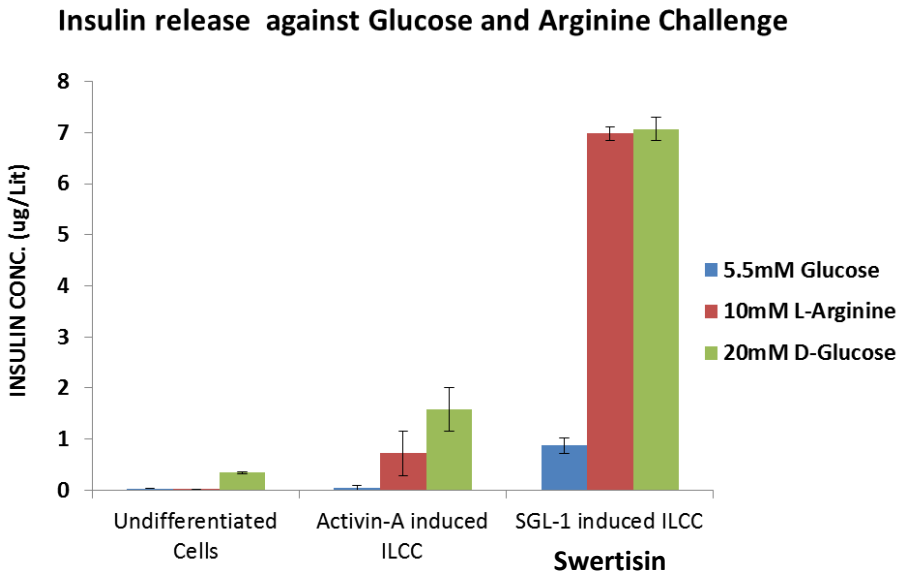


Fig 4.24: Glucose responsive insulin release assay: Fig 4.24 represents glucose responsive insulin release from SFM, Activin-A and swertisin mediated ILCC with increasing glucose and L-arginine concentration. Results are expressed as mean ± SEM of three independent observations. N=3.

4b.3.8 *In-vitro* insulin release in response to a glucose challenge:

Further to check the functional maturity of swertisin induced ILCC, *in-vitro* glucose challenge induced insulin release was monitored in concentration-dependent fashion (Fig.-4.24). The mean insulin secretion by 100 clusters (swertisin mediated) was 0.87 ± 0.022 µg/mL in response to 5.5 mM glucose, 6.98 ± 0.42 µg/mL in response to 10 mM L-Arginine and 7.07 ± 0.23 µg/mL in response to 20 mM glucose concentration, which is significantly higher than SFM alone (0.025 ± 0.009 µg/mL in 5.5 mM, 0.004 ± 0.052 µg/mL in response to 10 mM L-Arginine and 0.337 ± 0.15 µg/mL in 20 mM glucose concentration) and activin-A (0.035 ± 0.003 µg/mL in 5.5 mM, 0.716 ± 0.442 µg/mL in response to 10 mM L-Arginine and 1.584 ± 0.12 µg/mL in 20 mM glucose concentration). Swertisin differentiated ILCC showed 20.9 and 4.46 fold increase in insulin release compared to SFM and activin-A induced ILCC respectively with 20 mM glucose challenge. The result represents mean of three observations and differences were found statistically significant ($P < 0.01$).

4b.3.9 STZ mediated diabetes induction and Islet Transplantation in Renal Capsule

To check the functionality of swertisin induced NIH3T3-ILCC for insulin release *in-vivo*, STZ induced diabetic mice model were used. Multiple dose of STZ in balb/c mice produced effective TD1 mice with greatly elevated blood glucose levels. Induced diabetic mice also showed impaired IPGTT on forth week post diabetes induction (Fig. 4.25). The efficacy of glucose lowering tendency from swertisin induced ILCC was monitored from transplanted renal grafts. All the mice received renal grafts showed significant reduction in FBS within one week of transplantation (Fig. 4.25).

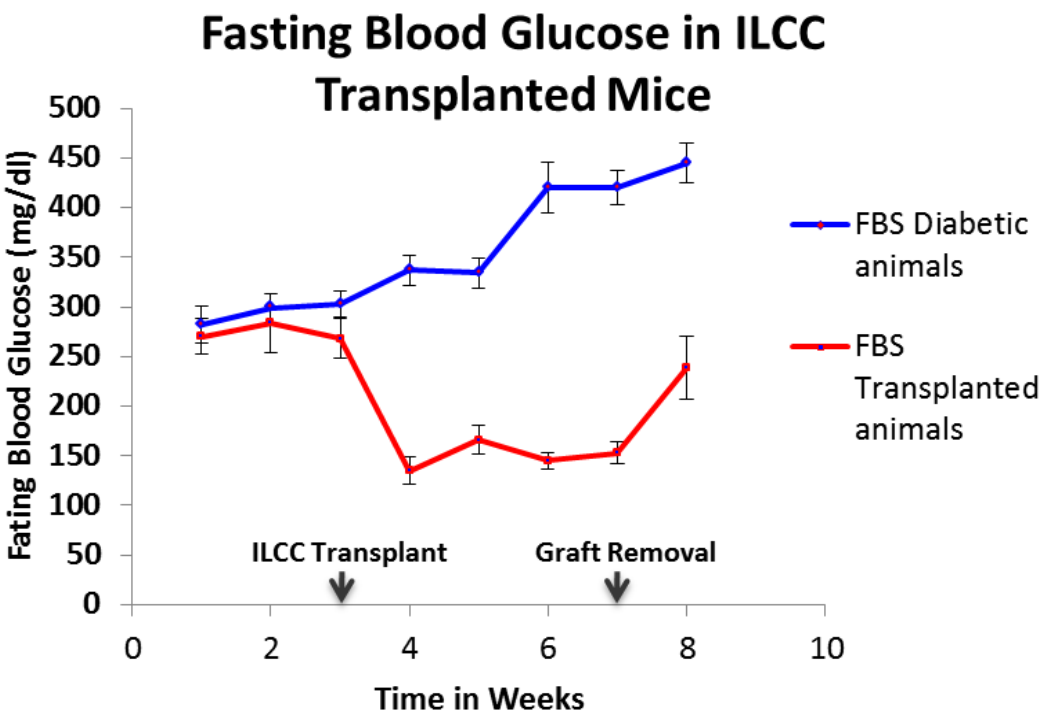


Fig 4.25: FBG in balb/c mice after transplantation: Fig 4.25 represents fasting blood glucose in time dependent manner from swertisin mediated transplanted ILCC and diabetic control mice. Glucose was monitored for 7 weeks, and 1 week after graft removal. Results are expressed as mean ± SEM of 5 transplanted animals.

4b.3.10 H&E staining and Immunohistochemistry of transplanted renal grafts

To demonstrate the viability and functional integrity of transplanted islet grafts *in-vivo*, after stabilization period of four weeks, renal capsule having transplanted ILCC grafts were excised, sectioned and histologically examined for intact islet architecture using H&E stain. Deep eosin cytoplasmic with less nuclear hematoxylin stain showed compact islet architecture. More of Eosin staining resembles the graft staining similar to pancreatic islet section stain. Presence of c-peptide, insulin, glucagon and somatostatin using immunohistochemistry was also assessed. Intense cytoplasmic staining for all the four hormones in excised tissue showed that transplanted ILCC graft was not only viable but also functional during transplanted period inside mouse kidney, whereas kidney cells did not stained for any islet hormone (fig 4.26).

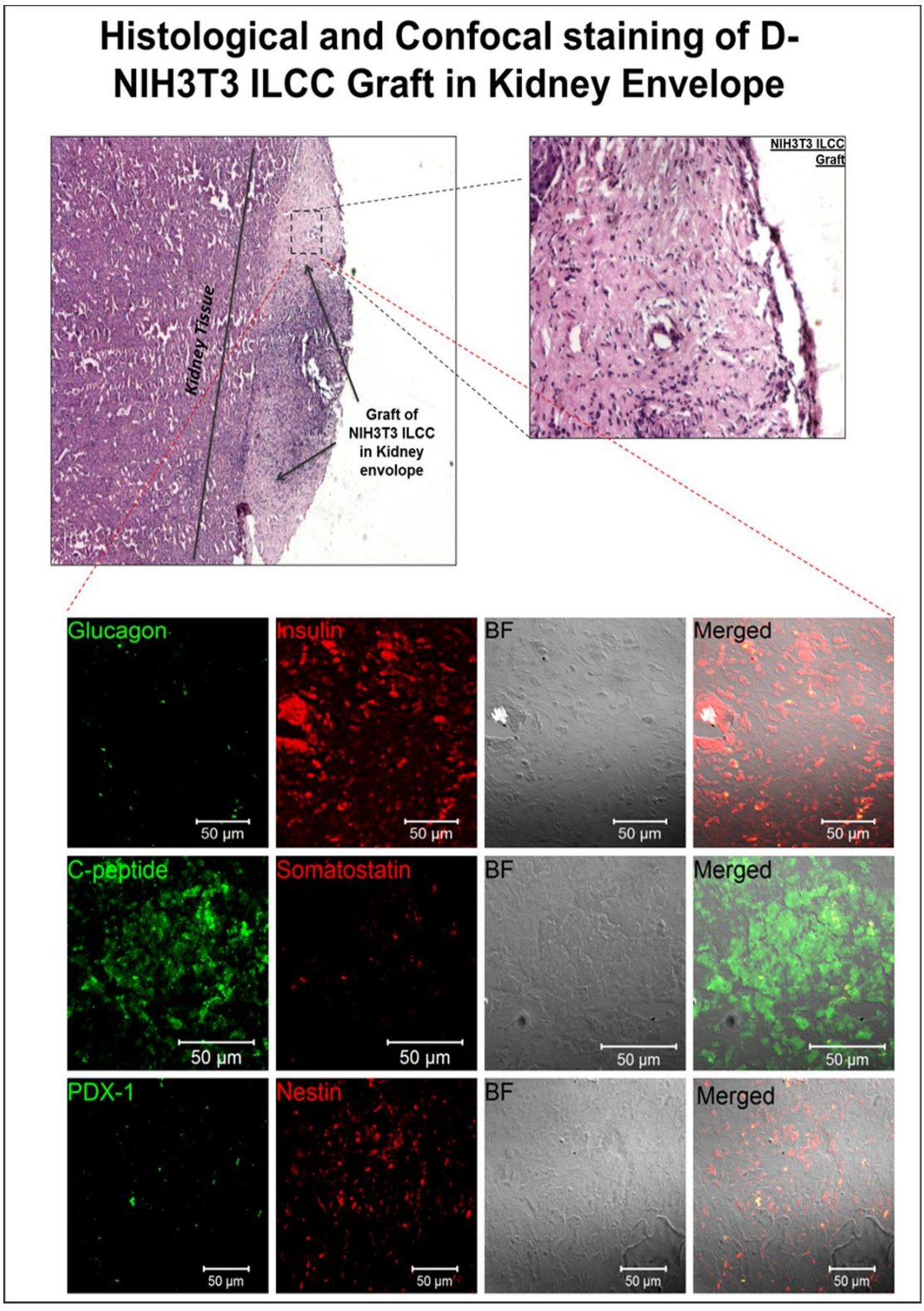


Fig 4.26: Histological and immunochemical staining of islet hormones in kidney-ILCC grafts: histological observation of kidney graft from transplanted swertisin mediated ILCC. Islet specific makers were also observed in these grafts, which show insulin (red), c-peptide (green), somatostatin (red), Pdx-1 (green) and Nestin (red).

4b.4 Discussion:

Many recent studies have demonstrated the possibility of generating insulin producing cells from embryonic as well as adult stem cells (Chandra et al., 2010; Jiang et al., 2007; Kadam et al., 2012; Lee and Stoffel, 2003; Sui Jing et al., 2011; Wang et al., 2011; Wang et al., 2010). Numerous growth factors had been screened to test stem cell conversion into islet cell types. Despite of their promising potential for differentiation, it is still difficult to obtain sufficient islet for autologous islet transplantation. One of the important obstacles is lack of potent differentiating agent which can yield enormous amount of islet cells from starting pool of stem cells.

To overcome this limitation we explored and opened up new venture to screen islet differentiation potential of bioactive ingredient from medicinal herbal plants. Identification of such potent herbal active ingredient may prove revolutionary therapeutic approach for achieving enormous islet yield for effective treatment of diabetic patients. Several *in-vitro* studies have demonstrated that NIH3T3 cells could be reprogrammed to adipocytes, oesteocytes and neural cells but differentiation in functional insulin-producing cells under certain culture condition using growth factors was recently demonstrated by us (Gupta, 2010). In this study we isolated various compounds form methanolic extract of E. littorale, evaluated for islet differentiating potential and characterized the most potent which was found to be, Swertisin a small flavonoid. It possesses immense potential to effectively differentiate tissue specific stem/progenitor cell like NIH3T3 which is extra-pancreatic in origin, into insulin producing cells without any genetic modification. It also regulates blood glucose levels in a murine model of IDDM showing significant decrease in hyperglycemia after transplantation of differentiated ILCC in renal capsule .Differentiation into insulin producing endocrine cells is induced by a cascade of events controlled by several transcription factors such as Nestin, PDX-l, Neurogenin-3 and PAX-4 (Murtaugh, 2007).which was expressed both at gene and protein level in characterstic time dependent manner.

Kim and associates (Kim and Hebrok, 2001) reported that rat pancreatic extract can provide regeneration factors that induce pancreatic regeneration. This finding was also confirmed by Choi and associates (Choi et al., 2005). Nicotinamide is a poly ADP-ribose synthetase inhibitor and could induce pancreatic progenitor cells into insulin producing cells (Tang et al., 2004). Whereas exendin-4 stimulate both β-cell replication and neogenesis from ductal progenitor cells and inhibit apoptosis of β-cell (Tang et al., 2004). It is needless to say that insulin production by islet-like clusters should be confirmed. Furthermore, their gene expression should be similar to that of pancreatic endocrine tissue. Insulin production was confirmed by staining with zinc chelating agent (DTZ). These cells were not only capable of insulin production but also of its release in a dose dependent fashion according to the glucose concentration. The expression of insulin, glucagon and somatostatin genes has been demonstrated in the differentiated clusters. Evidence has also been provided that PDX-l and NeuroD1 were up regulated. Some investigators suggested that, part of insulin detected may have been derived from insulin added to the culture media in certain protocols or insulin present in serum. The stepwise increase in the insulin release as a function of the glucose concentration does not support these contentions. In addition; detection of C-peptide in the cell clusters, immuno-histologically as well as insulin mass balance, confirms that insulin release was the result of endogenous synthesis. ILCC thus generated from NIHT3T also regulate blood glucose levels in a murine model of IDDM showing significant decrease in hyperglycemia after transplantation of ILCC in renal capsule.

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Chapter 5

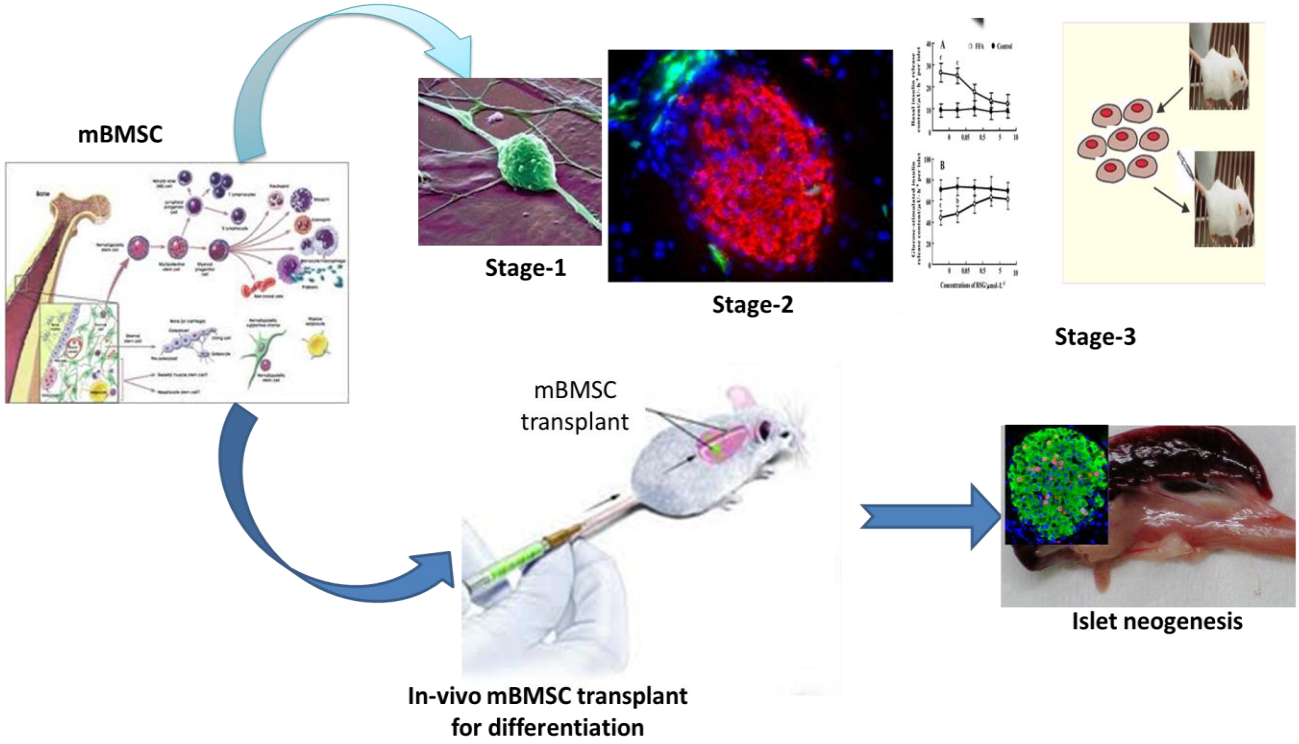
Title

"Differentiation of Mouse Bone Marrow Mesenchymal Stem cells into insulin producing cells using potential bioactive ingredient Swertisin (SGL-1) from *Enicostemma littorale* Blume"

Part-1:- *In-vitro* differentiation of mBMSC into insulin positive cells.

&

Part-2:- Endogenous *in-vivo* differentiation of mBMSC cells into insulin positive cells.



5.1 Introduction

5.1.1 Bone marrow mesenchymal stem cells (mBMSC)

The bone marrow consists of three main cellular systems; first hematopoietic, second endothelial and third part is stromal. Bone marrow, traditionally is composed of two main distinct lineages, hematopoietic giving rise to all blood cells and the mesenchymal lineage (a supporting hematopoiesis, as well provide stem/progenitor for tissue specific cells) (fig 5.1). BMSC's are the one which can differentiate into variety of cell type (Becker et al., 1963) such as osteoblasts (Brighton and Hunt, 1991), chondrocytes (Brighton and Hunt, 1997), and adipocytes (Qian et al., 2012).

Mesenchymal stem cells are characterized morphologically by small cell body with few long and thin cell processes. Cell body consists of large round nucleus with prominent nucleolus and remaining body consists of small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. (Brighton and Hunt, 1991).

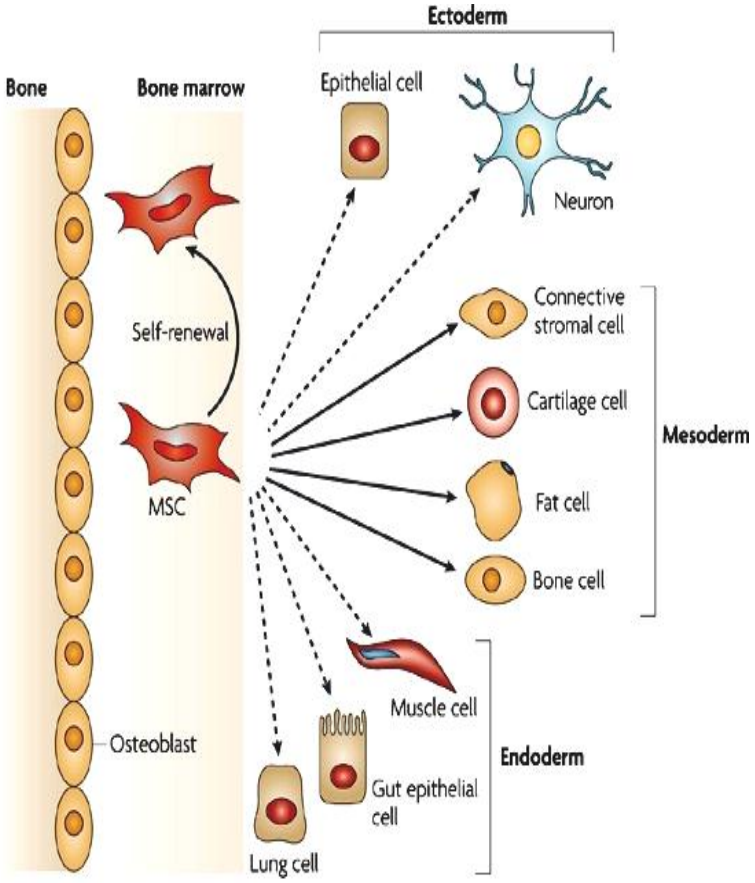


Figure 5.1 shows the ability of mesenchymal stem cells (MSCs) in the bone-marrow cavity to self-renew (curved arrow) and to differentiate (straight, solid arrows) towards the mesodermal lineage. The reported ability to trans-differentiate into cells of other lineages (ectoderm and endoderm) is shown by dashed arrows, as transdifferentiation is controversial in vivo (Antonio Uccelli et al., 2008).

5.1.2 Characteristics of BMSC

The isolation of a "pure" population of BMSC's remains elusive and the immuno-phenotypic identification is still based on a combination of negative and positive markers, rather than on a precise and specific subset of antigens. There is a strong consensus that both human and murine BMSCs do not express CD45 (Lai et al. 1998; Deans et al. 2000) while CD34, the primitive haematopoietic stem cell (HSC) marker, is rarely expressed in hBMSCs, although it can be positive in mice (Peister et al. 2004). CD117, a haematopoietic stem/progenitor cell marker, are almost always absent on hBMSCs (Pittenger et al. 1999; Colter et al. 2001), whereas they are present on murine (Baddoo et al. 2003). In particular, some antigens have had particular relevance and have been used for positive selection of BMSCs, such as Stro-1 (Simmons et al. 1991), CD105 and CD73 (Haynesworth et al. 1992), CD271 (Quirici et al. 2002), SSEA-1 and SSEA-4 (Anjos-Afonso et al. 2007; Gang et al. 2007) and more recently, CD146, which is the only one identified on BMSCs *in vitro* and *in vivo* (Sacchetti et al. 2007).

Stro-1 is by far the best-known BMSC marker (Simmons et al. 1991). The cell population negative for Stro-1 is not capable of forming colonies, whereas Stro-1- positive cells can become HSC-supporting fibroblasts, smooth muscle cells, adipocytes, osteoblasts, and chondrocytes (Gronthos et al. 1994). The functional description of the tri-lineage differentiation potential of BMSCs was provided by Pittenger and colleagues (Pittenger et al., 1991).

5.1.3 Self-renewal

Stem cells share the defining characteristics of self-renewal, which maintains or expands the stem-cell pool, and multi-lineage differentiation. These processes are influenced by the convergence of intrinsic cellular signals and extrinsic micro-environmental clues from the surrounding stem-cell niche (Watt et al. 2000). Embryonic stem (ES) cells maintain pluripotency by transcriptional factors such as Nanog, Oct-4 and Sox-2(Boiani et al. 2005). In the last few years several groups have shown the expression of Oct-4, Nanog and Sox2 in BMSCs (Jiang et al. 2002; Anjos-Afonso et al. 2007; Beltrami et al. 2007; Gonzalez et al. 2007; Greco et al. 2007; Kobayashi et al. 2008).

5.1.3.1 Oct4 Transcription factor

The octamer-bonding transcription factor 4 gene encodes a nuclear protein (Oct-4, also known as Pou5F1 and Oct3/4) expressed in embryonic stem cells and plays a critical role in maintaining pluripotency and self-renewal (Scholer et al. 1990). Several authors tried to identify the role of Oct-4 in BMSC's. Greco et al. demonstrated that Oct-4 provides certain regulatory circuitries of hBMSC and hESC specific genes and regulate BMSCs cell cycle progression (Greco et al. 2007). Moreover few recent papers dispute the role of Oct-4 as stemness marker and its expression in BMSCs. Liedtke et al. raise the issue of six known Oct-4 pseudogenes as a source of confusion in stem cell research (Liedtke et al. 2007; Liedtke et al. 2008). Later Kotoula et al. demonstrated that the second isoform of hOct-4, hOct-4B, which is related neither to stemness nor pluripotency, can account for much of misinterpretation of experimental data (Kotoula et al. 2008). Therafter Lengner et al. again demonstrated, specific inactivation of Oct-4 in bone marrow *i.e.* the absence of Oct-4 protein which does not interfere with BMSCs self-renewal or differentiation (Lengner et al. 2007).

5.1.3.2 Sox-2 Transcription factor

Sox-2 is a member of the HMG-domain DNA-binding-protein family that is implicated in the regulation of transcription and chromatin architecture (Pevny et al. 1997). Sox-2 forms a ternary complex with either Oct-4 or the ubiquitous Oct-1 protein on the enhancer DNA sequences of fibroblast growth factor 4 (Fgf-4). Recently the expression of Sox-2 has been also been reported in BMSCs (Beltrami et al. 2007; Gonzalez et al. 2007; Kobayashi et al. 2008). It has been observed that Sox-2-expressing BMSCs showed much higher expansion potential than control cells in both high and low serum conditions and higher osteogenic differentiation abilities (Go et al. 2008).

5.1.3.3 Nanog Transcription factor

Nanog is a transcription factor expressed in ES cells and is thought to be a key factor in maintaining pluripotency (Chambers et al. 2003; Mitsui et al. 2003). Similar to Oct-4, it has been proposed that Nanog might regulate differentiation

through the transcriptional repression of genes that promote differentiation (Mitsui et al. 2003). Very poor data are available about the role of Nanog for BMSC's, with few reports on Nanog expression in BMSCs (Jiang et al. 2002; Anjos-Afonso et al. 2007; Beltrami et al. 2007; Gonzalez et al. 2007; Greco et al. 2007; Kobayashi et al. 2008). More recently it has been demonstrated that Nanog-expressing BMSCs show much higher expansion potential, higher osteogenic differentiation abilities and higher chondrogenic differentiation abilities (Go et al. 2008; Liu et al. 2008).

5.1.4 Multilineage differentiation potential

It is always a difficult process to identify various specific signaling networks and 'master' regulatory genes that govern unique BMSC differentiation. During osteogenesis, multipotent BMSCs undergo asymmetric division and generate osteoprecursors, which then progress to form osteoprogenitors, preosteoblasts, functional osteoblasts and osteocytes. The bone morphogenic protein (BMPs) pathway has a well-known role in promoting osteogenesis (Chen et al. 2004). Chondrogenic differentiation of BMSCs in vitro mimics that of cartilage development in vivo. The transcription factor Sox-9 is considered the master gene of chondrogenesis and is responsible for the expression of extra cellular matrix (ECM) genes such as collagen types II and IX, aggrecan, biglycan, decorin, and cartilage oligomeric matrix protein (Goldring et al. 2006).

While in adipogenesis, the nuclear hormone receptor peroxisome proliferators activated receptor γ (PPARγ) is a critical adipogenic regulator promoting BMSC adipogenesis (Rosen et al. 2006).

5.1.5 Therapeutic Uses for MSCs

Several possible therapeutic functions exist for MSCs. First, they may directly participate in cell repopulation via expansion and differentiation by directing the differentiation of a patient's own stem and progenitor cells into the depleted cell types (Burroughs et al., 1994). A second possible role for MSCs is as a carrier for delivering a therapeutic transgene (Cantz et al., 2008). The dysfunctional allele(s) that may be responsible for a disease can be circumvented by the insertion of a functional gene into the patient's stem cells, followed by

transplantation into an appropriate tissue where they can propagate and produce the therapeutic gene product(s) (Caplan, 1991). A third possibility for MSCs in tissue repair is an indirect role in support of other cell types (Chen et al., 2005). MSCs supply physical support and cyto-chemical direction by producing growth factors and cytokines, likely providing the essential cues for cell proliferation and differentiation (Bianco et al., 2001). A more recent hypothesis suggests a similar role for those MSCs found to reside in other tissues undergoing repair and re-growth (Choi et al., 2009).

5.1.6 BMSC in diabetes and pancreas regeneration

Implication of islet neogenesis is targeting to diabetic therapeutics where scientist are indulge in generating new islet required for transplantation. For therapeutic purpose, whole islet therapy is considered a better option than isolated β-cells, because latter lack normal physiological control over glucose homeostasis due to absence of glucagon producing α-cells (Halban et al., 2010; Levetan, 2010). The islet transplantation therapy for type I diabetes patients pioneered in 2000 by the Edmonton group Shapiro et al. (Shapiro et al., 2000) demonstrated its potential with remarkable freedom from exogenous insulin-dependence for majority of patients for up to 1-2 years. Although there are limitations of this approach because more than 50% of the patients were back on insulin in five years, their need for much lower doses of insulin indicated some continuing function of residual grafts (Ryan et al., 2005).

Thus islet therapy has a great promise but there is a shortage of cadaveric islets. Hence, methods have been developed to create in vitro large quantity of glucose-responsive functional islets from pancreatic stem or precursor cells under well-defined stimulus conditions (Phadnis et al., ; Ramiya et al., 2000) or more famously from human embryonic stem cells by “Novocell protocol” of sequential introduction of specific differentiating agents to direct the stem cells towards hormone expressing group of endocrine cells or “islets” (D'Amour et al., 2006). It is ideal that islets created *in- vitro* have all the cells present in natural islets, but at the least they must have a majority of insulin-releasing β-cells mixed with small fraction of α, γ, and δ cells so that the “new islet cluster” can

independently maintain glucose homeostasis by tightly regulating the release of insulin in response to glucose.

BMSCs offer an excellent source of stem cells and provide clinical option for pancreas regeneration or islet differentiation and transplantation for curative and definitive treatment of insulin-dependent diabetes. By far now, bone marrow mesenchymal stem cells (BMSCs) have been shown to ameliorate diabetes in animal models. The mechanism, however, remains largely unknown. BMSC can be obtained with relative ease from each patient, allowing potential circumvention of allograft rejection. Few earlier studies have shown that mouse BMSCs spontaneously differentiate into endocrine pancreas cells *in-vivo* (Ianus et al., 2003), but still a major unanswered question remains, whether BMSCs are able to differentiate into β-cells *in-vivo*, or they are able to mediate recovery and/or regeneration of endogenous β-cells by facilitating growth and differentiation of other tissue specific progenitors.

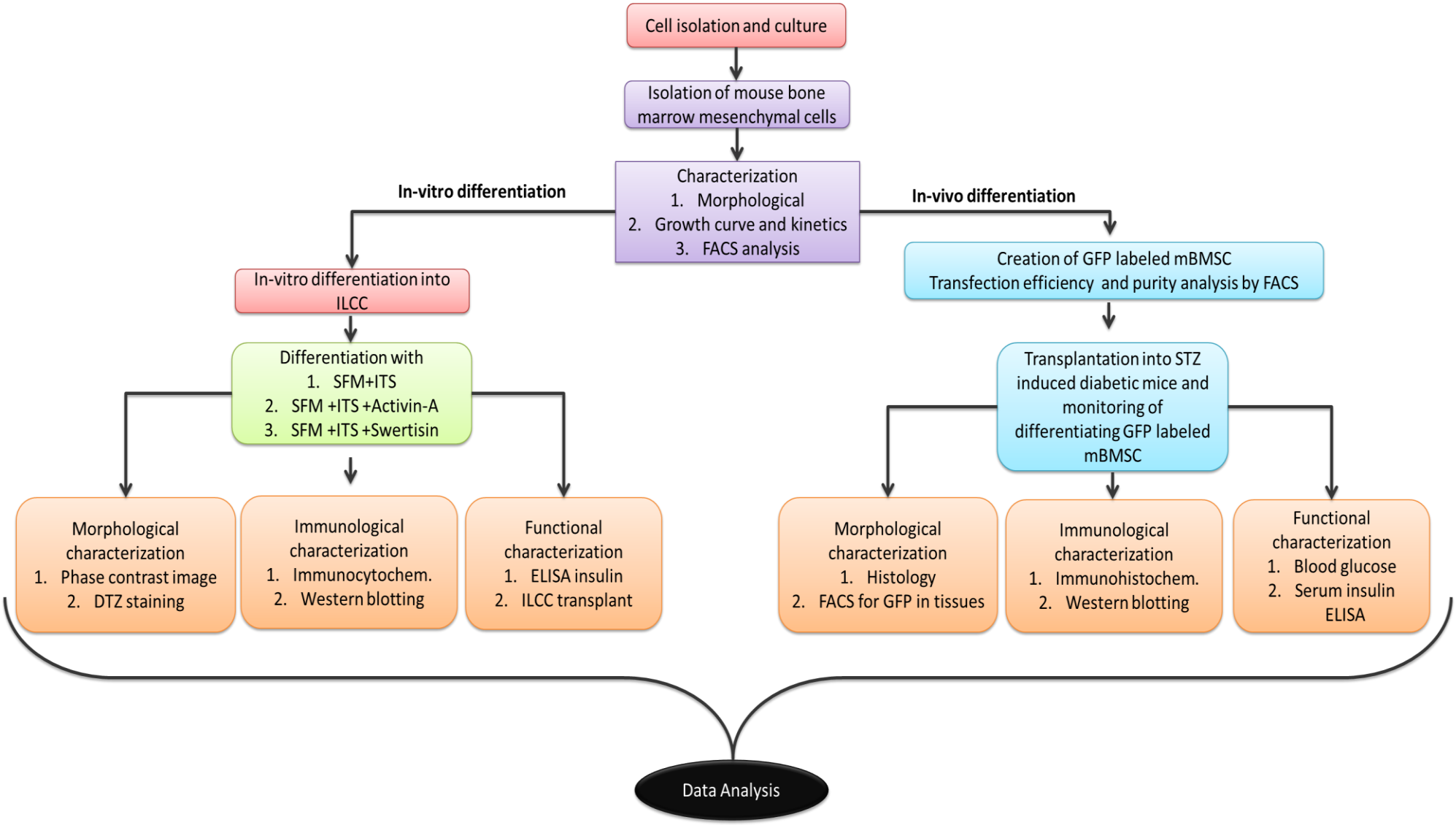
In recent times couple of evidences are there which suggested BMSCs differentiation into insulin-producing cells *in vitro* (Choi et al., 2005; Phadnis et al., 2010; Xie et al., 2009) with the capacity to engraft and improve islet function in animal models of diabetes (Mathews et al., 2004; Lee et al., 2006; Sordi and Piemonti, 2010). However, few studies have assessed the therapeutic effects resulting from the *in-vivo* differentiation of BMSCs into pancreatic parenchyma and mesenchymal cells after pancreatic injury. Hess et al. reported a lowering of blood glucose levels within a week after intravenous infusion of green fluorescence protein (GFP)-tagged allogenic bone marrow into STZ-induced diabetic mice (Hess et al., 2003). Only a low frequency of donor-derived insulin-producing cells was found in the host; islets were found to be largely replenished by endogenous cells. The authors suggested that donor GFP-positive bone marrow stem cells "only" guided the endogenous growth of islets. Ianus et al. showed that bone marrow-derived cells can differentiate into insulin-producing islet cells with glucose-dependent and incretin-enhanced insulin secretion when transplanted into lethally irradiated mice (Ianus et al., 2003). However, other groups working on bone marrow stem cells have not

observed such an anti-diabetic effect after bone marrow infusion (Beilhack et al., 2003; Choi et al., 2003; Lechner et al., 2004).

An approach for *in-vivo* endogenous differentiation of any stem cell type is the actual therapeutic intervention for any diabetic patient in treatment. Drugs or agents which can be capable of enforcing organ stem cells or circulating stem cells like bone marrow mesenchymal stem cells to form functional islets into diabetic individuals endogenously will promise an attractive option. Aiming this, we attempted BMSC differentiation into islets both under in vitro and in vivo condition with swertisin as positive differentiating agent.

The Experimental design of work done under this chapter is described in figure below

Experimental Design



5.2 Material and Methods:

5.2.1 Chemicals and cell culture media: All chemicals, media and antibodies used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA. Molecular biology reagents and cDNA and PCR kits were procured from Fermentas Inc. All plasticwares were purchased from Nunc Inc. (Naperville, IL, USA).

5.2.2 Animals: Male *Balb/c* mice, 3-4 weeks old, weighing around 25-30 grams, were used for transplantation and bone marrow cell isolation experiments. All animal experiments were performed in accordance with our institutional ''Ethical Committee for Animal Experiments'' and CPCSEA guidelines and regulations.

5.2.3 Isolation of marrow derived mesenchymal stem cells: Bone marrow derived mononuclear cells were isolated from 4 week old *Balb/c* mice according to Zhu et al. (Hsiao et al., 2010; Zhu et al., 2010). Mice were sacrificed after proper anesthesia. Briefly, tibia and femur bones were dissected out under sterile conditions. The metaphyseal ends of the bones were cut, and the marrow plugs were flushed out by passing L-DMEM (Sigma) through a needle inserted into one end of the bone. Bone marrow cells from mouse were collected, big tissue debris removed with help of a cell strainer and then seeded in low glucose RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) for 2 days.

5.2.4 Purification and propagation of mesenchymal stem cells: Mesenchymal stem cells (MSCs) were purified as described earlier (Hsiao et al., 2010; Zhu et al., 2010). Briefly, the cultured cells were seeded at a density of ten thousand cells/ml in complete medium (high glucose DMEM supplemented with 10% FBS (Sigma), penicillin (100 IU/ml), streptomycin (100 IU/ml) (MP Biological Inc. USA)) in polystyrene T25 culture flasks and incubated in a humidified 5% CO2 incubator at 37°C for 48 hrs. Loosely attached hematopoietic cells were discarded upon trypsinizing adherent cells for 5 min. Cells, that remain adhered, were trypsinized for another 7 min (Hsiao et al., 2010) and then cultured in the complete medium for 10 days. The cell media was replaced with fresh one every

3rd day. The cells were trypsinized using Trypsin-EDTA (Sigma-Aldrich, USA) and passaged in T75 flasks until second generation became 70-90% confluent. Cultured mBMSC were then characterized and used for comparative study with NIH3T3 after ensuring their pluripotency.

5.2.5 Cell count and growth curve study: Cell count and growth curve and doubling time of the purified mBMSC were performed as explained earlier in chapter-4, section 4a.2.4 and data was analyzed with mean±SEM of observations from three independent experiments.

5.2.6 Characterization of mBMSC by Flowcytometry, immunocytochemistry and western blot: For flowcytometry analysis again the cells were stained in similar process explained earlier in chapter-4, section 4a.2.13. Unlabeled mBMSC, GFP labeled mBMSC and single cell suspension from both pancreatic (collagenase digested; 1mg/ml) and liver tissues (with trypsin; 0.05%) was analysed for various BMSC markers on FACS ARIA III (BD biosciences).

5.2.7 Differentiation of mBMSC into ILCC (*in vitro*): mBMSC were induced for the formation of ILCC using activin-a at 2ng/ml and swertisin at 15ug/ml dose. The method for islet differentiaton was followed exactly same as mentioned earlier in chapter-3, section 3.2.4.

5.2.8 Transfection and GFP labeling of mBMSC: Before carrying out *in-vivo* transplantation experiment for insulin cell endogenous differentiation, mBMSC were transfected with eGFP harboring piggyback plasmid pPBGFP and pCYL43-PBase (gifted from Sanger Institute, UK) map shown in fig 5.2 for monitoring the niche and endogenous differentiation into new islet cell. Breifly, one hundred thousand cells were transfected with 1µg pPBGFP along with 2 µg of pCYL43-PBase vector DNA using lipofectamine 2000 (Invitrogen) as a transfecting agent in 1:3 volume ratio in 3.5 cm^2 dish, according to manufacturer's protocol. Following the transfection, stably GFP expressing clones were selected by growing them in media containing puromycin at 300µg/ml concentration for first 2 days and thereafter in 900µg/ml for following 7 days. Clones were purified from isolated colonies using clonal discs, scaled up

and analyzed by FACS for transfection efficiency and purity before transplanting intravenously into STZ induced diabetic *balb/c* mice.

