Histone deacetylase inhibitor valproic acid in pancreas differentiation

Inaugural Dissertation

Submitted to the
Faculty of Medicine
In partial fulfillment of the requirements
For the Ph.D.-degree
Of the faculties of Veterinary Medicine and Medicine
Of the Justus Liebig University Giessen

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Giessen 2020

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Date of Doctoral Defence 27.08.2020

Dedicated to my Family

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Summary

Use of histone deacetylase inhibitors as small molecules is a promising approach to increase the differentiation efficiency of various cell types. In the present study, efficiency of the Histone deacetylase inhibitor Valproic acid (VPA) to induce endocrine differentiation in human exocrine pancreatic ductal adenocarcinoma cell line (Panc-1) was investigated. Panc-1 cells were cultured and treated with different concentrations of VPA and using quantitative real-time polymerase chain reaction regulation of pancreatic developmental genes were studied. The real-time PCR studies revealed an enhanced expression of pancreatic developmental genes Pdx1, Sox17, Ngn3, Pax6, Isl1, whereas very low regulation was observed in Foxa2 expression. Regulation of Ngn3 and Pdx1 were further looked at protein level by Western blots. Glucagon expression was found in cells treated with VPA, which was confirmed at protein level by Western blot, immunocytochemistry and measured glucagon content in the lysates by enzyme-linked immunoassay. Results from Western blots demonstrate enhanced acetylation of histones H3 and H4, which marks in the most cases active chromatin, indicating that the action of VPA on pancreatic differentiation occurred through the prevention of deacetylation of histones H3 and H4.

The results collectively show that VPA induces the differentiation of Panc-1 cells into glucagon producing endocrine-like cells by induction of pancreatic genes through histone acetylation. Further understanding of the underlying mechanisms will highlight the current findings in the field of diabetes, and thus these cells can serve as tools for identifying compounds that convert alpha to beta cells as novel strategy for treatment of diabetes. VPA can also be interesting in diabetes studies that are focused on glucagon regulation or studies looking for mechanisms underlying glucagon dysregulation.

Zusammenfassung

Die Verwendung von niedermolekularen Histon-Deacetylase Inhibitoren ist ein vielversprechender Ansatz, um die Differenzierungseffizienz verschiedener Zelltypen zu erhöhen. In der vorliegenden Arbeit wurde die Wirksamkeit des Histon-Deacetylase Inhibitors Valproinsäure (VPA) auf die endokrine pankreatische Differenzierung untersucht. Hierzu wurde die humane, exokrine, aus einem duktalen Adenokarzinom stammende Zelllinie Panc-1 verwendet.

PANC-1 Zellen wurden mit ansteigenden VPA Konzentrationen kultiviert, und die für die Regulation der Entwicklung des Pankreas wichtigen Gene mittels Real-Time Polymerase Kettenrektion (qRT-PCR) gemessen. Die Real-Time PCR Ergebnisse zeigten eine erhöhte Expression der pankreatischen Entwicklungsgene Pdx1, SOX17, Ngn3, Pax6 und Isl1, während nur eine sehr geringe oder keine Regulation der Foxa2 Expression beobachtet wurde. Die Regulation von Ngn3 und Pdx1 wurde im Folgenden mittels Western blot auf Proteinebene überprüft. Bei den mit VPA behandelten Zellen wurde zusätzlich die Expression von Glukagon gefunden, welche auf Proteinebene immunozytochemisch und über die Messung des Glukagongehalts in den Zelllysaten mittels Enzym-linked Immunoassay bestätigt wurde. Die Western blot Ergebnisse zeigten eine Erhöhung der Acetylierung der Histone H3 und H4. Dieser Vorgang führt in den meisten Fällen dazu, dass Chromatin aktiviert wird indem es für Transkriptionsfaktoren zugänglich wird. Das ist ein Hinweis, dass die Wirkung von VPA auf die pankreatische Differenzierung über die Acetylierung der Histone H3 und H4 erfolgt.

Zusammengefasst zeigten die Ergebnisse, dass VPA, über die Aktivierung von pankreatischen Genen durch Inhibition der Histondeacetylierung, die Differenzierung von Panc-1 Zellen in Glukagon- produzierende Zellen induziert.

Die vorliegenden Erkenntnisse führen zu einem besseren Verständnis der zugrunde liegenden Mechanismen auf dem Gebiet der Entwicklung von insulinproduzierenden Zellen. Die Ergebnisse können zur Identifizierung von Substanzen dienen, die Alpha- in Beta Zellen konvertieren und damit neue Strategien in der Diabetesbehandlung eröffnen. VPA könnte auch für Diabetesstudien interessant sein, die sich mit den Mechanismen der Regulation und Dysregulation des Glukagons beschäftigen.

1. Introduction

1.1. Diabetes mellitus

Diabetes mellitus is a metabolic disease marked by elevated blood glucose levels due to absence/ insufficient insulin production (type 1 or juvenile diabetes) or by the ineffectiveness of the insulin produced (type 2 diabetes). Currently the patients are treated with exogenous insulin, which has increased the quality of life of diabetic patients but could not completely control the fluctuations in blood glucose levels leading to hypo- and hyperglycemic conditions. However, increasing a patient`s beta-cell mass could potentially improve or cure their condition. In this context, islet transplantation has become an alternative approach to treat patients with type I diabetes, but the limited amount of donor organs is a major obstacle for this therapy. In recent years other treatment options like cell replacement therapies have received much attention. Understanding of the in vivo pancreas development, beta-cell differentiation and regeneration would allow in generating an unlimited supply of beta-cells from stem or precursor cells that can be used for transplantation.

1.1.1. Development of pancreas

The pancreas is a complex endoderm derived mixed gland that possesses exocrine and endocrine functions. The exocrine compartment that accounts for the major part of the pancreatic mass has acinar cells which secrete digestive enzymes and ductal cells which transport these enzymes into the duodenum. The endocrine compartment, the islets of Langerhans, which is only 2-3% of the pancreatic cell population comprises hormone secreting cells. The islets of Langerhans consist of five different cell types: alpha-cells (α-) - secreting glucagon, beta-cells (β-) - secreting insulin, delta-cells (δ-) - producing somatostatin, PP/gamma cells (γ-) - secreting pancreatic polypeptide and, epsilon-cells (ε-) - producing ghrelin [1]. The percentage and arrangement of each cell type varies between species but usually β-cells form the majority, followed by alpha-cells. It is around 20-30% of alpha-, ~60% beta-, 10% delta-, <5% gamma- and 1% epsilon-cells in humans [2].

Mouse pancreas originates at e8.5 to e9.5, by the formation of dorsal and ventral buds from a prepatterned endodermal epithelium of the foregut. By e10.5-e12.5 the epithelium of these two buds branches into ducts and undifferentiated epithelium, called first developmental transition. The endocrine cells are arrayed in the undifferentiated epithelium as single cells. Further the dorsal and ventral buds begin to differentiate into endocrine and exocrine lineages and

proliferate and expand by e14 (second development transition). By e15 to e19 the dorsal and ventral pancreases rotate, fuse and form a nearly fully developed pancreas and the endocrine cells begin to organize into isolated clusters that condense into islets of Langerhans (third development transition). The islets undergo additional remodeling, maturation and their acquisition of full nutrient responsiveness continues for two to three weeks after birth. A hierarchy of transcription factors play a key role in regulating specification, growth and differentiation into exocrine and endocrine cells during the pancreas development [1, 3].

1.1.2. Pancreatic cell differentiation

The process of endocrine cell differentiation is a quite complex pathway requiring specification of pancreas versus other endodermal organs, endocrine cells versus exocrine cells and beta-cells versus non-beta-endocrine cells [4]. This pathway involves cascade of transcription factors that work together in a precise and sequential manner at appropriate time to bring out a fully mature and functional beta-cell (Figure 1.1). Thus, understanding and identifying the molecular regulators of beta-cell differentiation and proliferation pave the way for cell replacement therapy [3, 5].

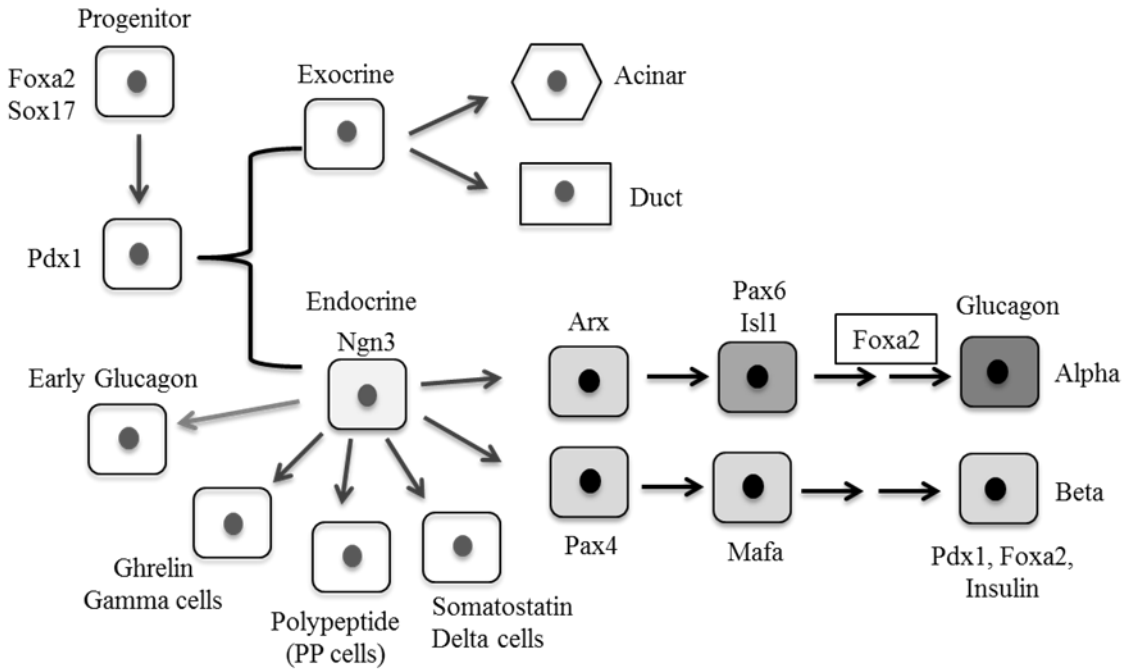


Figure 1.1: Hierarchy of pancreatic transcription factors, expressed during pancreas development and beta-cell differentiation. Pdx1+ progenitor cells differentiate into exocrine-with duct and acinar cells and Ngn3+ endocrine progenitors. These Ngn3+ endocrine progenitors further give rise to islets with alpha, beta, delta, gamma, and epsilon cells. Modified according to [3, 5].

1.1.3. Transcription factors in pancreatic and endocrine cell differentiation

Pancreas development and endocrine cell differentiation is coordinated by a transcriptional network that work in a precise and sequential manner to bring a fully matured endocrine cell [3, 5] . Transcription factors that have been studied in the present study are listed below.

Winged-helix/forkhead member A2, Foxa2:

Foxa2, formerly known as hepatocyte nuclear factor 3-beta, is expressed in the foregut endoderm, before and at the onset of pancreatic development and persists to adulthood, where it is expressed throughout the islet cells. Forkhead box A transcription factors plays multiple roles at different stages of pancreatic development and differentiation [6-8]. Endoderm-specific ablation of Foxa2 resulted in absence of mature alpha-cells and a reduction of Pdx1 expression and beta cell differentiation [9, 10]. In recent study it has been demonstrated the involvement of Foxa2 in regulating alpha-cell differentiation, glucagon synthesis and secretion [11].

Sex determining region Y- box-17 (Sox17):

Sox17 is a Sry-related HMG box factor that is expressed as early as embryonic day 5.5-6.5 in mouse and regulates endoderm formation. Sox17 null mutation in mice leads to depleted gut endoderm and ectopic pancreas formation [12]. In a recent study it has been shown that Sox17 is involved in the regulation of insulin trafficking and secretion in adult beta-cells both in normal and diabetic states [13].

Pancreatic duodenal homeobox gene 1 (Pdx1):

Pdx1 is considered as master regulator of pancreatic development since targeted disruption of the Pdx1 gene resulted in complete agenesis of pancreas [14, 15]. Pdx1 expressing progenitor cells further differentiate into both exocrine and endocrine progenitor cells [16]. It is highly expressed throughout the entire pancreatic epithelium during the early stages of development however, in later stages its expression becomes more restricted to beta-cells with high level of expression and low level of expression observed in alpha-cells [17]. A study has described that forced expression of Pdx1 in Ngn3+ endocrine progenitor cells altered alpha- and beta-cell ratios in both embryo and adult pancreas [18]. Pdx1 regulates beta-cell identity by activating genes essential for beta-cell and repress those associated with alpha-cell identity [19].

Neurogenin 3 (Ngn3):

Basic helix-loop-helix family transcription factor Ngn3, is key transcription factor required for development of all endocrine cells, and acts as marker for islet precursor cells [20-22]. In mice targeted disruption of Ngn3 expression showed no endocrine cells, and in contrast its overexpression showed increase in endocrine formation mostly glucagon producing cells, thus indicating its expression is essential for the development of all islet cells [20, 23]. A study has reported, Ngn3 expressing cells in the human exocrine pancreas, mark a dedifferentiating cell population with endocrine fate [24].

Paired homeodomain factor 6 (Pax6):

Pax6 is expressed early in developing pancreas and later restricted to mature endocrine- alpha, beta-, delta- and polypeptide cells and regulates their hormone expression [25]. Mice with mutant Pax6 gene show an abnormal islet morphology and decreased islet endocrine cell number. This indicate its critical role in these cell differentiations, especially of the alpha-cells, and their hormone expression [25, 26]. A recent study has reported, that it co-ordinates

glucagon gene expression, synthesis and secretion independent of the transcription factors Foxa2 and Arx that are critical for alpha-cell differentiation [27].

Islet1 (Isl1):

The LIM-Homeodomain protein, formerly known as islet1 is required for the survival, maturation and proliferation of the endocrine pancreas [28]. Loss of Isl-1 from the pancreatic epithelium leads to a severe reduction in hormone- expressing cells and the eventual loss of islet mass [29]. Additionally, MafA and Arx transcription factors required for beta and alpha-cell development are regulated by Isl1 [28, 30]. Recent study has found that Isl-1 is essential for postnatal beta-cell function [31].

Further specification and differentiation of islet cells include additional transcription factors like Arx, Pax4, Nkx2.2, Nkx6.1, MafA, and MafB, Rfx3 and 6, Glis3 [5]. Pancreas from Arx-mutant mice showed a complete absence of alpha-cells accompanied by increase in beta and delta-cell number. It has been reported that ectopic expression of Arx in insulin producing cells is enough to convert those cells into glucagon and PP producing cells, thus highlighting the role of Arx in promoting alpha-cell fate [32, 33]. Table 1.1 summarizes some of the transcription factors involved in pancreatic development and altered phenotype in knockout mice of each transcription factor.

Transcription factors	**Pancreas altered phenotype in knockout mice**	**References**
Foxa2	Absence of alpha-cells	[8]
Sox17	Absence of endoderm	[12]
Pdx1	Absence of Pancreas. Initial dorsal bud formation	[14] [15]
Ngn3	Absence of endocrine cells and endocrine precursors	[20]
Pax6	Absence of alpha-cells	[25, 26]

Isl1	Absence of differentiated islet cells and lack of dorsal pancreatic mesoderm	[29]

Table 1.1: Transcription factors involved in pancreatic development and altered phenotype in knockout mice. Modified according to [3, 5].