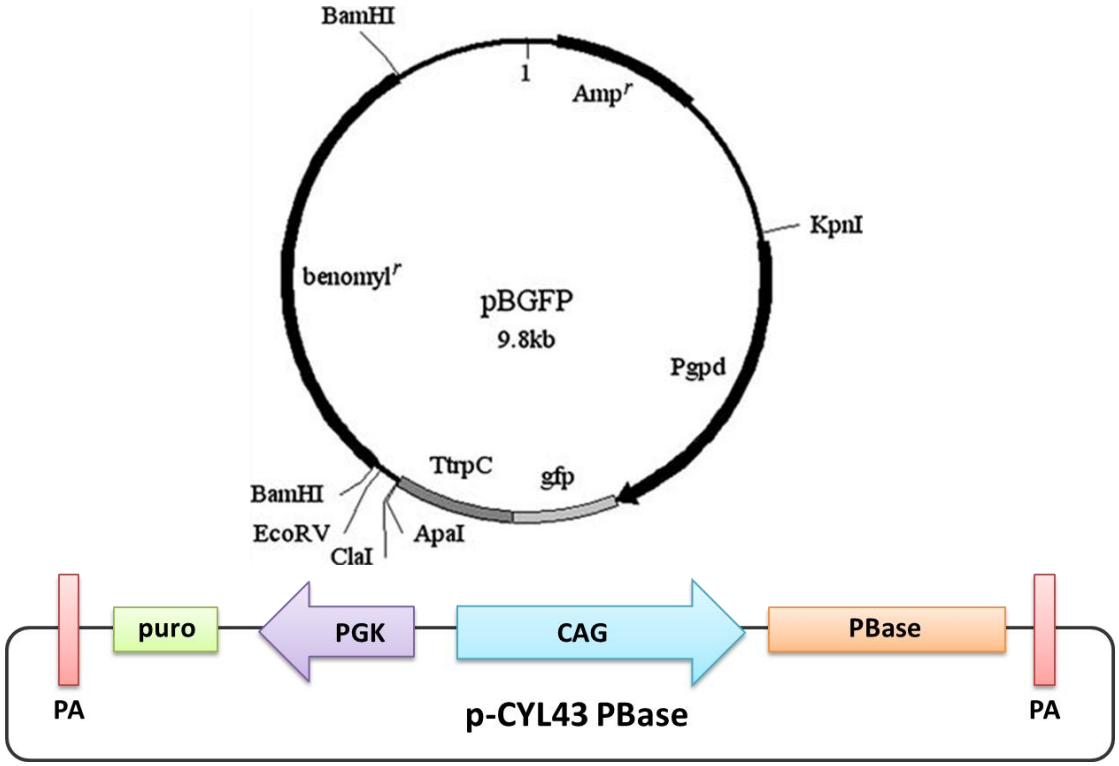


Fig 5.2 showing map of pPBGFP and pCyL43 Pbase, providing combination of a non-integrating and constitutively expressing GFP and piggyBac transposase (PBase).

5.2.9 Diabetic Induction using Streptozotocin (STZ) in *balb/c* mice:

For inducing diabetes, mice were fasted overnight prior to STZ (70 mg/kg bwt) injection. STZ was freshly prepared with Na-Citrate buffer at pH 4.5 as vehicle and given continuously for five days. Blood glucose was measured every week till a month to ensure the diabetic condition stability in injected mice.

5.2.10 Transplantation of GFP labeled mBMSC: One million GFP^{+ve} mBMS cells were suspended in sterile saline and incubated with activin-A (2.5µg/kg.b.wt) and Swertisin (2.5µg/kg.b.wt) for 30 minutes to initiate endocrine signaling and thereafter injected intravenously into STZ induced diabetic *balb/c* mice in respective groups. A subset of mice (mBMSC alone group) received only GFP labeled mBMS cells without any inducing agent, served as control. The cells were

transplanted only once on the 15th day of STZ treatment, whereas the dosing of both activin A and swertisin continued from day 14 till day 29 (i.e. 15 days).

5.2.11 Characterization of ILCC (*in vitro*) and pancreatic tissue (*in vivo*) by flowcytometry, immunohistochemistry and western blot

For flowcytometry analysis single cell suspension from both pancreatic (collagenase digested; 1mg/ml) and liver tissues (with trypsin; 0.05%) were analysed for various BMSC markers on FACS ARIA III (BD biosciences). mBMSC and ILCC clusters obtained after differentiation were imaged by confocal microscopy for endocrine markers as described in chapter-4, section 4a.2.10 (for stem cells features) and chapter-4; section 4b.2.8. Later the pancreatic tissues obtained from the eGFP labelled mBMSC transplantation experiment were subjected to immunohistologial staining for monitoring insulin and GFP co-expressing cells. Staining for the section was again performed as described in chapter-4, section 4b.2.14. For proteomic characterization of BMSC cells and islet differentiation pathway proteins in pancreatic tissues of eGFP-MBMSC transplanted animals were probed for various markers after bradford protein quantification using chemiluminiscence based western blot procedure in a similar manner as described earlier in chapter-4, section 4.2.14.

5.2.12 Functional Characterization: For the estimation of insulin in *in-vitro* mBMSC differentiated ILCC (SFM, Activin A and Swertisin) challenged with varying glucose concentration and mice serum samples collected at various time point from mBMSC transplanted animals *(in-vivo)* were analysed using Insulin ELISA kit from Mercodia according to the mentioned protocol in chapter-4; section 4b.2.11. Blood glucose was also monitored in transplanted animals before and at the end of experiment.

5.3 Results: *In-vitro* differentiation of mBMSC

5.3.1 Bone Marrow Mesenchymal Stem Cell isolation provided purified Mesenchymal Stem Cells (MSC)

We successfully isolated mBMSC from bone marrow of mice employing surgical procedure used by Hasio and Cheng et.al. (Hsiao et al., 2010). Homogeneous

population of mBMSC, without hematopoietic or macrophage contaminations was achieved by differential trypsinization technique; one of the major steps in isolation and purification procedure, and as a result of it, long term cultures of mBMSC could be established. Number of nucleated marrow cells obtained after isolation was around $3.26 \pm 0.31 \times 10^7$ per mouse; comparable to that reported by Hasio and Cheng et.al. 2010 (Awad et al., 2004). One of the most important factors in determining the establishment of a long term culture of mBMSC was the cell density in the starting culture. mBMSC were seeded at a density of 0.2 million nucleated cells/cm^2 and then allowed to attach to the surface of the plates for two days in low glucose RPMI-1640 medium supplemented with 10% FBS which prevented the adherence of contaminating undesired cells. Non-adherent cells were easily removed when medium was changed on day 3 and confluent colonies were observed by day 8 (P#1). Once passaged, in addition to mBMSC, a large number of contaminating small spherical cells, called as lymphohaematopoietic cells, engaged on mBMSC colonies as a second layer were also observed (Fig 5.3 P#3-P#5). Purification of mBMSC was achieved with property that they can be removed from plastic surface within 5 min in response to trypsin, while hematopoietic ones takes 8 min. However, trypsin-lifted cells still contained few hematopoietic cells. When seeded at density of fifty thousand cells/cm^2 in the next passage; these hematopoietic cells did not adhere to the plastic surface and were eradicated from the culture upon subsequent passaging. (Fig 5.3: P#3 to P#10 for gradual purification of mBMSC). The differential trypsinization steps were repeatedly done till passage#8. To ensure pure population of mBMSC different characterization and comparative studies were carried out on cells obtained after P#10.

5.3.2 Growth curve; Cumulative doubling time measurement of mBMSC

Healthy cells from sub-confluent cultures of both cell types were plated in 24-well plates and used for determining the population doubling potential, progression and proliferative activity. Cumulative population doublings were calculated by considering initial number of mBMSC cells seeded at 0 hrs and number of cells harvested at each destined time point respectively without passaging. These observations provide a theoretical growth curve that is directly

proportional to the cell number. With the help of curve, doubling time was found to be 21.87±2.39 hrs for mBMSC. Log scale graph gave a trendline that agreed with linear curve equation i.e. y=mx+c. , with R^2 value) of 8.2.

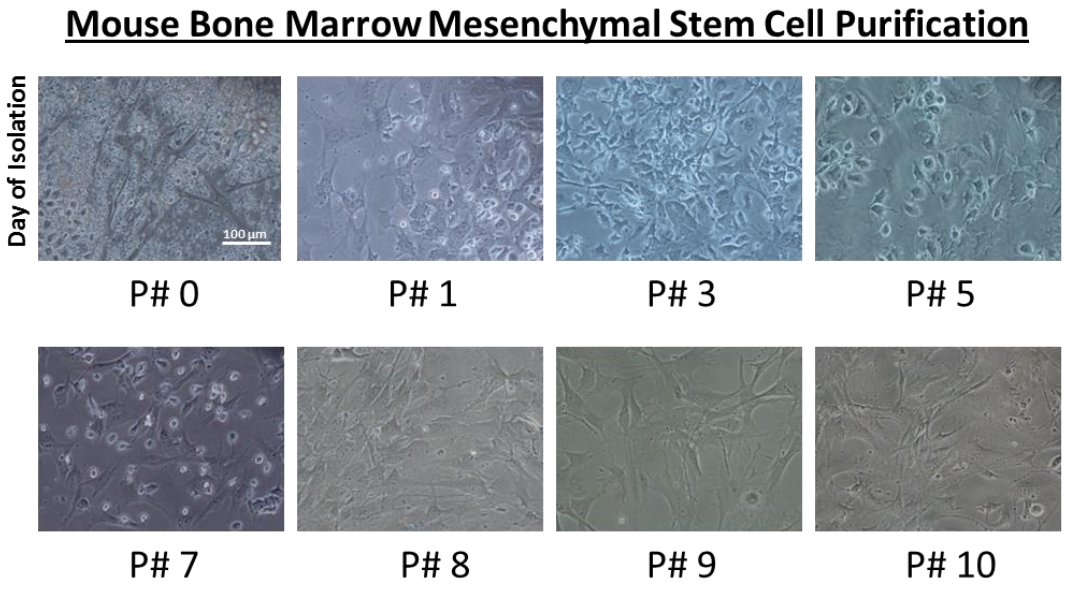


Fig 5.3 showing phase contrast images demonstrate step-wise purification of mBMSC from P# 0 to P# 10. Day if cell isolation represents heterogeneous population of cells while other subsequent images represent step wise purification and gradual depletion of hematopoietic from cultures of mesenchymal cells (passage 1 to passage 9). Last Phase contrast photomicrograph at passage#10 represents purified population of bone marrow mesenchymal cells.

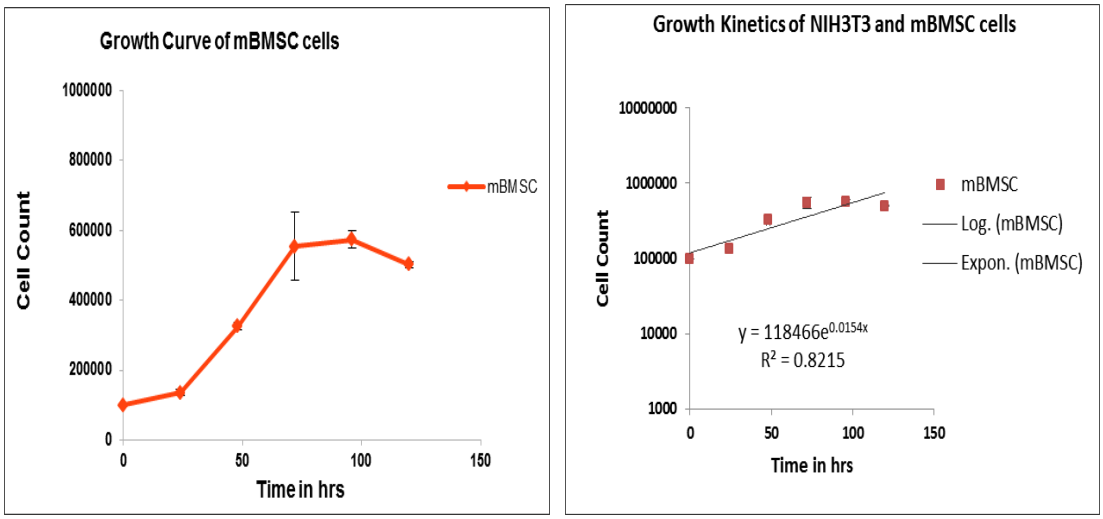


Fig 5.4 shows growth curves and kinetics of mBMSC: figure represents sigmoid growth pattern and proliferation of mBMSC. Data are represented as mean ±SEM of four independent experiments. N=4.

5.3.3 Immunophenotyping of mBMSC using flowcytometry

mBMSC cells were analyzed for specific mesenchymal stem cells surface markers growing in log phase culture. mBMSC cells were probed with CD45, CD34, CD44, CD117, CD49b, vimentin, SMA, Nestin and PDX-1. We found that mBMSC cells at passage no-14 showed immuno-positivity for CD 44^{+ve} (99%), CD 34^{+ve} (91.4%), CD 90^{+ve} (90%), CD 117^{+ve} (4.23%), $nestin^{+ve}$ (65.8%), $vimentin^{+ve}$ (97.1%), SMA^{+ve} (98.4%), $PDX\text{-}1^{+ve}$ (15%) while negative for CD45 (3%), CD49b (1%).

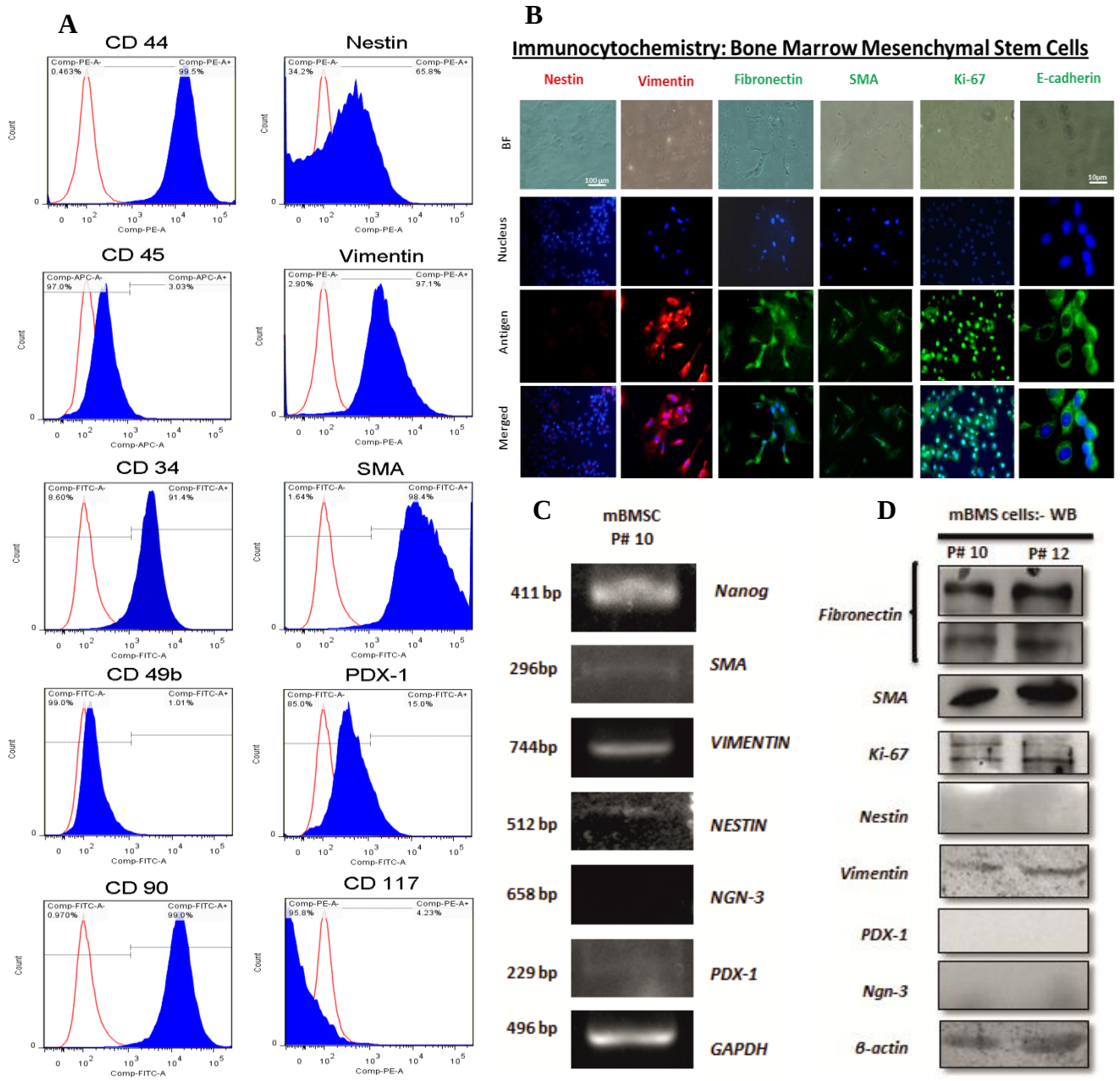


Fig 5.5 Characterization of mBMSC by FACS, ICC and western blot. Fig 5.5a shows FACS profile of key BMSC surface markers. Fig 5.5b shows immunocytochemical images for various stem/progenitor markers. Fig 5.5c represents RT PCR profile key stemness markers. Fig 5.5d demonstrates western blot profile of various stemness proteins.

5.3.4 Four stage differentiation of mBMS cells into insulin producing cells

To drive differentiation of bone marrow stem cells towards pancreatic lineage insulin producing cells, we developed a culture procedure comprised of four steps as described earlier in chapet-3. In the first step the mBMSC cells were cultured for 2 days prior differentiation followed by 10 day differentiation mediated by activin-a as positive control and swertisin compound. mBMS cells were subjected to SFM alone; SFM with ITS; SFM with activin-A and SFM with swertisin for 8 days. We observed effective cell clustering started on day 2 translated aggregates by 3rd day, demonstrating zone of activation (fig 5.6). Same clusters after 8th day induction turned into mature islet like cell clusters, showed presence of insulin granule with DTZ staining on 10th day. Both activin and swertisin exhibited intense deep crimson red or brownish staining whereas those of SFM alone showed less staining (fig 5.6).

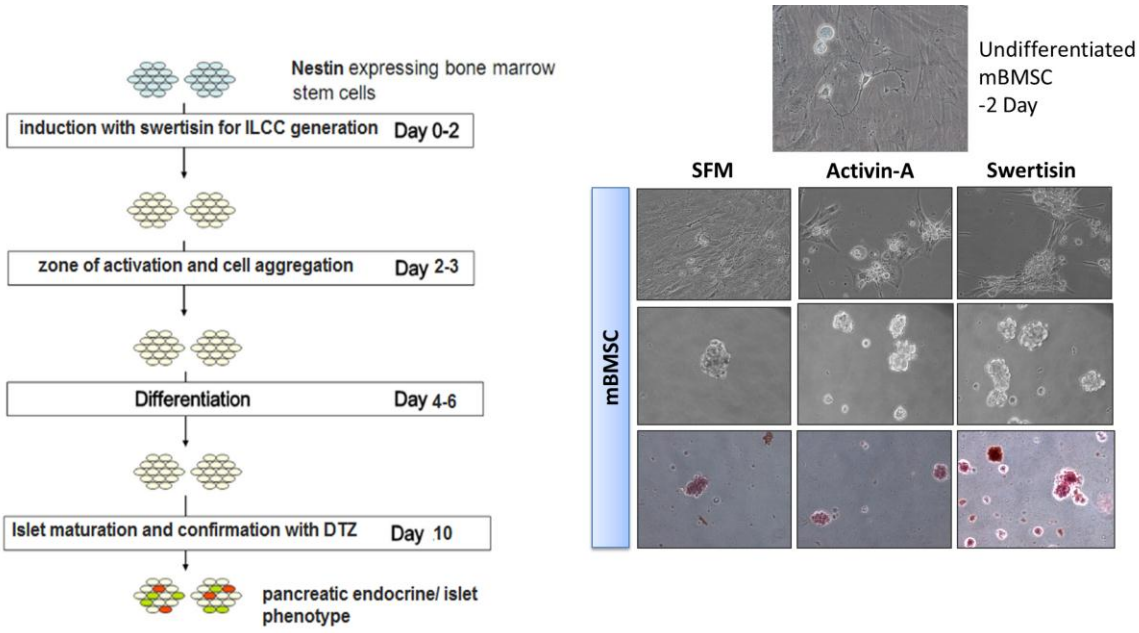


Fig 5.6: Cluster formation of insulin-positive islet-like clusters from mBMS cells. mBMSC were maintained for 10 days in serum-free medium (SFM) with or without insulin-transferrin and selenium (ITS) and/or Activin A, and swertisin (SGL-1) and stained with DTZ on 10th day of differentiation.

5.3.5 Immunocytochemical characterization of swertisin mediated ILCC

For determining the islet phenotype in neoislets, ILCC generated with swertisin induction were examined using immunocytochemical study, confirmed the presence of insulin (green), C-peptide (green), glucagon (red), somatostatin

(red) along with other key important transcription factors (nestin, pdx-1, neuroD and ngn-3) in swertisin mediated clusters (fig 5.7). At this time point (10 day), we were able to identify pancreatic master gene (PDX-1), together with other genes important in the early pancreatic development (nestin, ngn-3, neuroD). Many cells were positive for PDX1 along with nestin as assayed by immunocytochemistry (Fig 5.7). Subsequently, the neuroD positive cell population along with ngn-3 positive cells was also seen (Fig 5.7).

5.3.6 *In-vitro* mechanistic action of swertisin for islet differentiation with mBMS cells

In order to understand the mechanism of action of swertisin for islet differentiation from mBMSC, we observed key regulatory proteins implicated in TGF-β ligand Activin-A mediated signaling. When mBMSC were examined in time dependent manner (0-8 days), we found islet differentiation not proceeding by activin-a mediated pathway. In all groups (SFM alone, Activin-A and Swertisin), there was no up regulation of Ngn-3, on other side phospho-smad2 fails to express at all. Smad-7 expression was also found to be uniform from day 2 till day 8, except elevated levels of nestin expression which was observed high from day 2 till day 7 in swertisin clusters while modestly expressed in Activin-A. Further all groups ILCC did not showed PDX-1 expression as well.

5.3.7 *In-vitro* insulin release from mBMSC ILCC in response to a glucose challenge

In order to check the functional maturity of swertisin induced ILCC from mBMS cells, *in-vitro* glucose challenge induced insulin release was monitored (Fig.-5.9). Mean insulin secretion by 50 clusters (swertisin mediated) was 1.05 ± 0.009µg/mL in response to 5.5 mM glucose, 2.6 ± 0.03 µg/mL in response to 20 mM glucose concentration, which is significantly higher than SFM alone (0.05 ± 0.002 µg/mL in 5.5 mM, 0.07 ± 0.001 µg/mL in response to 20 mM glucose concentration) and activin-A (0.15 ± 0.003 µg/mL in 5.5 mM, 0.76 ± 0.001 µg/mL in response to 20 mM glucose concentration). Swertisin differentiated ILCC showed 3.7 fold increase in insulin release compared to activin-a with 20 mM glucose challenge. Result expressed as mean of three observations ($P < 0.01$).

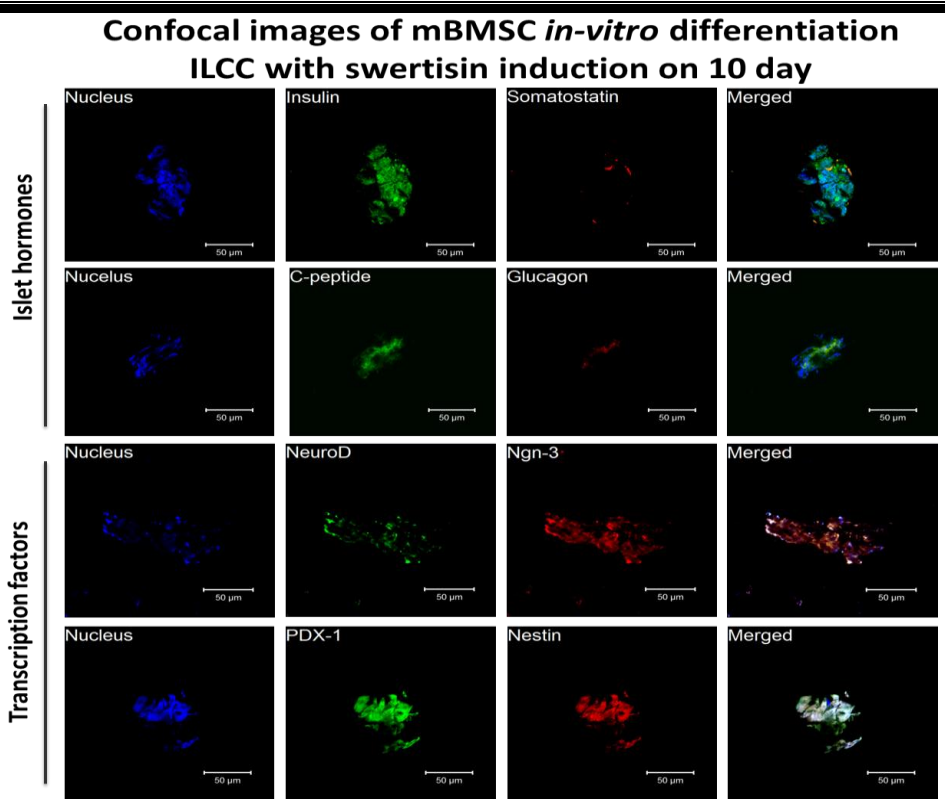


Fig 5.7 **Confocal images of various islet markers in swertisin induced ILCC: Fig 4.22 depicts intense positive staining for Insulin (red color), C-peptide (Green color), Glucagon (Red color) and Somatostatin (pink color). Nucleus of all the clusters was stained with DAPI shown in blue color.**

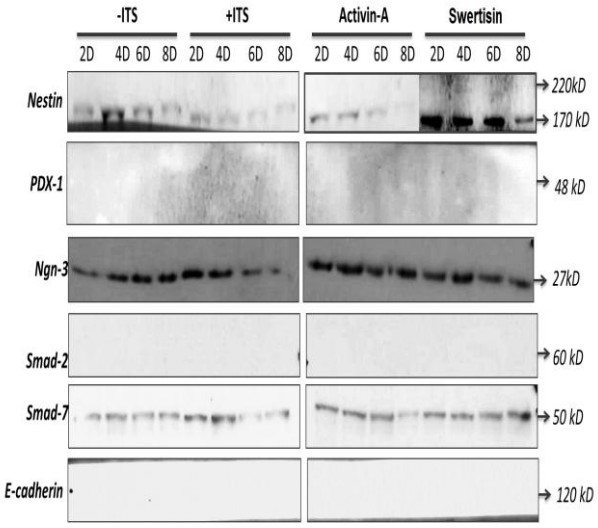
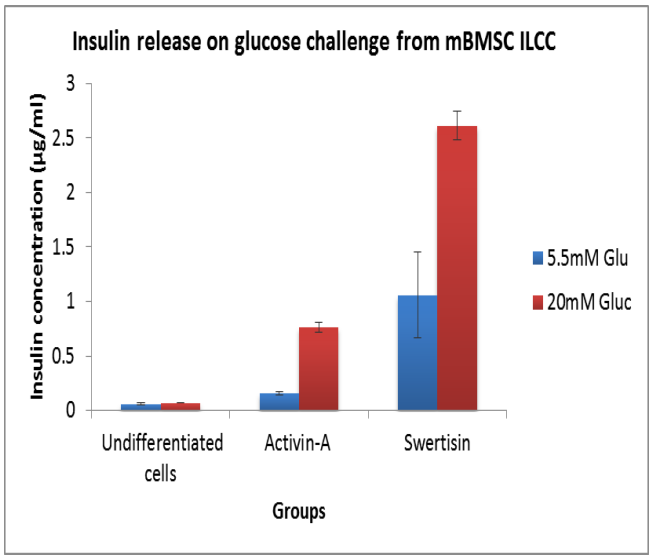


Fig 5.8: Time dependent Western blot study of key stem/progenitor and islet differentiation pathway protein during differentiation protocol ranging from 0 to 8 days.

Fig 5.9 represents in-vitro insulin release from undifferentiated mBMSC, activin-A and swertisin induced ILCC on 10th day of differentiation. Results are expressed as mean ±SEM n=3 *** p< 0.001 vs undifferentiated and activin-A.



5.3.8 STZ mediated diabetes induction and Islet Transplantation

To check the functionality of swertisin mediated mBMSC-ILCC *in-vivo*, STZ induced diabetic mice model were used. Multiple dose of STZ in balb/c mice produced effective TD1 mice with greatly elevated blood glucose levels (Fig. 5.10). The *in-vivo* functional integration of swertisin induced ILCC was monitored from transplanted renal grafts. All the mice received renal grafts showed significant reduction in FBS within one week of transplantation and presence of insulin and glucoagon hormones in recovered grafts after 7 weeks (fig 5.11).

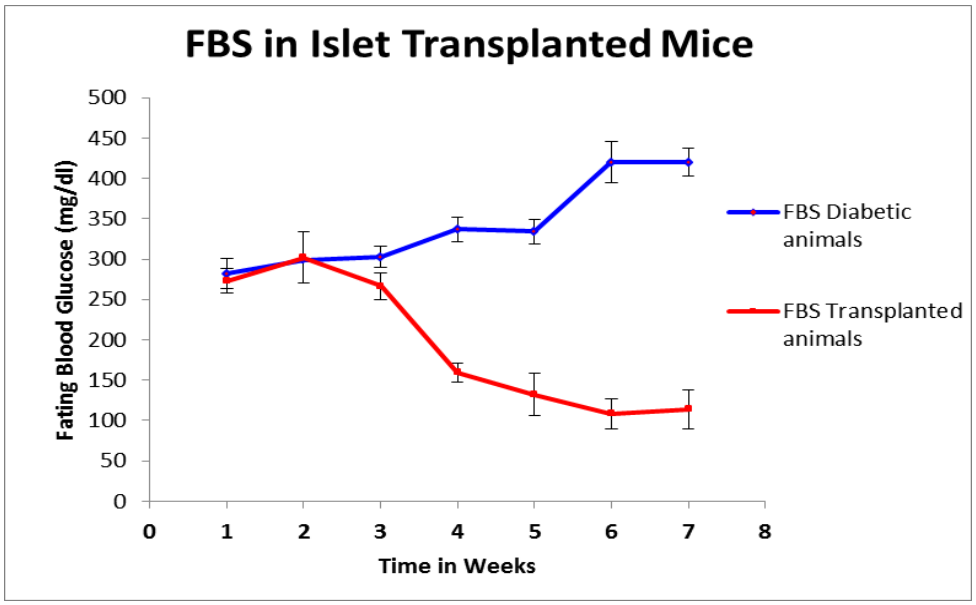


Fig 5.10: plasma blood glucose of diabetic control and swertisin mediated mBMSC ILCC transplanted balb/c mice in time dependent manner from wee 1-7. Results are expressed as mean ± SEM of 5 transplanted animals.

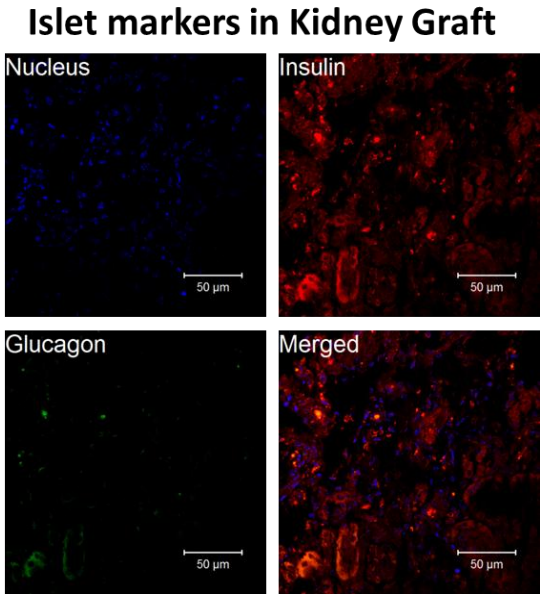


Fig 5.11 shows immuno-histological image of swertisin mediated ILCC transplanted in kidney capsule. Image show presence of insulin (red) glucagon (green) protein in pancreas tissue with nucleus by DAPI (Blue).

5.3.9 Creation and characterization of GFP labeled mBMS cells

In quest of investigating endogenous differentiation of mBMS cells, fully characterized mBMSC were labeled with GFP protein by transfecting with permanently genome integrative plasmid piggybac. Piggybac plasmid works by transposes enzyme and transposomal elements. GFP gene cloned between the two transposomal elements was permanently getting incorporated into host cell genome with help of transposes enzyme catalytic activity. We successfully created GFP labeled mBMSC and confirmed the transfection efficiency and purity by FACS analysis. Transfected cells showed more than 95% GFP positive cells maintained on antibiotic selection medium. Fluorescent imaging of these cells also showed presence of green fluorescing GFP protein with huge efficiency (fig 5.12).

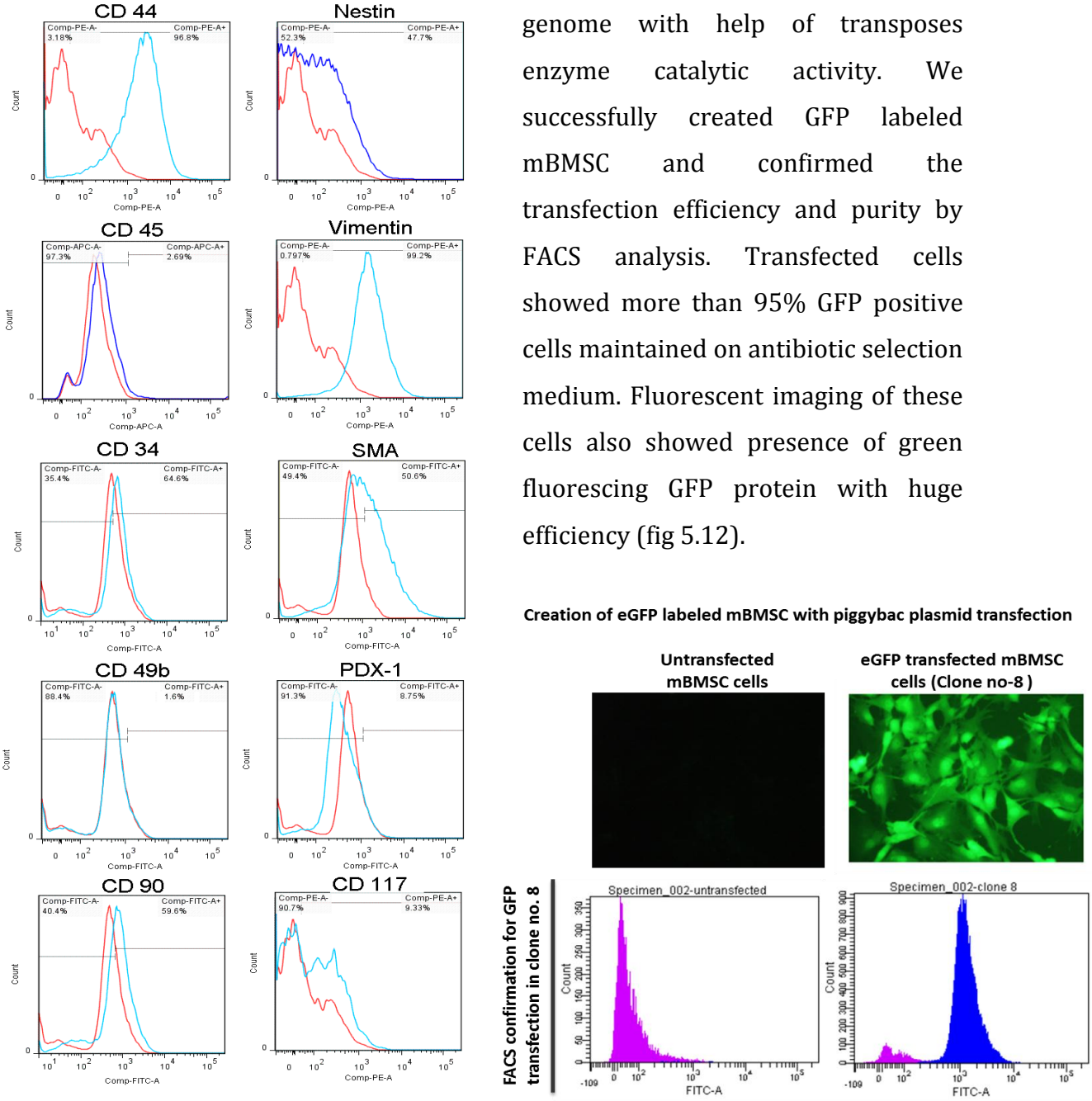


Fig 5.12 representing creation of eGFP labeled mBMSC cells using piggybac vector transfection. Clone no-8 shows GFP (green) protein in fluorescent image and purity of transfected clone in FACS profile (blue peak).

After successfully integrating GFP protein, we then checked the key stemness related markers in GFP labeled transfected BMSC clone, in order to ensure the integrity of stemness property after transfection. We found these GFP^{+ve} mBMSC cells positive for all mesenchymal stem cells related CD markers. CD 44^{+ve} (96.8%), CD 34^{+ve} (64.6%), CD 90^{+ve} (59.6%), CD 117^{+ve} (9.33%), nestin^{+ve} (47.7%), vimentin^{+ve} (99.2%), SMA^{+ve} (50.6%), PDX-1^{+ve} (8.75%) while negative for CD45 (2.65%), CD49b (1.6%) (fig 5.12).

5.3.10 Monitoring of GFP^{+ve} mBMSC transplanted cells into STZ induced diabetic mice for endogenous islet cell differentiation

For investigating homing of GFP labeled mBMSC cells after transplantation and treatment of 15 days with activin-a and swertisin, three groups of diabetic mice (mBMSC-GFP alone, mBMSC-GFP with activin-A and mBMSC-GFP with swertisin) received mBMSC-GFP cells intravenously, while the fourth group served as diabetic control group. After 30 days of monitoring, percentage population of GFP^{+ve} cells in both pancreas and live tissues was analyzed. In our study we observed that diabetic control mice and those which received only mBMSC-GFP, showed very less GFP^{+ve} population (2.6% diabetic control; 1.6% mBMSC alone) which could be with little bit of RBC contaminating cell signals (as hemoglobin also give signal overlapping with GFP). Whereas mice received both GFP labeled cells with activin and swertisin showed high percentage of GFP^{+ve} infiltrating cells (5.7% mBMSC with activin-A; 12% mBMSc with swertisin). Further to identify the phenotype of these GFP^{+ve} cells, CD44 was probed along with GFP and found that diabetic control and mBMSC alone did not showed any significant number of GFP with CD44^{+ve} duel positive population (0.1% diabetic control and 1% mBMSC). However increase CD44 percent cells in mBMSC transplant pancreatic tissue signified that mBSMC tends to move towards pancreas in response to STZ insult. Further a significant increase in CD44^{+ve} cells was observed with Swertisin treated animals (3.1 %), while those of activin-A treated showed only 1.2%. An interesting thing about this is all CD44 positive cells found within the pancreatic tissues was not GFP positive, which showed endogenous differentiation of GFP labeled mBMSC in both activin and swertisin treated animals (fig 5.13).