1.2. Sources for beta-cell regeneration

The limitations in the treatment options led to search for sources of beta-cells. Studies have explored the use of several alternative sources for generating functional beta-cells for transplantation. By differentiation of stem cells into insulin producing cells, proliferation of existing beta-cells, by inducing differentiation in pancreatic progenitor cells (neogenesis), by inducing transdifferentiation (conversion from one cell type to other) of alpha-cells, exocrine-consisting acinar and ductal cells, and from non-pancreatic -hepatocytes and gut cells [34, 35].

1.2.1. Stem cells as source of pancreatic beta-cells

The successful culture of human embryonic stem cells (hES cells) opened the door for developing methods for generating islet cells [36]. With advances in the field, discovery of induced pluripotent stem cells (iPSCs) derived from skin fibroblast cells by transfections have raised the possibility of patient-specific treatment [37]. Stem cells from different sources include MSCs (mesenchymal stem cells) isolated from umbilical cord, adipose tissue, bone marrow are used to differentiate into insulin producing cells under specific culture conditions or by genetic manipulation [38, 39].

Early studies developed stepwise protocols using combinations of inducing growth factors and chemicals and differentiated hES into islet cells with mixed hormone expression. Together with studies on embryonic pancreatic development they paved the way for developing better differentiation protocols [38, 40]. More recently efforts have been made for the production of islet clusters that morphologically and functionally resemble to pancreatic islets and can respond to glucose, further entering into clinical trials [41].

1.2.2. Existing beta-cells and progenitor cells

Another approach to generate numerous beta-cells endogenously is from expansion of preexisting adult beta-cells through cell division. Studies showed a high percentage of beta-cell proliferation in young mice and a rapid decline with age. In addition, conditions like obesity and pregnancy were reported to enhance beta-cell mass by islet hyperplasia in adult mice [42]. Proliferation in human beta-cells was controversial in the past, however studies with material from a large pancreas organ bank demonstrated that replicating events are rare, but significantly augmented by pregnancy and even in the diabetic pancreas [43-47]. In recent years more advance has come from high throughput compound screens for identifying agents that can stimulate beta-cell proliferation [48, 49].

Existence of pancreatic stem or progenitor cells is a long-standing hypothesis and the formation of new islets from them is designated as neogenesis. Studies in rodents reported the pancreatic ducts as the preferred niche for endocrine progenitors suggesting neogenesis from ducts, but it remains unclear and still under investigation whether this can be translated to the human pancreas [50-54].

1.2.3. Pancreatic exocrine to beta-cell reprogramming

Exocrine tissue, consisting of ductal and acinar cells, represents the major part of the pancreas. Therefore much attention was given on the possibility to generate new beta-cells from these cell types [55]. It was reported that viral transfection of only three transcription factors Pdx1, Ngn3, MafA directly reprogrammed mouse pancreatic acinar cells to beta-cells. This was the first study to confirm that beta-cells could be generated from pancreatic exocrine cells [56]. A combined action of transcription factors Ngn3 and +MafA, converted acinar cells to alpha- like cells and sole action of Ngn3 to delta- like cells. This indicated that three major islet endocrine cell types can be generated by acinar reprogramming [57]. Ngn3 expressing cells in human exocrine pancreas were shown to have the capacity for endocrine cell fate [24] .

Human duct cells could be reprogrammed to insulin producing cells by overexpression of Ngn3 [58]. Another study reported that adult human ductal cells were converted into endocrine cells by the ectopic expression of pancreatic genes MafA, Ngn3, Pdx1 together with Pax6 [59]. The investigators concluded that duct cells harbor endocrine potential upon Ngn3 induction, which was sufficient to induce latent endocrine programs and proposed them a potential source for replacement of beta-cells [60]. In the present study the non-endocrine pancreatic ductal

adenocarcinoma cell line Panc-1 was used. Besides of its cancer properties this cell line has been used in several studies as a model system for studying the process of endocrine lineage development [61-63].

Over the last decade studies have showed that more cell types of endodermal origin from liver and gastrointestinal tract were reprogrammed into insulin producing cells with ectopic expression or deletion of some of the transcription factors. This research opened even more promising alternatives for cell-based replacement therapy of diabetes [64-67].

1.2.4. Alpha-cells as a source for beta-cell regeneration

The second highest number of cells after the beta-cells in the islets are alpha-cells, and glucagon secretion is their primary role [68]. In mouse islets alpha and delta cells reside at the periphery of the islet. The alpha-cell arrangement in human islets is different, where they are randomly distributed throughout the islet along with the other beta and delta cells, sharing a close lineage relationship with beta-cells [69]. Dysregulation of alpha-cells with enhanced glucagon secretion contributed to both type 1 and type 2 diabetes [70, 71]. Ectopic expression of transcription factor Pax4 in the mouse pancreas converted progenitor cells into alpha and subsequently beta-cells [72]. Studies from cell lineage tracing experiments showed that the direct origin for regenerated beta-cells were adult alpha or delta-cells after near to complete beta-cell loss, however, the molecular basis of this reprogramming was not elucidated [73, 74]. A recent study reported that enhanced direct alpha- to beta-cell transdifferentiation was observed with activin A. This finding pointed to a completely different mechanism and that signalling regulation was effective under specific conditions [75]. Thus, over the recent years cell culture protocols of differentiation either along lineage or transdifferentiation of alpha to beta-cells have grabbed the attention of researchers as therapeutic approach for restoring beta-cell function in type 1 diabetic patients.

Further understanding of the heterogeneity and epigenetic status of endocrine cells opens a completely new field of research focusing on genetic and epigenetic manipulation to attain reprogramming towards the beta-cell fate. Findings from recent study demonstrated that human pancreatic islet cells display cell-type–specific epigenomic plasticity, indicating that epigenomic manipulation could provide a path to cell reprogramming and replacement-based therapies for diabetes [76].

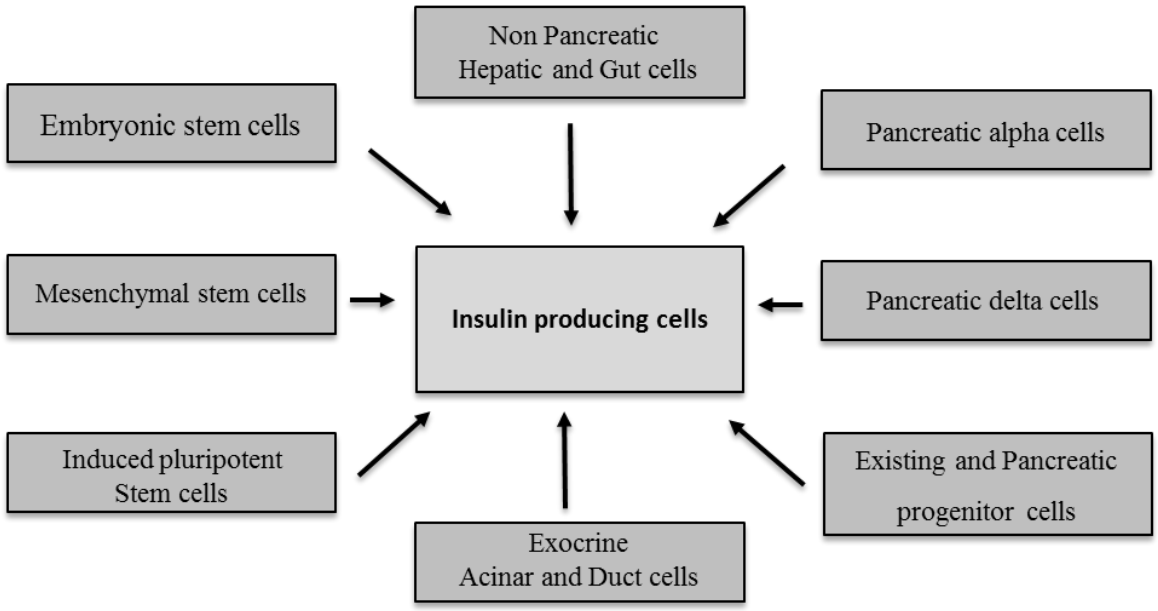


Figure 1.2: Multiple cell sources for beta-cell reprogramming: A supply for beta-cell, reprogramming may be derived by inducing differentiation of embryonic, induced pluripotent and multipotent mesenchymal stem cells, by inducing transdifferentiation in related cell types such as non-pancreatic hepatic or gut cells, from pancreatic exocrine-acinar, duct cells, endocrine non beta – alpha and delta-cells into insulin producing cells modified according [55].

1.3. Epigenetics

Epigenetics is the study of heritable changes in gene expression without involving any modification in the DNA sequence. Epigenetic changes determine cell fate, differentiation of cell types and contribute to complex diseases such as immune disorders and some types of cancer. Epigenetic effector mechanisms shown to be important for regulation of cellular functions are further classified into three mechanisms such as DNA methylation, posttranslational histone tail modifications and non-coding RNAs [77, 78].

Knowledge of chromatin organization is essential to understand the mechanisms behind epigenetics. In eukaryotes, transcriptional regulation occurs within the chromatin and is influenced by post-translational histone modifications. The basic structure of eukaryotic chromatin is the nucleosome. Each nucleosome consists of approximately 146 bp of DNA wrapped around a core of eight basic proteins, called histones, consisting of two copies of each H2A, H2B, H3, and H4. These histones have long C or N terminal tails, which are subjected

to several, post translational modifications like acetylation/deacetylation, methylation, phosphorylation, sumoylation and ubiquitination that affect chromatin structure and further regulates gene expression [79, 80]. Among these currently established histone modifications are acetylation and methylation, of which methylation can lead to transcriptional activation and repression. Acetylation of histone tails mostly enhances gene expression [81, 82]. For example, acetylation of H3, H4 and trimethylation of histone H3 lysine 4 (H3K4-me3) are associated with active transcription [79, 83].

Acetylation of histones is accomplished by transferases (HATs) and neutralizes the positive charge of histones, generating a relaxed open chromatin allowing transcription factors to access target DNA sequences. Deacetylation of histones by histone deacetylases (HDACs) make them bind tightly to the phosphate backbone of DNA, compacting the chromatin thereby and repressing the transcription. Thus, HATs and HDACs bring changes in chromatin structure and thereby modulate cell proliferation/differentiation in various tissues [82, 84, 85]. Recent advances in phylogenetic analysis showed that molecular function of HDACs is not only restricted to histone deacetylation. They regulate the activity of a wide range of non-histone proteins which include transcription factors and regulators, signal transduction mediators, DNA repair enzymes, nuclear import regulators, chaperone proteins, structural proteins, inflammation mediators and viral proteins, that are involved in numerous cell pathways including regulation of gene expression, cell proliferation, differentiation, DNA repair, migration and apoptosis [86]. Eighteen different human HDAC isoforms have been identified so far which are further grouped into four different classes. HDACs 1, 2, 3 and 8 constitute class I. HDACs 4, 5, 6, 7, 9 and 10 form class II. Class III constitutes seven sirutins and HDAC11 form class IV [84, 85, 87]. Studies from recent years have explained the role of HDACs in many diseases like neurodegenerative disorders, cardiovascular dysfunction, autoimmunity, diabetes mellitus and most importantly in cancer initiation and progression. Thus, targeting HDACs became a promising therapeutic strategy in the treatment of these diseases. Histone deacetylase inhibitors (HDACis) are small epigenetically active molecules that inhibit HDACs. They prevent deacetylation of the lysine residues of histones, as well as non-histone proteins, resulting altered gene expression in response to physiological changes in cells [88].

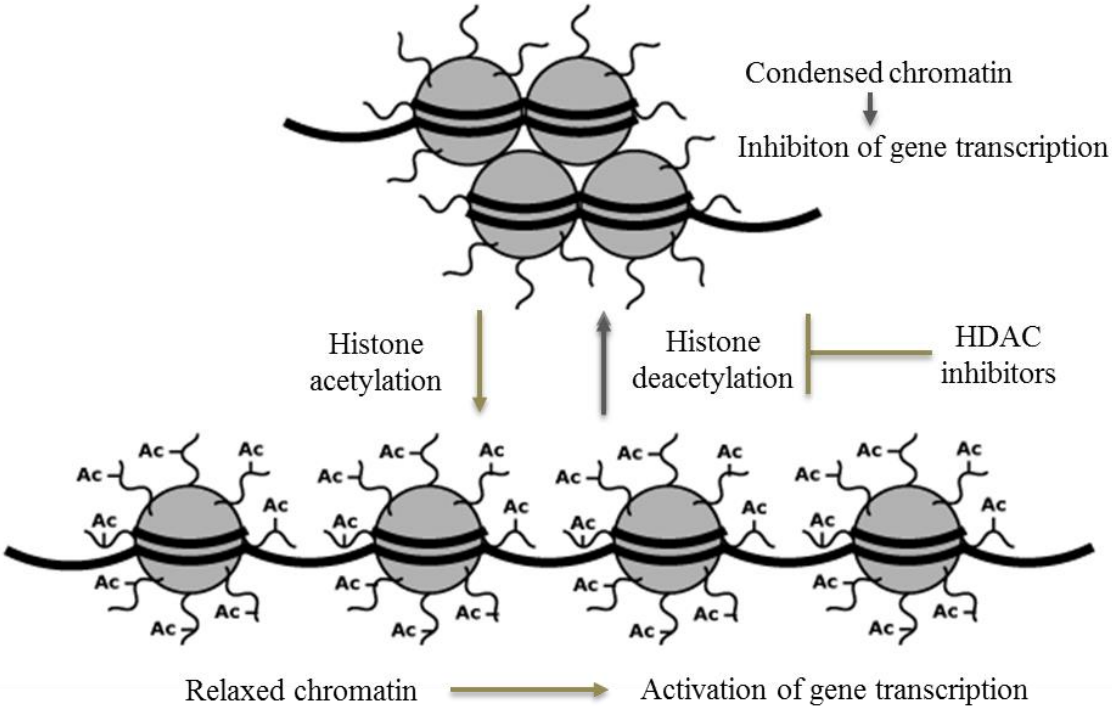


Figure 1.3: Chromatin remodelling by histone acetylation and deacetylation: Acetylation of histone proteins is catalysed by the action of HATs and is reversed by the action of HDACs. Acetylation involves attachment of acetyl groups to lysine residues in tails of histone proteins, thereby neutralizing the positive charge of histone tails and decreasing their affinity for DNA. This result is more relaxed chromatin structure that is associated with activation of gene transcription. The opposite reaction (deacetylation) by HDACs removes acetyl groups from lysine residues in the tails of histones resulting in condensation of chromatin associated with inhibition of gene transcription.

1.3.1. Epigenetics in pancreatic differentiation

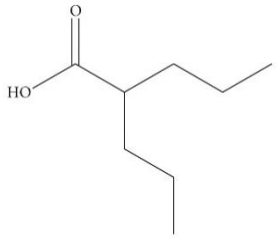
Studies have stressed the role of epigenetic mechanisms in pancreatic lineage development and showed that these epigenetic signatures are critical to proper beta-cell development and function [76, 89]. Among cellular reprogramming strategies, the small molecule approach is aimed to have better clinical prospects, as it does not involve genetic manipulation. Several small molecules targeting certain epigenetic enzymes and signaling pathways have been successful in helping to induce pancreatic beta-cell specification [90]. In an mouse model of intrauterine growth restriction it was demonstrated that HDAC1 is involved in silencing of Pdx1 leading to repression of Pdx1 transcription and further failure in beta-cell development

and subsequent beta-cell dysfunction [91]. The function of beta-cells to release insulin is also regulated by HDACs and HATs. At high glucose levels Pdx1 associates with the histone acetyltransferase p300 leading to increased acetylation of histone H4 in the insulin promoter and further transcription of preproinsulin [92, 93]. Moreover, selective inhibition of HDAC3 protected pancreatic beta-cells and improved glycaemia and insulin secretion in obese diabetic rats [94].