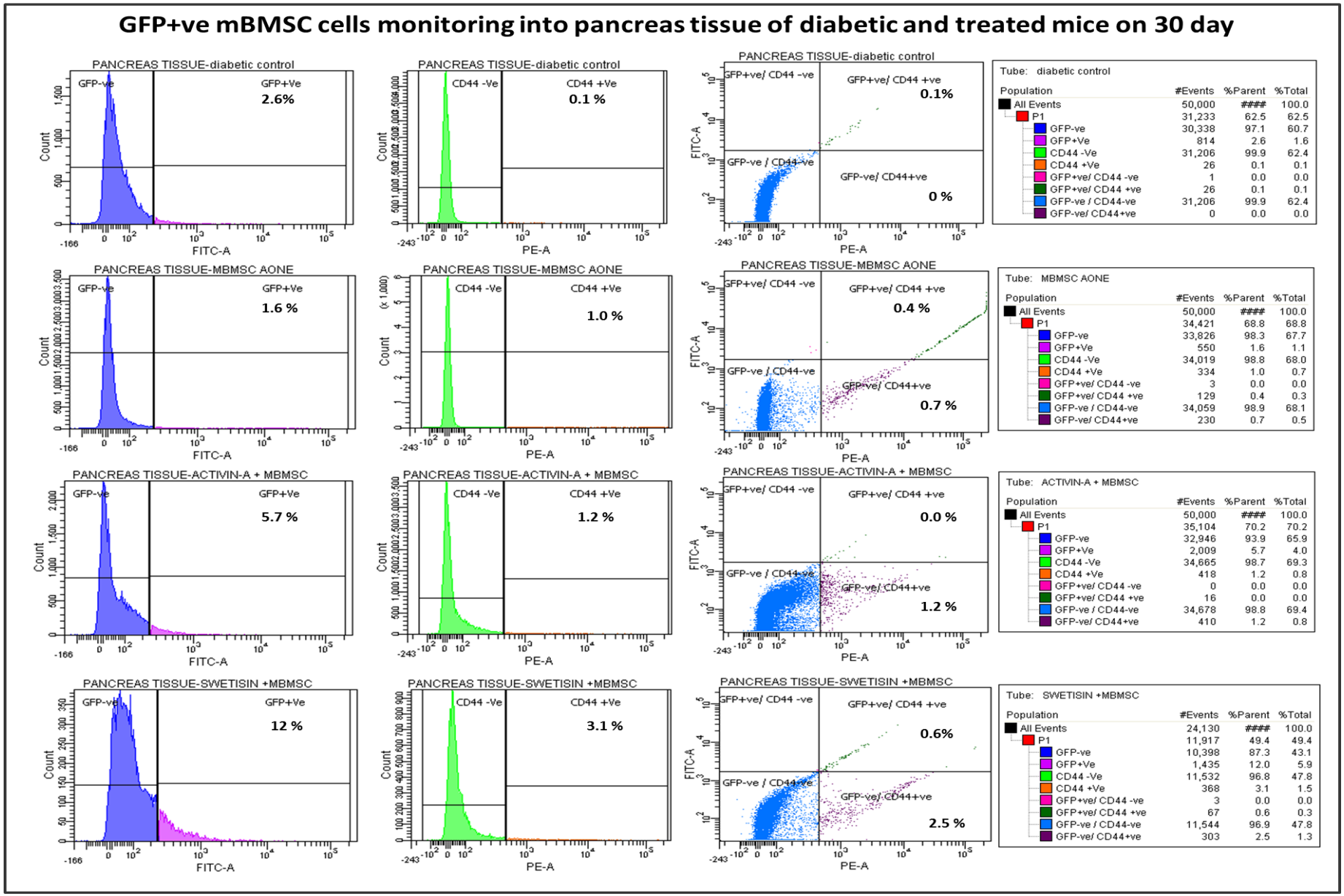


Fig 5.13 show flowcytometry data of GFP labeled mBMSC cells in pancreas tissues from mBMSC alone, activin and swertisin treated animals. Histograms with blue and green peak show non GFP and CD44-ve cells in pancreas, While pink and orange peek denotes GFP+ with CD44+ve mBMSC cells. Dot plots represents identification of GFP and CD44 duel population.

Further the localization of mBMSC cells except pancreas was also observed in liver cells to confirm the migration of mBMSC-GFP cells in any other tissue except pancreas. Results of liver cells Flow-Cytometry analysis revealed no significant homing of GFP labeled BMSC cells in liver tissue in all mice groups (fig 5.14).

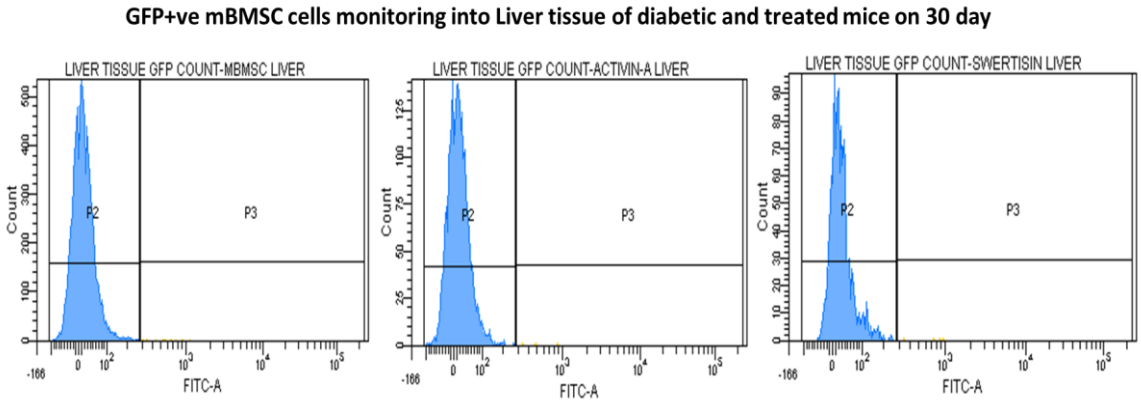


Fig 5.14 show flowcytometry data of GFP labeled mBMSC cells in liver tissues from mBMSC alone, activin and swertisin treated animals. Histograms show P2 population of non GFP liver cells while P3 denotes GFP+ mBMSC cells.

5.3.11 Biomonitoring of insulin and GFP duel positive cells within pancreatic tissues from transplanted animals

Pancreas from all animal groups were sectioned and stained for insulin protein to identify GFP with insulin co-localized cells denoting endogenous differentiation of transplanted mBMSC cells. The migration of GFP labeled BMSC cells in pancreas indicated that mBSMC cells are enforced to form new islets in response to experimentally induced diabetes. Immunohistochemical slides in diabetic control animals did not showed any GFP expression also insulin protein was extremely low indicating destroyed beta cells by STZ induction. Further few GFP positive cells was observed in acinar region of mBMSC alone transplanted animals but they were not able to show insulin co-expression reflecting no differentiation into islets from transplanted mBMS cells alone. However when tissues of activin-A and swertisin treated animals were observed, we could find high ratio of GFP^{+ve} cells in all acinar ductal and islet. Though activin-A treated animals showed GFP internalization, but those GFP cells were not found to be co-expressing insulin denoting that animals are reverting back to normoglycemia by Activin-A treatment and not by mBMSC cell differentiation.

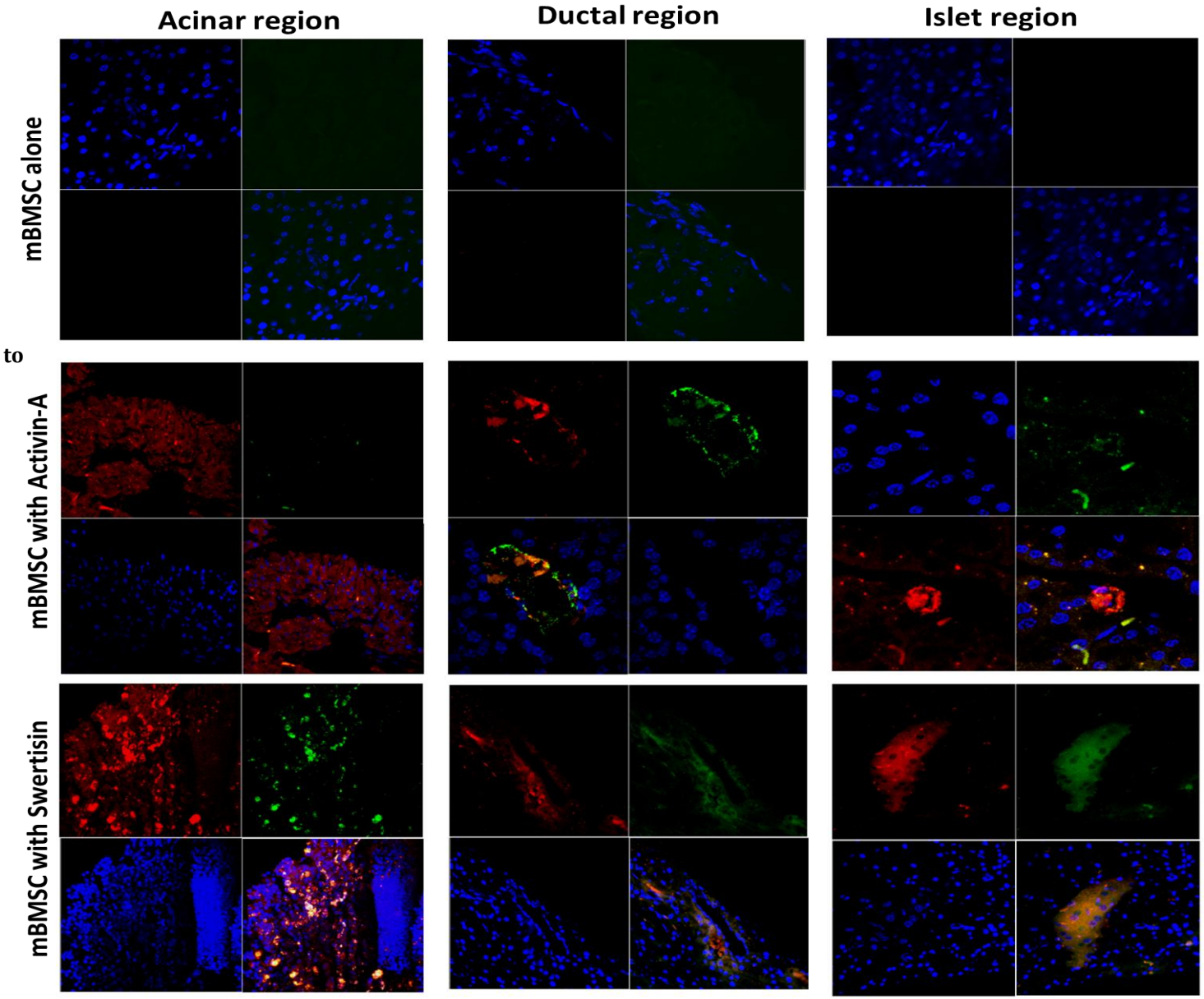


Fig 5.15 shows confocal images of pancreatic tissue sections of mBMSC-GFP cells transplanted alone and in combination with activin-A and swertisin for differentiating mBMSC endogenously. Three different region of each pancreatic tissue was observed in order monitor GFP and insulin co-labeled cells. Insulin is showing in red while GFP in green and nucleus was stained with DAPI in blue.

Whereas with swertisin huge GFP positive cells were found in all three regions and interestingly all GFP cells were found to be co-expressing insulin. Scattered GFP and insulin co-localized cells found in acinar tissues, many ductular structures also showed duel protein expressing cells and also within the islets large percent of GFP insulin co-expressing cells were observed. These above results strongly indicate that swertisin enforce mBMSC cells into insulin producing cells endogenously and function to reduce hyperglycemia (fig 5.15).

5.3.12 *In-vivo* functionality of transplanted mBMS cells for endogenous islet differentiation

For evaluating the functional status of transplanted BMSC cells, plasma blood glucose and serum insulin was performed after 30 days treatment in all groups of mice. Plasma blood glucose was monitored in tie dependent manner from day 1 to day 30, while serum insulin was estimated on 30th day after sacrifice of animals. The blood glucose profile showed STZ induced animals did not reverted to normoglycemia even after 30 days and remain diabetic, while mBMSC alone transplant animals somewhat tends to revert back but failed to do so, while swertisin and activin treated animals showed improved glycaemia state after 30 days treatment and swertisin treated animals showed best result and completely revert back to normal (fig 5.16a).

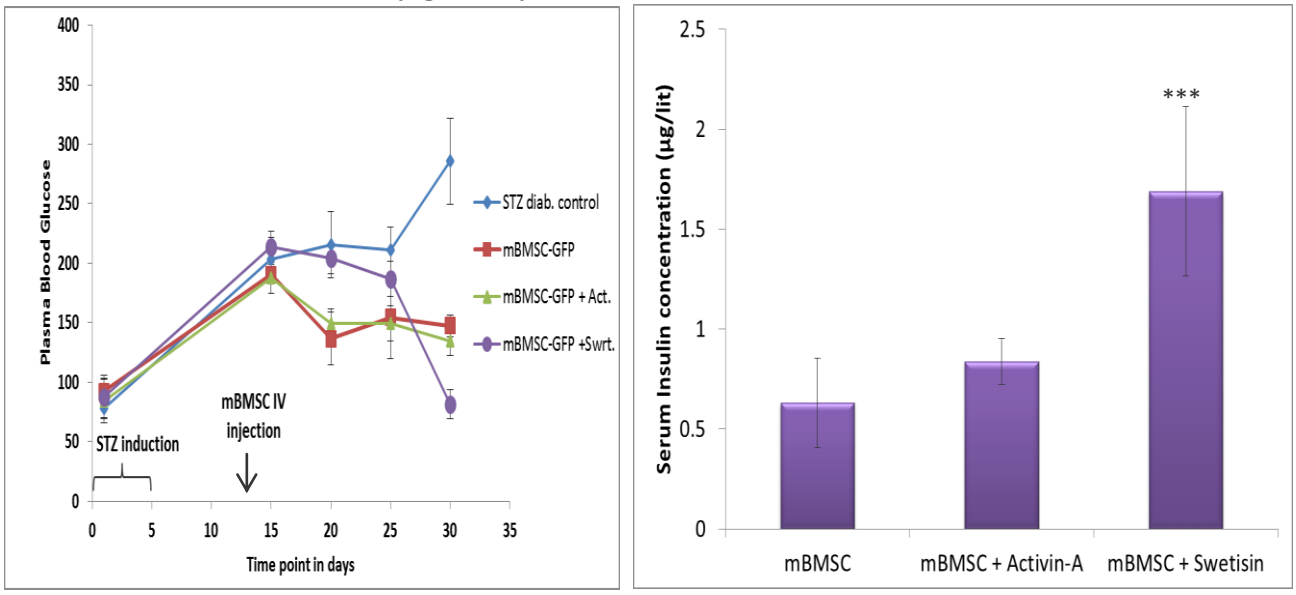


Fig 5.16a and b: Blood glucose and insulin profile of mBMSC-GFP transplanted animals. Fig 5.16a shows plasma blood glucose in mBMSC-GFP transplanted animals in time dependent manner. Fig 5.16b represents serum insulin levels in these transplanted animals on 30th day of the study. Data represents mean ±SEM. N=3 animals in each group, ***p< 0.001.

We also examined serum insulin levels in these transplanted animals and found that swertisin effectively showed significantly high insulin levels compared to activin-A and mBMSC alone transplanted animals (Fig 5.16b).

5.4 Discussion

Many studies in some recent times have demonstrated the possibility of generating insulin producing cells or islets from various cellular sources including embryonic stem cells (Shi et al., 2005), Induced Pluripotent Stem Cells (IPS cells) and MSCs (Chao et al., 2008; Gao et al., 2008). In spite of their promising potential, there are still some significant challenges, like the efficiency of differentiation or normal functioning of newly formed β-cells for insulin synthesis and secretion and more than that lack of potent differentiating agents. Bone marrow mesenchymal cells (MSC) reside in bone marrow and are multipotent, and can differentiate into lineages of mesenchymal tissues, such as bone, cartilage, fat, tendon, muscle, adipocytes, chondrocytes, osteocytes(Florio et al., 2000; Hill, 2001). MSCs as differentiation donors are of advantages compared with other stem cells.

In present study we attempted to answer these questions using bone marrow mesenchymal stem cells and tried to differentiate them into insulin producing cells/islet to mitigate hyperglycemia upon transplantation in diabetic mice model. Our results demonstrated that cells isolated, purified and characterized from mouse femur and tibia bones exhibited typical MSC characteristics phenotypically: a fibroblast-like morphology, plastic-adherent capability, and the expression of a typical set of surface proteins which also previously reported (Jensen et al., 2000).

In order to find the efficacy of swertisin compound for the differentiation of mBMSC into ILCC, we carried out comparative study using SFM alone, activin-A and swertisin as an inducing agent. In our study, by use of swertisin we could differentiate mBMSC into new ILCC, which were positive for C-peptide, insulin, glucagon, nestin, pdx-1, ngn-3 and neuroD. More than this we found these clusters were also able to release insulin in response to glucose induction. For

determining the mechanistic pathway of swertisin mediated islet differentiation we tried to analyze the molecular signaling in time dependent manner (0-8 day) from mBMSC clusters. As per our earlier observation, swertisin showed islet differentiation mechanism mimicking ACT-MAPK-TKK pathway Here, when we analyzed various signaling protein, surprisingly we did not find swertisin to produce new islet by previously examined ACT-MAPK-TKK pathway (with NIH3T3; chapter-4b and Panc-1 cells chapter-6). We did not observe ngn-3 up regulation and neither phospho smad-2 from day 2 till day 8. Also BMS cells failed to express PDX-1 in all groups. Nestin, a neurofilament protein is regarded as a marker of pancreatic progenitor cells (Lumelsky et al., 2001; Zulewski et al., 2001). In our study, we found high nestin expression with swertisin clusters right away form day 2 till day 8, suggesting that MSCs could differentiate into islet cells by some other mechanistic pathway. Benjamin et a l., in 2009 reported that KGF, an another islet differentiating agent, differentiate pancreatic progenitors by MEK-ERK pathway. KGF induced high ductal cell proliferation and alternatively enforces insulin cell differentiation by PI3K/AKT pathway. Some other alternative signaling might be operative in BMSC cell differentiation with swertisin induction and need to be further properly validated using specific ERK and PI3K/AKT inhibitors. It is interesting to note that swertisin mediated mBMSC ILCC showed timely regulated insulin release upon glucose induction and showed improved blood glucose parameters with presence of insulin and glucagon hormones in ILCC grafts retrieved from renal capsule.

Few earlier studies have assessed the therapeutic effects resulting from the *in-vivo* differentiation of BMSCs into pancreatic parenchyma and mesenchymal cells after pancreatic injury. Hess et al. reported a lowering of blood glucose levels within a week after intravenous infusion of green fluorescence protein (GFP)-tagged allogenic bone marrow into STZ-induced diabetic mice (Hess et al., 2003). With quantitation of GFP insulin cells it was postulated that only 0.5-2 % cells getting migrated to pancreas and getting differentiated. Later in some other report no significant transdifferentiation of BMSC happen into insulin producing cell *in-vivo* (Lechner A et al., 2004) In this quest we designed an experiment to

look for role of differentiating agents additionally with transplanted stem cells, to assess the effects of BMSCs in promoting islet regeneration, and to explore the mechanisms involved in this process. Pancreatic regeneration after STZ induction has been well characterized using different animal models (Bonner-Weir et al., 1993; Hardikar et al., 1999). In our study, the transplanted GFP labeled mBMSCs exhibited therapeutic benefits via a significant increase in migration and differentiation into islet cells in combination with swertisin compound rather than with mBMSC transplant alone.

Our results also show that transplanted BMSCs underwent differentiation into insulin positive cells and migrate maximally to pancreas with swertisin action while activin-a does not support this. Flowcytometry data of GFP positive cell population in whole pancreas cell suspension on 30 day showed 5% and 12 % migration of GFP^{+ve} mBMSC, while mBMSC alone showed approx. 1-2%. Our data for mBMSC alone goes in accordance with data reported by Hess et al. (Hess et al., 2003). Wang et al. (2006) demonstrated that, after transplantation of GFP^{+ve} bone marrow cells into neonatal mice, up to 40% goes to exocrine and endocrine compartment derived from donor bone marrow (Wang et al., 2005). Our data also show that almost all GFP^{+ve} cells migrate toward pancreas and gets differentiated with both Activin-A and swertisin as evident from quantitative dotplots of $CD44^{+ve}$ (undifferentiated BMSC marker) co-expressing with insulin (beta cell marker) which suggest that all transplanted BMSCs migrated towards pancreas contribute to the islet regeneration by losing their stemness features more significantly with swertisin than with activin-A.

One earlier report states that BMDCs contribute to the population of pancreatic stem cells in mice and rats leading to development of fibrosis have also indicated (Iwano M, 2002; Russo, 2006; Sparmann, 2012; Watanabe, 2009). However, the cells responsible for these results could not be identified in our case, because all the previous publications used the transplantation of whole bone marrow populations and for us we used well characterized and purified population of BMSC.

In conclusion, the results indicate that BMSCs alone fails to produce any significant endogenous islet differentiation while the same was achieved in combination with activin and swertisin treatment. Taken together, the data presented in this study indicated that mBMSC proves to be best source with their fascinating advantages such as a large potential donor pool, rapid availability, absence of discomfort to the donor with low risk of rejection, could be enforced to differentiate into ILCC *in-vitro*, and endogenous differentiation of BMS cells *in-vivo* upon proper differentiation. Also, Swertisin proved to be the most potent inducing agent to be used as potential therapy along with BMSC in diabetes.

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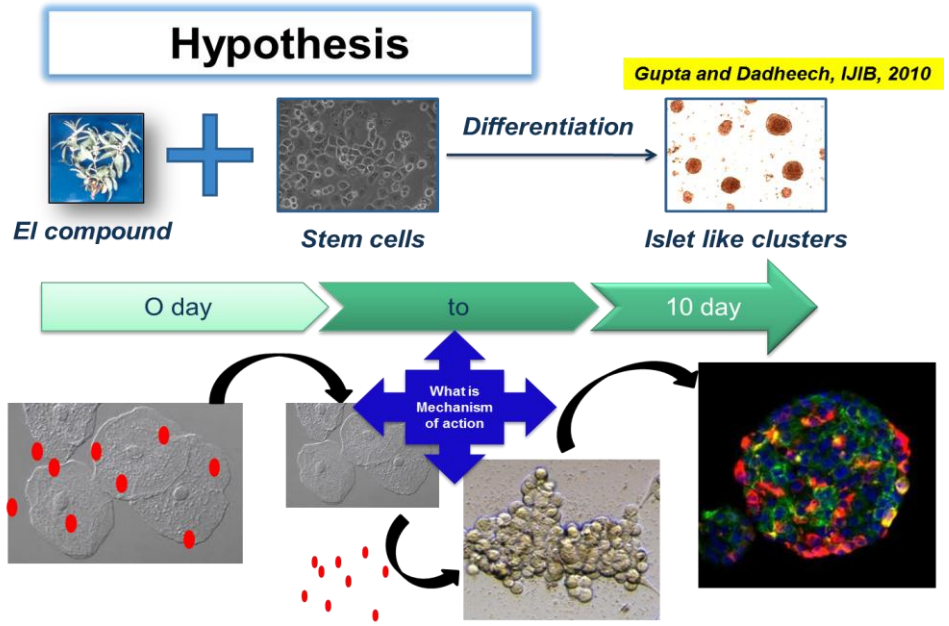
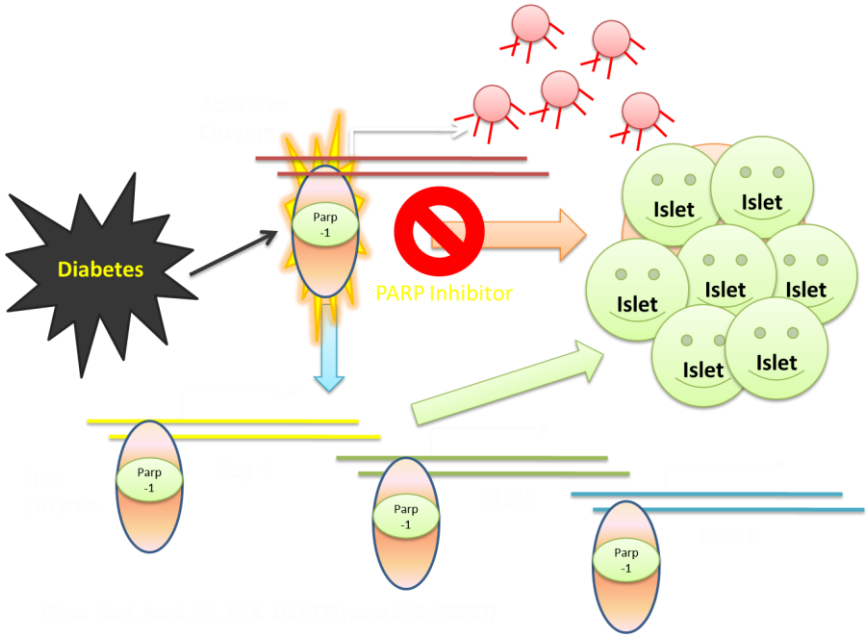
Chapter 6

Title

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



Role of PARP in regeneration or islet differentiation



6.1 Introduction

6.1.1 Islet Neogenesis

Islet Neogenesis refers to generation of new β-cells from progenitor cells. Since insulin producing β-cells, which form bulk of the islets (65-80%), are targeted for destruction at early stage in type I diabetes and at an advanced stage in type II diabetes, there is a need to understand how new islets or new β-cells are formed. During embryonic development, both pancreatic endocrine and exocrine cells are derived from pancreatic endoderm; however, post-natal origin of pancreatic β-cells was a controversial issue for a long time. In 2004, Melton's group conducted an elegant lineage tracing experiment to strongly argue that pre-existing terminally differentiated β-cells retain a strong proliferative capacity in vivo and they are the major source of new β-cells during adult life and after partial pancreatectomy in mice (Dor, Brown et al. 2004). Their study challenged the notion that adult pluripotent stem cells could have a significant role in β-cells replenishment. Although subsequently, the same group produced equally clear evidence that in vivo one could reprogram mature exocrine cells to become functional β-cells through delivery of a series of key developmental regulators, namely Ngn-3, PDX-1 and Maf-A (Fig. 6.1a). (Zhou, Brown et al. 2008).

In parallel, Xu et al. (Xu, D'Hoker et al. 2008) produced equally strong evidence that new β-cells can be generated in injured pancreas of adult mouse from its endogenous (pancreatic) progenitor/stem cells. They showed that after partial ligation of tail duct of pancreas, both exocrine and endocrine cells were dying, but new Ngn-3 positive islets were formed starting from hormone negative ductal cells within the tail region. The current balanced view (Granger and Kushner 2009) is that there are three distinct mechanisms to account for β-cells regeneration: (i) trans-differentiation of exocrine cells into endocrine β-cells; (ii) emergence of new β-cells from pancreatic ductal epithelium; and (iii) replication of pre-existing β-cells and (iv) Stem cell differentiation from various tissue sources. (Fig. 6.2).

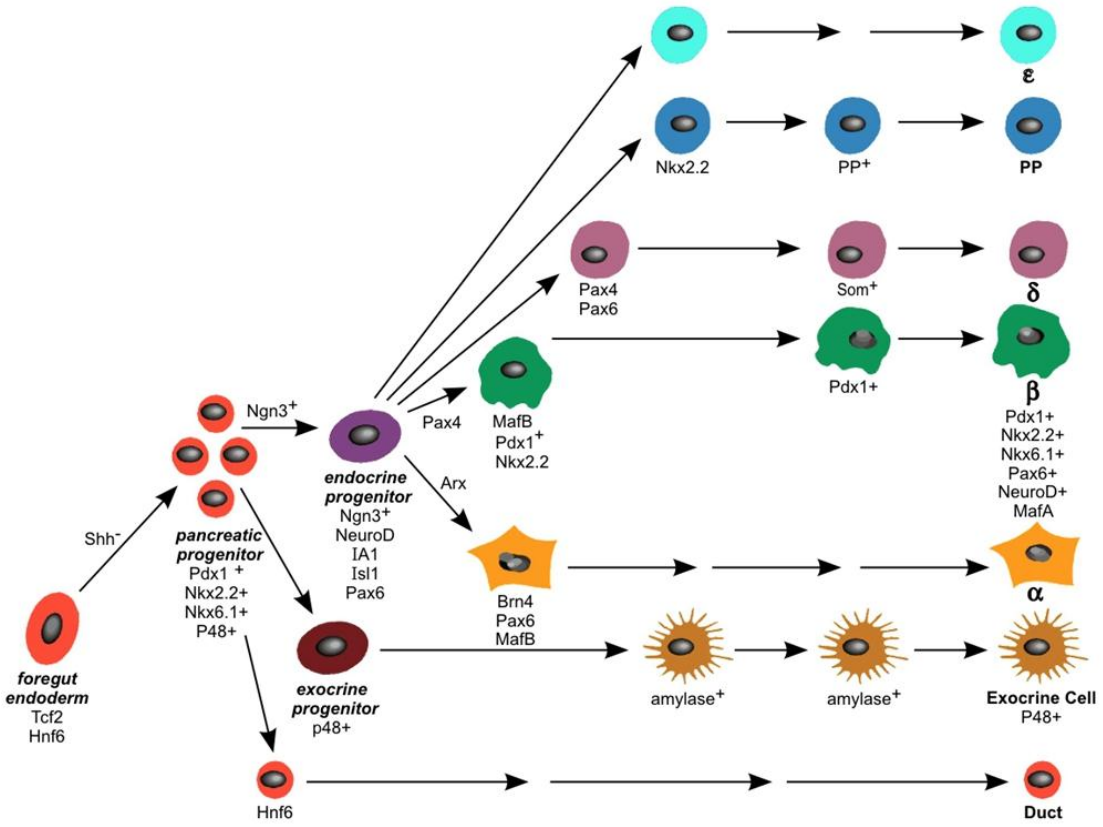


Fig 6.1a: shows islet differentiation pathway and key transcription factors involved in conversion of stem/progenitor into insulin producing cells.

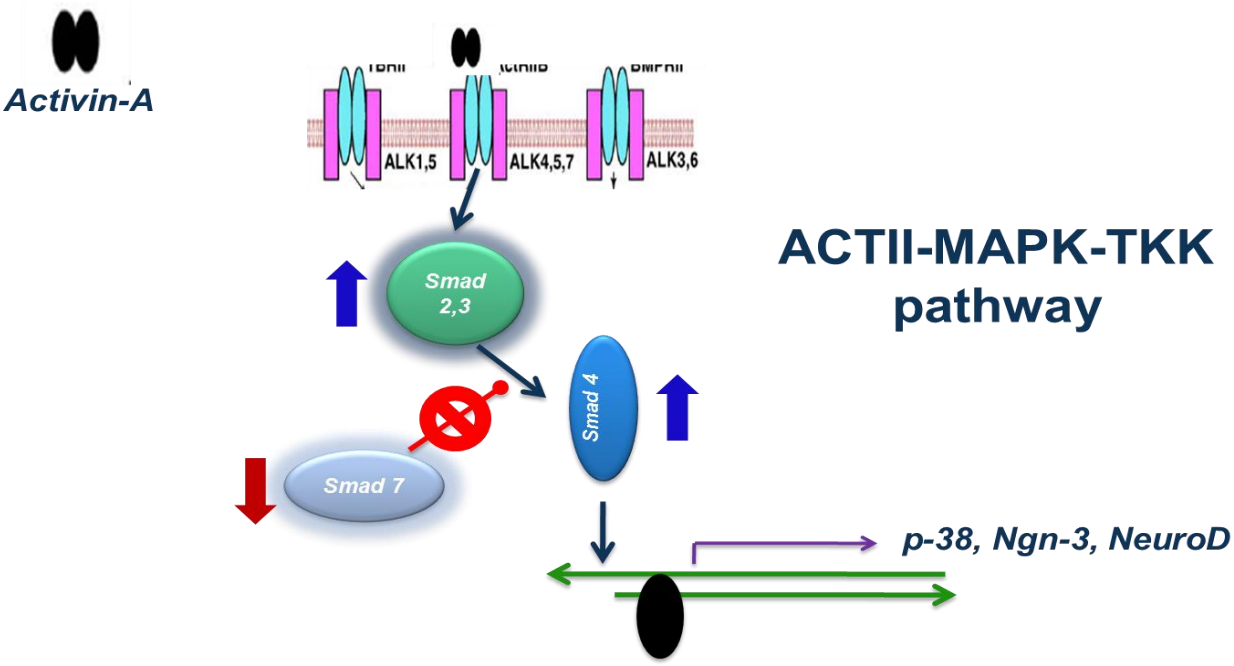


Fig 6.1b: shows mechanism of Activin-A mediated islet differentiation pathway ACT-MAPK-TKK. Activin-A acts via activin receptors ACT-3/5/7 and does high phosphorylation if smad-2 protein and simultaneously I inhibits smad- 7activation in differentiating cells. P-smad2 in turn leads to smad 4 activation and together this complex thereafter activates p38 phosphorylation and ngn-3 up regulation (Mellissa et al., 2003).

To expedite the process of *in-vitro* islet neogenesis from various types of progenitor cells, we need to have a better understanding of different factors and their mode of action that can influence this process. Many studies have focused on the role of small peptides, cytokines and proteins in stem cell differentiation to obtain insulin-producing cells (Soria, Skoudy et al. 2001). Some of the compounds have been instrumental in islet differentiation protocols, such as HGF, IGF, Activin-A, Exendin-4, GLP-1, INGAP, Betacellulin etc. (Fig. 6.1c). In this sense, numerous molecules have been used by various investigators in order to drive the differentiation of stem cells towards β-cell-like phenotype, as well as to promote the proliferation and differentiation of β-cell precursors isolated from adult tissues. Till date only two of the molecule Activin-A and Keratinocyte growth factor has been explored for their mechanism of differentiation (Benjamin Uzan 2009; Melissa L. Brown and Schneyer 2010). Activin-A promote islet differentiation via ACT-MAPK-TKK pathway mediated through activin ACTIII receptor (Fig. 6.1b). High phosphorylation of p38 leading to over expression of Ngn-3 (Itaru Kojimaa and Umezawab 2006). Movassat et al., demonstrated that KGF promotes beta-cell regeneration by stimulating duct cell proliferation in vivo. Moreover, KGF directly induces the expression of PDX1 in some ductal cells thus inducing beta-cell neogenesis. The molecular mechanism involved in this process was shown that the effects of KGF on duct cell proliferation are mediated by the MEK-ERK1/2 pathway, while the KGF-induced cell differentiation is mediated by the PI3K/AKT pathway (Benjamin Uzan 2009).

In addition, very few investigators have used herbal products as replacement of known differentiation factors to obtain insulin-producing cells. Kojima et al., used herbal agent named as conophylline. The compound showed generous differentiation of pancreatic acinar cells AR42J into insulin producing cells. As a result they obtained insulin positive cells, as well as expression of other pancreatic factors such as Pdx-1, Ngn3 and GLUT-2 (Umezawa, Hiroki et al. 2003). The group has also provided some evidences to focus on mechanism of islet differentiation targeting Activin-A induced islet differentiation pathway via p38 mediated neurogenin-3 up-regulation. It is pertinent to note that despite the efforts and major advances that have been made in generating islet cells using

class of differentiating agents, the mechanism of action of these small molecules in the process of stem cell differentiation demands the strong desire for use of such molecules as a therapeutic drug. All together better strategies must be taken in account to develop viable options for generating enormous islet yield which can overcome the limitations of transplantation.

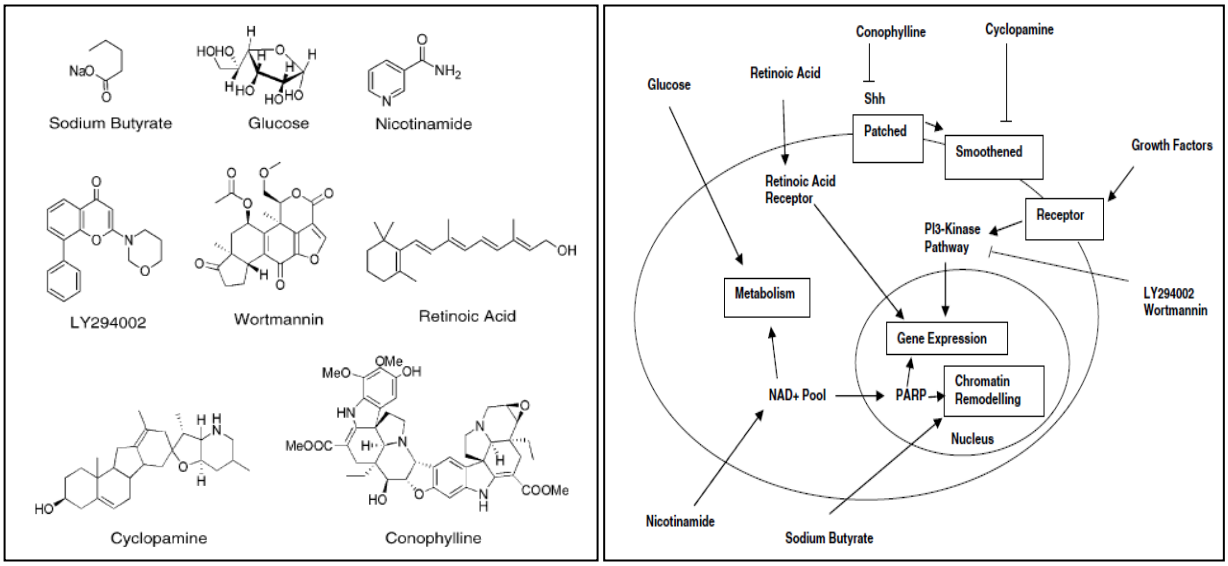


Fig 6.1c: representing structure of molecules facilitating islet differentiation and their possible mechanistic action to produce insulin positive cells from stem cells.

In last few years, our lab has also examined anti-diabetic, hypolipidaemic, islet protective activity of *E. littorale*. The plant has been examined for its capacity 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 (Gupta 2010) chapter-3 and Bone marrow mesenchymal stem cell chapter-5 with most potent differentiating compound swertisin isolated from *E. littorale.* This led us to investigate the molecular mechanism of this potent bioactive agent swertisin (SGL-1) for its effective islet cell differentiation property. In the present chapter we investigated role both by in vitro and in vivo differentiation.

6.1.2 Poly (ADP-ribose) (PAR) polymerase (PARP)-1

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 (Akiyama, Takasawa et al. 2001)(Fig. 6.4).

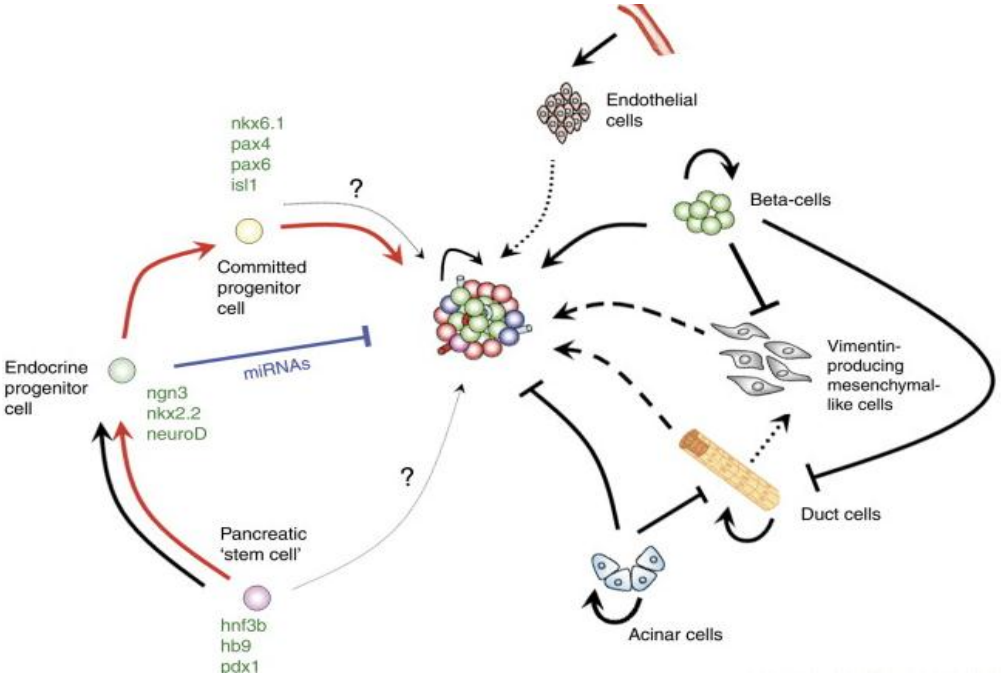


Fig 6.2: representing possibility of islet neogenesis from various cell types to for new functional islet cells. Various cells have different fate and genes involved for the conversion into insulin producing cells. Endothelial cells, mesenchymal cells and endocrine precursor cells form islet by total differentiation, whereas ductal cells, acinar cells does by transdifferentiation.