Expression of HDACs was reportedly upregulated in embryonic pancreas, and administration of HDACi shifted the lineage of pancreatic precursors from acinar to islet cell phenotype. This suggested that HDACi were effective tools to examine a putative connection between chromatin effects and cell lineage specification [89, 95]. Utilization of HDACis was proposed for embryonic stem (ES) cell culture. Early events of pancreatic specification were stimulated in ES cells with sodium butyrate (NaB), while Trichostatin A (TSA) was repressive [96, 97]. Study in pancreatic explants showed that treatment with VPA lead to differentiation into glucagon positive cells, while treatment with TSA resulted in insulin and somatostatin positive cells [98]. HDACi have distinct structures and thereby might have functions independent of the inhibitory action on HDAC activity and additionally, the action of HDACi might vary with concentration. Efforts have been undertaken to get a better idea on the importance of HDAC subtypes and dose finding studies are going on with specific HDACi [89, 99]. In the present study valproic acid (VPA), a potent inhibitor of class I and II histone deacetylases was used.

1.3.2. Valproic acid

In the present study, we used valproic acid (VPA) which is also termed 2-propylvaleric acid, 2-propylpentanoic acid or n-dipropylacetic acid, naturally produced by valerian (*Valeriana officinali*s). It is a branched, short-chain fatty acid derived from valeric acid and was first synthesized in 1882 by Burton.



Structure of valproic acid

It has a half-life time of 9-16 hrs and at room temperature it forms a clear liquid. For over 40 years it has been used for the treatment of patients with epilepsy and other neuropsychiatric disorders [100, 101].

1.3.3. Action of VPA

VPA acts through enhanced acetylation of histones by inhibiting HDACs from class I and class IIa most likely through binding to the catalytic site and further regulating gene expression by increased acetylation of histones and in part by shifting HDACS into proteosomal degradation [102, 103]**.** It was reported to differentiate transformed haemopoietic progenitor cells by inhibition of HDACs and subsequent hyperacetylation of the N-terminal tails of histones H3 and H4 [104]. A study to survey the effect of VPA on mouse salivary gland cells showed that VPA treatment increased phenotypic plasticity of these cells into pancreatic cells by inducing the pancreatic genes Ngn3, Pax4 and Ins1/2. However, the exact mechanism of VPA action in this commitment remained unknown [105]. Treatment of human induced pluripotent stem cells with VPA promoted differentiation into hepatocyte like cells by inhibiting HDAC activity [106]. Similar kind of phenotypic plasticity was observed in mouse salivary gland cells. When pretreated with VPA they differentiated into endodermal and hepatic-like lineage [107]. VPA pre-treatment of canine adipose tissue-derived stem cells decreased the proliferation in a dose dependent manner and promoted neurogenic differentiation [108]. A study in juvenile diabetic rat demonstrated that VPA improved beta-cell proliferation and function as well as reduced beta-cell apoptosis through HDAC inhibition [109]. Dose-dependent effects of VPA on cell lines were summarised in Table 1.2.

VPA (mM) concentrations used	**Cell lines**	**Effect of VPA treatment on cell differentiation**
2 mM	Human induced pluripotent cells	Hepatocytes [106]
2, 4, 8 mM	Canine Adipose tissue Derived Stem cells (ADSCs)	Neurogenic differentiation [108]
2.5, 5, 10 mM 5 mM	Human umbilical cord derived stem cells	Converted to hepatic lineage [110]

	Human bone marrow cells	Differentiation into hepatic lineage [111]
1, 5, 10 mM	Thyroid cancer cells	Redifferentiated [112]
1 mM	Salivary gland cells	Increased phenotypic plasticity of cells [105]
1 mM	Mouse Pancreatic explants	Glucagon positive endocrine cells [98]

Table 1.2: VPA in millimolar range of concentration and its effect on endodermal or pancreatic differentiation in various cell lines.

1.4. Pancreatic cancer

Pancreatic adenocarcinoma is one of the most aggressive human cancers, with a five year survival rate of <7% [113]. The disease is usually diagnosed at advanced stage and the treatment options are insufficient. The lethal nature of pancreatic ductal adenocarcinoma (PDAC) is due to its rapid dissemination to the lymphatic system and extensive tumor invasion to distant organs. Despite of efforts made in the recent years, conventional treatment approaches such as surgery and chemotherapy had little impact. Due to anatomic and biologic reasons, such as hypovascularization, expression of drug metabolizing enzymes and the presence of pancreatic cancer stem cells this disease remains hard to be diagnosed and treated effectively [114, 115].

1.4.1. Molecular and epigenetics of pancreatic cancer

Early studies have defined mechanisms of oncogenesis in PDAC such as mutations in onco-genes, altered expression of tumor suppressor genes, changes in pathways. An updated genomic analysis characterized PDAC as one of the most heterogenous malignant diseases because of the diverse genetic events occurring in each pancreatic tumor [115-117]. In addition to the involvement of genetic alterations studies have demonstrated that epigenetic changes can also alter gene functions. This includes DNA methylation, histone modifications and non-coding RNAs [78, 118].

HDACs were found to be involved in various cell pathways including control of gene expression, regulation of cell proliferation, differentiation, migration and cell death. Studies reported overexpression of HDACs in several types of human cancers, including PDAC [85, 119]. Hence, targeting histone deacetylases became a promising approach and increased interest in treating pancreatic cancer. It was shown that HDACi induced differentiation and cell cycle arrest in proliferating cancer cells. They activated pathways of apoptosis and inhibited invasion and angiogenesis in various cancer cell lines [120, 121]. Therefore, HDACi´s emerged as anticancer drugs and VPA as an anticancer drug has been in phase1 and 2 clinical trials [122]. In wide range of hematological malignancies HDAC inhibitors became promising anticancer agents as disease remissions were observed. However, the results in solid tumors have been disappointing [123]. Studies from recent years showed that HDACi treatment could lead to epithelial-to-mesenchymal transition (EMT) in prostate cancer cells and in head and neck squamous cancer cells. Further, suggesting application of HDACi as anticancer agents requires caution and it is important to select appropriate drug for different tumors [124, 125].

1.4.2. Epithelial to mesenchymal transition

EMT is a biologic process first recognized to be active during embryogenesis and is vital for morphogenesis in embryo development. Aberrant activation of this process acts as a trigger for tumor progression and metastasis. Studies have shown its importance in cancer biology and been implicated in conversion of early stage tumors to invasive malignancies [126, 127]. During EMT epithelial cells undergo morphologic changes by losing polarity, cell-cell adhesion and further the epithelial phenotype associated with down regulation of e-cadherin. Moreover, they acquire migratory potential with upregulation of mesenchymal markers such as vimentin, fibronectin and n-cadherin. This process is mediated by a group of key transcription factors such as snail 1/2, slug, zinc finger homeodomain family zeb1/2 and twist. In tumor, this transition from epithelial to mesenchymal phenotype was shown to be associated with cancer progression, that included increased cell invasion, angiogenesis, chemo resistance, and formation of cancer stem cells [128, 129]. Activation of EMT program is found to be involved in multiple signaling pathways and several epigenetic and post translational mechanisms as well [127, 130].