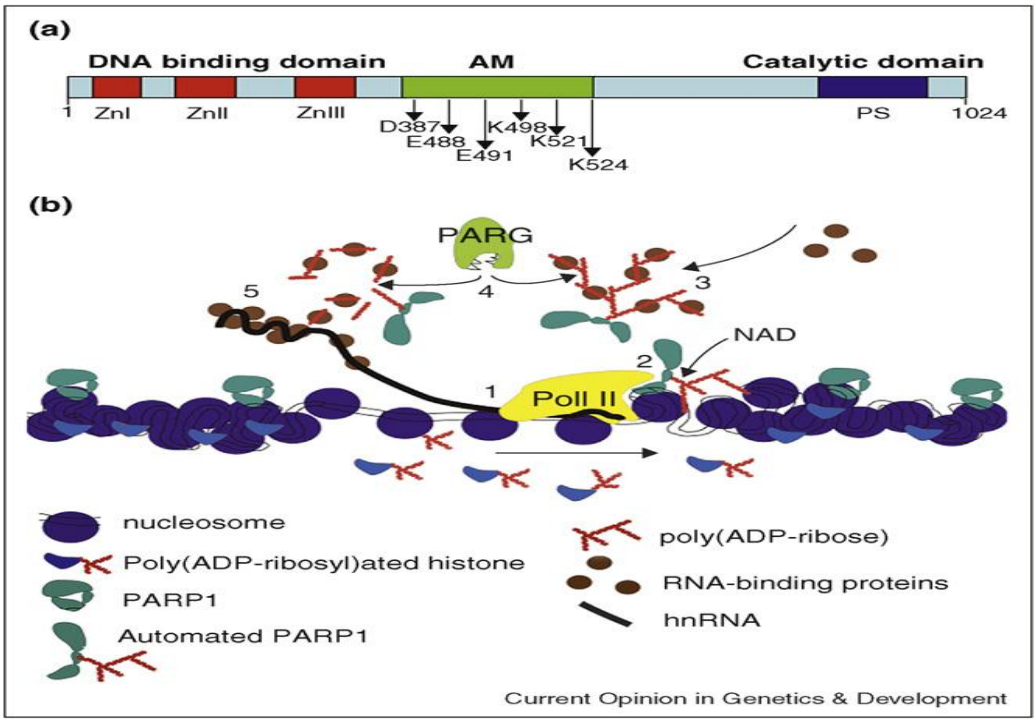


Fig 6.3: shows general structure of PARP-1 enzyme and its genral role of catalysis of pADPr. PARP-1 is made up of three domains namely, DNA binding, Auto-modification and Catalytic.

Earlier observations in a simpler model of only β-cell lines revealed that PARP-inhibitor could promote transcription of *Reg-1* (Akiyama, Takasawa et al. 2001) and *Maf-A* (Yelamos, Monreal et al. 2006) 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.

Poly (ADP-ribose) polymerase-1 (PARP-1 or simply PARP) is a nuclear enzyme in higher eukaryotes that is catalytically activated in response to DNA damage to consume NAD and form polymers of ADP-ribose (pADPr) that post-translationally modify key target proteins and transiently alter their functions until the polymers are removed from the proteins (Fig. 6.3) (Le Rhun, Kirkland et al. 1998). These reactions, i.e., PARP-activation, pADPr-modification of proteins and ATP-depletion as a consequence of NAD-depletion, have been most frequently studied in conjunction with two of the key DNA damage responses, namely DNA repair and cell death (Krishnakumar and Kraus; Le Rhun, Kirkland et al. 1998; Yelamos, Monreal et al. 2006). However, more recent studies are showing that PARP also has other roles that are not obviously linked to overt DNA damage, such as that in the transcriptional activation or repression and in chromatin remodeling as well as in differentiation (Rouleau, Patel et al.; Umezawa, Hiroki et al. 2003). Couple of recent evidences has also depicted role of PARP-1 in diabetes and pancreas regeneration (Yonemura, Takashima et al. 1984).

6.1.3 PARP and its structure:

Poly (ADP-ribose) (PAR) polymerase (PARP)-1 is a nuclear enzyme which catalysis the polymerization of PAR from cleaved NAD+ moieties. It has a pivotal role in DNA damage repair and cell death. PARP is composed of four domains namely, N terminal domain, DNA-binding domain, central auto-modification domain (caspase-cleaved domain domain) and C terminal catalytic domain. The DNA-binding domain (44kD) is composed of two zinc finger motifs F1 and F11. F1 is known to interact with double stranded nick whereas F11 recognize and binds single stranded nicks (Fig. 6.3). It also contains a NLS sequence which is

required for the nuclear translocation of PARP protein and is cleaved off at DEVD site upon activation of caspase 3 and 7. The central auto-modification domain (16kD) is responsible for releasing the protein from the DNA after catalysis, this region is rich in glutamine, serine and leucine residues and serves as a major site for various protein–protein interactions essential for cancer, cell death and apoptosis and cell cycle progression. Also, it plays an integral role in cleavage-induced inactivation. The last C terminal catalytic domain (55kD) is required for the catalysis of ADPr from reduced NAD+ species and its polymerization into linear or branch chain polymers(Kim, Zhang et al. 2005).

6.1.4 PARP Family Members: PARP-1 and the PARP Family

Poly (ADP-ribosyl)ation reactions and PARP-like genes have been identified in a wide variety of single and multicellular eukaryotes, from fungi to mammals (but surprisingly not the yeast like *S. cerevisae and S. pombe*), as well as eubacteria, archaebacteria, and double-stranded DNA viruses. In mammalian cells, the bulk of the PAR production is catalyzed by PARP-1, although recent studies have begun to characterize the structure and function of related PARP proteins. Till now it has been considered that PARP has 18 proteins in its family, but recent finding and reviews clearly insight 17 proteins only (Kim, Zhang et al. 2005).

6.1.5 How does PARP functions

PARP is found in the cell's nucleus, the main role is to detect and signal single strand DNA breaks (SSB) to the enzymatic machinery involved in the SSB repair. PARP activation is an immediate cellular response to metabolic, chemical, or radiation-induced DNA SSB damage. Once PARP detects a SSB it binds to the DNA, and, after a structural change, begin the synthesis of a poly (ADP-ribose)chain (PAR) as a signal for the other DNA repairing enzymes such as DNA ligase III (LigIII), DNA polymerase beta (polβ) and scaffolding proteins such as x-ray repair complementing gene 1 (XRCC1). After repairing, the PAR chains are degraded via PAR glycohydrolase (PARG). Interestingly, NAD^+ is required as substrate for generating ADP-ribose monomers. The over activation of PARP may deplete the stores of cellular NAD^+ and induce a progressive ATP depletion leading to necrotic cell death. In this regard, PARP is inactivated by

caspase-3 cleavage (in a specific domain of the enzyme) during programmed cell death. The catalytic domain is responsible for Poly (ADP-ribose) polymerization. This domain has a highly conserved motif that is common to all members of the PARP family. PAR polymer can reach lengths up to 200 bp before inducing apoptotic processes. PARP enzymes are essential in a number of cellular functions, including expression of inflammatory genes.

6.1.6 PARP in Embryonic Development and Cell Differentiation process

Although PARP-1 knockout mice develop normally (Klein, Z.Ling et al. 2003), the embryonic lethal phenotype of PARP-1/PARP-2 double knockout mice indicates that PARPs are critical for embryonic development via maintenance of genomic stability (Menissier de Murcia J, Ricoul M et al. 2009). The extent to which they control other specific developmental processes is not clear, although new studies have suggested roles for PARP-1 in stem cells and differentiation. PARP-1, as well as PARP-2, has been implicated in the differentiation of many cell types. For example, in a model of neuronal differentiation, PARP-1 is required for the exchange of corepressors or coactivators at the promoters of genes regulated by the transcription factor HES1 (Ju BG, Solum D et al. 2004). PARP-1 is also required for T cell dependent immunoglobulin class switching in B cells (Ambrose, Willimott et al. 2009), (Morrison C, Smith GC et al. 1997) and PARP-1 deficiency promotes the differentiation of regulatory (CD4+/CD25+/Foxp3+) T cells (Nasta, Laudisi et al.). In a model of endodermal differentiation, PARP-1 and PARP-2 play distinct roles in interactions with the heterochromatin-associated proteins HP1 and TIF1b: PARP-2 is required for differentiation of mouse embryonal carcinoma cells into primitive endoderm-like cells in response to retinoic acid, while PARP-1 is required for subsequent differentiation into parietal endoderm-like cells in response to retinoic acid and dibutyryl cAMP (Quenet, Gasser et al. 2008). PARP-2 is required for adipogenesis, spermiogenesis (Dantzer, Mark et al. 2006), and T cell survival during thymopoiesis (Yelamos, Monreal et al. 2006).

6.1.7 PARP-1 in Stem Cell Function and Differentiation

Embryonic stem cells (ESCs) fromPARP-$1^{-/-}$ mice, showed altered expression of about 10% of genes compared to only 3% genes in liver from the same animals (Ogino, Nozaki et al. 2007). The number of genes down regulated by PARP-1 knockout was about 2-fold more than the number of upregulated genes in both cases, indicating a major role for PARP-1 in keeping genes active in ESCs and liver cells (Ogino, Nozaki et al. 2007). The large panel of genes whose expression is dependent on PARP-1 in ESCs suggests a role for PARP-1 in the developmental programming of these cells. A recent study has revealed some of the molecular mechanisms whereby PARP-1 might help to promote the differentiation of stem cells (Gao, Kwon et al. 2009). Specifically, PARP-1 antagonizes the DNA binding transcription factor Sox2 to stimulate expression of the gene encoding fibroblast growth factor 4 (FGF4), which promotes differentiation. In response to appropriate cellular signals, PARP-1 PARylates Sox2 at the FGF4 enhancer, leading to dissociation and degradation of Sox2 with enhanced expression of FGF4. These results indicate that PARP-1 can regulate the pluripotent state of ESCs by controlling the activity of key stem cell transcription factors

6.1.8 PARP-1 in diabetes

Role of PARP in diabetes is being explored since last thirty years with early efforts employing PARP-inhibitors (Yamamoto, Uchigata et al. 1981) and later studies using PARP-$1^{-/-}$ mice or cells (Burkart, Wang et al. 1999; Pieper, Brat et al. 1999; Klein, Z.Ling et al. 2003; Ogino, Nozaki et al. 2007). The results could be summarized under four broad categories:

(A) The best outbreak established is the role of PARP in the destruction of pancreatic β-cells in the models of diabetes treated with streptozotocin or alloxan via direct or indirect oxidative DNA damage which activates PARP (Yamamoto, Uchigata et al. 1981; Uchigata, Yamamoto et al. 1982), depletes NAD and consequently ATP leading to β-cell death. Hence mice in which PARP function is inhibited (Yamamoto, Uchigata et al. 1981) or PARP-gene is knocked out (Burkart, Wang et al. 1999; Pieper, Brat et al. 1999; Ogino, Nozaki et al. 2007) are resistant to drug-induced diabetes development.

(B) Second role of PARP is in hyperglycemia-induced diabetic complications (Garcia-Soriano, Virag et al. 2001; Brownlee 2005), such as diabetic changes in endothelial functions, (Garcia-Soriano, Virag et al. 2001) neuropathy (Obrosova, Li et al. 2004) and retinopathy (Zheng, Szabo et al. 2004). Mechanism involved is mediated via hyperglycemia induced oxidative DNA damage which causes excessive activation of PARP that results in death of damaged endothelial cells (Garcia-Soriano, Virag et al. 2001). This explains micro-vascular damage (Zheng, Szabo et al. 2004); a contributory factor associated with various diabetes complications. In addition, PARP-activation can also up-regulate expression of inflammatory genes such as NF-κB that can cause death of endothelial cells (Garcia-Soriano, Virag et al. 2001; Zheng, Szabo et al. 2004) or can upregulate expression of adhesion molecules such as ICAM-1, E-selectin and IL-6 in endothelial cells which can lead to blockage of blood vessels (Piconi, Quagliaro et al. 2004). PARP-activation is also known to play a role in death of neuronal and glial cells in brain, which is thought to be partly responsible for neuropathic complications of diabetes (Obrosova, Li et al. 2004). Finally, PARP-activation by hyperglycemia-induced oxidative DNA damage is implicated in pADPr-modification and inactivation of GAPDH, which results in three major pathways implicated in diabetic complications (Du, Matsumura et al. 2003; Brownlee 2005).

(C) The role of PARP in proliferation of β-cells has been comparatively much less examined (Akiyama, Takasawa et al. 2001). In RINm5F insulinoma-derived β cells, it was shown that only in the native (unmodified) state PARP can bind in vitro to the promoter region of proliferation-associated gene Reg-1 and increase its expression, PARP must not be activated (because automodification of PARP with pADPr) and reduce its affinity to bind to the DNA (Akiyama, Takasawa et al. 2001). This was indirectly supported by an earlier data that treating rats after partial pancreatectomy with weak PARP-inhibitors (e.g., nicotinamide) results in enlarged islets with large β-cell mass (Yonemura, Takashima et al. 1984). Supporting the concept that non-activated PARP can have a role in transcriptional regulation of key genes .

(D) The least examined is the role of PARP in insulin biosynthesis: In the INS-1 β-cells, weak PARP-inhibitors (e.g. nicotinamide) could increase insulin biosynthesis by increasing expression of its controlling gene Maf-A (Yelamos, Monreal et al. 2006). However, more potent PARP-inhibitor (PJ-34) failed to increase insulin biosynthesis.

While above studies highlight the role of PARP in development of diabetes, insulin secretion and regeneration of isolated pancreatic β-cells, none have addressed the possible role of PARP in islet neogenesis from precursor cells. In the present study it will be examined in context of islet neogenesis from precursor cells by using PARP inhibitors and generating PARP -/- precursors.

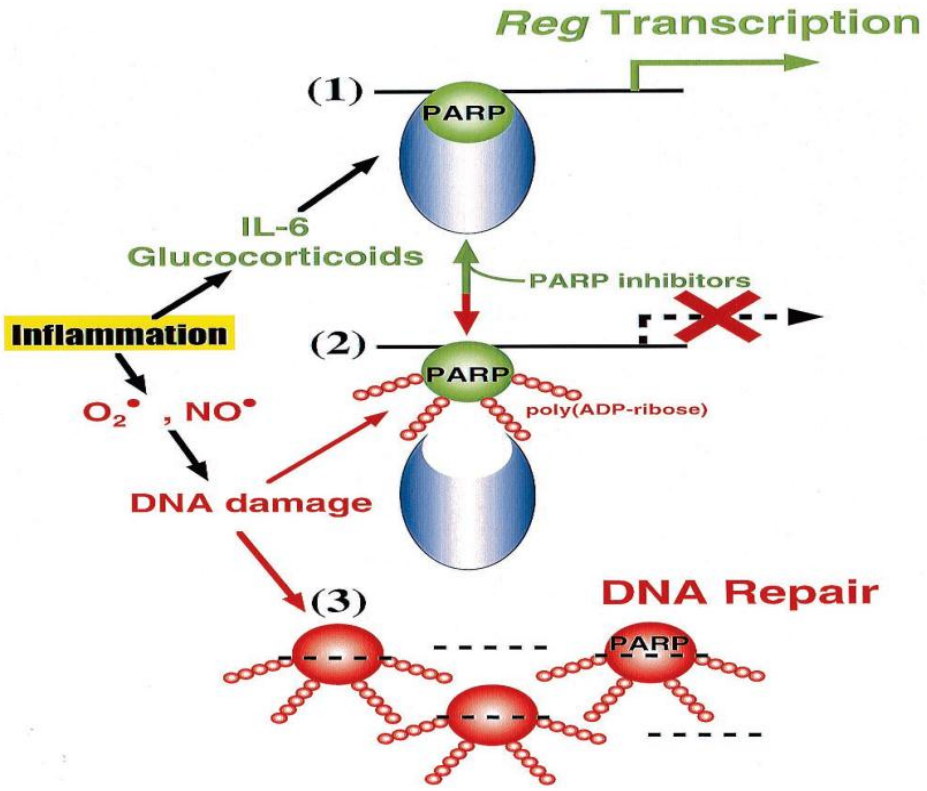
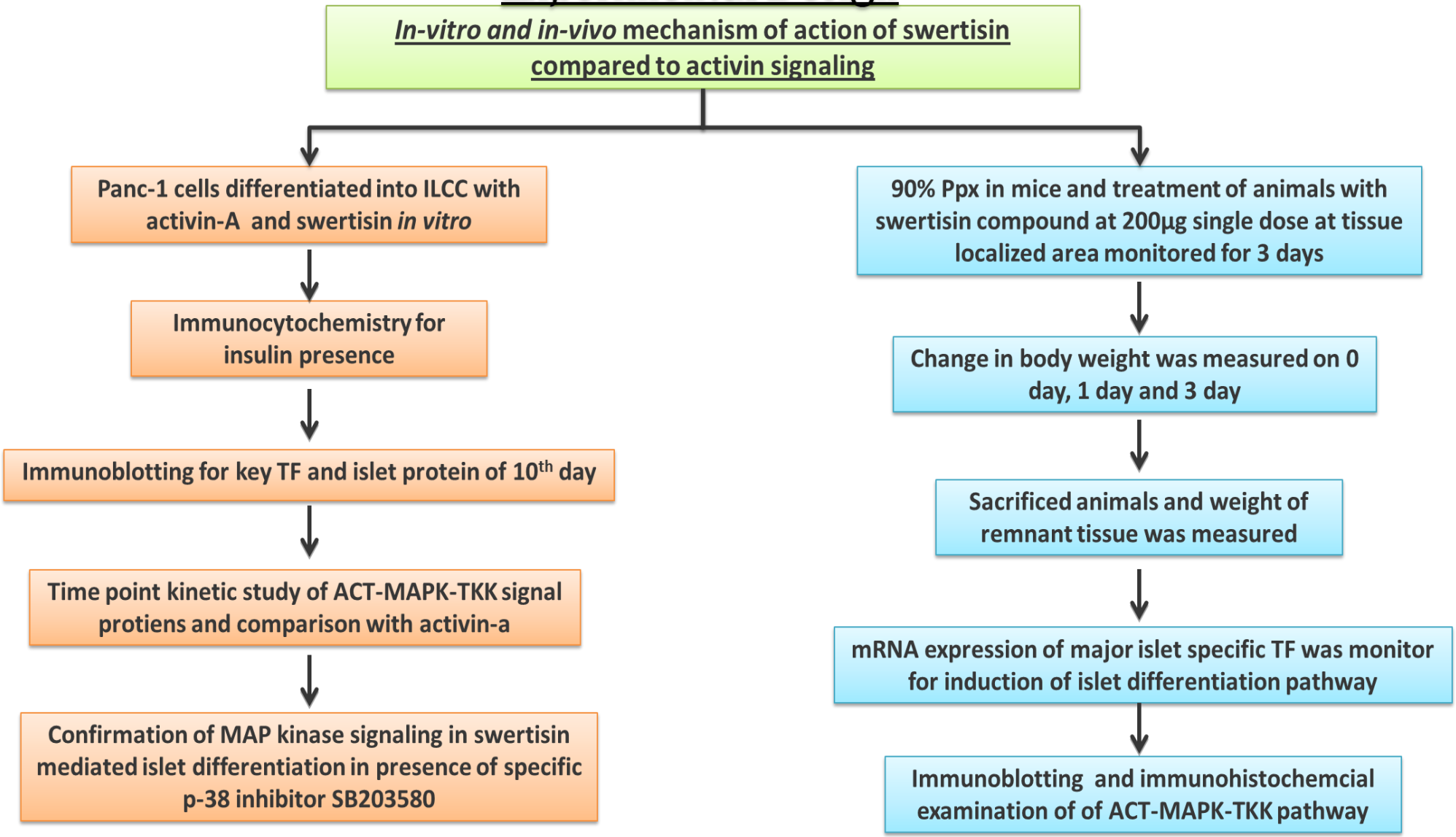


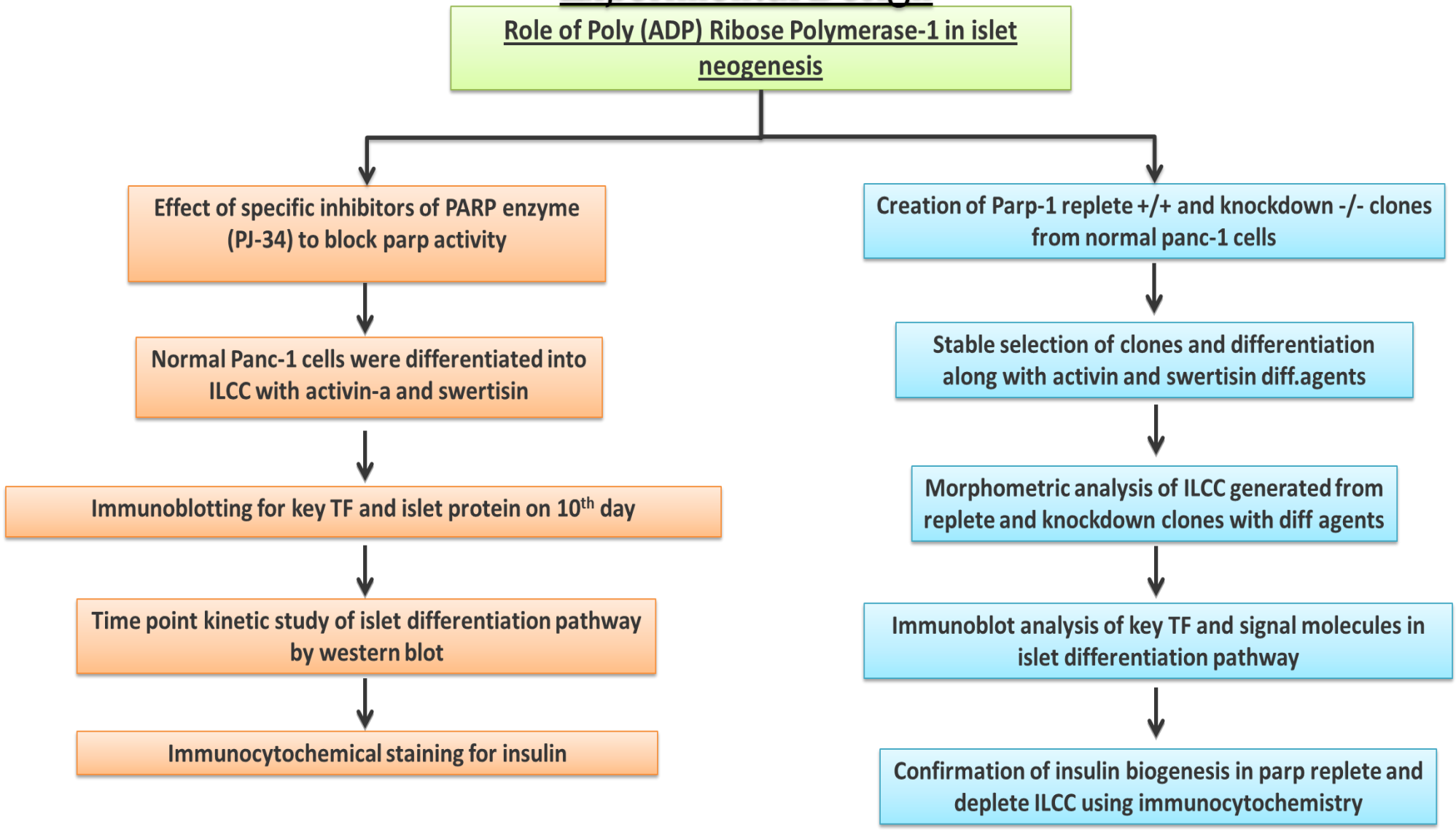
Fig 6.4: Regulation of Reg gene expression by PARP-1 in pancreas regeneration (Okamaoto et al., 2001; 2002).

The Experimental design of work done under this chapter is described in figure below.

Experimental Design



Experimental Design



6.2 Material and Methods:

6.2.1 Cell culture maintenance and differentiation: PANC-1 cells were obtained from Dr. Girish M Shah's Lab. Universite Laval, Quebec, Canada (work was carried out as a part of research project under Canadian Commonwealth Scholarship, awarded in 2010) and maintained in high glucose DMEM supplemented with 10% Fetal Bovine Serum. PANC-1 cells were cultured and maintained. These cells were allowed for differentiation in presence of swertisin as described earlier in as discussed in chapter-3, section 3.2.4. Kinetics of differentiation to ILCC was monitored along with mechanism of action in comparison to activin dependent pathway.

6.2.2 Animals: Male *balb/c* mice, 3-4 weeks old, weighing around 25-30 grams, were used for 90% partial pancreatectomy to understand the mechanism of pancreatic regeneration process *In-Vivo* with swertisin treatment. All animal experiments were performed in accordance with our institutional ''Ethical Committee for Animal Experiments'' and CPCSEA guidelines and regulations.

6.2.3 Partial Pancreatectomy: 90% partial pancreatectomy was performed according to Bouwens et al., and Bonner-weir et al. (Bonner-Weir, Taneja et al. 2000; Lardon, Rooman et al. 2002) Briefly, Mice were anesthetized by administration of ketamine and Xylazine (100 mg/kg and 50 mg/kg bwt; i.p.). Hairs were cleaned and the abdomen was opened through a left lateral incision. The entire splenic portion and most of mesenteric portion of the pancreas was surgically removed, resulting in a ~90% pancreatectomy, confirmed by weighing the removed and remnant portions. Sham operation was performed by opening the abdomen while leaving the pancreas intact. The incision was closed using 4-0 silk treads for the peritoneum and sutured back for the outer skin as well. Animals were given injections of pain reliever for 3 days and wounds were dressed with tropical skin ointment.

6.2.4 Creation of PARP-1$^{-/-}$ PANC-1 cells using stable RNA*i* knockdown technology: RNA silencing is a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (transcriptional gene silencing [TGS]) or by activating a sequence-specific RNA degradation process.

Permanently knockdown of any gene occurs by cleavage of mRNA recognized by specific shRNA sequences in a fashioned mechanism shown in figure 6.5a. To understand the mechanism of PARP mediated islet differentiation, PARP-1 gene was knockdown using specific shRNA against PARP-1 gene. DNA vector-based RNAi approach, established in Dr. Girish M Shah lab (Le Rhun, Kirkland et al. 1998), to stably and very significantly knock down PARP-1 in PANC-1 cells (PANC-SiP clone) and control PARP-1-replete (PANC-U6 clone) having only vector construct without shRNA. The vector map and sequence of shRNA chosen against PARP-1 gene are shown in figure below 6.5b and 6.5c.

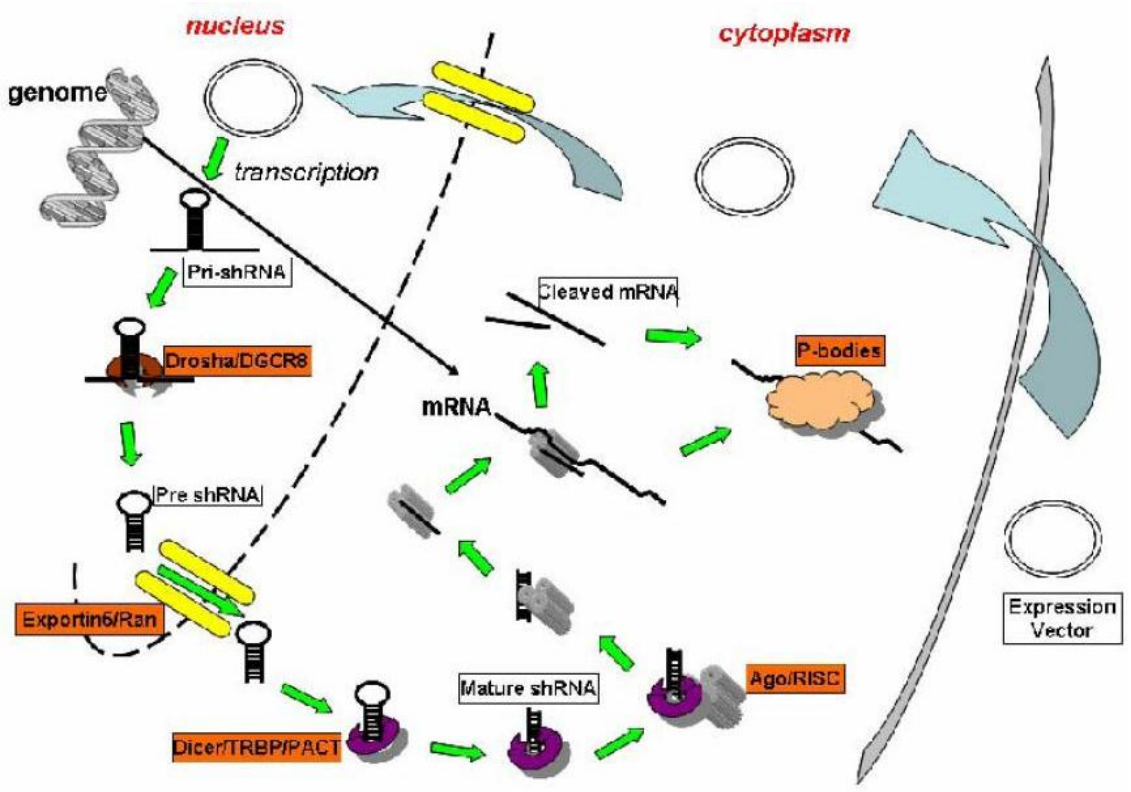
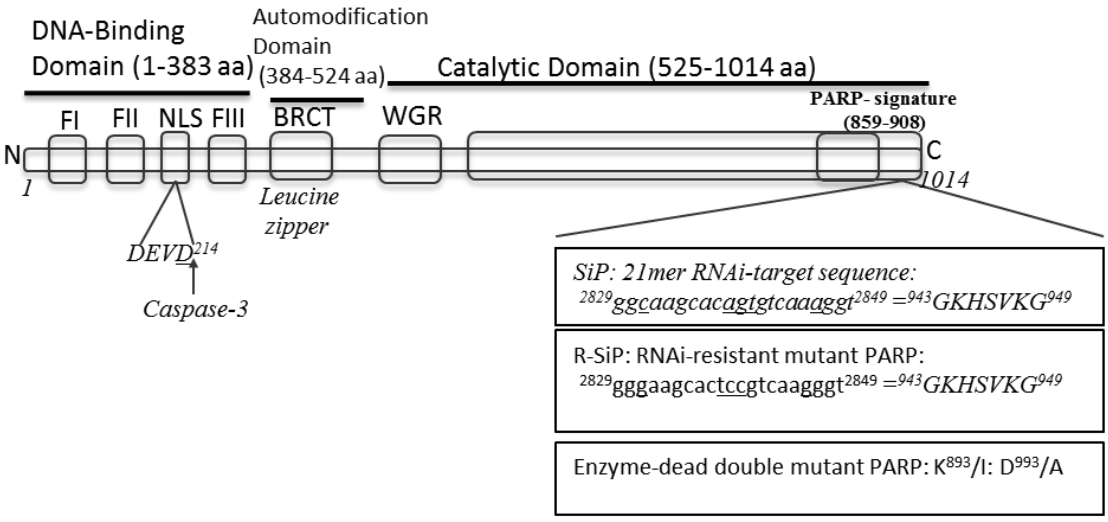


Fig 6.5a representing mechanism of shRNA mediated gene knockdown and cleavage of mRNA. shRNA transcribes from viral plasmid, express as pri shRNA and recognized by Drosha to form pre shRNA by dicer. The pre shRNA was transported to cytoplasm by dicer/TRBP/PACT complex and processed again as mature shRNA. This mature shRNA recognize target gene mRNA and attracts nucleases to cleave mRNA and knockdown gene expression.



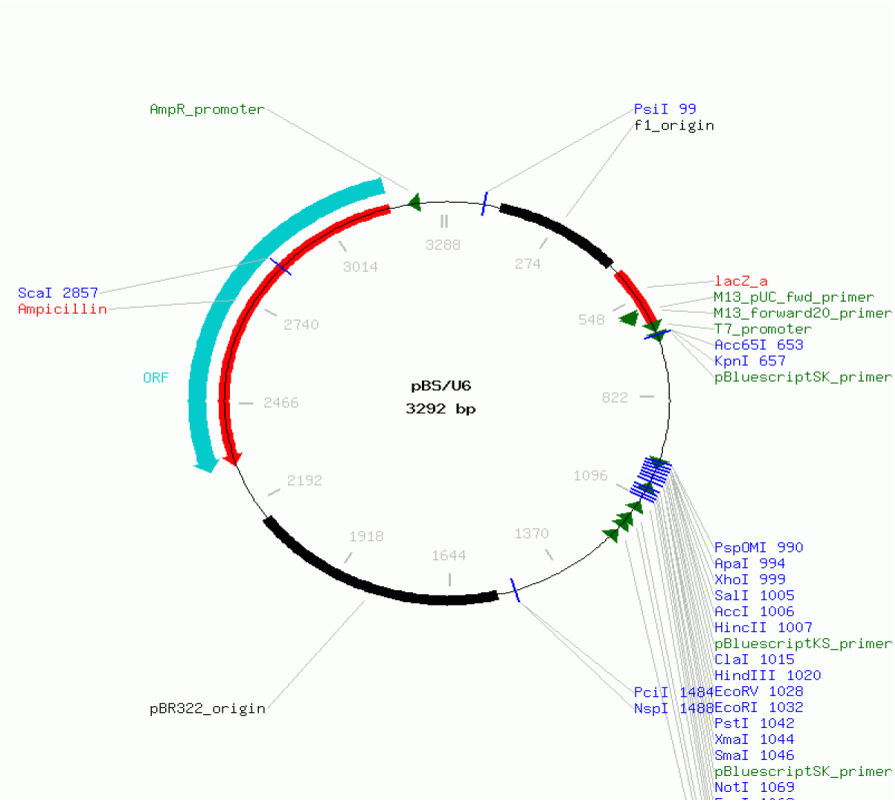


Fig 6.5b: representing general structure of PARP-1 gene and site from which the shRNA sequence was cloned. Fig 6.5c show Map of pBS/U6 plasmid where PARP-1 targeted shRNA sequences 5'-GGGCAAGCACAGTGTCAAAGG-3' was introduced under the control of U6 promoter to create SiP912 shRNA against PARP-1 gene. The forward double-stranded oligo pair was cloned in pBS-U6 at ApaI (blunt)–HindIII sites, followed by cloning of the reverse oligo pair at HindIII–EcoRI sites. This DNA would direct cellular RNA polymerase III to create an RNA that would form a short hairpin (shRNA) with the two underlined complementary sequences forming double-stranded stem and intervening AAGCTT sequence (HindIII site) forming the loop of the hairpin. (Rashmi G Shah et al., 2005).

6.2.5 Transfection: Panc-1 cells were transfected with plasmid pBS/U6 harboring PARP shRNA. Transfection was done using lipofectamine 2000 as described in chapter 4, section 4.2.7; except the selection for the positive panc-1 PARP-1-/- clones was done on hygromycine at the concentration of 200 µg/ml.

Clones were purified from isolated colonies using clonal discs and scaled up. These clones were confirmed using Immunoblotting using anti-PARP antibody and were maintained for further experimentation.

6.2.6 Differentiation of normal U6 replete and Sip deplete PANC-1 cells into ILCC: Normal Panc-1 cells were differentiated using Activin-A 2ng/ml and SGL-1 15μg/ml as differentiating factors in a eight day differentiation protocol discussed in chapter 3, section 3.2.4. In order to better comprehend the differentiation procedure of islet neogenesis, differentiation of Panc-1, in presence and absence of PJ-34 (Inhibitor of PARP activity) and SB203580 (Inhibitor of p-P38-MAPK) was performed. In one of the groups pj-34 at 10μM concentration was added and in another group SB203580 at 10μM conc. was added. The results from the above experiment shed light of PARP-1 playing an important role in islet neogenesis. Hence, further differentiation studies were performed using PARP U6 replete and PARP Sip deplete cells.

6.2.7 Immunohistochemistry/Immunocytochemistry: In order to understand the mechanism of Swertisin action in regeneration pancreas after 90% pancreatectomy, Immunohistochemistry of the following protein markers: Ki-67, Nestin, P-Smad-2, Smad-7, C-peptide, Glucagon, Ck-19 and Ngn-3 were done as described in chapter 4, section 4.2.10. Insulin and Glucagon immunocytochemistry staining was done to characterize ILCCs obtained after differentiation of panc-1 cells. Immunocytochemistry was performed as described in chapter 4, section 4.2.10. Similarly, Immunocytochemistry for insulin was done for the groups containing pj-34 and SB203580, and PARP-1 U6 replete and PARP-1 Sip deplete cells.

6.2.8 RNA extraction and semi quantitative Reverse Transcriptase PCR (RT-PCR): The pancreatic tissue of normal mice with and without Swertisin(SGL-1) on the 3rd day after pancreatectomy were dissected and subjected to RNA isolation, C DNA preparation followed by gene expression profiling by RT-PCR of Nestin, PDX-1, Ngn-3, INS and G6PDH.

6.2.9 Protein extraction and Western blotting: In order to understand the mechanism of SGL-1 the pancreatic tissue on day 3 after pancreatectomy was harvested and homogenized, its protein was estimated which was followed by the western blot profiling of the following proteins was done: Nestin, Ck-19, E-Cadherin, PDX-1, Neurogenin-3. Smad-7, P-Smad-2, PARP-1 and Caspase-3. Also, Western analysis was done in order to understand the effect of PARP-1 depletion by RNAi on expression of key regulatory events during Activin-A inducted differentiation of ILCC from panc-1 cells. The Panc-1 cells were differentiated and harvested on 10^{th} day. Protein expression levels of PARP-1, Nestin, E-cadherin, PDX-1, P-p38 and Ngn-3 were monitored. Western blotting analysis was done as discussed in chapter 4, section 4.2.14.

6.3 Results: *In-Vitro* and *In-Vivo* mechanistic action of swertisin

The mechanism of action of Activin-A in islet differentiation was described earlier by Demeterco and Melissa (Demeterco, Beattie et al. 2000; Brown and Schneyer 2010). In brief, Activin A binds to its receptors ActRIIb which results in stimulation/phosphorylation of SMAD-2 and SMAD-3 which in turn prevents action of SMAD-7. The activated SMAD-2 and SMAD-3 in-turn phosphorylate SMAD-4 and this complex will go to nucleus and stimulate transcription of MafA, Ngn-3, NeuroD1 and PDX-1 via phospho-p38 pathway, resulting in direct regulation of insulin gene transcription (Brown and Schneyer 2010). The intervening genes in this pathway also have independent role in endocrine cell differentiation and are thus implicated in conversion of precursor cells to insulin producing functional cells. In order to understand the mechanism of islet cell differentiation mediated by our isolated molecule swertisin (SGL-1), we carried out experiments tracing signaling pathway using human pancreatic adenocarcinoma cells PANC-1 and differentiated them with swertisin (SGL-1) along with Activin-A. The rational for using activin–a is to compare that whether swertisin also favors same pathway for differentiation as Activin-A does.