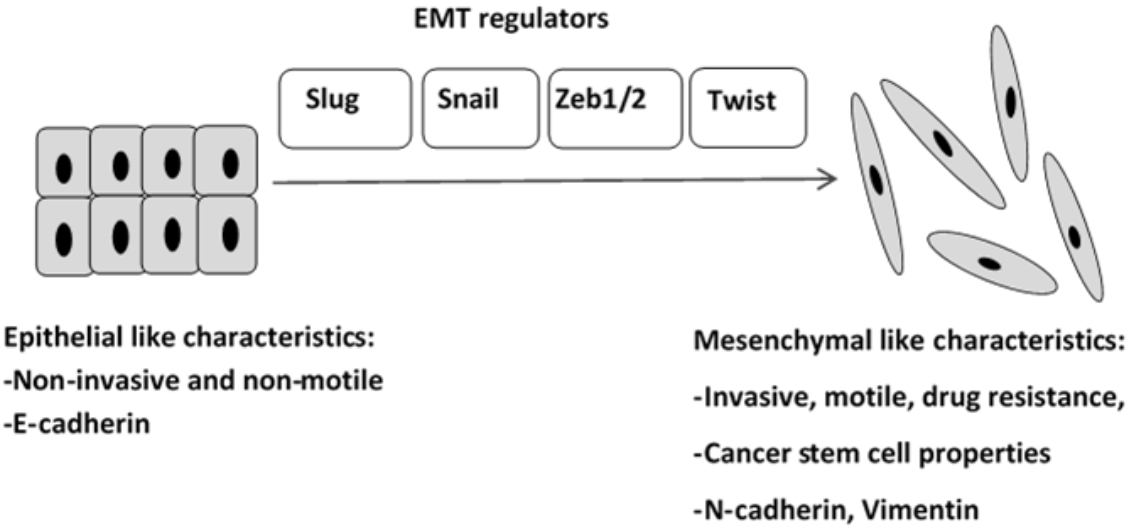


Figure 1.4: Epithelial to mesenchymal transition (EMT): Transcriptional regulators transform tumor cells from epithelial to mesenchymal like cells with suppression of epithelial and expression of mesenchymal markers - resulting in cancer metastasis, drug resistance and cells with features of cancer stem cells.

1.4.3. Cancer stem cells (CSCs) in PDAC

Cancer stem cells are proposed to be a small population of stem-like cells that have the ability to self-renew and differentiate into new diverse tumor cells and further, leading to disease progression, metastases and drug-resistance [131]. In many tumors such cancer stem cells are identified and isolated by using surface markers. Stem cells of pancreatic cancer are identified by using markers like CD 44+/ CD24+/ epithelial-specific antigen (ESA), CD133+ / CXCR4+ /ABCG2 [132-134]. Studies showed that a relationship exists between EMT and cancer stem cells. The EMT endows cancer stem cells with the ability to metastasize [129, 135]. Accumulating evidence suggested that these CSCs exhibit abundant expression of drug transporters like ABCG2 and further, show increased resistant to chemotherapy and are implicated in tumor metastasis [136].

1.5. Aims

Studying the role of epigenetic mechanisms in pancreatic lineage development, demonstrated that epigenetic signatures contribute to cell fate decisions and proper beta-cell development [76, 89]. These discoveries open the exciting possibility that, by understanding the mechanisms it might be possible to induce beta-cell regeneration in diabetic patients through cellular reprogramming strategies, with small molecule approach being most favourable.

The aim of this study is to investigate the ability of the Histone deacetylase inhibitor valproic acid (VPA) to promote endocrine differentiation of human Panc-1 cells and study the effects on endocrine pancreatic gene expression. The aims of the study in detail are:

1) To study if the action of VPA is going through acetylation of histones.
2) To investigate if VPA can induce endocrine gene expression in Panc-1 cells.
3) To study the effect of differentiation concentrations of VPA on pancreatic gene expression.
4) To observe and characterize the morphological changes in the cells upon treatment with VPA.
5) To find out whether VPA induces epithelial to mesenchymal transition and modulates cancer stem cell markers.
6) To study differentially expressed genes and pathways between VPA treated and control by RNA sequencing.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Product	Company
Ammonium persulfate (APS)	Sigma
Acrylamide-bis	Serva
Bovine serum albumin (BSA)	Sigma
Bromphenolblue	Merck
Dimethyl sulfoxide (DMSO)	Merck
DMEM D5671 Medium	Sigma
DNAse 1	Qiagen
Donkey serum	Jackson Immunoresearch
ECL Western Blotting Detection reagent	ThermoScientific
Ethanol	Merck
EDTA	Fluka
Fetal calf serum (FCS)	Sigma
Glycerol	Acros Organics
Glycine	Roth
Glutamine	Life technologies
Hoechst 33342	Sigma
Human serum	Biowest
Hydrochloric acid 30% (HCL)	Merck
IGEPAL CA-630	Sigma
Magnesium chloride (MgCl2)	Merck
b-Mercaptoethanol	Fluka

Methanol	Merck
Molecular Weight Marker	Fermentas
Oligo (dt) 20	Invitrogen
Paraformaldehyde	Merck
Penicillin/Streptomycin	Life technologies
Phosphate Buffered Saline (PBS)	B Braun
Potassium chloride (KCL)	Fluka
Prolong Gold	Invitrogen
Proteinase Inhibitor	Thermo scientific
Protein standard	Fermentas
RNAse-Free Water	Invitrogen
Skim milk powder	Merck
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Bio-Rad
Sodium Hydroxide (NaOH)	Fluka
SYBR Green	Bio-Rad
Tetra-methyl-ethylenediamine (TEMED)	Merck
Tris-base	Sigma
Tris-HCL	Sigma
TritonX-100	Sigma
Trypan Blue	Sigma
Trypsin/EDTA	Life technologies
Tween20	Merck
Valproic Acid	Sigma

2.1.2. Instruments

Instrument	Company
Laminar flow hood	Kendro
Incubator	Thermo electron corporation
NanoDrop 1000 Spectrophometer	Peqlab
StepOne plus Real-Time PCR system	Applied Biosystems
Thermo cycler	VWR International
SDS electrophoresis set	Peqlab
Power supplier	Consort
Transfer chamber	C.B.S *Scientific CO
Cassettes	Cronex 18*24
Gel Doc Bio visible	Vilber Lourmat
Fluorescent Microscope	Leica Microsystems
Light Microscope	Ernst Leitz
ELISA Reader (Mithras LB940)	Berthold Technologies

2.1.3. Software

Software	Company
Statistical Analysis	Graphpad Prism
Microsoft Office	Microsoft
Leica Application Suite	Leica
Motic Image Plus 2.0	Motic
Bio 1D	Vilber Lourmat
Image J software	Image J
EndNote	Thomson Reuters

2.1.4. Kits

Kits	Company
Bio-Rad Protein Assay Kit	BioRad
Glucagon ELISA kit	DRG Instruments
RNA isolation kit	Qiagen
cDNA synthesis kit	Invitrogen

2.1.5. Human Forward and Reverse Primer sequences for real-time PCR

ABCG2_for	GGTTACGTGGTACAAGATGATGTTG
ABCG2_rev	AGCCGAAGAGTCGCTGAGAA
CD24_for	ACCCACGCAGATTTATTCCA
CD24_rev	GAGCTTTCTTGGCCTGAGTC
CD44_for	ACAGCACAGACAGAATCCCTG
CD44_rev	TCTTCTGCCCACACCTTCTCC
CD133_for	TCAGGATTTTGCTGCTTGTG
CD133_rev	GCAGTATCTAGAGCGGTGGC
CXCR4_for	CACCGCATCTGGAGAACCA
CXCR4_rev	GCCCATTTCCTCGGTGTAGTT
E-CAD_for	AGGAATTCTTGCTTTGCTAATTCTG
E-CAD_rev	CGAAGAAACAGCAAGAGCAGC
ESA_for	GGAAGCTGAGTGCAAGAAGG
ESA_rev	GCTGCACAACCTCAATCTCA
FOXA2_for	GGGAGCGGTGAAGATGGA
FOXA2_rev	TCATGTTGCTCACGGAGGAGTA