6.3.1 Differentiation of normal PANC-1 cells into ILCC

Panc-1 cells were differentiated into islet like clusters in same protocol as described earlier in chapter 3; section 3.2.3. Panc-1 cells were subjected to SFM

alone; SFM with ITS; SFM with Activin-A and SFM with swertisin for 8 days. We observed effective cell clustering started on 3rd day, demonstrating zone of activation. Same clusters by 8th day turned into mature islet like cell clusters, showed presence of insulin granule with DTZ staining at 10th day. Both activin and swertisin exhibited intense deep crimson red or brownish staining whereas those of SFM alone failed to show stain. The same clusters when examined by immunocytochemical study, confirmed the presence of insulin (red) in activin and swertisin clusters but not in SFM alone (Fig 6.6).

We also quantitated insulin fluorescence signal per unit cytoplasm and found that both Activin-A and swertisin able to shoot insulin expression upon 8 day differentiation. The scatter plot, analyzed between intensity of insulin fluorescence alexa 488 and nuclear signal of DAPI, depicted number of positive cells for insulin. When observed in control SFM alone, cells were negative for alexa 488 signals, while SFM with ITS somewhat initiated the differentiation and thereby showed little insulin pixels. A dramatic shift in alexa signals was observed with both Activin-A and swertisin mediated clusters (fig 6.7).

6.3.2 *In-vitro* mechanistic action of swertisin for islet differentiation

In order to understand the mechanism of action of swertisin for islet differentiation, we compared swertisin and Activin-A mediated signaling pathway during differentiation. It has been evident from the cited literature that activin carried new islet formation via ACT-MAPK-TKK pathway. There is one report for herbal compound conophylline to possess islet neogenic property by mimicking same Activin-A pathway. Therefore based on available information, we attempted to trace ACT-MAPK-TKK pathway signaling proteins along with key transcription factors implicated in islet differentiation pathway upon swertisin induction. Normal Panc cells in undifferentiated state show high proliferation by persistent ki67 expression, with basal PDX-1 and Ngn-3 expression. Since ACT-MAPK-TKK pathway involves high phosphorylation of p38 MAPK protein, undifferentiated Panc-1 cells were found to express very low phospho-p38 with abundance of native p-38 protein. The presence of high e-cadherin but not n-cadherin expression postulated progenitor nature of

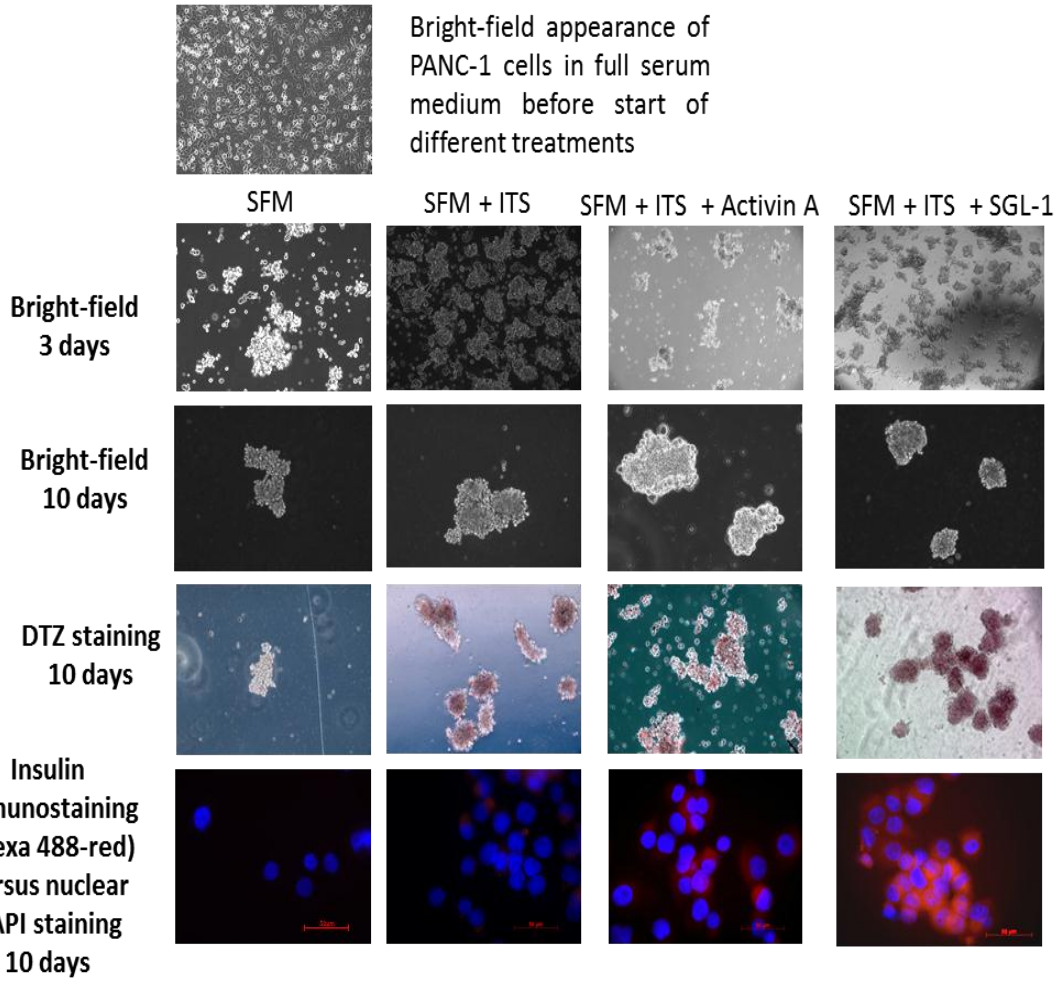


Fig 6.6: Cluster formation of insulin-positive islet-like clusters from PANC-1 cells. PANC-1 cells were maintained for 10 days in serum-free medium (SFM) with or without insulin-transferrin and selenium (ITS) and/or Activin A, and swertisin (SGL-1). The development of islet-like clusters were monitored daily under microscope and islet-like clusters were harvested at 10th day for staining of insulin by dithizone (DTZ) and immunocytological staining for insulin (red). The nuclear DNA was stained with DAPI (blue).

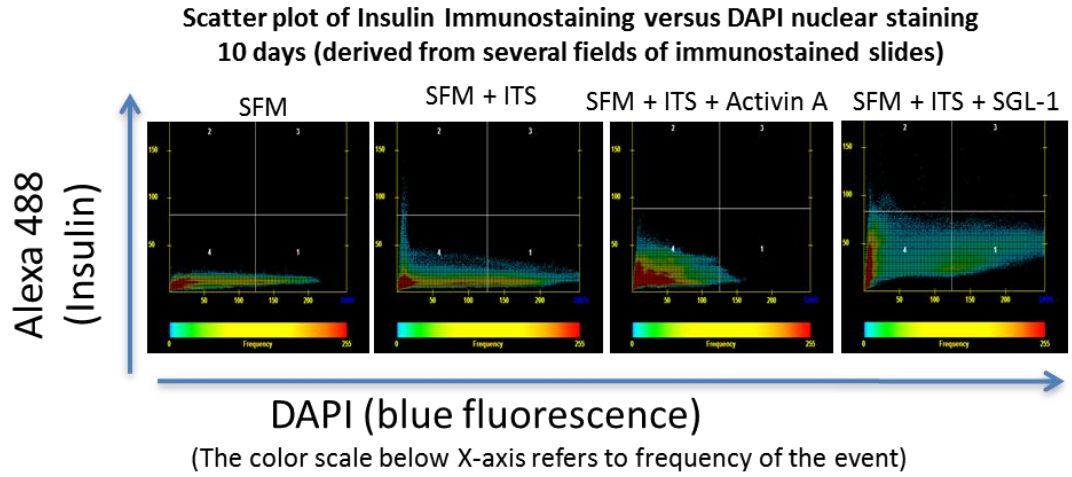


Fig 6.7: Quantitative scatter plot of insulin fluorescence in ILCC: The data from 3-5 fields after immunostaining for insulin were pooled to create the scatter plot representing intensity and number of islet-clusters that are positive for insulin (red).

undifferentiated panc-1 cells (fig 6.8). ILCC protein of 10th day differentiated clusters, when probed for various TF and MAP kinase signal pathway revealed substantial inhibitory effect on proliferation rate in all groups (SFM; ITS, Activin-A and swertisin) demonstrating induction of differentiation process. However an elevated expression of nestin with ngn-3 protein in Activin-A and swertisin group indicated that panc-1 cells are enforced to acquire endocrine phenotype similar to islet cells.

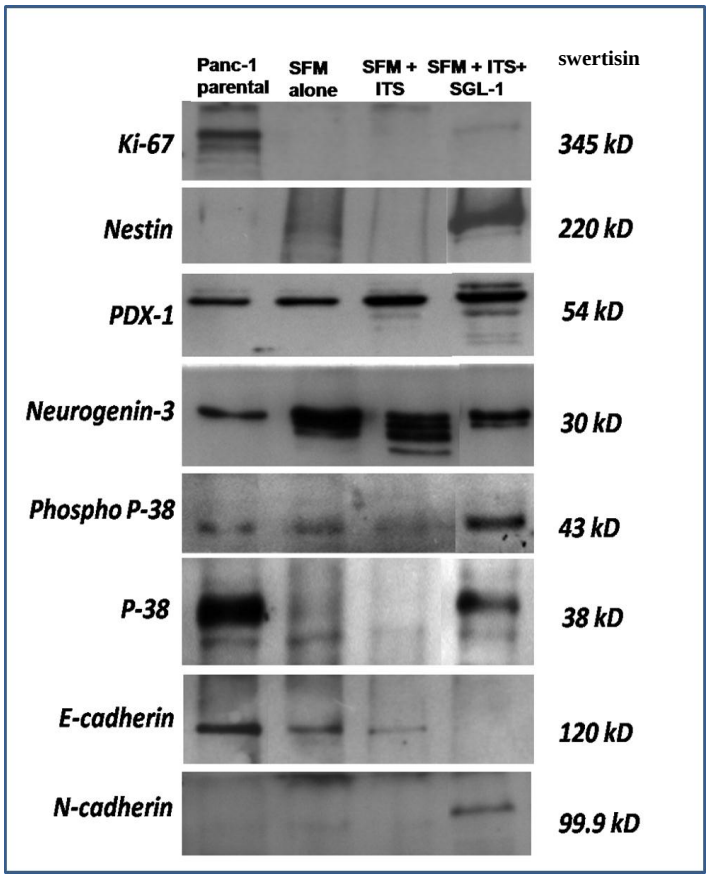


Fig 6.8: demonstrating protein expression of various islet differentiation pathway transcription factors and MAP kinase pathway proteins. ILCC from all groups (SFM; ITS; Activin-A and Swertisin) was collected at 10th day, and probed to understand the islet differentiation mechanism mediated through MAP kinase pathway.

As reported, activin picks up high ngn-3 expression due to increased p-38 phosphorylation; we observed that swertisin also proceeds with same signal cascade. On 10th day swertisin clusters showed high p-38 phosphorylation with high native p-38 expression suggesting the fate of islet differentiation may follows p-38 mediated over activation of ngn-3 leading to new functional islet cells. On other hand, SFM and SFM+ITS clusters failed to shoot nestin expression inspite of increased ngn-3 expression. Also they do not exemplify p-38 phosphorylation as evident with activin and swertisin groups (fig 6.8).

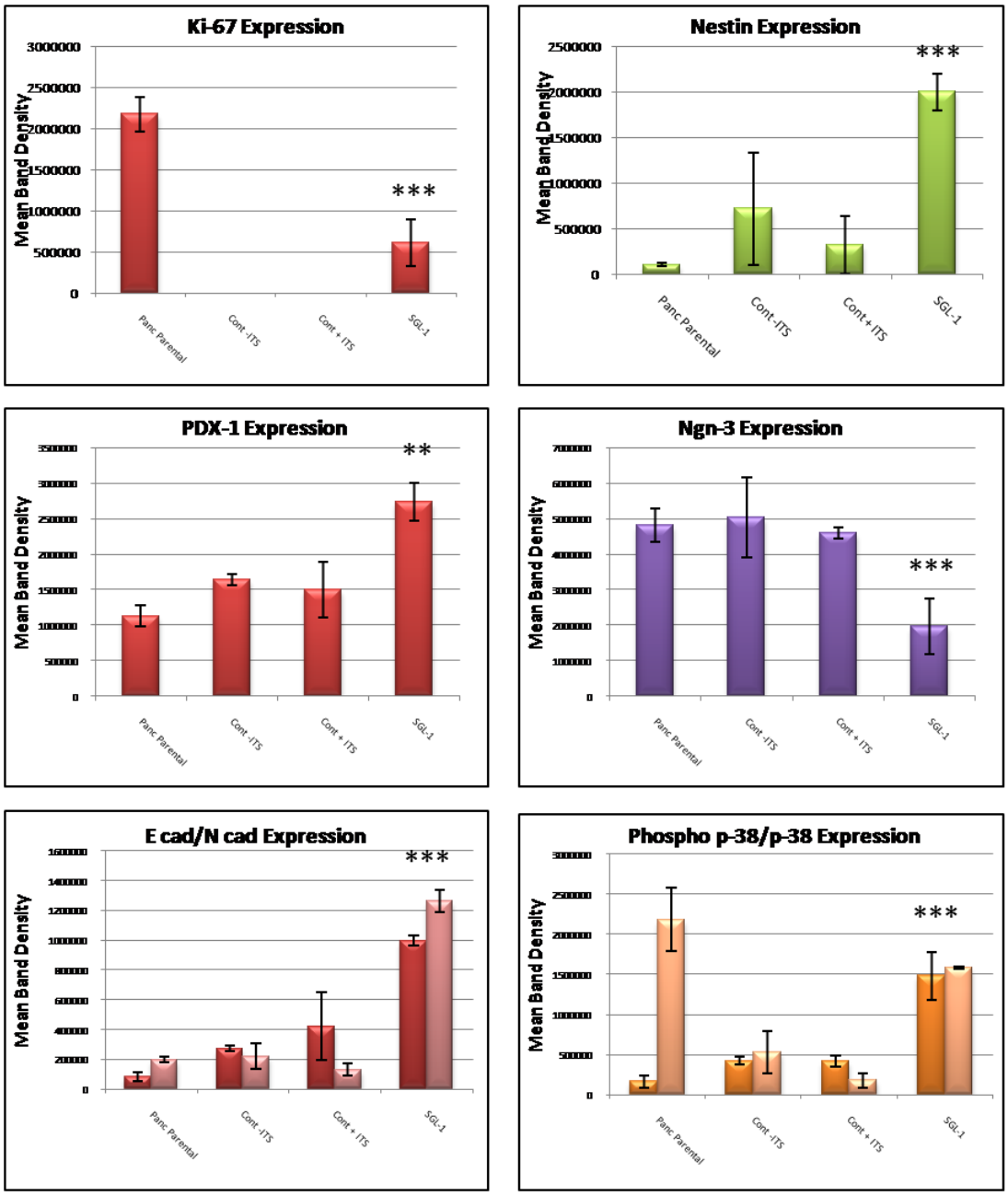


Fig 6.9: Comparative graphs of protein expression of various TF's and MAPK pathway proteins probed at 10th day of differentiation process. Graph shows expression of Ki-67; nestin; PDX-1; Ngn-3; Ratio of Ecad/ Ncad and P-p38 / p-38. Data represents mean±SEM of three independent observations. *** P<0.001 ; ** P<0.01 and * P<0.05 vs. Control +ITS.

The comparative statistical protein expression analysis revealed that swertisin at 10th day able to shoot nestin, PDX-1 expression significantly high compared to SFM+ITS, while that of Ngn-3 significantly down regulates (fig 6.10). Moreover swertisin also showed high E-Cad to N-Cad ratio and phosphor-p38 to basal p-38 ratio.

6.3.3 Kinetic protein expression study for examining *in-vitro* mechanistic action of swertisin

Immunoblot study on 10 day process of differentiation indicated that swertisin may facilitate differentiation process by activin mediated pathway. To understand this mechanism in more detail and provide enough evidence for swertisin targeting ACT-MAPK-TKK pathway, a ten day time course kinetic study was performed with SFM+ ITS, Activin-A and swertisin. Samples were harvested on each day and then probed for nestin, ngn-3, pdx-1, phospho-p38 and E-Cadherin. In this time course study we examined changes in key parameters that would indicate that precursor PANC-1 cells are being converted to islet-like endocrine cells via p-p38 pathway (fig 6.10). We noted that Nestin, a marker for precursor/stem cells increased up to 2-3 days after Activin A treatment and subsequently declined by 7 days, while that in swertisin it shoots by day 2 and remain till day 9, indicating that these cells are changing from precursor type to differentiated cells. The pancreatic duodenal homeobox gene 1 (PDX-1), a marker of pancreatic endodermal cells remained constant up to six days with a small increase in its expression from 7-10 days and same is the case with swertisin as well. Further as expected, the expression of activin-targeted key islet differentiating gene Ngn-3 was strongly upregulated from 2nd day and persisted up to 10th day whereas with swertisin it increased right away by day one and peaks at day 4-6 and declined by day 9 (fig 6.10) . In parallel, since the effect of activin on Ngn-3 expression is mediated via p38 MAP kinase, we noted that phospho-p38 MAP kinase signal increases by second day and remains high from 5-9 days. Similar trend was also observed with swertisin where Phospho-p38 MAP kinase signal increases by second day, peaks at day 4 and then declined by day 7, leading to activation of Ngn-3 in consequence (fig 6.10).

6.3.4 Confirmation of *in-vitro* swertisin mode of action via MAPK-TKK pathway using p38 MAP kinase specific inhibitor SB203580

In order to confirm the fact, that swertisin does mediate islet differentiation *via* MAP kinase pathway we again attempted to carry differentiation experiment in presence and absence of specific p-38 MAP kinase inhibitor i.e. SB203580.

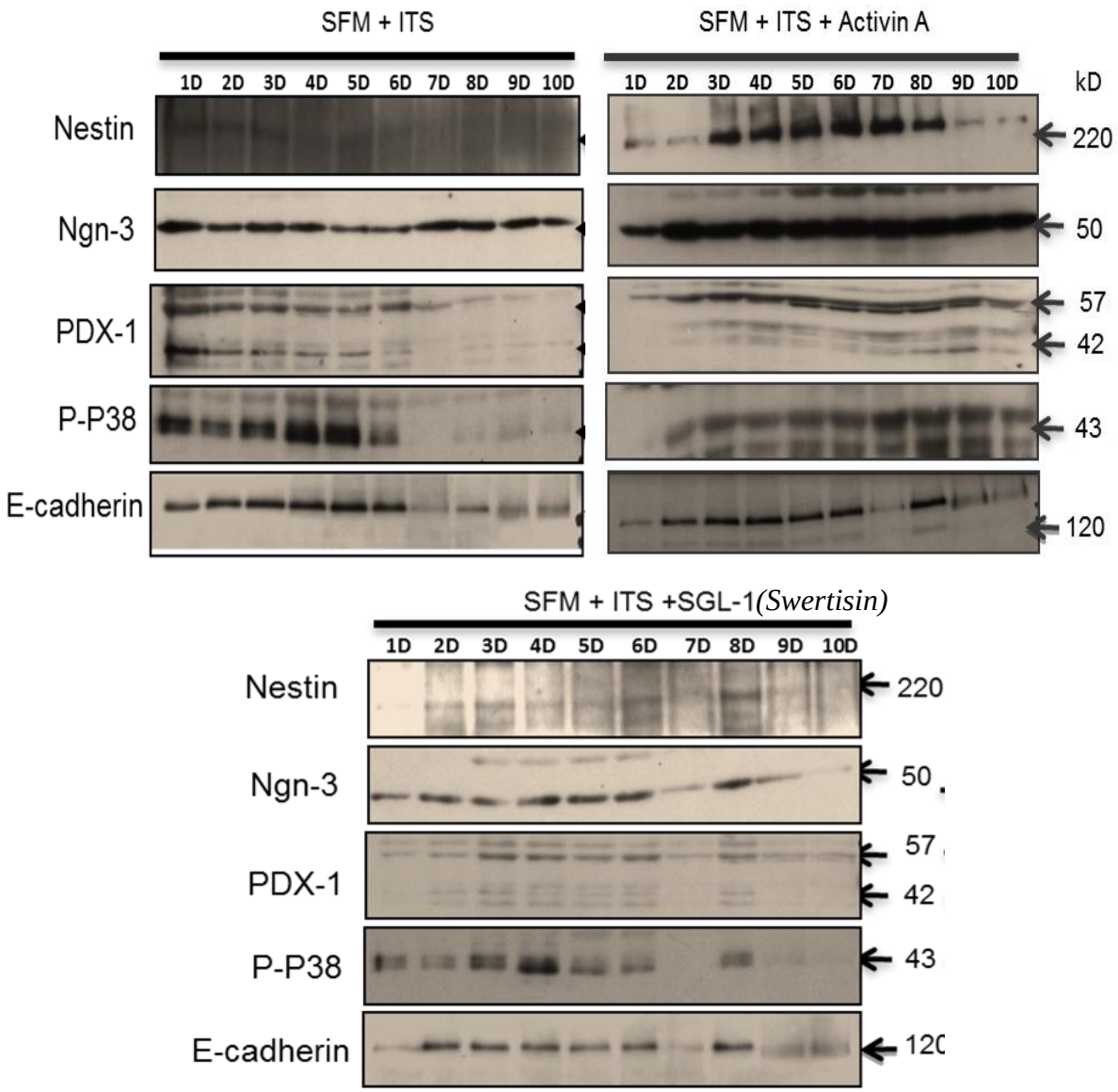


Fig 6.10: Long term kinetic study (1 to 10 day) for various Tf and MAPK protein expression with SFM; Activin-A and SGL-1 (Swertisin) mediated ILCC differentiation. Samples at each day collected and probed for nestin, ngn-3, pdx-1, p-p38 and e cadherin proteins.

Herein we observed that panc-1 cells when differentiated normally with Activin-A and swertisin induction, showed deep cytoplasmic insulin staining (green) in immunocytochemical analysis. Cell in SFM ILCC fails to stain for insulin, while those of SFM+ITS showed some insulin positive cells. As evident from literature that Activin-A process inhibits if p-30 MAPK pathway was blocked, so we observed significant abolishment of insulin positive cells when Activin-A clusters were differentiated with SB203580 (inhibitor of p-38 MAP kinase) (fig

6.11). Surprisingly, we also observed that swertisin induced panc clusters also fail to synthesis insulin hormones in presence of SB203580, which had earlier showed intense insulin presence in absence of it (fig 6.11).

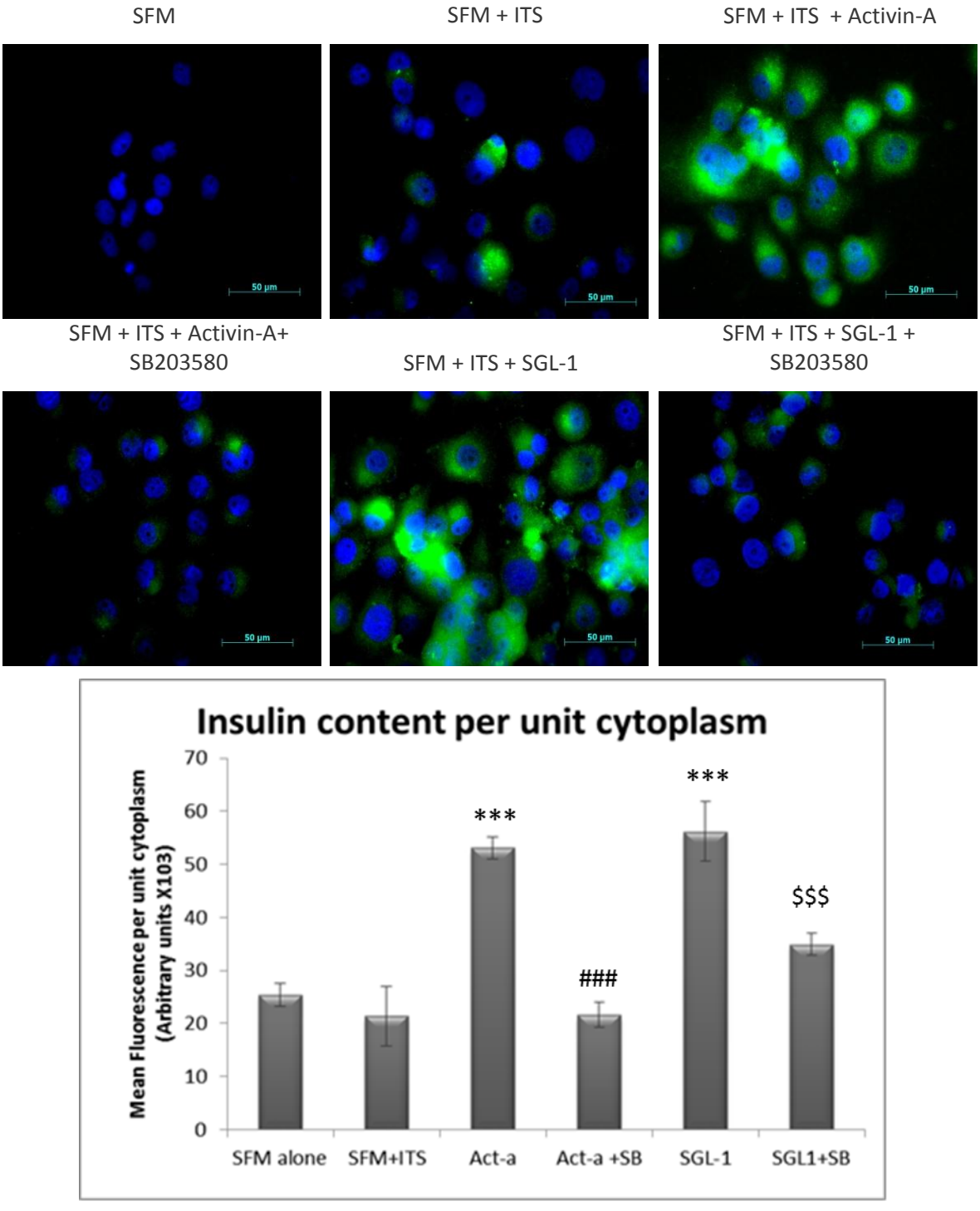


Fig 6.11: representing immunocytochemical images of insulin expression in panc-1 cells after induction with ITS alone; Activin-A and SGL-1(Swertisin) along with conjunction of p-38 pathway inhibitor SB203580. The graph represents insulin content per unit cytoplasm. ***P<0.001 vs. SFM+ITS; ###P<0.001 vs. Act-A and $$$P<0.001 vs SGL-1.

6.3.5 *In-vivo* mechanistic action of swertisin for islet differentiation in 90% partial pancreatectomy mice

90% partial pancreatectomy (Ppx) has been reported to involve beta cells replenishment by islet neogenesis from stem/precursor cells. In present experiment we tried to investigate and reconfirm the mechanistic action of swertisin induction for new islet formation mediated through ACT-MAPK-TKK pathway, under *In-Vivo* condition in surgically induced diabetic pancreatectomized mice.

We observed that mice undergone 90% Ppx (Fig. 6.12a) with and without swertisin did not demonstrate any change in body weight during three days post ppx, compared to normal mice (fig 6.12b). An increasing trend in remnant pancreas tissue weight has been observed in swertisin treated animals on 3rd day, notifying small volume formation of new pancreas tissue. Although it is not statistically significant compared to control (fig 6.12c)

In order to confirm the islet differentiation, mRNA expression for various islet specific TF was observed using RT-PCR. Semi quantitative gene expression data reveled that Ppx alone animals favored hyper activation of pancreatic progenitor gene –PDX-1 and not reached to endocrine fate commitment till 3rd day post Ppx. On the other hand, swertisin treated Ppx mice identified low PDX-1 but more Ngn-3 expression suggesting early induction and endocrine fate regeneration signals (fig 6.12d). None of the animal group had shown nestin expression, but swertisin treated ppx mice reveled significantly elevated insulin gene mRNA transcripts compared to Ppx alone (fig 6.12d).

Protein extracted from regenerated pancreas tissue on 3rd day was probed for islet specific and Activin-A signaling protiens. In western blot analysis we found that Ppx alone animals fails to induce nestin expression as noted in mRNA data, whereas swertisin treated Ppx animals persist some nestin expression leading to early differentiation signaling (fig 6.12e). Further the markers of ductal progenitor cells Cytokeratin-19 (CK-19) was highly upregulated in Ppx alone mice while significantly down regulated in treated ones.

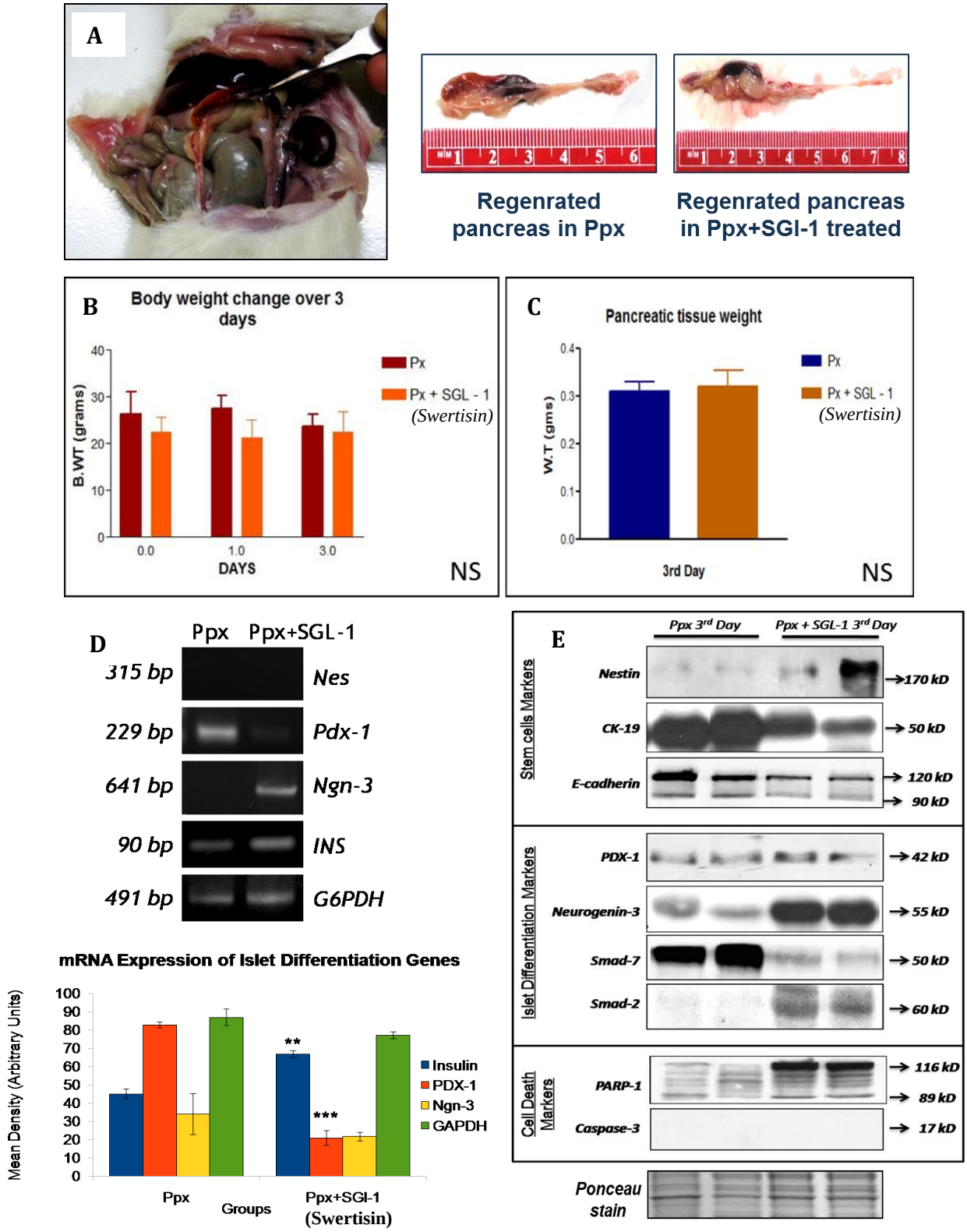
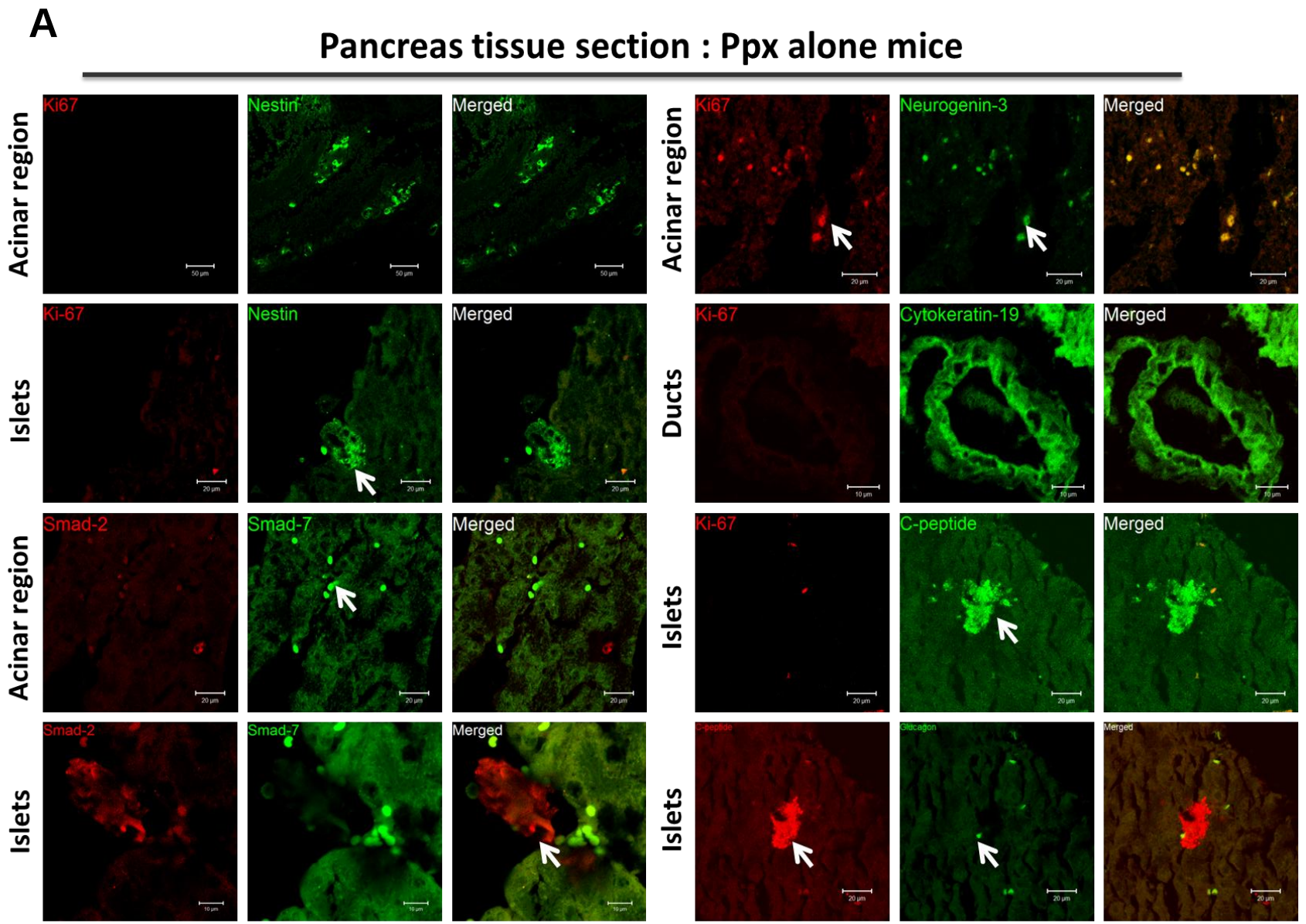


Fig 6.12a showing partial pancreatectomy in mice removing splenic and mesenteric pancreas and pictorial representation of regenerated pancreas. Fig 6.12b demonstrate change in body weight on 0, 1st and 3rd days post-surgery. Fig 6.12c shows change in pancreas weight calculated on 3rd day after sacrifice. Fig 6.12d show gene expression data for various islet specific markers in regenerating pancreas tissue. Fig 6.12e represents western blot profile of key transcription factors and signal molecules in activin induced islet differentiation pathway. All data seta are represented as mean ± SEM and calculated from 3-5 independent animal observations. NS=No significance.

E-cadherin which is known to express more in progenitor cells compared to differentiated cells has been observed in high proportion in Ppx alone animals but low in treated animals. Interestingly, at protein levels there was no change in PDX-1 expression in both animal groups in contrast to mRNA levels. ngn-3 a trigger of Map kinase signaling demonstrated slightly elevated expression in Ppx alone mice, but dramatic increase in ngn-3 expression in swertisin treated animals confirming that its role as trigger for ACT-MAPK-TKK signaling. To further confirm this, the downstream TF like smad 2 and 7 were analyzed. When compared we found Ppx alone mice expressing significantly high smad 7, While high samd-2 phosphorylation in swertisin treated animals which is a prerequisite for induction of islet differentiation pathway (fig 6.12e).

6.3.6 Activation of ACT-MAPK-TKK signaling with swertisin induction confirmed by confocal microscopy of regenerated pancreas tissue

We then observed and confirmed the activation of p-38 mediated activation of ngn-3 expression leading to islet neogenesis *In-Vivo* with swertisin treatment by staining the section of regenerated pancreas tissue from Ppx alone and swertisin treated animals. Various markers for islet differentiation pathway and activin signaling were analyzed. In Ppx tissues we found that nestin expression (green) was randomized in some patches near acinar tissue, but few islet cells showed nestin positivity as well. None of these areas showed ki67 expression (red) showing less proliferation of acinar or islet cells. A persistent proliferating population of ngn-3 positive (green) cells was observed with dual ki67 staining (red). Moreover Ppx animals also showed intense Ck19 (green) staining in epitheloid ductular structures. Most important observations were that ppx animal tissue had deep smad-7 (green) staining and very weak p-smad 2 (red) staining. The presence of low c-peptide with few scattered glucagon positive cells and compact size islet indicating slower differentiation process. On other side, swertisin treated animal tissue showed large number of dual stained population of ki-67 and nestin within both acinar and islet tissue. These sections also showed low Ck19 staining in ductular tissues, but enormously high p-smad-2 staining within acinar and islet area as well. The staining of smad-7 goes lower in treated animals showing less presence of progenitor nature of cells (fig 6.13).