GLUCAGON_for	CCCAAGATTTTGTGCAGTGGTT
GLUCAGON_rev	CAGCATGTATCTCAAATTCATCGT
HPRT_for	TCAGGCAGTATAATCCAAAGATGGT
HPRT_rev	AGTCTGGCTTATATCCAACACTTCG
SL1_for	CAACTGGTCAATTTTTCAGAAGGA
ISL1_rev	TTGAGAGGACATTGATGCTACTTCAC
INSULIN_for	GCAGCCTTTGTGAACCAACA
INSULIN _rev	TTCCCCGCACACTAGGTAGAGA
NGN3_for	CTATTCTTTTGCGCCGGTAGA
NGN3_rev	CTCACGGGTCACTTGGACAGT
N-CAD_for	CCCACACCCTGGAGACATTG
N-CAD_rev	GCCGCTTTAAGGCCCTCA
NOTCH1_for	GGACCTCATCAACTCACA
NOTCH1 _rev	GGTGTCTCCTCCCTGTTGTT
OCT4_for	CACGAGTGGAAAGCAACTCA
OCT4_rev	AGATGGTGGTCTGGCTGAAC
PDX1_for	TGATACTGGATTGGCGTTGTTT
PDX1_rev	TCCCAAGGTGGAGTGCTGTAG
PAX6_for	TGCGACATTTCCCGAATTCT
PAX6_rev	GATGGAGCCAGTCTCGTAATACCT
SOX17_for	GGCGCAGCAGAATCCAGA
SOX17_rev	CCACGACTTGCCCAGCAT
SOMATOSTATIN_for	GATGCCCTGGAACCTGAAGA
SOMATOSTATIN_rev	CCGGGTTTGAGTTAGCAGATCT

SLUG_for	ACACACACACACCCACAGAG
SLUG_rev	AAATGATTTGGCAGCAATGT
SNAIL_for	ACCCCACATCCTTCTCACTG
SNAIL_rev	TACAAAAACCCACGCAGACA
ZEB_for	GCACAACCAAGTGCAGAAGA
ZEB_rev	CATTTGCAGATTGAGGCTGA

2.1.6. Antibodies

Primary Antibody	**Dilution**	**Company**
Ngn3 (WB)	1:2000	Abcam
Pdx1 (WB)	1:1500	Millipore
Glucagon (WB)	1:500	Anaspec
E-cad (WB)	1:3000	Abcam
β actin (WB)	1:3000	Abcam
Vimentin (WB)	1:3000	Abcam
Glucagon (ICC)	1:100	Novusbio
Histones (WB)	1:1000	Cell signalling
Secondary Antibody	**Dilution**	**Company**
Peroxidase-conjugated Goat anti-mouse-IgG (WB)	1:3000	Dako
Peroxidase-conjugated Goat anti-rabbit-IgG (WB)	1:3000	Dako
FITC-APure Donkey Anti-Rabbit IgG (ICC)	1:500	Jackson Immuno Research
Rhod Red-X-APure Donkey Anti-Rabbit IgG (ICC)	1:500	Jackson ImmunoResearch

2.2. Methods

2.2.1. Cell line and Culture conditions

Panc-1 cell culture

Panc-1, is a human pancreatic ductal cell line derived from an adenocarcinoma in the head of the pancreas which invaded the duodenal wall and metastasized in one peripancreatic lymph node. Cells have a doubling time of 52 h and grow as monolayer in culture. The cells are epithelioid, large and multinucleate with 57-64 chromosomes [137], American Type Culture Collection (ATCC, Feb 2012) .

The cell line Panc-1 was purchased from American Type Culture Collection (ATCC, distributor LGC Standards GmbH, Wesel, Germany). The cells were maintained as a monolayer in 25-mmol/L glucose Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum supplemented with 100 units/ml penicillin, 100mg/ml streptomycin, 2 mmol/L-glutamine. Cells were cultured at 37°C with 5% of CO_2 and 95% air humidity.

Cells were passaged by trypsinization. They were washed once with PBS (without Ca^{2+} and Mg^{2+}) and treated with pre-warmed 0.05% trypsin/EDTA (1x) solution. After a short incubation period of 3-5 minutes at 37°C the cells detached were diluted with warm DMEM culture medium, centrifuged for four minutes at 1000 rpm and the supernatant with traces of trypsin were removed. Cell suspension was diluted with culture medium and cells were seeded into new flask for maintenance and one part of cells was taken to experiment studies. For freezing the cells, the trypsinized cells were centrifuged and supernatant was removed. Concentrated cell suspension was diluted with freshly prepared freezing medium (80% FCS and 20% DMSO) and incubated at -20°C for 1hr followed by -80°C overnight incubation. Finally, the frozen cells were stored in liquid nitrogen. To thaw the cells, a vial was transferred from liquid nitrogen quickly to a water bath (37°C) for two to three minutes, cells were suspended directly in fresh medium for centrifugation, the supernatant with DMSO was removed and then cells were plated with fresh culture medium.

VPA treatment

Cells were plated in culture dishes and incubated overnight. On the next day, cells with about 50-60% confluence, were treated with VPA in doses ranging from 1 mM to 6 mM for five or three days. VPA dissolved in water and filtered was used for the treatments. The solvent used

for VPA was used for control treatments. After treatment samples were collected for further analysis.

Amount of VPA-solution (1M)/5ml medium	End concentration
Control	0 mM
2.5 µl	0.5 mM
5 µl	1 mM
10 µl	2 mM
20 µl	4 mM
30 µl	6 mM

2.2.2. Isolation of RNA

Total RNA was isolated from samples using RNeasy Mini kit according to the manufacturer's instructions (Qiagen). For this purpose, after treatment cells were harvested and lysed using RLT buffer with 1% beta-mercaptoethanol. Then the lysates were mixed with equal ratio of 70% ethanol in a tube. This lysis solution was then transferred into RNeasy spin column and centrifuged for one minute at 13,000 rpm. After this and following centrifugation steps the flow-through was discarded. 500 µl of RWI buffer was added into the column followed by another centrifugation. Next 500 µl of RPE was added into the column, followed by centrifugation under conditions mentioned above. The sample was then washed by adding 500 µl of 80% ethanol, followed by another centrifugation. At this stage the spin column was placed into a fresh collection tube, then 20 µl of RNAse free water was added and centrifuged for two minutes at 13,000 rpm. The purified RNA remained in the collection tube and was stored at -80°C until further processing. To prevent contamination of RNA by RNAses standard precautions were carefully taken, such as cleaning the working area with RNAase decontamination solution, using RNAse/DNAses free tubes, using RNAse/DNAses free water and cleaned gloves.

Measurement of RNA Concentration

Using NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington) the quality and quantity of RNA was measured. The ratio of sample absorbance at 260 and 280 nm (260/280) was measured to check the purity of RNA which should be around 2.0 for pure RNA. Sample concentration was given in ng/µl based on its absorbance at 260 nm.

DNAse treatment

To eliminate any genomic DNA contamination all RNA samples were treated with DNAse. In a microfuge tube 1µg RNA was mixed with 1µl DNase I (1U/µl), 1µl 10x DNAse reaction buffer and RNAse/DNAse free water diluted up to 10 µl. After an incubation at 37°C for 15 minutes. 1 µl of 25mM EDTA was added to inactivate DNAse I by an incubation at 65°C for 15 minutes. The reaction was collected by a brief centrifuge and used for further cDNA synthesis.

RNA-1µg
10X Reaction Buffer-1 µl
DNase I (1 U/µl) - 1 µl
RNAse/DNAse free water-up to 10 µl

cDNA synthesis

For synthesis of cDNA, DNAse treated mRNA samples were used. By using Oligo (dT) primers, and reverse transcriptase, mRNA was reverse transcribed into cDNA. The mRNA sample was mixed with 9 µl of master mix that contains 4 µl of 5 x firststrand buffer, 1µl of 10mM dNTP mix, 1µl Oligo (dT) 20 (0.5 µg/ µl), and 2 µl of 0.1 M DTT and 1 µl of SuperScript III RT (200 U) and the resulting 20 µl solution was incubated at 42°C for 50 minutes and heated at 70°C for 15 minutes. Further the synthesized cDNA was used for qRT-PCR.