A
Pancreas tissue section : Ppx alone mice
Acinar region
Ki67
Nestin
Merged
Acinar region
Ki67
Neurogenin-3
Merged
Islets
Ki-67
Nestin
Merged
Ducts
Ki-67
Cytokeratin-19
Merged
Acinar region
Smad-2
Smad-7
Merged
Islets
Ki-67
C-peptide
Merged
Islets
Smad-2
Smad-7
Merged
Islets
C-peptide
Glucagon
Merged

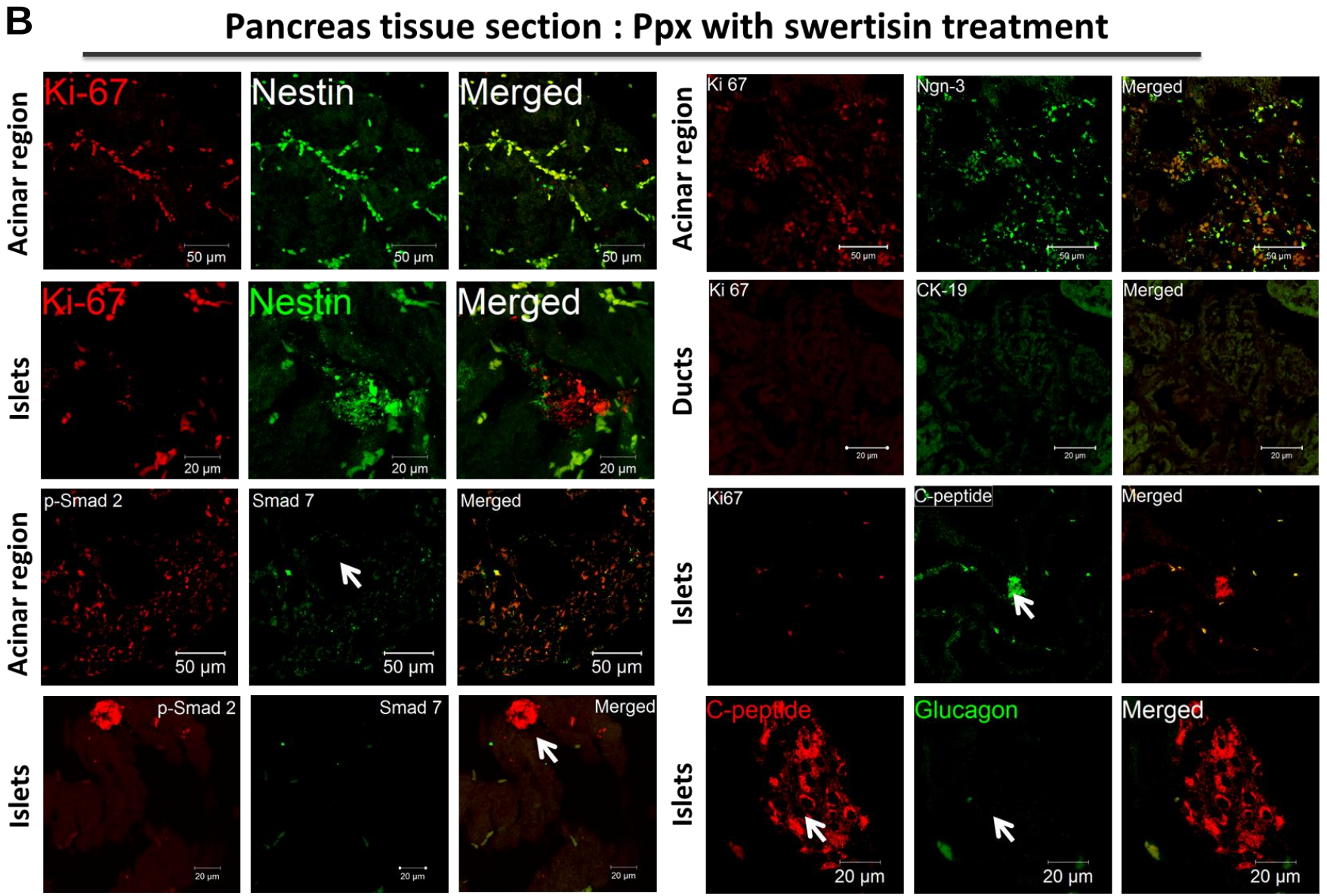


Fig 6.13a shows confocal images of various islet transcription factors and signaling proteins from tissue of Ppx alone animals while Fig 6.13b shows same protein confocal images from pancreas tissue of Ppx with Swertisin treatment.

6.4 Results: Role of poly (ADP) Ribose polymerases in islet differentiation

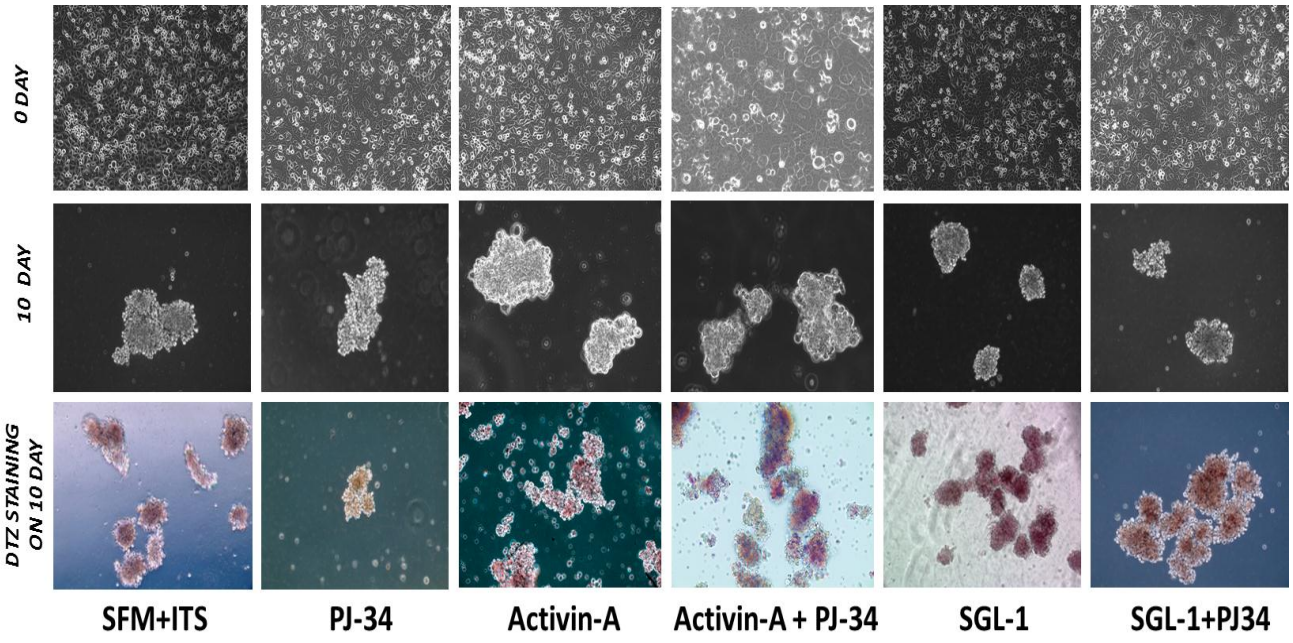
PARP-1 a nuclear enzyme has been shown to play important role in diabetes. Whether it is insulin signaling or pancreas regeneration both are affected with PARP protein, when catalytically blocked using either weak or strong inhibitors. Few recent reports had shown that PARP does play modulatory role in stem cell differentiation as well. So far PARP-1 enzyme had been observed with increasing reg-1 and Maf-A expression in Ppx and INS cell line model systems.

To investigate that whether PARP-1 also has significant modulatory role in islet neogenesis (differentiating stem/progenitor cells into insulin producing cells), we carried out differentiation study using pancreatic progenitor model, PANC-1 cells in presence and absence of specific PARP inhibitor PJ-34, which block activity of all PARP family enzymes. Further we also attempted to create PARP-1 replete and deplete cells using DNA vector based RNA*i* technology, and PARP-1 role was again monitored in absolute absence of PARP-1 during differentiation.

6.4.1 Differentiation of normal Panc cells with strong PARP inhibitor PJ-34

Human pancreatic carcinoma cells, Panc-1 showing pancreatic progenitor features has already been monitored for new islet cluster formation earlier in chapter 3 from both Activin-A and swertisin compounds. To test effect of PARP inhibitor on islet differentiation pathway, panc-1 cells were differentiated in presence of PARP inhibitor PJ34 alone and in combination with Activin-A/ swertisin compound as well.

After induction with same four stage protocol till 10 day we found that PJ-34 showed effective retardation in islet cluster yield and less intense DTZ staining (fig 6.14). Whereas, the same when added in combination with Activin-A showed same islet cluster yield but intense DTZ staining even more than Activin-A alone does. Similarly, we also found decrease in islet cluster yield when PJ-34 added in combination with swertisin but we do not found much difference in DTZ staining unlike activin-A with PJ-34 (fig 6.14).



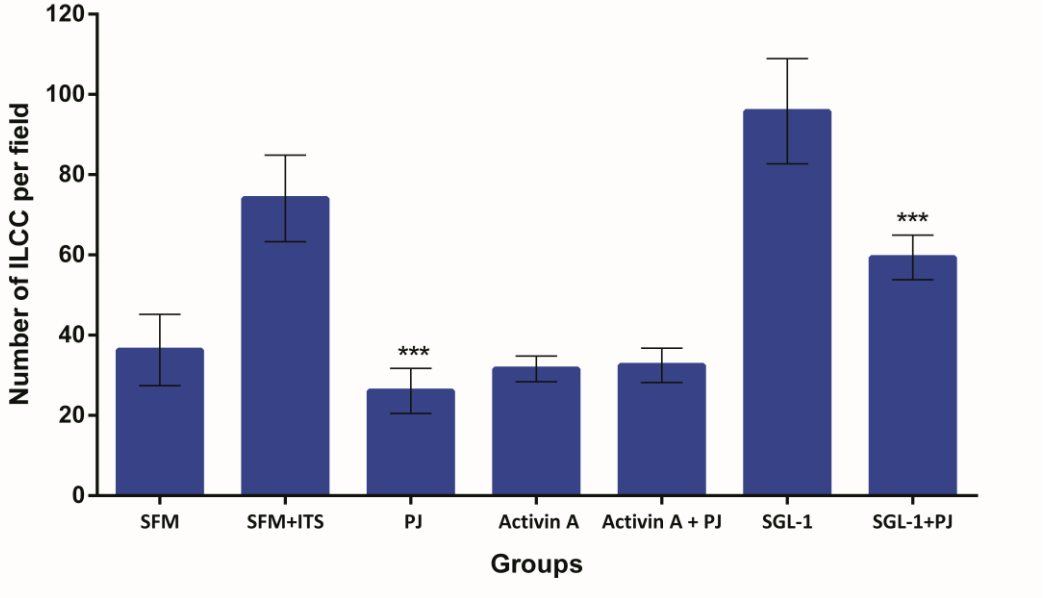


Fig 6.14a shows differentiation of normal panc-1 cells into ILCC in presence and combination with Activin-A and SGL-1(Swertisin). Pictures include stage representation at 0 day, 10 day and DTZ staining (10 day). Fig 6.14b represents graph for yield of ILCC generated with use of PARP inhibitor PJ34 and in combination with Activin-A and swertisin. Results are expressed as mean ± SEM. N=10 observations. * indicate p<0.001 vs SFM+ITS group for PJ-34 alone and SGL-1 for SGL-1+PJ-34.**

6.4.2 Immunoblotting of islet transcription factor for determining role of PARP-1 in islet differentiation by PJ-34 inhibition.

For understanding the mechanism and impact of PARP inhibition on differentiation pathway, we carried out Immunoblot profile of key transcription factors, where we found that PARP inhibition with PJ-34 alone without any

differentiating agent abrogate the differentiation process. Down regulation of PDX-1 and phospho-p38 suppression indicate that differentiation process was dramatically retarded or probably slower down. Initial but transient increase in ngn-3 expression with persistent nestin expression also supports the delayed response of cells for differentiation in presence of PARP inhibitor. Also PARP-1 was found to up-regulate till 5th day and then declines by 10 day (Fig 6.15).

Moreover, when differentiation was carried in combination with Activin-A, we observed that PJ-34 augmented islet differentiation. Compared to PJ34 alone, in combination with Activin-A, cells showed improved pdx-1 expression like activin-A alone till 10 days. Phosphorylation of p-38 MAPK was also found to increase with this combinatorial effect and persisted till 10 day. Up-regulation of ngn-3 by day 2, peaks at day 5-6 and then declining to maintain basal levels even till 10 day demonstrate efficient islet differentiation and additive effect of PJ-34 with activin-A (Fig 6.15). Nestin expression was only seen in last days of differentiation (7-10 day) Unlike PJ-34 alone, here the PARP-1 expression was sustained higher till 10 days indication evident involvement of PARP-1 in differentiation process.

The same combination when observed with swertisin molecule, we found slightly different results. First we did not observed pdx-1 expression which was quite evident with Activin-A combination, seems the effect was more similar to PJ-34 alone. Also the ngn-3 expression was quite abrupt due to inaccurate p-38 phosphorylation pattern. The nestin expression was also suggested the slower pace of differentiation pattern as it prevailed till 8 days. More surprisingly we found that the PARP-1 protein expression was initially up-regulated but it declines very shortly within 4 days and never shoots again, whereas the same thing also seen with PJ-34 alone but there the expression persisted till 6 days and then declined (fig 6.15).

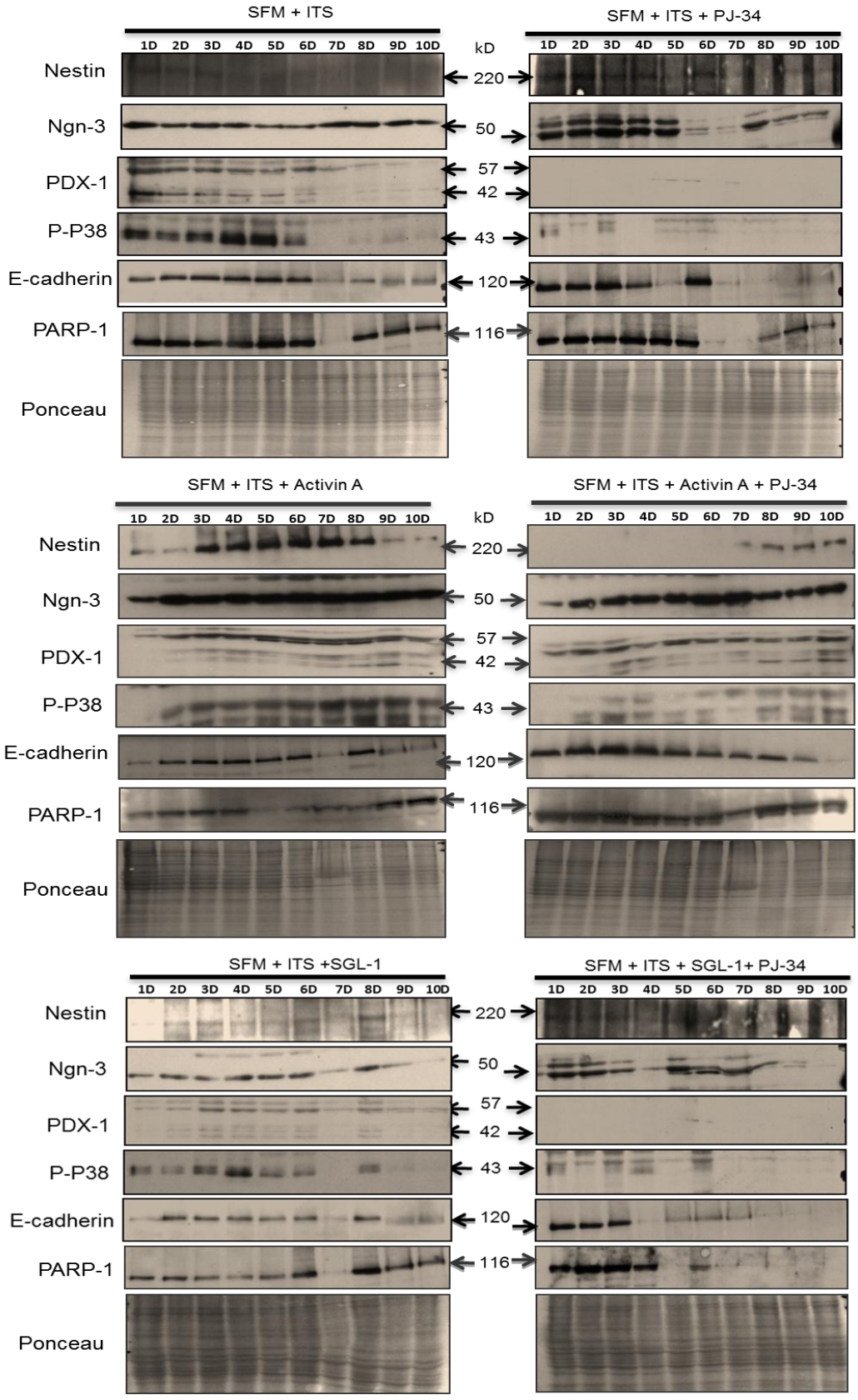


Fig 6.15 represents time dependent kinetic (day 1-10) study of differentiated ILCC and carried immunoblotting profile of various islet specific transcription factors and MAPK pathway protein along with PARP-1 in presence and combination of PJ-34 with Activin-A and swertisin agents.

6.4.3 Immunocytochemical analysis of effect of PARP inhibition on islet differentiation

Further we carried immunocytochemistry for insulin protein in differentiated ILCC with PJ-34 alone and in combination to understand the endpoint biological phenomenon i.e. biogenesis of insulin. The effect of PARP inhibition on insulin biogenesis reveled that clusters generated with PJ-34 alone failed to differentiate and produce insulin after 10 day treatment, which was also observed earlier with DTZ and western profile. While the cells when differentiated with combination of Activin-A and PJ-34, very intense insulin staining was observed within the clusters. This highlighted that PJ-34 with Activin-A facilitates islet biogenesis and more differentiation in panc-1 cells. When we observed clusters with swertisin and PJ-34, the effect was reverse very less insulin staining was found in these clusters, this again was observed earlier in western blot data depicting here PJ-34 inhibition doesn't supports islet neogenesis (Fig.6.16).

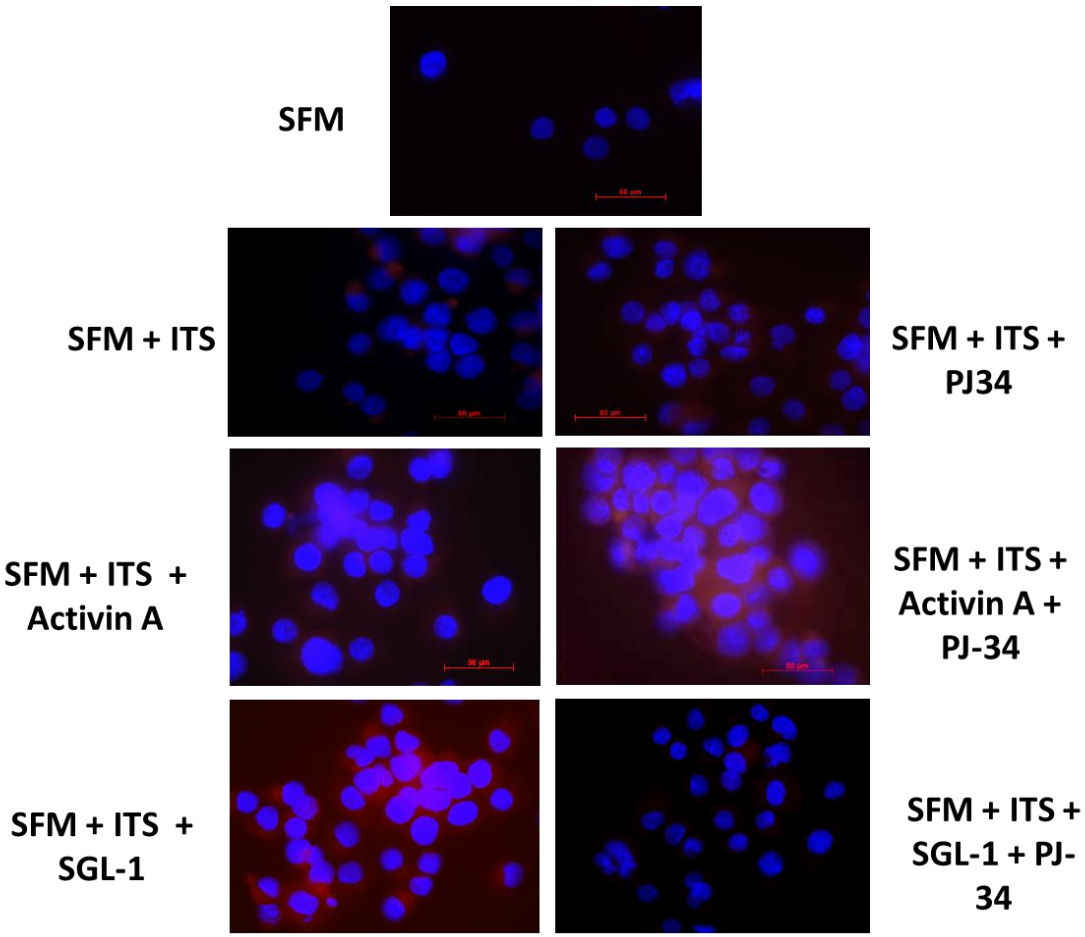


Fig 6.16 demonstrating immunocytochemistry for insulin protein in ILCC differentiated in presence and combination of PJ-34 with Activin-A and swertisin agents. Insulin in red showed cytoplasmic staining and DAPI nuclear.

6.4.4 Effect of PARP-1 knockdown on islet neogenesis pathway

PARP inhibition study have showed crucial role of PARP-1 protein in process of islet differentiation when monitored with use of specific and strong PARP inhibitor PJ-34. Those above experiments had denied the possibility of role of PARP-1 enzyme activity in differentiation process. Additionally, it raised a question and valid point to check whether PARP-1 as per protein does have any impact on islet differentiation pathway (Fig. 6.17).

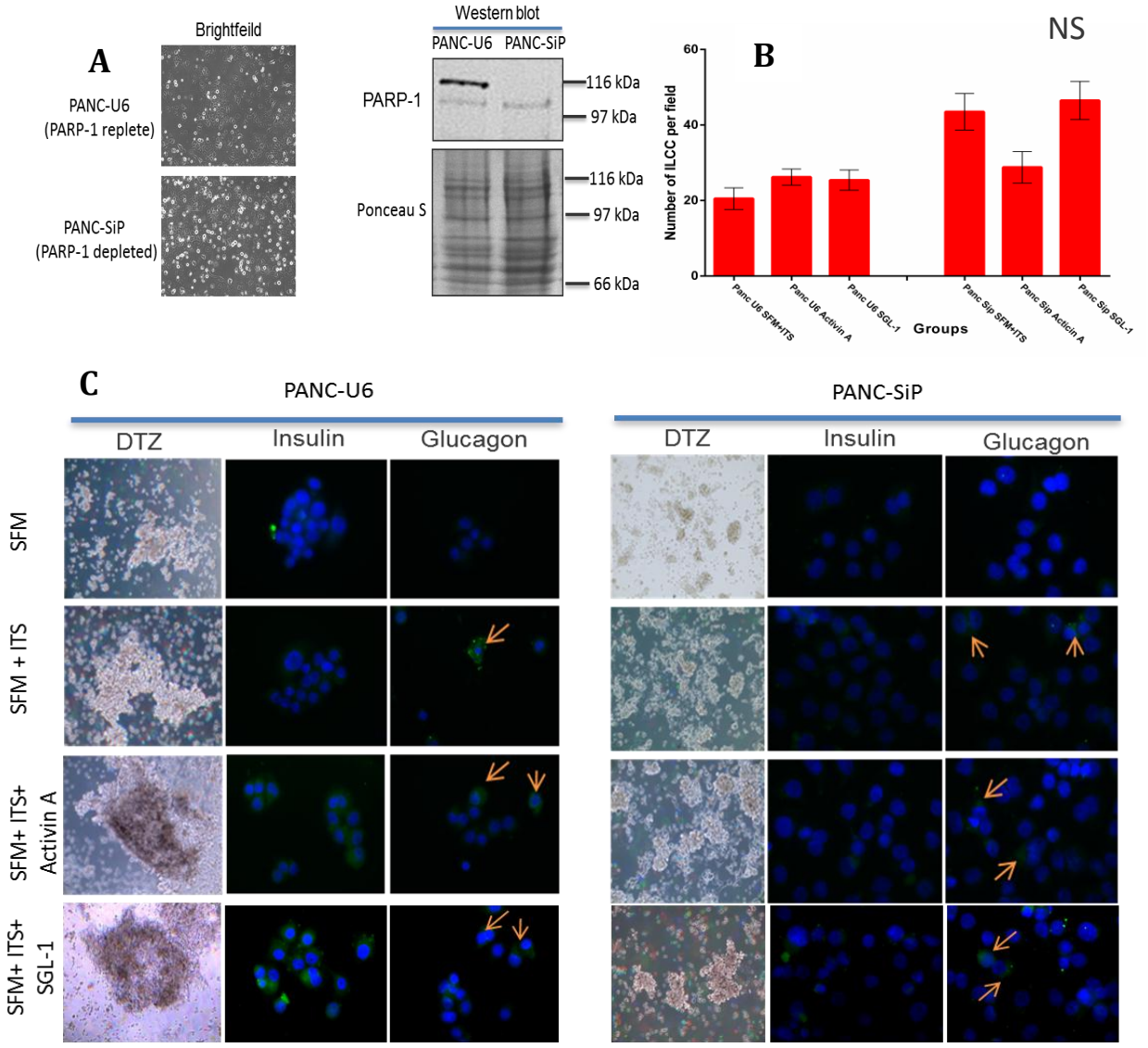


Fig 6.17a represents clone confirmation from panc-1 U6 (PARP-1 replete) and SiP (PARP-1 deplete) using Immunoblotting. Fig 6.17b shows ILCC yield from U6 and SiP clones on 10th day. Fig 6.17c denotes cluster formation with DTZ staining in ILCC from U6 and SiP clones and immunocytochemistry pictures for insulin and glucagon in clusters generated from U6 and SiP clones on 10 day differentiation using Activin-A and swertisin compounds. Presence of insulin is observed as deep cytoplasmic staining (green) and glucagon positive cells (green) are shown by orange arrows heads.

In order to answer these questions we created PARP-1 knockdown using DNA vector based shRNA against PARP-1 enzyme as explained in material and method section earlier in this chapter. With vector control for shRNA we generated PARP-1 U6 replete clones and PARP-1 knockdown SiP clones using shRNA SiP912 against catalytic domain of PARP-1 protein. Immunoblotting data for U6 and Sip clones showed more than 95 % knockdown of PARP-1 protein in panc-1 cells (fig 6.17a). Morphologically also we have not observed any change in PARP-1 deplete cells and those are similar in growth and shape to PARP-1 U6 replete cells.

Further when these U6 and SiP cells were differentiated with SFM+ ITS, Activin-A and swertisin compounds, the total ILCC yield was quantified. Anonymously PARP-1 Sip cells did not showed any significant effect on cluster yield generation, but more importantly the clusters were deformed, with loosen cells, smaller in size and immature with undeveloped architecture compared to U6 cells, where PARP-1 protein was present (fig 6.17b and c). Therefore it becomes more important to check insulin and glucagon positive cells in these clusters using immunocytochemistry. Panc-1 U6 cells showed normal differentiation induction and depicted presence of both insulin and glucagon positive cells after 10 day from both activin-A and swertisin induction, whereas panc-1 SiP cells failed to produce insulin but not glucagon, from both activin-A and swertisin after 10 day treatment. These result indicated and evident role of PARP-1 protein in insulin cell differentiation but not on glucagon cells within newly developed islets (fig 6.17c).

6.4.5 Mechanism of islet differentiation hampers in PARP-1 deplete panc-1 cells but not in replete panc-1 cells

Proteins from panc-1 U6 and SiP ILCC when probed for key islet differentiation pathway proteins; both activin and swertisin were able to show phosphor-p38 up regulation leading to ngn-3 over activation and decline of e-cadherin proteins (fig 6.18). Whereas panc-1 SiP cells failed to show this mechanism and confirmed their sustained progenitor state even after 10 day induction with Activin-A and swertisin. SiP clones showed high e-cadherin till 10 days and inspite of persisted

p-38 phosphorylation, ngn-3 expression was not at all upregulated, with pdx-1 expression declined in SiP clusters (fig 6.18). These all mechanistic evidences confirm with insulin imaging data that PARP-1 has essential role in islet neogenesis pathway, regardless of its activity.

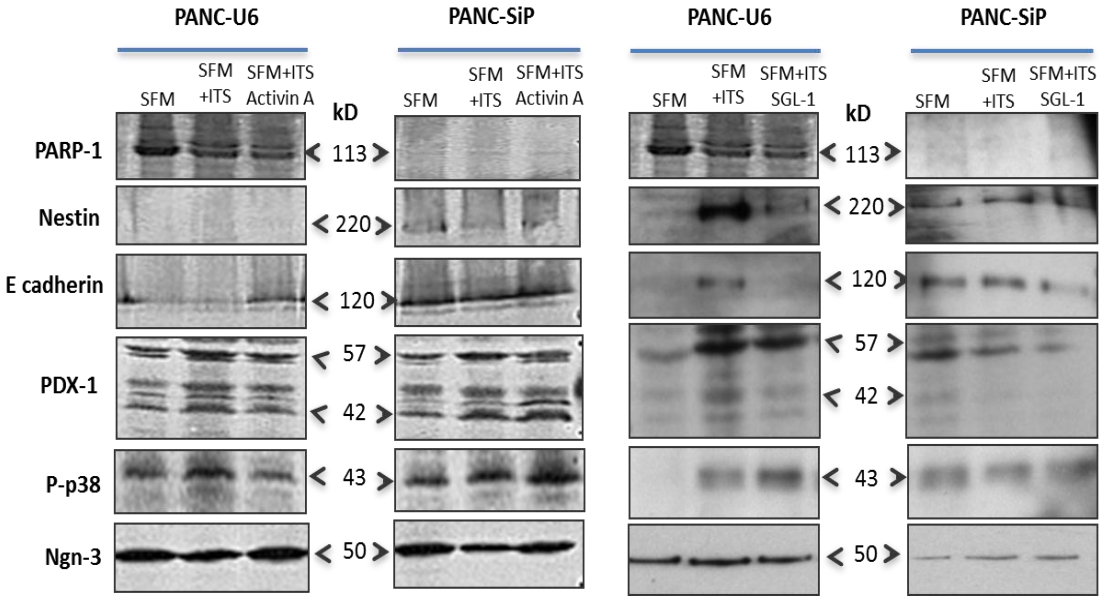


Fig 6.18 demonstrate western blot profile of islet differentiation pathway signaling in 10 day differentiated ILCC with Activin-A and swertisin induction.

6.5 Discussion

Pancreas (organ transplant) or Cadaveric islet transplantation treatment strategies have many limitations (Stratta and Alloway 1998). Stem cell to islet differentiation provide alternative approach to cure the disease (Pittenger, Taylor-Fishwick et al. 2009). Currently efforts are being made to generate insulin-producing cells from stem cells or tissue specific progenitor cells (Bonner-Weir, Taneja et al. 2000; Lumelsky, Blondel et al. 2001). Very few reports are there which show pancreatic regeneration using herbal extract or compounds. One of such study done by Kojima et al. showed insulin positive cell differentiation from AR42J cells using conophylline isolated from *Ervatamia microphylla* (Ogihara, Watada et al. 2003). *Enicostemma littorale* is one such anti-diabetic plant which has been examined for it's capacity of promoting islet neogenesis as demonstrated earlier in chapter-3, 4 and 5 of this thesis. *In-Vitro* formation of islet-like cell clusters containing both β and α cells starting from

NIH3T3, PANC-1 cell lines, primary cultured mBMSC and adult rPSC, when treated with herbal bioactive agent swertisin isolated from *E. littorale* methanolic extract (Gupta 2010). This led us to investigate the molecular mechanism of this potent bioactive compound swertisin for its effective islet cell differentiation property. We hypothesized that swertisin may differentiate progenitor cells undergoing pathway same as that of Activin-A mediated.

To test the mode of action of swertisin, we used panc-1 cells and compared the pattern of protein expression of ILCC generated from swertisin with Activin-A in time course manner (fig 6.19). In our experiment, swertisin effectively differentiates panc-1 cells with highest ILCC yield even more than activin. Further we could show that within ten days of treatment with Activin-A and swertisin, islet-like clusters were formed that could be stained with DTZ or immunostained for insulin. We have also noted that Nestin, a key marker in islet differentiation signaling, peaks by 4 day subsequently declined by 7-8 days, with strong up regulation of *Ngn-3* between 4-6 day in both Activin-A and swertisin mediated ILCC protein blots.

According to the literature, pathway for islet differentiation starts with either nestin progenitor cells (Lardon, Rooman et al. 2002; Selander and H.Edlund 2002; Klein, Z.Ling et al. 2003) or by activation of master key gene Ngn-3 for islet endocrine cells(Baeyens, Bonne et al. 2006; Murtaugh 2007). Nestin was found to be expressed at different levels in the acinar component, as well as in ductal structures and islets to some degree (Corbett and Berger 2004). A number of investigators have taken this approach of generating islet, using Pdx1 as a candidate master regulator. The Pdx1 protein directly binds and activates the insulin promoter (Ohlsson, Karlsson et al. 1993), as well as that of at least one acinar enzyme gene (Swift GH, Liu Y et al. 1998). Further Baeyens et al., in 2006 demonstrated that *in vitro* growth factor stimulation can induce recapitulation of an embryonic endocrine differentiation pathway in adult dedifferentiated exocrine cells *via* Ngn-3 ectopic expression and over activation (Baeyens, Bonne et al. 2006). We also observed increased phospho-p38 MAP kinase expression prior to *Ngn-3* expression. Few other reports says that Activin-A a member of

TGF-β family of proteins that helps in growth and differentiation of β-cells (Demeterco, Beattie et al. 2000; Brown and Schneyer 2010) and promotes expression of pro-endocrine gene Neurogenin 3 (Ngn-3) by upregulating p38 MAP kinase (Ogihara, Watada et al. 2003) by smad 7 downregulation and activation smad2 and 4 phosphorylation, thereby facilitates islet neogenesis (Melissa L. Brown and Schneyer 2010). Since the signaling pathway of both Activin-A and swertisin found to be mediated through pro-endocrine gene Ngn 3 (Ngn-3) by up regulation of p38 MAP kinase (Ogihara, Watada et al. 2003), we then confirmed whether SB203580, an inhibitor of p38 MAPK could abolish islet-promoting action. With inhibition of MAP kinase signaling activin-A failed to form islets. The same was observed with swertisin which confirmed that swertisin do form islets mediated by ACT-MAPK-TKK pathway.

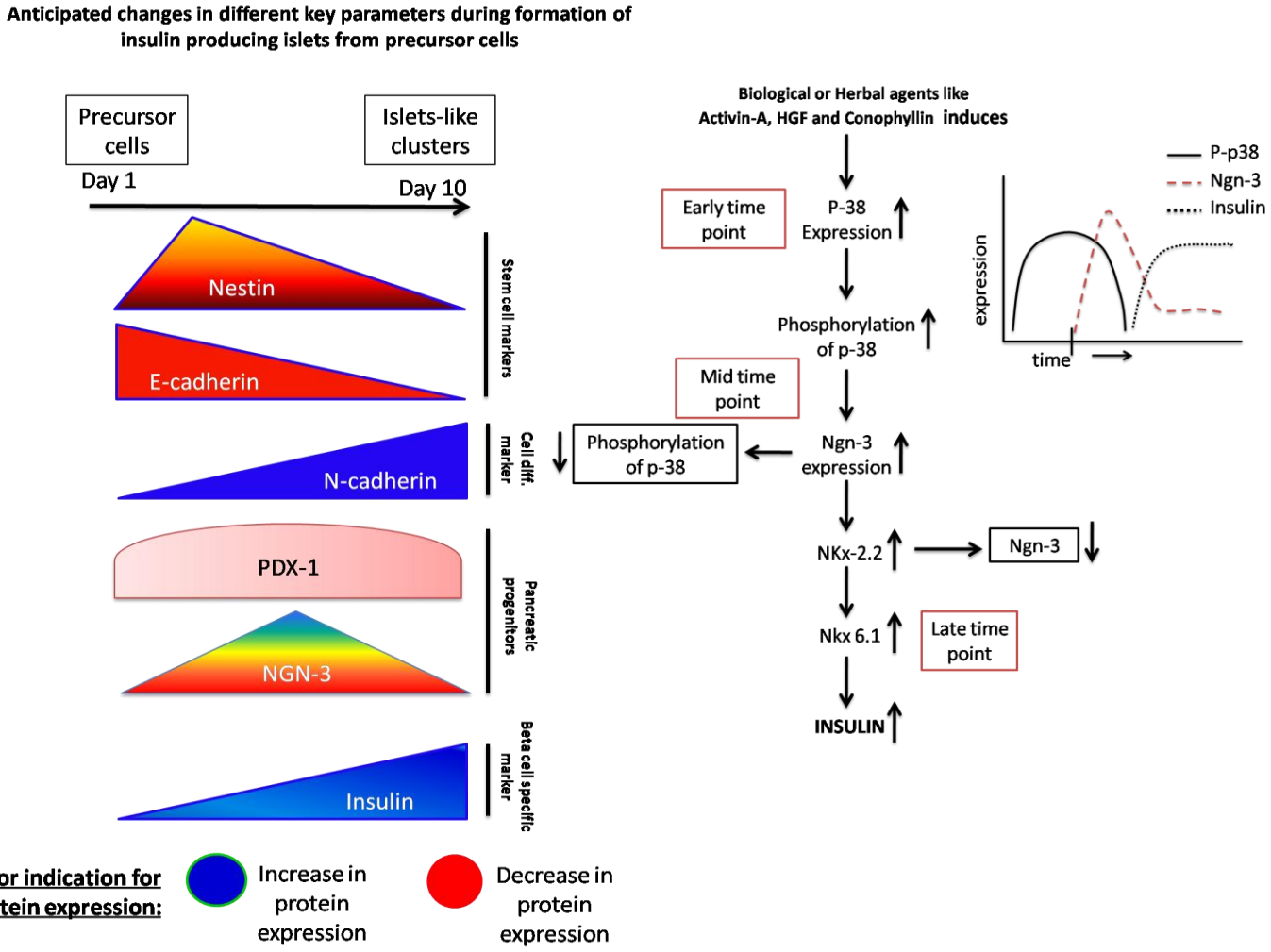


Fig 6.19 demonstrate anticipated changes in key parameters during islet differentiation pathway from 0 to 10 day and mode of action of Activin-A; HGF; and conophylline like compounds. P-38 activation in early time point with ngn-3 activation at mid time which translate to insulin in late time point.

Partial pancreatectomy Ppx is well known model for the study of β-cell regeneration. The mechanism by which regeneration occurs in this model has been controversial, with some claiming that islet and β-cell neogenesis is important, while others claim that β-cell replication is predominant. The process and mode of regeneration efficiency that follows with Ppx injury depends on the degree of surgical insult. In 50-7-% Ppx, β-cell mass expansion is mainly due to replication of pre-existing β-cells, but no ductal cell proliferation or Ngn-3 induction. However with 90% Ppx, the β-cell mass expansion has been attributed to neogenesis with the proliferation of putative ductal precursors in main pancreatic ducts, and induction of Ngn3 expression, since there is a wave of proliferation initiated in main duct, which is followed in smaller ducts and finally in islet β-cells, With transient up-regulation of PDX-1 in ductal cells along with nestin and ngn-3 (Bonal and Herrera 2008). On this basis we again tried to identify and reconfirm the mechanistic action of swertisin in *In-Vivo* condition with the choice of using 90% Ppx mice model, as it provides evidences to study islet neogenesis process endogenously. Thus when 90% Ppx animals were treated with single injection of swertisin in pancreas on day 0, they showed high ngn-3 expression by 3rd day with elevated p-samd2 and low samd-7, indicating ACT-MAPK pathway activation. Moreover less e-cadherin and ck-19 expression again reconfirms low ductal cell proliferation. All *in-vivo* results were found in accordance with earlier *in-vitro* observation by us and other groups (D'Amour, Bang et al. 2006; Kojima and Umezawa 2006).

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. Role of PARP in diabetes is being explored since last thirty years with early efforts employing PARP-inhibitors (Yamamoto, Uchigata et al. 1981) and later studies using PARP-1-/- mice or cells (Burkart, Wang et al. 1999; Pieper, Brat et al. 1999; Klein, Z.Ling et al. 2003; Ogino, Nozaki et al. 2007). Major emphasis by the scientific community overlaid in exploring the role of DNA repair enzyme PARP-1 is centered over beta cell death, survival, physiology and insulin secretion, however least attempts were made to

understand the possible mechanism of this enzyme in beta cell regeneration or differentiation.

Islet neogenesis is one such pathway for new islet cell differentiation and now observed to be dramatically influenced by PARP activity and DNA-Protein interaction with various key transcriptional factors involved in islet formation such as NGN-3, PDX-1, MafA, Reg-1 etc. (Akiyama, Takasawa et al. 2001; Ye, Tai et al. 2006). As this concept is still unsolved and neglected since long, we in this chapter made an attempt to answer this question, using specific PARP inhibitor PJ-34 and DNA vector based RNA-i mediated PARP-1 knockdown model in human pancreatic progenitor cells panc-1.

With the logic of inhibiting the whole PARP activity we use PJ-34 and observed that absolute PARP inhibition facilitate islet neogenesis with Activin-A but not with swertisin, while stable knockdown of PARP-1 protein completely abolish islet cell differentiation pathway. PARP-inhibitor PJ-34 is a nonspecific strong inhibitor which does not allow PARP-1 or PARP-2 to carry DNA repair function, while it executes the free protein to interact with various possible target protiens and modulate other physiological pathways apart from DNA damage induced repair (Ji and Tulin; Krishnakumar and Kraus; Rouleau, Patel et al.; Le Rhun, Kirkland et al. 1998; Schreiber, Dantzer et al. 2006). In our study, PJ-34 alone hampers the islet neogenesis pathway while along with Activin-A combination it accelerated the formation of islet-like clusters with intense DTZ staining suggesting insulin biogenesis and recruitment of essential transcription factors/ signaling proteins in islet differentiation. However, we could not observe same result of PJ-34 in combination with swertisin. SFM medium cause mild DNA damage leading to PARP over activation and no islet differentiation. Hence with inhibition of PARP activity free PARP protein interact and up regulate Ngn-3 expression as observed in our study as well.

But in combination with swertisin and PJ-34, islet neogenesis is not accelerated. This is due to the fact that swertisin earlier has been reported to have antioxidant potential (Vasu TV et al, 2005; Thirumalai T et al., 2011; Shibano M et al., 2008; Kumarasamy Y et al., 2004), which might have scavenged the free

radicles and did not allow upregulation of PARP-1 as observed in Activin-A mediated islet differentiation.

Earlier results with PJ-34 showed that PARP-inhibition stimulates formation of islet-like clusters, hence next we examined if PARP is necessary at all for neogenesis of islet-clusters. To confirm this we used DNA vector-based RNAi approach, established by Shah GM et. al., (Le Rhun, Kirkland et al. 1998), to stably and very significantly knock down PARP-1 in PANC-1 cells (PANC-SiP clone) as compared to control PARP-1-replete PANC-U6 clone. We found that PARP-depleted (RNAi) PANC-SiP cells lose the capacity to form insulin producing cells. When treated with activin A and swertisin , PARP-1-replete U6 cells formed large islet-like clusters, which were strongly positive for insulin, as measured by DTZ staining and immunofluorescence. In contrast, PARP-1-depleted PANC-SiP cells failed to form insulin-positive islet-like clusters of significant size. In fact the smaller clusters formed by PANC-SiP cells were similar in size with or without treatment with Activin-A and swertisin both The analyses of various transcription factors involved in endocrine differentiation, revealed that PARP-depleted PANC-SiP clusters at 10 days showed higher expression of precursor genes like Nestin and E-cadherin indicating that they did not differentiate as much as PARP-replete U6 cells. Interestingly, although Activin-A and swertisin treatment upregulated phospho-p38 in SiP cells, it did not translate to strong up-regulation of Ngn-3 or an increased expression of insulin. The results with PARP-1-depleted SiP cells clearly show that PARP-1 protein is required and indispensible for proper formation of islet-like clusters from PANC-1 cells.

Based on our results with PANC-1 model, we hypothesize that the presence of PARP protein, but not its catalytic activation, is essential for formation of functional islet clusters from Activin-A and swertisin. We also confirmed that PARP-activation would negatively influence this event. Our results reflect two earlier observations in a simpler model of only β-cell lines, that PARP-inhibitor could promote transcription of Reg-1 (Akiyama, Takasawa et al. 2001) and MafA (Ye, Tai et al. 2006) genes, which are implicated in β-cell proliferation or insulin

gene expression, respectively. Unlike these studies that involved only mature β-cells, our model involves differentiation of precursor cells to islet-like cluster of insulin and glucagon producing cells. In addition, since PARP is now known to have a role in chromatin remodeling and transcriptional activation or repression in different contexts of the genome, we believe that the native (non-activated) PARP is implicated in reprograming transcription of many genes that are implicated in formation of islet-like clusters from precursor cells.

Our results with strong and specific PARP-inhibitor PJ-34 and our data with PARP-1 knockdown (RNAi) cells allow us to conclude that PARP-1 protein specifically plays a role in islet-like cluster formation from precursor cells.

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Chapter- 7

Summary

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Conclusion

7.1 Summary

The present study revolves from demonstrating islet neogenic property of herbal plant *Enicostemma littorale* blume. Methanolic extract of *Enicostemma littorale* was used to differentiate four stem/ progenitor cell systems. Two were cell line possessing stem cell like features PANC-1, NIH3T3 and the other two were primary culture systems mBMSC and rPSC with isolated differentiating agent form El plant. The quest of identification of most potent compound was achieved by preliminary screening of all cell systems with methanolic extracts and later on active principle was purified and characterized. The compound, which showed tremendous islet neogenic potential and significant islet yield compared to control (SFM) and known differentiating agents like KGF or activin-A with morphological, molecular, immunological and functional levels identical to normal rodents islets was most potent compound which was identified as swertisin (SGL-1), an active principle for islet neogenesis.

Several lines of evidence in our study for the first time lend credence for the pluripotent nature of NIH3T3 cells. The most striking evidences that provided functional confirmation for their pluripotency were strong alkaline phosphatase (AP) staining, basal *Nanog* expression and high teratoma forming capacity of these cells in mice without any growth supplements. The acquisition of pluripotency in NIH3T3 cells was concurrent with the lower levels of *Nanog* expression in comparison with mESC rather than complete silencing. Their potential to differentiate into insulin producing cells with activin-A and swertisin furnished functional evidence of their pluripotentiality. Rapid induction for islet differentiation by Swertisin, a novel herbal biomolecule provides low cost and readily available differentiating agent.

BMSC serves as a wonderful source of pluripotent/multipotent stem cells and have the potential to undergo any lineage. In our *in-vitro* study we found swertisin to differentiate bone marrow mesenchymal stem cells into ILCC very efficiently and on transplantation they can ameliorate hyperglycaemic condition

in STZ diabetic mice. Further we created GFP labelled BMSC and performed lineage tracing experiment. We successfully demonstrated newly generated islets in STZ induced mice pancreas which was maximum with swertisin treatment compared to activin or SFM group. This potential can be effectively translated as therapeutic tool for the treatment of diabetes.

To elucidate the mechanism of action of swertisin, differentiation pathway was traced using Panc -1 cells and 90 % PPx mice model. Swertisin differentiate stem/progenitor cells into insulin producing cells via ACT-MAPK-TKK pathway. However differentiation with BMSC indicated alternative signalling pathway.

Poly (ADP)Ribose polymerase-1 has a crucial role in islet neogenesis and is inevitable for beta cell differentiation. Thus to understand the role of PARP-1 and its protein interaction with key transcription factors like PDX-1, Ngn-3, MafA and reg -1, experiments were designed with Panc-1 cells by inhibiting Parp activity (PJ-34) and by creating parp deplete panc -1 stem progenitor cells . It was observed that catalytic activity of parp directly does not play any role in islet neogenesis but presence of PARP protein is essential for formation of functional islet clusters. Activin and swertisin behave in similar manner in Parp deplete cells suggesting its role as transcriptional regulator.

7.2 Conclusion

1. From this study we conclude stem cell differentiating potential of *Enicostemma littorale* for islet neogenesis which can be exciting therapeutic tool for effective treatment of diabetes. Swertisin, an iso-flavonoid compound isolated from *Enicostemma littorale* has been successfully demonstrated as a novel and the most potent differentiating agent for new islet like cell cluster formation.

2. NIH3T3 has been demonstrated to exhibit pluripotency and generated ILCC with swertisin suggesting its use as potential tool in screening various compounds for islet neogenic potential and their mode of action.

3. Swertisin showed best islet neogenic potential both *in-vitro* and *in-vivo* from mBMSC. Thus combination of BMSC with swertisin molecule can lead a very potent and novel therapeutic in near future for diabetic patients.

4. Swertisin followed TGF-β (activin-A) pathway for new islet clusters formation *in-vitro* and *in-vivo*. PARP deplete stem/ progenitor cell highlighted role of PARP and its mechanism in islet differentiation. This information thus seems to be very useful to design newer therapeutic approach, governing TGF-β mediated signalling pathway and PARP modulated stem/progenitor cell to treat diabetes.

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Table 1: Primers for Reverse Transcriptase PCR

Name of Gene	Gene Accession Number	Primer Sequence Forward	Primer Sequence Reverse	PCR Conditions (Tm)	Amplicon Size (BP)
Oct-3/4	*NM_013633.2*	*GGCGTTCG CTTTGGAA AGGTGTTC*	*CTCGAACCACA TCCTTCTCT*	*60*	*431*
Nanog	*NM_028016.2*	*TACCTCAG CCTCCAGC AGAT*	*GAAGTTATGGA GCGGAGCAG*	*57*	*411*
α-SMA	*NT_039687.7*	*AGTCGCCA TCAGGAAC CTCGAG*	*ATCTTTTCCATG TCGTCCCAGTT G*	*60*	*296*
Vimentin	*NT_039202.7*	*AGCGGGAC AACCTGGC CG*	*GGGAAGAAAAG GTTGGCAGAGG C*	*58*	*744*
Nestin	*NT_039240.7*	*GCGGGGC GGTGCGTG ACTAC*	*AGGCAAGGGG GAAGAGAAGGA TGT*	*58*	*326*
PDX-1	*NT_039324.7*	*CTC GCT GGG AAC GCT GGA ACA*	*GCT TTG GTG GAT TTC ATC CAC GG*	*55*	*229*
Ngn-3	*NT_039500.7*	*ACTAGGAT GGCGCCTC ATCCCTTG*	*GGTCTCTTCAC AAGAAGTCTGA GA*	*57*	*658*
GAPDH	*NT_166349.1*	*CAAGGTCA TCCATGAC AACTTTG*	*GTCCACCACCC TGTTGCTGTAG*	*58*	*496*
Pax-4	*NM_011038.2*	*TGGCTTCC TGTCCTTCT GTGAGG*	*TCCAAGACTCC TGTGCGGTAGT AG*	*62*	*214*
Nkx-6.1	*NM_144955.2*	*ATGGGAAG AGAAAACA CACCAGAC*	*TAATCGTCGTC GTCCTCCTCGT TC*	*60*	*280*
Insulin	*NM_008386.3*	*GCCCAGGC TTTTGTCAA ACA*	*CTCCCCACACA CCAGGTAGAG*	*55*	*90*
Glucagon	*NM_008100.3*	*ATGAAGAC CATTTACTT TGTGGCT*	*GGTGTTCATCA ACCACTGCAC*	*58*	*243*
Reg-1	*NM_009042.1*	*AAGCTGAA GAAGACCT GCCA*	*TGTTAGGAGAC CCAGTTGCC*	*60*	*123*

Table 2: Primers for Real Time PCR

Name of Gene	Gene Accession Number	Primer Sequence Forward	Primer Sequence Reverse	PCR Conditions (Tm)
Oct-3/4	*NM_013633.2*	*AACCTTCAGGAGATATGCAAATCG*	*AACCTTCAGGAGATATGCAAATCG*	*54*
Nanog	*NM_028016.2*	*TACCTCAGCCTCCAGCAGAT*	*GAAGTTATGGAGCGGAGCAG*	*57*
Sox-2	*NM_011443.3*	*CTGCAGTACAACTCCATGACCAG*	*GGACTTGACCACAGAGCCCAT*	*54*
Beta Actin	*NM_007393.3*	*ACGAGGCCCAGAGCAAGAG*	*GGTGTGGTGCCAGATCTCCTC*	*57*

Table 3: Antibody for Flow Cytometery

Name of Antibody	Company	Mono/Polyclonal	Mol. Weight (kDa)	Source	Dilution for Flow Cytometry
Nestin-PE	*BD*	*Mono*	*177*	*Mouse*	*1:10*
Pdx-1	*BD*	*Mono*	*42*	*Rabbit*	*1:10*
Vimentin-Cy-3	*Sigma*	*Mono*	*53*	*Mouse*	*1:10*
CD 90-FITC	*BD*	*Mono*	*--*	*Mouse*	*1:10*
CD 34-FITC	*BD*	*Mono*	*--*	*Mouse*	*1:10*
CD 45-APC	*BD*	*Mono*	*--*	*Mouse*	*1:10*
CD 49b-FITC	*BD*	*Mono*	*--*	*Mouse*	*1:10*
CD 117-PE	*BD*	*Mono*	*--*	*Mouse*	*1:10*
CD44-PE	*BD*	*Mono*	*--*	*Mouse*	*1:10*
Sox-2-Alexa 647	*BD*	*Mono*	*--*	*Mouse*	*1:10*
Oct-3/4-PE	*BD*	*Mono*	*--*	*Mouse*	*1:10*
Nanog	*BD*	*Mono*	*--*	*Mouse*	*1:10*
SMA-FITC	*Sigma*	*Mono*	*--*	*Mouse*	*1:10*

Table 4: Antibody for ICC/IHC

Name of Antibody	Company	Mono/Poly clonal	Mol. Weight (kDa)	Source	Dilution for ICC
E-Cadherin	*Cell Signaling*	*Mono*	*135*	*Rabbit*	*1:200*
Fibronectin	*Sigma*	*Mono*	*220*	*Rabbit*	*1:800*
Ki-67	*Sigma*	*Mono*	*345/395*	*Mouse*	*1:800*
Neurogenin 3	*Santa Cruz*	*Mono*	*27*	*Mouse*	*1:100*
Nestin	*Sigma Aldrich*	*Mono*	*177*	*Rabbit*	*1:250*
Pdx1	*Cell Signaling*	*Mono*	*42*	*Rabbit*	*1:250*
α-Smooth muscle actin	*Sigma*	*Mono*	*42*	*Mouse*	*1:250*
Vimentin	*Sigma*	*Mono*	*53*	*Mouse*	*1/250*
Oct3/4-PE	*BD*	*Mono*	*32*	*mouse*	*1:200*
Nanog	*BD*	*Mono*	*42*	*mouse*	*1:200*
Sox-2-APC	*BD*	*Mono*	*38*	*Mouse*	*1:200*
CD44-PE	*BD*	*Mono*	*--*	*Mouse*	*1:200*

Table 5: Antibodies for Western Blotting

Name of Antibody	Company	Mono/Poly clonal	Mol. Weight (kDa)	Source	Dilution for WB
Beta Actin	*Thermo Scientific*	*Mono*	*42*	*Mouse*	*1:5000*
E-Cadherin	*Cell Signaling*	*Mono*	*135*	*Rabbit*	*1:1000*
Fibronectin	*Sigma*	*Mono*	*220*	*Rabbit*	*1:1000*
Ki-67	*Sigma*	*Mono*	*345/395*	*Mouse*	*1:1000*
Neurogenin 3	*Santa Cruz*	*Mono*	*27*	*Mouse*	*1:1000*
Nestin	*Sigma Aldrich*	*Mono*	*177*	*Rabbit*	*1:1000*
Pdx1	*Cell Signaling*	*Mono*	*42*	*Rabbit*	*1:1000*
α-Smooth muscle actin	*Sigma*	*Mono*	*42*	*Mouse*	*1:1000*
Vimentin	*Sigma*	*Mono*	*53*	*Mouse*	*1:1000*
Oct3/4-PE	*BD*	*Mono*	*32*	*mouse*	*1:1000*
Nanog	*BD*	*Mono*	*42*	*mouse*	*1:1000*
Sox-2-APC	*BD*	*Mono*	*38*	*Mouse*	*1:1000*
CD44-PE	*BD*	*Mono*	*~90*	*Mouse*	*1:1000*
CD-90	*BD*	*Mono*	*~25-37*	*Mouse*	*1:1000*

List Of Publications

Publications:

1. **Nidheesh Dadheech**, Abhay Srivastava, Muskaan Belani, Sharad Gupta, Rajarshi Pal, R.R. Bhonde, Anand S Srivastava and Sarita Gupta. Basal Expression of Pluripotency Associated Genes Can Contribute to Stemness Property and Differentiation Potential. Stem Cell and Development, 2012. (Under Minor Revision, IF-4.8).

2. Batul Yusuf, Renjitha Gopurappilly, **Nidheesh Dadheech**, Sarita Gupta, Ramesh Bhonde and Rajarshi Pal. Mouse embryonic fibroblasts represent a connecting link between embryonic and mesenchymal stem cells. Development Growth and Development, 2012. (Under Minor Revision, IF-2.21).

3. Sarita Gupta, **Nidheesh Dadheech**, Anubha Singh, Sanket Soni, R.R. Bhone. Enicostemma littorale: a new therapeutic target for islet neogenesis. IJIB, vol-9,pp 49-53, 2010. (Novel report on herbal therapeutics).

4. Review: **Nidheesh Dadheech**. Desiccation tolerance in Cyanobacteria. African Journal of Microbiology Research. Vol-4, June, 2010. (As Single and Corresponding Author, IF-1.5).

Manuscript In communication:

1. **Nidheesh Dadheech**, Sanket Soni, Abhay Srivastava, Sucheta Solanki, Anubha Singh, Gauri Sanghvi, RR Bhonde and Sarita Gupta. Swertisin, a Novel Bioactive Compound from Enicostemma littorale Potentiates Islet Cell Differentiation from Mouse Embryonic Fibroblast NIH3T3 for Transplantation in Diabetic BALB/c Mice. 2011. (Under Communication)

2. **Nidheesh Dadheech**, Sarita Gupta and Girish Shah. Role of PolyADP Ribose Polymerases in Activin induced islet cell differentiation from Human Pancreatic progenitor cells PANC-1. (Under preparation with Dr. GM SHAH, Canada).

3. **Nidheesh Dadheech**, Girish Shah and Sarita Gupta. SGL-1 a compound from Enicostemma littorale differentiated Human Pancreatic progenitor cells PANC-1 via Activin A mediated over expression of p-38 induced Ngn-3 trigger.(Under preparation with Dr. GM SHAH, Canada).

Short Communication

International Journal of Integrative Biology
A journal for biology beyond borders

IJIB
ISSN 0973-8363

Enicostemma Littorale: A new therapeutic target for islet neogenesis

Sarita Gupta [1,*], Nidheesh Dadheech [1], Anubha Singh [1], Sanket Soni [1], R R Bhonde [2]

[1] Molecular Endocrinology and Stem Cell Research Lab, Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Gujarat, India
[2] Stempeutics Research Pvt. Ltd, Bangalore, Karnataka, India

Submitted: 18 Jan. 2010; Accepted: 16 Feb. 2010

Abstract
Multipotent differentiation property of stem cells opens up new arena for the treatment of diabetic patients. Many chemical and biochemical compounds make stem cells to get differentiate into insulin producing cells. Present study highlighted islet neogenic property of one herbal plant *Enicostemma littorale* blume. An active herbal compound SGL-1 was isolated and purified from extract of *Enicostemma littorale* and used to differentiate two model stem cell lines PANC-1 and NIH3T3 which showed tremendous islet neogenic potential and significant islet yield compared to control serum free medium (SFM). Morphological, molecular and immunological characterization of newly generated islet like cellular aggregates (ICA's) proved them differentiated and positive for islet hormones. Functional characterization of ICA's confirmed significant glucose responsive insulin release. This preliminary data does offer exciting possibility of alternate source to increase islet mass which can be used for treatment of diabetic patients.

Keywords: *Enicostemma littorale*; Islet Neogenesis; Diabetes and Stem cells.

INTRODUCTION

Diabetes mellitus is a metabolic disorder that affects millions of people. The number of patients suffering from diabetes continues to increase all over the world. Both type 1 and type 2 diabetes results from an inadequate mass of functioning beta cells. The prevalence of both type 1 and type 2 diabetes mellitus is increasing throughout the world along with the ensuing morbidity and early mortality because of premature microvascular and macrovascular disease.

Type-I diabetes is a consequence of self destruction of insulin producing pancreatic β cells which creates a chaos state for which there is presently no cure. Clinical trials indicate that, in some instances, control of blood glucose can be restored by transplantation of cadaveric derived islets (Korsgen *et al.,* 2005). Stem cells both embryonic and adult open new vistas in islet therapy. Recently researcher look for fibroblast-like cells emerging from islets in culture due to gradual loss of endocrine cells as an alternative and potential source of beta cells (Scharfmann *et al.,* 2008; Schmied *et al.,* 2001; Zulewski *et al.,* 2001). Proper lineage, nature and origin of these fibroblast-like cells are still a matter of conflict. Various groups now started characterizing these cells as endocrine precursors (Dorisetty *et al.,* 2008; Zulewski *et al.,* 2001; Lars and Helena, 2002) but not many evidences have been generated so far to value them as islet progenitor cells. In fact queries still persist that whether these cells do have proliferative potencies or not and are they originate from beta cells or are they really nestin positive cells (NPC's). These cell based therapies may eventually provide new rays of hopes for a curative treatment. However, even if the hurdles in islet transplantation can be significantly improved, the availability of this treatment option will always be limited by the (1) dearth of cadaveric islet donors (Holland *et al.,* 2009), (2) generation of new islets from stem cell pool, (3) availability of islet neogenic or differentiating agents (Zhang *et al.,* 2005). Medical practitioners have started using islet transplantation therapy to treat diabetes in year 2000 (Bretzel *et al.,* 2004).

Stem cell pool is prodigious target that is being exploited to generate islets for clinical intention. Various differentiating agents for this rationale are now being rummage around. Biological growth factor like

Corresponding author:
Sarita Gupta, Ph.D.
Molecular Endocrinology and Stem Cell Research Lab, Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara- 390002, Gujarat, India
Email: saritagupta9@yahoo.com

KGF, FGF, GLP-1, and betacellulin (Abraham *et al.,* 2002; Katdare *et al.,* 2004; Meenal Banerjee *et al.,* 2005) and chemical agents like Nicotinamide, Activin-A, Exendin-4 (Chandra *et al.,* 2009; Banerjee and Bhonde, 2003; Xu *et al.,* 1999) are conveniently used up by researchers. But the yield of islets after differentiation is not sufficient to overcome the verge of demand and also the cost of therapy is very high. So people are now shifting to uncover the dramatic medicinal properties of herbal plants that may possess islet neogenic activity. Much work has not been done on this line, except a very recent work from a Japanese group (Itaru and Kazuo, 2006). One compound conophyllin isolated from plant *Evertiamia microphylla* do show islet regeneration. Kojima's idea was that the generation of new islets is *via* Activin-A signaling. Conophyllin acts as a ligand for Activin-A was proven by performing differentiation in presence of Activin-A antagonist. The antagonist blocked islet generation when incubated with Conophyllin (Itaru and Kazuo, 2006). This clearly provides evidence that herbal compounds do have potent role in islet differentiation.

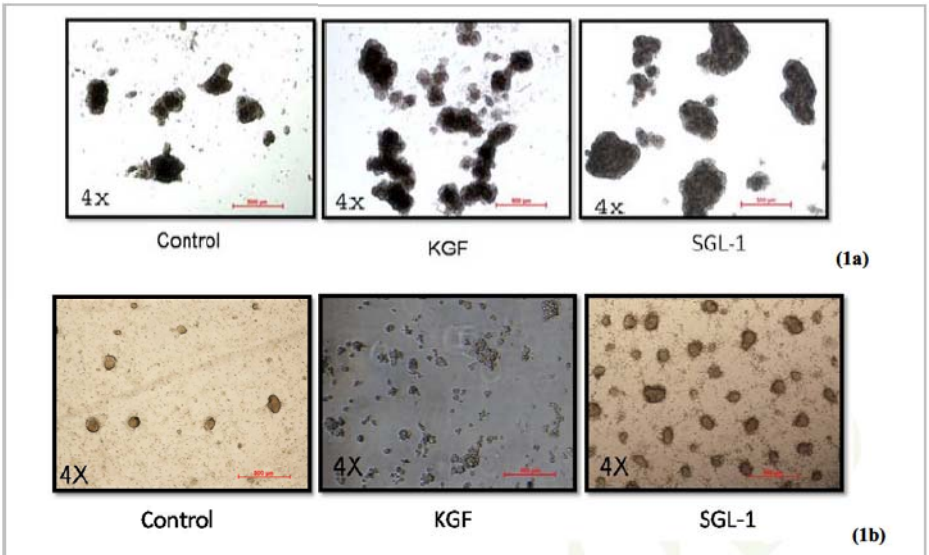

Figure 1: 4X bright field images of ICA's generated after islet differentiation. Fig 1a and 1b shows comparative images of ICA yield between control SFM medium, KGF (positive control) and SGL-1 compound from PANC-1 and NIH3T3 respectively..

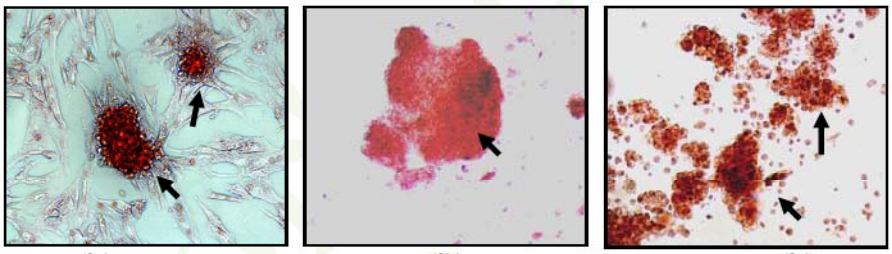

Figure 2: a) DTZ stained ICA's. fig 2a shows stained differentiated cluster (arrow marked) and undifferentiated cells without stain. B) shows stained ICA's from NIH3T3 cells and c) shows stained ICA's from Panc-1 cells respectively.

Enicostemma littorale (EL) is another such plant which is well characterized by the author. Antidiabetic, hypolipideamic, and antioxidant properties of *Enicostemma littorale* (EL) are very well established (Maroo *et al.,* 2003a; Maroo *et al.,* 2003b); Vijayvargia *et al.,* 2000; Vasu *et al.,* 2005). Author also demonstrated earlier that *Enicostemma littorale* have insulin secretory activity in isolated islets (Maroo *et al.,* 2002). Preliminary experiment with extract treated alloxan induced diabetic rats not only showed improved glucose status but also enlighted some blueprints for new islet generation histologically. This led the authors to hypothesize that there are some active components in either aqueous or methanolic extracts of the plant which are responsible for its islet neogenic activity.

MATERIALS AND METHODS

Chemicals and medium

All chemicals, medium and antibodies used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA. Molecular biology reagents and cDNA and PCR kits were procured from Fermentas Inc USA. All plastic wares were purchased from NUNC, USA (Naperville, IL, USA).

Plant material

Plant material was procured from sourastra region of Gujarat after proper identification with voucher specimen (Oza 51, 51 (a)) kept in the herbarium of Department of Botany MS University of Baroda, Vadodara.

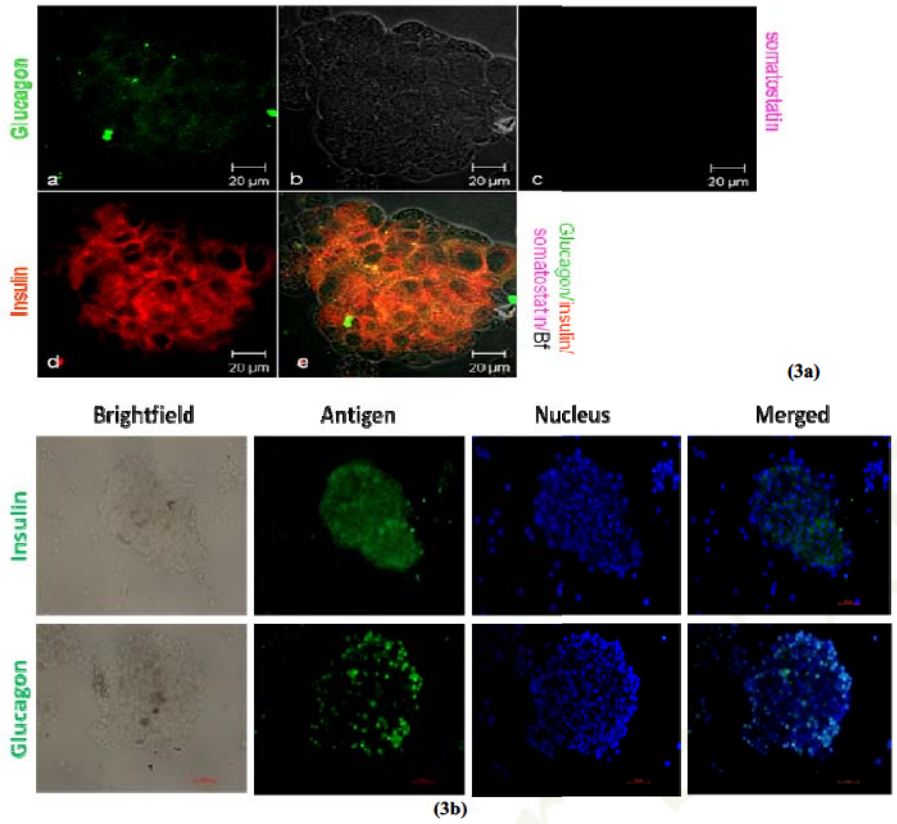

Figure 3: a) Islet like cluster generated from PANC-1 cells stained for guinea pig anti-insulin (red-Alexa-546), mouse anti-glucagon (green-FITC) and mouse anti-somatostatin (pink- alexa-633). ICA obtained was positive for Insulin and Glucagon but did not show any fluorescence for somatostatin. Fig-3b shows ICA generated from NIH3T3 cells stained for mouse anti-insulin (green-FITC), mouse anti-Glucagon (green-FITC).

Extract Preparation and Isolation of SGL-1

Plant material was dried and cut into small pieces. Methanolic extract from EL plant was prepared according to Maroo *et al.* (2003a). Compound SGL-1 was isolated by fractionating methanolic extract successively with chloroform, ethyl acetate and butanol. The precipitated powdered compound obtained was named SGL-1 (characterization is under process).

Cell culture and Induction of Differentiation

NIH3T3 and PANC-1 cell lines (generous gift from Dr. R.R. Bhonde, NCCS, Pune) was maintained and cultured in DMEM high glucose medium with 10% Fetal Bovine Serum. Cells were passaged and splitted into 1:1 ratio in new tissue culture flask upon reaching 90% confluency. Once confluent, the cells were then washed twice with DMEM medium and replaced by neogenic differentiating medium prepared with DMEM Ham's F-12 (1:1, 8mM Glucose) medium with no serum, containing cocktail supplements of Insulin 5mg/L, Transferrin 5ug/L and Selenite 5 ug/L (Sigma Aldrich, USA) and BSA 1.5g/L with antibiotics Penicillin 25ug/ml, Streptomycin 25ug/ml, Gentamycin 25ug/ml, Oxy-tetracyclin 25ug/ml and Amphotericin B 25ug/ml. The differentiation was carried out with control (SFM), positive control KGF (10ng/ml), EL extracts and SGL-1compound (15ug/ml). The medium was changed every alternate day till 6 days. Finally on 8th day cells were observed for islet like cellular aggregates (ICA's) in phase contrast inverted microscope (Nikon TE200, Japan) and confirmed with DTZ staining.

Morphometric analysis

To estimate the total yield of ICA's generated, medium along with suspended ICA's were centrifuged and resuspended in 1ml PBS and 100ul drops of this suspended clusters was stained with 10ul of DTZ. Brick red or crimson red stained clusters were counted in bright field inverted microscope in three independent experiments.

Molecular and Immunological characterization

ICA's were spun at 1500rpm (Eppendorf 5415R) and total RNA was isolated using TRizol (Sigma Aldrich). 1µg RNA was reverse transcribed using Mulv Reverse transcriptase, oligo dT, dNTPs, and ribolock inhibitor using manufacturer's manual instructions (Fermentas). Gene specific forward and reverse primer for insulin was used to amplify the desired cDNA product at 56^0C annealing temperature. Amplified product was confirmed on 15% polyacrylamide gel.

For immunocharacterization ICA's were fixed on glass coverslip with DMEM and 10% FBS for 30 min. Upon attachment on cover slip clusters were immediately fixed with 3.7% paraformaldehyde for

Table 1: Assessment of Islet like aggregates compared with control.

Islet like cellular aggregates with SGL-1 compound	Total % yield of ICA	% yield of 300-3000 µm size ICA	% increase in average area of ICA	Fold increase in insulin mRNA expression	Fold increase in glucose responsive insulin release
PANC-1	205	230	140	1.6	1.6
NIH3T3	375	138	110	5	162

immunocytochemistry. Fixed cells were incubated for 1 hour at room temperature in blocking solution (1% BSA and 1% horse serum in PBS). After incubation ICA were stained with anti-Insulin, Glucagon and Somatostatin primary antibodies (dilutions are 1:100, 1:4000 and 1:100 respectively) overnight at 4^0C. Coverslips were then washed for three times with washing buffer and further incubated with anti-mouse FITC (1:400), anti-guinea pig alexa 546 (1:100), anti-mouse FITC (1:400) and anti-mouse alexa-633 (1:100) conjugated secondary antibodies for 1 hour at room temperature and finally stained with DAPI and mounted with DABCO and glycerol mountant.

Functional characterization

To check the functional status of newly differentiated ICA's from both control and SGL-1 group, 30 ICA's from each group were incubated with 100µl of 10mM L-arginine and 20mM glucose respectively for 2 hours at 370C. After incubation supernatant was used to estimate insulin content released from the ICA's using Mercodia rat insulin ELISA kit according to manufacturer's manual instructions.

RESULTS

Cell lines NIH3T3 (mouse embryonic skin fibroblast) of extra-pancreatic nature and PANC-1 (Human pancreatic cancer stem cell) of pancreatic nature having stem cell like property were chosen to prove islet neogenic activity. The efficacy for islet differentiation from different compounds isolated from EL extract was then tested. The compounds were isolated in sequential fractionization. Three different compounds SGL-1, 2 and 3 were isolated. These compounds were tested for differentiation for new islet like clusters (ICA's) formation in four stage protocol in DMEM-F12 medium along with BSA and Insulin, Transferrin and Selenite cocktail with and without growth factors as control.

The generated ICA were deeply stained crimson red when stained with DTZ showed presence of insulin (Fig. 2). Morphometric analysis of these ICA's in all groups (Aqueous, Methanolic, SGL-1, 2 and 3 and KGF as positive control) were statistically compared with control (SFM). SGL-1 found to be the most potent compound and showed 375% higher yield of ICA's as compared to control with NIH3T3 cells and 205% in case of PANC-1 cell line. The ICA's with size 300-3000 µm were 138% more with NIH3T3 cells and 230% with that of PANC-1 cells. Furthermore the average area of these newly generated ICA's was 110% and 140% more with NIH3T3 and PANC-1 cells respectively, when compared with control (Table1). Following data provide evidences for the higher yield, intactness, maturity and normal tissue architecture of differentiated ICA's.

Molecular characterization of ICA's by Reverse Transcriptase PCR for Insulin mRNA expression profile of newly differentiated ICA's further confirmed the expression of endocrine markers. Insulin expression at 6 day was found to be 1.6 and 5 fold higher in PANC-1 and NIH3T3 differentiated ICA's respectively (Table 1).

Immunopositivity of ICAs for islet hormones proved them differentiated. Prominent insulin staining was seen in whole clusters whereas little stain for few glucagon positive cells was observed at the periphery of the islets. Stain for somatostatin was negative in these aggregates (Fig. 3a and 3b). This characterization implies that newly generated islets are more or less identical to that of normal islets. Functionality assessment of the ICAs was achieved by challenging them for glucose and L-arginine thereby measuring insulin release by ELISA. It has been reported that mature islets responds more to glucose while that of fetal nature responds to arginine (Banerjee and Bhonde, 2005) The newly generated ICAs from both cell lines, when challenged to glucose and arginine, showed higher response to glucose and not to arginine. NIH3T3 showed 162 fold while PANC-1 showed 1.6 fold higher response to glucose when compared with positive control (Table1). This clearly implies that newly generated islets are mature and fully functional to sense glucose shift extracellularly.

Hence SGL-1 compound isolated from *Enicostemma littorale* found to be the most promising candidate for differentiation of stem cells and endogenous pancreatic progenitor cells into islets from cell lines of both pancreatic and extra-pancreatic lineages, which provides new hopes for alternate medicine and therapeutic agent to increase islet mass which can be used for treatment of diabetic patients.

Acknowledgement

The authors would like to acknowledge Department of Biotechnology, Govt. of India, New Delhi for funding the project (Grant Number: BT/TR-7721/MED/14/1071/2006). Authors also thank Dr. Anandwardhan Hardikar for their generous help and discussions. Confocal facility was availed at National Centre for Cell Science, Ganeshkhind, Pune, Maharastra.

Abbreviations

EL, *Enicostemma littorale*, ICA's, Islet like Clusters Aggregates, NPC's, Nestin Positive Cells, KGF, Keratinocyte Growth Factor, FGF, Fibroblast Growth Factor, GLP-1, Glucagon Like Peptide-1, SFM, Serum Free Medium.

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Stem Cells and Development

Stem Cells and Development: http://mc.manuscriptcentral.com/scd

Basal Expression of Pluripotency Associated Genes Can Contribute to Stemness Property and Differentiation Potential

Journal:	*Stem Cells and Development*
Manuscript ID:	SCD-2012-0261
Manuscript Type:	Original Research Report
Date Submitted by the Author:	12-May-2012
Complete List of Authors:	Dadheech, Nidheesh; The MS University of Baroda, Biochemistry Srivastava, Abhay; The MS University of Baroda, Biochemistry Belani, Muakaan; The MS University of Baroda, Biochemistry Gupta, Sharad; Gupta Pathology Diagnostic Lab, Bhonde, Ramesh; Manipal regenerative Medicine Center, Manipal Univ. Srivastava, Anand; Global Institute of Stem Cell Therapy and Research, Gupta, Sarita; The M S University of Baroda, Biochemistry
Keyword:	Differentiation, Pluripotential, Bone Marrow
Abstract:	Pluripotency and Stemness, believed to be associated with high Oct-3/4, Nanog, Sox-2 (ONS) expression. Alike Embryonic Stem (ES) cells, high ONS expression eventually became measure of pluripotency in any cell. Threshold expression of ONS genes that contributes pluripotency, stemness and differentiation potential is still unclear. Therefore, we raised a question as to whether pluripotency and stemness is a function of basal ONS gene expression. To prove this, we carried out comparative study between basal ONS expressing NIH3T3 cells with pluripotent Bone Marrow Mesenchymal Stem Cells (mBMSC). Our studies on cellular, molecular and immunological biomarkers between NIH3T3 and mBMSC demonstrated stemness property of undifferentiated NIH3T3 cells similar to mBMSC. In-vivo teratoma with tri-germlayer formation, strengthen the fact that these cells despite of basal ONS gene expression can differentiate into cells of multiple lineages without any genetic modification. Conclusively our novel findings elucidated that pluripotency that imparts ability for multilineage cell differentiation is not necessarily a function of high ONS gene expression

Basal Expression of Pluripotency Associated Genes Can Contribute to Stemness Property and Differentiation Potential.

Nidheesh Dadheech[1], Abhay Srivastava[1], Muskaan Belani[1], Sharad Gupta[2], R.R. Bhonde[3], Anand S Srivastava[4] and Sarita Gupta[1*].

[1]Molecular Endocrinology and Stem Cell Research Laboratory, Department of Biochemistry, Faculty of Science, The M.S. University of Baroda, Vadodara-390002, Gujarat, India. [2]Gupta Pathology Laboratory, Dandia Bazar, Vadodara, Gujarat, India. [3]Manipal Regenerative Medicine Center, Manipal Hospital, Manipal, Karnataka, India, [4] Global Institute of Stem Cell Therapy and Research, 4370 La Jolla Village Drive, San Diego CA-92121, USA.

Running title: Mouse Embryonic Fibroblast Cell Possess Pluripotency.

[*]Corresponding author's address: Molecular Endocrinology and Stem Cell Research Laboratory, Department of Biochemistry, Faculty of Science, The M.S. University of Baroda, Vadodara-390002, Gujarat, India. Ph. No. +91-265-2795594, e-mail: saritagupta9@yahoo.com, First author e-mail: dadheech_21@yahoo.co.in

Authors' contributions: ND, RRB and SG[1]: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript; AS and MB: collection and/or assembly of data; SG[2]: Histological examination, data analysis, and data interpretation. AS[4]: data analysis, interpretation, and final approval of the manuscript.

Mouse embryonic fibroblasts represent a connecting link between embryonic and mesenchymal stem cells

Journal:	*Journal of Tissue Engineering and Regenerative Medicine*
Manuscript ID:	TERM-12-0281
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	26-Jul-2012

Complete List of Authors:	Pal, Rajarshi; Manipal University, Manipal Institute of Regenerative Medicine Yusuf, Batul; Manipal University, Manipal Institute of Regenerative Medicine Gopurappilly, Renjitha; Manipal University, Manipal Institute of Regenerative Medicine Dhadheech, Nidheesh; MS University, Molecular Endocrinology and Stem Cell Research Gupta, Sarita; MS University, Molecular Endocrinology and Stem Cell Research Bhonde, Ramesh; Manipal University, Manipal Institute of Regenerative Medicine

Keywords:	mesenchymal stem cells, fibroblasts, trilineage differentiation, proliferation, teratoma assay, pluripotency

Mouse embryonic fibroblasts represent a connecting link between embryonic and mesenchymal stem cells

Batul Yusuf[1], Renjitha Gopurappilly[1], Nidheesh Dadheech[2], Sarita Gupta[2], Ramesh Bhonde[1] and Rajarshi Pal[1*]

[1] Manipal Institute of Regenerative Medicine, Manipal University Branch Campus, Yelahanka, Bangalore- 560 065 Karnataka, India

[2] Department of Biochemistry, Faculty of Science, MS University of Baroda, Vadodara- 390 002 Gujarat, India

[*] Author for correspondence (tel: +91 80 25356661; fax: +91 80 25356662; e-mail: rajarshi.pal@manipal.edu)

KEYWORDS: Mouse embryonic fibroblasts, multipotent stem cells, pluripotency, tri-lineage differentiation.

RUNNING TITLE: Enhanced plasticity of MEFs

ABSTRACT

It is well established that fibroblasts and mesenchymal stem cells (MSC) share several characteristics with subtle differences. However, no study highlighting the versatile characteristics of fibroblasts beyond their multipotency has been reported so far. Mouse embryonic fibroblasts (MEF) are widely used as feeder layers to support the growth of embryonic stem cells (ESC). We hypothesized that MEF may retain ES- like features in concurrence to their developmental hierarchy in addition to their multipotent nature. Hence we performed a comparative assessment of MEF and ESC to determine their ability to differentiate into cell types other than mesoderm and capacity to form teratoma employing routine in vitro and in vivo techniques. MEF were derived by trypsin/EDTA digestion from E13.5 embryos after removing heads and viscera following plastic adherence. MEF robustly proliferated in culture until passage 15 and formed aggregates by hanging drop method. Flow cytometry, RT-PCR and

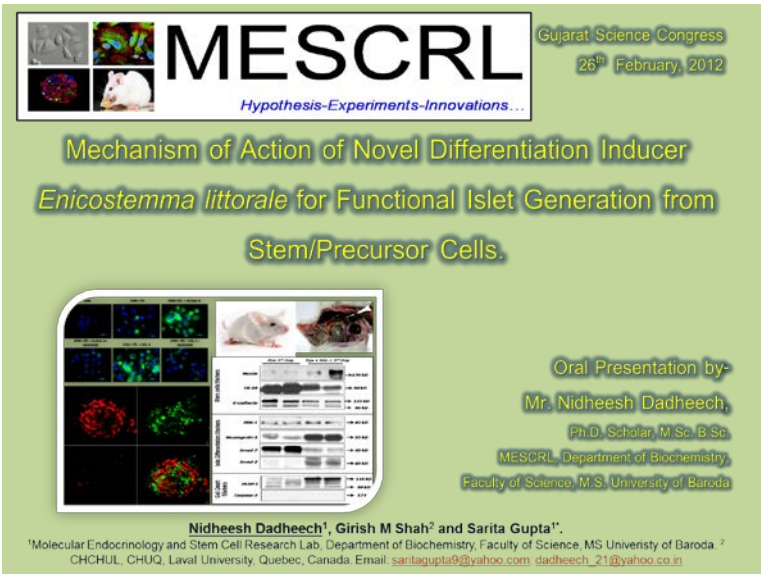
Abstracts, Posters and Conferences

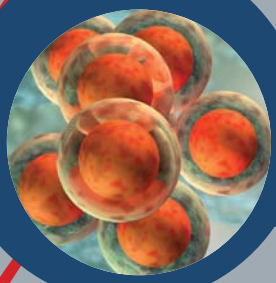
Gujarat Science Congress 2012

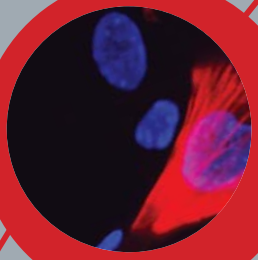
Mechanism of Action of Novel Differentiation Inducer *Enicostemma littorale* for Functional Islet Generation from Stem/Precursor Cells

Nidheesh Dadheech[1], Girish M Shah[2] and Sarita Gupta[1*].
[1]Molecular Endocrinology and Stem Cell Research Lab, Department of Biochemistry, Faculty of Science, MS Univeristy of Baroda. [2] CHCHUL, CHUQ, Laval University, Quebec, Canada.
Email: saritagupta9@yahoo.com; dadheech_21@yahoo.co.in

Objective: Insulin producing β-cells are targeted for destruction at early stage in T1D and at advanced stage in T2D. For this, there is an obvious need to understand how new islets or β-cells are formed and what mechanism regulated the formation of these cells. Inquisitiveness for identification of novel differentiating agent is prime requisite for development of stem cell based therapy for diabetes. *Enicostemma littorale* (El) gives a potent ingredient SGL-1 for new islet cells generation. Herein we investigated intracellular molecular signaling pathway involved in the mediation of pancreatic stem/precursors differentiation into endocrine cells *via* SGL-1.
Breif Results: *In-vitro* and *in-vivo* differentiation pathway of islet cell differentiation was monitored in time dependent manner by protein expression of key transcription factor, which showed crucial role of phopho-p38 Map kinase mediated over expression of Neurogenin-3 protein in transformation of precursors to functional β-cells. The implication of MEPK mediation in conversion and maturation of islet like cell clusters was elucidated. Potent increase in insulin expression through induced SMAD signaling in El (SGL-1) treated partial pancreatectomized mice at third day post surgery again confirmed mechanism of islet differentiation of SGL-1 targeting MEPK mediated pathway.
Conclusion: In present study we showed SGL-1effectively differentiate stem/precursors into islet like cell clusters. Moreover we also demonstrated SGL-1 mechanism of action for effective islet differentiation *in-vitro* and *in-vivo*, mimicking MEPK mediated Ngn-3 over expression pathway similar to Activin-A. These findings held importance for designing SGL-1 as potent pharmaceutical drug for effective cell based therapy in diabetic patients.







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stocks and so co-expressed Nurr1/GDNF subsequently expressed higher levels of TH and AADC compared to when either virus stock was applied. Our data suggest that Nurr1 and GDNF synergize to accelerate dopamine signaling pathways and that their strong co-presence can direct a substantial proportion of the transduced test cells toward dopaminergic fate.

Poster Board Number: 2504

GROWTH/DIFFERENTIATION FACTORS MAINTAIN TENDONGENIC CHARACTERISTICS OF HUMAN PERIODONTAL LIGAMENT DERIVED CELLS IN CULTURE

Sumita, Yoshinori[1], Xia, Dengsheng[2], Wakamatsu, Yuka[1], Kawasaki, Takako[1], Liu, Younan[2], Khalili, Saeed[2], Maria, Ola[2], Ohba, Seigo[1], Agata, Hideki[3], Kagami, Hideaki[3], Tran, Simon D.[2], Asahina, Izumi[1]

[1]Department of Regenerative Oral Surgery, Nagasaki University, Nagasaki, Japan, [2]Faculty of Dentistry, McGill University, Montreal, QC, Canada, [3]Division of Molecular Therapy, Tokyo University, Tokyo, Japan

Objective: Native periodontal ligament-derived cells (PDLCs) constantly express both osteogenesis and tendongenesis-related genes, but when passaged in culture, PDLCs have a tendency of de-differentiation. Although it is well known that dexamethasone or BMP2 can induce/enhance osteogenic characteristics of PDLCs, little is known about effective factors that can induce/enhance tendongenic characteristics of PDLCs. In this study, we examined whether GDFs can enhance or maintain tendongenic characteristics of human PDLCs being expanded in culture during several passages for potential use as a cell source for tissue engineering of tendons and ligaments. Methods: Human PDLCs were obtained from extracted teeth, digested with collagenase, and the isolated cells subcultured until passage 6 for experiments herein. PDLCs from passages 3-6 were cultured until 70% confluency and subsequently placed in serum-free media with or without various concentrations of recombinant human GDF5 (100 ng/ml) and/or GDF7 (1-200ng/ml). Then, these treated cells were evaluated using cell proliferation (WST8), alkaline phosphatase (ALP) activity, reverse transcription -polymerase chain reaction (RT-PCR) and quantitative real-time PCR to determine the expression of tendongenic- and osteogenic-related genes. Human mesenchymal stem cells (MSCs) and dental pulp-derived cells (DPCs) were used as controls. Results: GDFs reduced ALP activity in cultured PDLCs, but its effect was reduced during passages. In contrast, ALP levels in MSCs and DPCs were increased by treatment with GDF5. Consistent with this result, the expression of osteogenic-related genes was down-regulated in GDFs-treated PDLCs, while its expression was up-regulated in MSCs or DPCs. On the other hand, the expression of tendongenic-related genes was up-regulated in GDFs-treated PDLCs. In particular, tenomodulin expression (a late marker of tendongenesis) was maintained in GDFs-treated PDLCs even at passage 6, contrasting with its disappearance in non-treated PDLCs. This suggests that GDFs stimulation rescued tendongenic characteristics in de-differentiated PDLCs. We are currently investigating additional inducible tendongenesis characteristics by GDFs in cultured PDLCs.

Poster Board Number: 2506

NOVEL POPULATION OF NESTIN POSITIVE PROGENITORS IN RAT ADULT PROSTATE DIFFERENTIATES INTO INSULIN PRODUCING CELLS FROM SGL1: A BIOACTIVE COMPONENT OF ENICOSTEMMA LITTORALE

Gupta, Sarita Sharadchandra[1], Dadheech, Nidheesh[2], Prajapati, Akhilesh[2], Singh, Anubha[2], Soni, Sanket[2], Gupta, Sharad[3], Bhonde, Ramesh[4]

[1]M.S University, Vadodara, India, [2]Biochemistry, M.S University, Vadodara, India, [3]Pathology Laboratory, Vadodara, India, [4]NCCS, Pune, India

By 2010 the number of people with Diabetes Mellitus has reached upto 350 million worldwide. Major hurdles in treatment of diabetes are the current treatment of exogenous insulin supply which is not fully capable of achieving tight control over glucose regulation, leading to long-term complications. Recent success in islet transplantation-based therapies for diabetes mellitus and storage of pancreatic islets have motivated sprouting efforts to develop renewable sources of islet replacement tissue. A major impediment to our understanding of the biology of stem cells is the inability to distinguish them from their homing niche and multipotent differentiating property which makes them usable for islet replacement therapy. We made efforts to make use of association of stem cells with adult prostate to isolate a novel population of prostatic stem/ progenitors that express higher levels of nestin, a neuroendocrine protein and vimentin, mesenchymal stem cell marker, enabling a transformative link for endocrine cell differentiation. Enicostemma littorale, an antidiabetic medicinal Indian herb recently reported from our lab to have islet neogenic potential from various multipotent stem/ progentiors. In present study we analyzed selected population from prostate tissue which has a basal phenotype of fibroblastic mesenchymal with high proliferative and mulitpotential nature, as determined by expression of Nestin, Vimentin, SMA proteins by confocal microscopy and has a greater ability to form islet like clusters in-vitro upon differentiation with SGL-1 bioactive component from Enicostemma littorale. Furthermore the presence of dithizone staining and immunopositivity for islet hormones in post differentiated prostate progenitors suggests that these cells share common regulating pathways with pancreatic stem cells during developmental fate in organogenesis. Our study provides an evidence for distinct source for neuroendocrine progenitors which can be isolated and used for diabetic patients who are undergoing prostectomy

Poster Board Number: 2508

FGF2 BUT NOT WNT3A OR BMP2 INHIBIT MOUSE MESENCHYMAL STEM CELL DIFFERENTIATION BY INDUCING TWIST2 AND SPRY4 AND BLOCKING ACTIVATION OF EXTRACELLULAR REGULATED KINASE

Krishnappa, Veena[1], Lai, Wen-Tzu[2], Phinney, Donald G.[1]

[1]Molecular Therapeutics, The Scripps Research Institute, Jupiter, FL, USA, [2]National Institute of Health, Bethesda, MD, USA

Cell signaling pathways that regulate adipogenic, chondrogenic, and osteogenic differentiation of mesenchymal stem cells (MSCs) are well described but those that maintain cells in an undifferentiated state remain largely unexplored. Previously we reported that FGF2 reversibly inhibited multilineage differentiation of primary mouse MSCs. Herein we identify a unique compliment of signaling proteins expressed in MSCs that are dynamically regulated by FGF2 and contribute directly to its inhibitory effect on cell differentiation. FGF2 significantly induced expression of Twist2, Sprouty4 (Spry4) and Cyclin D1 (Ccnd1) in MSCs and down regulated expression of

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RAT ADULT PANCREATIC STEM CELLS DIFFERENTIATION INTO NEW ISLETS: AN *IN-VIVO* STUDY IN PARTIAL PANCREATECTOMY RATS WITH ENICOSTEMMA LITTORALE

Sarita Gupta, Nidheesh Dadheech, Anubha Singh, Sanket Soni, Arpita Dave, Veena Kinare and R.R. Bhonde

In mammals, organs such as pancreas and liver are well known to inherit a strong regeneration potential. The remnant pancreas can regenerate and recover the function of insulted pancreas after pancreatitis or partial pancreatectomy. Every organ do sustain an inherit population of homing stem/ progenitors. During injury they contribute to partial or full organ generation by differentiating into respective functional cell types. Proliferation and Differentiation are the two possible modes by which pancreatic regeneration occurs. Pancreatic stem cells contribute to islet generation upon differentiation when properly stimulated. Growth factor and hormones such as Gastrin and GLP-1 showed increased pancreatic regeneration. Antidiabetic herbal plants so far been tested for pancreatic regeneration have not been clearly characterized for their mode of action resulted in increased islet mass.

Enicostemma littorale an Indian herbal plant showed antidiabetic effects and also demonstrated to have increased islet formation *in-vivo* in alloxan induced diabetic rats and stem cells differentiation into islets like cellular aggregates *in-vitro* which was well characterized and reported by authors lab earlier. In this study the proliferation and differentiation of pancreatic stem cells in remnant pancreas during regeneration in partial pancreactectomy rats was studied and the fate of pancreatic stem cells and preexisting islets (beta-cells) was determined *in-vivo* with Enicostemma littorale treatment.

In present study, we investigated the proliferation of pancreatic stem cells in early days of surgical insult in adult rats following the partial pancreatectomy, and detected the mechanism of pancreas regeneration, and found the site of pancreatic stem cells in tissues and their fate of differentiation in presence and absence of Enicostemma littorale.

Sarita Gupta, **Nidheesh Dadheech**, Anubha Singh, Sanket Soni, Arpita Dave and Veena Kinare. Rat adult pancreatic stem cells differentiation into new islets: an in-vivo study in partial pancreatectomy rats with Enicostemma littorale. Abstract Book, 8th annual meting, ISSCR, Moscone West, San Francisco, CA, USA. 2010.

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a suspension culture without stromal cells. RBCs have negatively charged surfaces which make them repel each other and the nucleus which turns highly condensed during terminal erythropoiesis would head towards the bottom. Thus, we supposed that the electrostatic force of negatively charged culture plate could be an enhancer for enucleation by pushing out the cytoplasm more than nucleus. Cord blood CD34+ cells (n=4) were cultured in IMDM media with several reagents and cytokines of stem cell factor, erythropoietin and interleukin-3 to purely differentiate to erythroid cells for 18 days on the standard tissue culture treated polystyrene. On day 18, the same amount of cells was distributed to a well whose bottom surfaces were coated with DTOPV and UV exposed DTOPV (DTOPV+UV), respectively. The enucleation rate was counted after Wright-Giemsa staining on day 24 and the viability was analyzed after trypan blue staining. Polymer films, diphenylamino-s-triazine bridged p-phenylene vinylene polymer (DTOPV), were exposed to high intensity UV light for 60 min to change the surface property from hydrophobicity to hydrophilicity with negative charges via photo-oxidation. The enucleation rates of erythroblasts cultured on DTOPV and DTOPV+UV were 46.8 ± 21.3 (mean ± SD) and 71.8 ± 10.6, respectively, showing 1.53-fold higher rate in DTOPV+UV. The viability was 67.1 ± 29.7, and 60.9 ± 25.6 revealing no significant differences. The vinylene group in the DTOPV backbone structure was broken to give carbonyl compounds making DTOPV more hydrophilic and more negative in electrostatic charge. The water contact angles of the film decreased from 97.1° to 83.1° by exposure to UV. The roughness and depth of DTOPV and DTOPV+UV were very similar with differences of 0.12 nm and 1.1 nm. Even though erythroid cells live in suspension, the more enucleactions were occurred on the more hydrophilic, negatively charged culture surfaces. This might be caused by the repelling force between erythroblasts and culture surfaces, which accelerated the nucleus escape motion by pushing cytoplasm away. This result reveals that electrostatic force exists in microenvironments and affects the cell motion. By characterizing the invisible electric interaction using bio-materials, it would be possible to study the better culture conditions as well as the more efficient RBC production eliminating robust co-culture methods.

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PRODUCTION OF LIVE PROGENY OF THE BORDER LEICESTER DONOR GENOTYPE FOLLOWING TRANSPLANTATION OF TESTICULAR GERM CELLS TO MERINO RECIPIENT RAMS

Herrid, Muren[1], Jackson, Michael[2], Suchowerska, Natalka[3], Stockwell, Sally[4], Davey, Rhonda[1], Hutton, Keryn[1], Olejnik, Jeanette[1], Hope, Shelly[4], Lehnert, Sigrid[4], Hill, Jonathan[1]

[1]CSIRO Livestock Industries, Armidale, Australia, [2]Faculty of Medicine, University of Sydney, NSW, Sydney, Australia, [3]School of Physics, University of Sydney, Sydney, NSW, Australia, [4]CSIRO Livestock Industries, Queensland Biosciences Precinct, Brisbane, QLD, Australia

The immunological response to germ cell transplantation is of great interest, with contrasting results presented for allogeneic transplants in rodent and non-rodent species. In rodents, successful germ cell transplantation requires recipients to be either immuno-suppressed or genetically related to the donor animal. In goats, successful allogeneic transfers have been achieved without using infertile recipients or immuno-suppressed recipients. This also appears to be a case in testis germ cell transplantation in sheep. We have reported the production of offspring of the donor genotype following transplantation of testis germ cells to recipient sheep of the same breed. It is uncertain whether success in testicular germ cell transplantation depends on the privileged immune status of the testis or on immunological tolerance between donor and recipient. This study investigated the viability of testis germ cell transplantation between Border Leicester rams acting as donors and Merino rams acting as recipients. In the first experiment, testicular germ cells were transferred 5 weeks after irradiation of recipients. Testes of two recipients with scrotal circumferences (SC) 21 cm at the stage of pre-pubertal (spermatogenesis initiated) were treated with a single dose of 9 gray (Gy) with a 6MV photon beam irradiation. In the second experiment cells were transferred 6 weeks after irradiation of 2 recipients (one SC=20, other SC=21) with 15 Gy irradiation. The SCs of donor testes at the time germ cells harvested were 22 and 23 cm respectively. Between 200 and 250 million cells were transferred to each recipient testis. For a period of two years after transplantation, semen samples were collected routinely from the rams and analyzed using microsatellite markers to identify donor DNA. Three out of four recipients (75%) demonstrated the presence of donor DNA in their ejaculates. The percentages of donor sperm in the recipient ejaculate fluctuated with time. In order to investigate the fertility of the donor-origin sperm in recipient ejaculates, two groups of 50 ewes were artificially inseminated with semen from a positive ram from each transfer experiment. Six lambs (6/41, 14% of progeny produced) were sired by donor-derived sperm produced in the recipient ram in the second experiment, while no donor-derived offspring was detected amongst 34 progeny from the recipient in the first experiment. This study is the first report of the production of live progeny following transplantation of testis germ cell between breeds of sheep. Breed and race are considered to be fairly arbitrary constructs for describing the genetics of populations. We would expect that the donors and recipients would possess different histocompatibility antigens. Successful transfer across this transplantation barrier in sheep is in agreement with result obtained in pigs and goats. However, it remains unclear whether immuno-tolerance between donor and recipient is necessary for successful transplantation of testicular germ cells between breeds of sheep.

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EVALUATION OF DIFFERENTIATION POTENTIAL OF INDIAN HERB ENICOSTEMMA LITTORALE INTO NEOISLET FORMATION FROM HUMAN PANCREATIC CANCER STEM CELL LINE PANC-1 AND EXTRA-PANCREATIC MOUSE EMBRYONIC FIBROBLAST CELL LINES NIH3T3

Gupta, Sarita S., **Dadheech, Nidheesh**, Singh, Anubha, Gupta, Shivika, Solanki, Sucheta, Sanghvi, Gauri

Biochemistry, M.S. University of Baroda, Vadodara, India

Objective: One of the major challenges in islet therapy is pancreatic vulnerability towards inflammation, shortage of donor organs and requirement of enough number of islets for transplantation, which reflects the impetus for intense research in this field. Till date not many biochemical or chemical agents having differentiating potency into new islets generation has been reported. Hence in light of this, it is worthwhile to explore natural products as differentiating agents which can increase the yield of islet mass from stem / progen tor cells by new islet formation. Considering this point we have chosen some model cell lines like NIH3T3 (mesenchymal) and PANC-1 (epithelial) for our study. Earlier study in our laboratory has clearly demonstrated Enicostemma litorale (EL) as an effective antidiabetic herb. Diabetic rats when treated with extracts of this herb histologically showed increased islet mass. Thus EL is found to be promising candidate as differentiating agent for new islet formation from stem / progenitor cells present in these cell lines. Methods: Both these cell lines were cultured till 95% confluency separately in different groups and then subjected for differentiation medium in presence and absence of compound SGL-1 and SGL-2 isolated from our herbal plant EL and observed generation of new Islet like Cluster Structure (ICS). These ICS were then characterized for all endocrine hormones via immunostaing and molecular techniques like RT-PCR. The functional maturity of these ICS was assessed by in-vitro insulin release. Alloxan induced diabetic animals were also transplanted with these ICS and observed for reversal of hyperglycemia. Result and Discussion: Our result clearly showed tremendous increase in new Islet like Cluster Structures (ICS) generation ranging from 5 to 8 fold increase as compared to control. ICS were found to be fully functional and mature enough for glucose responsive insulin release. Transplantation of these ICS into alloxan induced diabetic animal, recovered within 1 week post transplantation and showed reversal of hyperglycemia. OGTT curve of transplanted animal was found to be comparable with control healthy animal. Conclusively our study showed good differentiating potential of compounds isolated from EL plant to obtain new islets structures, which can be used for therapeutic purpose in treatment of diabetes.

EVALUATION OF DIFFERENTIATION POTENTIAL OF INDIAN HERB *ENICOSTEMMA LITTORALE* INTO NEOISLET FORMATION FROM HUMAN PANCREATIC CANCER STEM CELL LINE PANC-1 AND EXTRA PANCREATIC MOUSE EMBRYONIC FIBROBLAST CELL LINES NIH3T3

Gupta, Sarita S[1*]., Dadheech, Nidheesh[1], Singh, Anubha[1], Soni, Sanket [1], Gupta, Shivika[2], Solanki, Sucheta[1], Sanghvi, Gauri[1] and Bhonde , R.R[3].

[1] MOLECULAR ENDOCRINOLOGY AND STEM CELL RESEARCH LAB, DEPARTMENT OF BIOCHEMISTRY, THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA, VADODARA, GUJARAT, INDIA. [2] HISLOP COLLEGE OF BIOTECHNOLOGY, NAGPUR UNIVERSITY, NAGPUR, MAHARASTRA, INDIA. [3] LAB NO.4, NATIONAL CENTRE FOR CELL SCIENCE, PUNE, MAHARASTRA,INDIA.

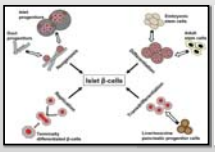
*Corresponding author- Email: saritagupta9@yahoo.com.

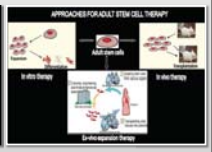
ABSTRACT

One of the major challenges in islet therapy is pancreatic vulnerability towards inflammation, shortage of donor organs and requirement of enough number of islets for transplantation, which reflects the impetus for intense research in this field. Considering this point we have chosen some model cell lines like NIH3T3 (mesenchymal) and PANC-1 (epithelial). Cells were subjected for differentiation medium in the presence and absence of E.L. compound and observed for generation of new Islet like Cluster Structure (ICS), which were characterized positive for endocrine hormones via immunostaining and RT-PCR. The functional maturity of neoislet was established through glucose responsive insulin release. Alloxan induced diabetic animals were further transplanted and showed reversal of hyperglycemia.

INTRODUCTION

Generation of ample number of insulin producing cells remains a major curb towards success in cell replacement therapy for diabetes. In addition not enough pancreatic mass is available to gratify the demand of islets required for transplantation in Diabetic patients. Multiple approaches are now being utilized to attain large number of insulin producing cells *in-vitro (fig below).*



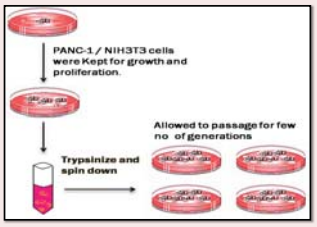


The discovery of agents which induces differentiation of pancreatic progenitors to β cells would be useful to develop new therapeutic approach to treat diabetes. Indian herb *Enicostemma littorale* have been demonstrated in our lab to have hypoglycemic, antioxidant, hypolipidemic activity. Rats treated with the crude extracts of this plant also showed increased islet mass histologically. Hence, we believe that there are some active compounds in E.L. extract which do have potential for new islet generation from stem/progenitor cells. To test this hypothesis, SGL-1 a compound isolated from methanolic fraction of E.L. plant was used for differentiation with two cells lines PANC-1 and NIH3T3 which are of pancreatic and extra-pancreatic lineages. The present study therefore highlights the differentiating property of the active compound isolated from EL plant, which can be used as a novel differentiating agent for islet formation in treatment of Diabetes.

MATERIAL AND METHODS

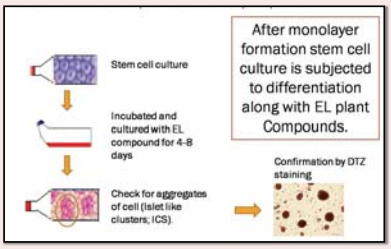
➢ **Proliferation and maintenance of cell line:**

Cells were cultured and maintained in DMEM with 10% serum at 5% CO_2 and 37ºC. Cells were allowed to passage into number of generation before differentiation in order to obtain 90% confluency. Medium was replenished after every two days.



➢ **Differentiation of cells to generate islets:**

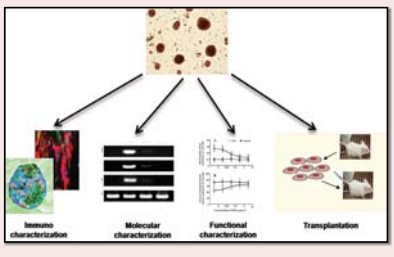
Both stem cell line were then subjected to conditioned serum free differentiating medium DMEM-Ham's F-12 with 1.5g/l BSA and ITS cocktail. Cells were kept for 4-6 days and then observed for ICS generation. ICS were then stained for insulin presence with Dithiazone (DTZ) stain.



➢ **Characterization of islets for all parameters:**

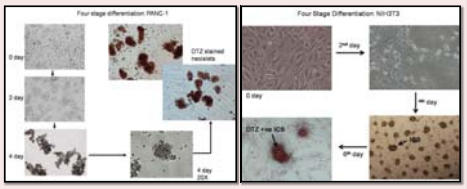
ICS once generated, were then characterized for –

- **Morphology** - on the basis of number and area of islets.
- **Molecular Profiling** – by mRNA expression of endocrine genes and islet specific markers.
- **Immunochemistry**- On basis of staining for endocrine hormones insulin, glucagon and somatostatin.
- **Functional Analysis** - Glucose responsive insulin release estimated by ELISA.
- **Transplantation**- Monitoring the reversal of diabetes by transplanting islets in renal capsule of diabetic animal.



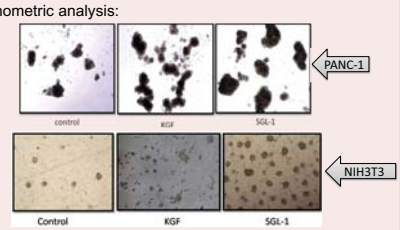
RESULTS

➢ Four stage differentiation procedure:



Four step differentiation was carried out in DMEM Ham's F12 medium without serum along with ITS and BSA. Panc-1 cells were kept for 4 days while NIH3T3 cells were kept till 6 days for differentiation. Finally Islet like clusters were stained with DTZ stain which specifically stains insulin granules.

➢ Morphometric analysis:



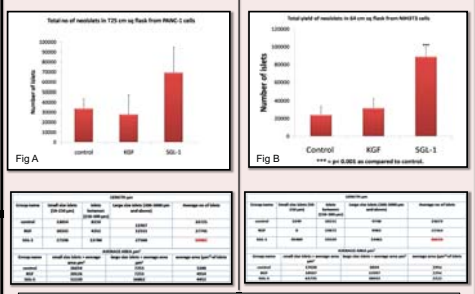
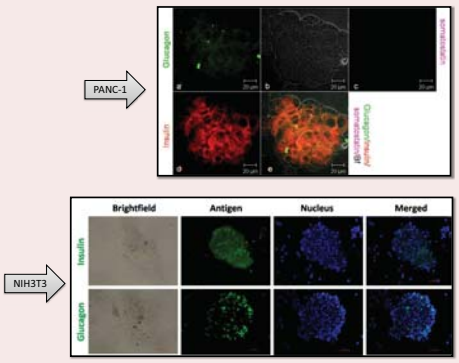
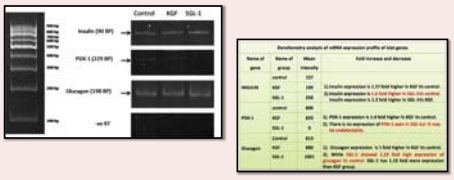


Fig A show total yield of islets generated from PANC-1 cell line. Fig B shows total yield of islets generated from NIH3T3 cell line. The total number of islets generated from SGL-1 compound is 205 % higher as compared to control in PANC-1 cell line while in case of NIH3T3, it is 375% higher in SGL-1 as compared to control.

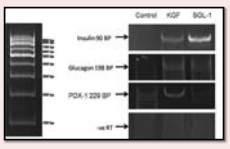
➢ Immunological characterization of neo-islet:



➢ Molecular characterization of neo-islet:



Molecular characterization: PANC-1 neoislets



Molecular characterization: NIH3T3 neoislets

➢ Functional characterization of neoislet:

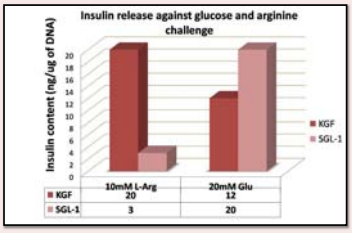
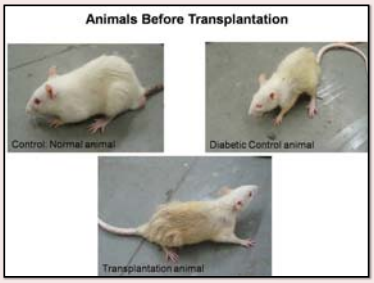
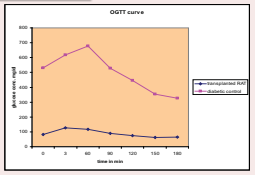


Fig shows insulin release against glucose and L-Arginine from PANC-1 neoislets. SGL-1 islets show more response to glucose than arginine which clearly depicts more maturity than KGF.

➢ Transplantation of neo-islet:



Status	Glucose conc (mg/dl)	Insulin doses	Weight in gms
Before transplantation	1st week:- ± 190mg/dl 2nd week:- ± 280mg/dl 3rd week:- ± 356mg/dl	No No No	75
prior to transplantation	1st day:- ± 261mg/dl 2nd day:- ± 136mg/dl 3rd day:- ± 85mg/dl	10 IU 10 IU 5IU	100
Post transplantation	1st week:- ± 83mg/dl 2nd week:- ± 75mg/dl 3rd week:- ± 65mg/dl	No No No	150



DISCUSSION

1. **PANC -1 and NIH3T3 cell line, when subjected for differentiation with SGL 1 compound demonstrated property of differentiation by forming islet like clusters.**
2. **Total number of islet generated with SGL1 is much higher compared to control and KGF, a known differentiating agent**
3. **Islet like clusters (neoislets) exhibited intense fluorescence for insulin while little for glucagon reveled that new islets are identical as normal islets.**
4. **Newly generated islets demonstrated good expression of insulin and glucagon while lesser expression of PDX -1 as depicted by RT PCR which showed proper maturity of islets.**
5. **Functionally, newly generated islets are mature and depicted glucose responsive insulin release. Like adult islets.**
6. **Transplanted rat showed improved glucose status within 1 week post transplantation. Fasting blood glucose levels of animal remain concurrent with normal animal. OGTT curves of transplanted animal over 180 minutes depicted normal curve 4 week post surgery.**

CONCLUSION

SGL1, a compound isolated from *Enicostemma littorale* has been successfully demonstrated as a novel differentiating agent for new islet like cluster structure formation and thus it is a promising candidate to increase islet mass which can be used for islet transplantation in diabetic patients.

ACKNOWLEDGEMENT

We would like to acknowledge Department of Biotechnology, New Delhi for funding the project. We are greatful to Dr. R.R. Bhonde and Dr. Anandvardhan Hardikar for their expertise, support and guidance. We would also like to thank National Centre for Cell Science, Pune for providing confocal imaging facility and other technical support.

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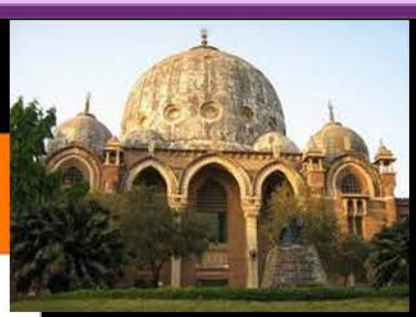
Presented at 7th Annual Meeting, International Society for Stem Cell Research, 8th to 11th July, 2009 Barcelona, Spain.

Government of Canada | Gouvernement du Canada

Canada

Collaboration

Awarded with
Canadian Commonwealth Fellowship to
work for Ph.D. Research in
Dr. Girish Shah's lab,
Laval University Quebec, Canada.



MESCRL

CERTIFICATE OF ATTENDANCE

This is to certify that

Nidheesh Dadheech

attended the

International Society for Stem Cell Research
7th ISSCR Annual Meeting
July 8 – 11, 2009
Convencions Internacional Barcelona
Barcelona, Spain

Nancy Witty
Executive Director

Fiona M. Watt
President

ISSCR

International Society for Stem Cell Research

Certificate of Achievement for

Nidheesh Dadheech

2009 ISSCR Travel Award Recipient

2008-2009 ISSCR President
Fiona M. Watt

ISSCR Executive Director
Nancy Witty

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

Nidheesh Dadheech, M.Sc.

Under Supervision of
Prof. Sarita Gupta
Molecular Endocrinology and Stem Cell Research Lab

Department of Biochemistry
Faculty of Science
The M. S. University of Baroda
Vadodara- 390 002

From:
Nidheesh Dadheech
Dept. of Biochemistry,
Faculty of Science,
The M. S. University of Baroda,
Vadodara - 390 002.

02 October 2011

To
The Registrar (Academic Section),
The M. S. University of Baroda,
Vadodara - 390 002.

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 life style 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 clinician 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 (Maroo et al., 2003a), it was decided to start *in-vitro* assay with 10^6-10^7 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 its 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 oesteocytes 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; Reiisi 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 immnunoblotting 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 neoislets 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 indispensible 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 like 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.

Obj-4: **Assessment of molecular mechanism of SGL-1, a bioactive ingredient of *Enicostemmalittorale* 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 vinca 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 *Enicostemma littorale* for islet cell differentiation which can be exciting therapeutic tool for effective treatment of diabetes. SGL1, a compound isolated from *Enicostemma 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. SGL-1 compound differentiate stem/progenitor cells via ACT-MAPK-TKK pathway, and Poly (ADP)Ribose polymerase-1 has a crucial role and is inevitable for islet cell neogenesis.

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2. **Nidheesh Dadheech**. Desiccation tolerance in cyanobacteria. African Journal of Microbiology Research, Vol. 4(11), 4 June, 2010.

Manuscript in communication:

1. **Nidheesh Dadheech**, Abhay Srivastava, Muskaan Belani, Sharad Gupta and Sarita Gupta. Mouse Embryonic Fibroblast NIH3T3 Inheriting Pluripotent Stem Cell like Phenotypes. 2011.
2. **Nidheesh Dadheech**, Abhay Srivastava, Sanket Soni, Shivika Gupta, R.R. Bhonde, Anubha Singh and Sarita Gupta. SGL-1: A Novel Bioactive Compound from *Enicostemma littorale* Potentiates Islet Cell Differentiation from Mouse Embryonic Fibroblast NIH3T3 for Transplantation in Diabetic BALB/c Mice. 2011.

Abstract Published:

1. **Nidheesh Dadheech**, Anubha Singh, Sanket Soni, Shivika Gupta, Sucheta Solanki, Gauri Sanghvi, R.R. Bhonde and Sarita Gupta. 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. Abstract Book, 7th annual meeting, ISSCR, Barcelona, Spain, 2009.
2. Sarita Gupta, **Nidheesh Dadheech**, Anubha Singh, Sanket Soni, Arpita dave and Veena Kinare. Rat adult pancreatic stem cells differentiation into new islets: an in-vivo study in partial pancreatectomy rats with Enicostemma littorale. Abstract Book, 8th annual meeting, ISSCR, Moscone West, San Francisco, CA, USA, 2010
3. **Nidheesh Dadheech**, Akhilesh Prajapati, Sanket Soni and Sarita Gupta. Identification of Novel Population of Nestin Positive cells from Rat Prostate for Insulin Producing cell differentiation. Abstract Book, 9th annual meeting, ISSCR, Toranto, Canada, 2011.

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.
2. Attended International Conference on Adult Stem Cells- ADNAT, organised by CCMB, Hyderabad, India, 22-24th Feb., 2008.

Date: October 2, 2011 **Signature of the candidate**

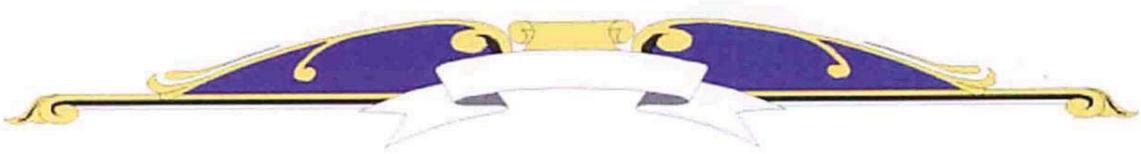
Nidheesh Dadheech

(Prof. Sarita Gupta)

Guide

Head **Dean**

Biochemistry Department **Faculty of science**



This is not just an end but it's a

"Beginning....."