Quantitative Real -Time PCR (qRT-PCR) analysis

Real-time PCR or qRT-PCR is used for the quantitative detection of PCR amplification of a specific target sequence from cDNA in real time using the fluorescent dye SYBR Green on the Step One Plus real-time polymerase chain reaction system.
The reaction mixture consists of:

SYBR Green Master Mix	5 µl
cDNA template	1 µl
Primers (F+R) 20 pmol/µl	0.5 µl
RNAse/DNAse free H2O	3.5 µl

PCR was carried out using the following program:

Steps	**Temp**	**Time**	**No. of cycles**
Enzyme activation	95°C	10 min	1 cycle
Denaturation	95°C	15 sec	40 cycles
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

After amplification of the products, melt curve analysis was performed to analyze the specificity of the products by using the following steps.

Steps	**Temp**	**Time**	**No. of cycles**
Denaturation	95°C	15 sec	1 cycle
Starting Temp	60°C	60 sec	1 cycle
Melting step	60-95°C in steps of max 0.3°	Temperature change after 15 sec	1 cycle

The expression of each gene was measured in triplicate for each sample. The threshold line is the point at which the PCR reaction reaches the fluorescent intensity above the background level. The cycle threshold value (Ct) for each individual PCR product was calculated by the instrument's software. Ct values for each target gene were assessed for each sample in triplicate and the mean was calculated. The relative changes of mRNA expression in treated and untreated cells were calculated by the comparative ΔCt method. ΔCt value was calculated by subtracting the mean Ct value of the reference gene (HPRT) from the mean Ct value of the

target gene from each sample. Primers used, and their sequences are summarized in Table (2.1.5). Among all genes, few genes expression was analyzed at protein level by Western blot.

2.2.3. Enzyme- Linked immunosorbent assay (ELISA)

Glucagon secreted from cells was quantified by EIA kit (DRG, Germany). The EIA kit is based on a competitive enzyme immunoassay and the antibody used is specific against the C-terminal fragment (19-29) of pancreatic glucagon and there is no cross reactivity with intestinal glucagon, GLP-1 or GLP-2. Following the manufacturer's instructions, the assay was carried out. The results were standardized with the protein content measured by Bio-Rad protein assay in the respective samples.

2.2.4. Immunohistochemistry

Fixation

Panc-1 cells were grown on slides and were treated with VPA and water as control for five days. After five days, the medium was removed and the slides with cells were washed twice in cold PBS. Then the cells were fixed with Zamboni solution for 15 minutes, followed by washings with PBS three times for five minutes.

Blocking and incubation

Afterwards, cells were blocked with blocking buffer (PBS, 10 % Donkey serum) for 20 minutes at room temperature. After blocking the cells were probed with the primary antibody diluted, as per the ratio mentioned in data sheet, in PBS containing 1% donkey serum and 0.1% triton 20 for overnight at 4°C. Next day, followed by washing the slides three times for 5 minutes in PBS. Then slides were incubated at room temperature with the fluorescence dye-coupled secondary antibody diluted in PBS (950 µl PBS+50 µl human serum+2.5 µl secondary antibody) for 1hour. The slides were washed again twice for 5 minutes in PBS. Then they were incubated with Hoechst dye for 5 minutes (1:1000 in Tris pH 7.6) by which the nuclear DNA is stained, further slides were given final wash in PBS for 5 minutes and continued with mounting.

Mounting

Then slides were mounted with cover slip with a drop of prolong gold and pressed gently. The slides were left overnight at 4°C. The slides were viewed and photographed under fluorescent microscope. (Leica DMLB, Germany).

2.2.5. Western blot

Protein extraction

Cell extraction in NP-40 lysis buffer

Cells were washed with cold PBS and by using the cell scrapper cells were collected. These cells were suspended in 300-350 ml of NP-40 lysis buffer, with freshly added protease inhibitor and were centrifuged for 20 min at 13000 rpm at 4°c. The total cell extract contained in the supernatant was collected and stored at -80°c for further use.

Lysis buffer	20mM	Tris/HCL pH 7.5
	150mM	NaCL
	1% (v/v)	Nonidet P-40

Measurement of protein concentration:

Protein concentration was measured by using the Bio-Rad Protein Assay. This assay is a dye-binding assay based on the Bradford method. It measures differential color change of dye-Coomassie Brilliant Blue G-250 in response to various concentrations of protein in solution. The dye binds to primarily basic and aromatic amino acid residues, especially arginine. The maximum absorbance for the dye is at 465 nm, but shifts to 595 nm when binding to protein occurs. The protein concentration of the test samples was obtained by comparing to a standard curve with known concentrations of BSA (protein standard, Sigma). The OD595 value of test samples were measured with Mithras LB 940 Multimode Microplate reader (Berthold technologies)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

By using SDS-PAGE equivalent amount of protein was separated based on their molecular size. The system consists of two gels: a stacking gel with a low pH (6.8) and low level of cross linkage thus allowing proteins to enter the gel and compact without smearing and a separating gel with higher pH (8.8), where the proteins are separated according to molecular size. The following solutions were used for making a gel.

	Resolving gel (10%)	Stacking gel (5%)
H2O	5.93 ml	3.4 ml
Acrylamid-Bis (30%)	5 ml	0.83 ml
1,5 M Tris/HCL pH 8.8	3.75 ml	0
1 M Tris/HCL pH 6.8	0	0.63 ml
APS (10%)	150 µl	50 µl
SDS (10%)	150 µl	50 µl
TEMED	15 µl	5 µl
5x SDS running buffer (1000 ml):-	72g Glycine 15g Tris-base 5g SDS pH 8.3 fill up to 1000ml with Distilled water	

Resolving gel was incubated for overnight. Followed by next day with preparation of stacking gel and loaded over the resolving gel provided with a comb for making wells. Equivalent amount of protein of each sample combined with 4x sample buffer were denatured by heating at 95°C for 5 min, and immediately cooled on ice. By brief centrifugation samples were collected and equal amounts of protein were loaded into wells on a gel. The protein marker was loaded into the first lane. The electrophoretic separation of proteins was carried out in a

vertical chamber with 1 x SDS running buffer. After the electrophoresis run, gels were washed three times in transfer buffer and is further used for Western blot analysis.

4 x Sample buffer	2.4 ml 1M Tris, pH 6.8 0.8 g SDS 4 ml Glycerol (100%) 0.2 ml Bromophenol blue (0.5%) 2.8 ml Distilled water
1x Transfer buffer (1000 ml)	5.8 g Tris-base 2.9 g Glycine 0.37 g SDS 200 ml Methanol fill up to 1000 ml with Distilled water

Thus, the proteins separated were electrically transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Millipore) by electro blotting at 93 V for 30-40 mins. After the electrotransfer, membrane was washed with TBS-T for 15min and were blocked for 1 hour at room temperature with 5% non-fat milk powder or BSA dissolved in 1 x TBST (TBS containing 0.1% Tween 20). After blocking the membrane was incubated with the appropriate primary antibody for overnight at 4°C. Next day the membrane was washed three times for 15 mins in 1 x TBST and then incubated with horseradish peroxidase conjugated secondary antibody diluted in milk powder at room temperature for 1hour, followed by three washes in TBST. The proteins bound to the membrane were then detected by using Amersham chemiluminescence system (ECL). The membrane was exposed to X-ray films and further was developed and fixed.

10 x TBS	24.23 g Tris/HCL pH7.6 80.06 g NaCL pH 7.6 fill up to 1000 ml with Distilled water
1 x TBST (1000 ml)	100 ml 10 x TBS

10 x TBS	24.23 g Tris/HCL pH7.6 80.06 g NaCL pH 7.6 fill up to 1000 ml with Distilled water 899 ml Distilled water 1 ml Tween-20

2.2.6. In vitro wound healing (scratch) assay

Panc-1 cells were seeded in plates and allowed to grow to approximately 70% confluence. VPA was added to the plate to a final concentration of 2 mM. Aquadest was used as control. After 24 hours of incubation with VPA the cells were treated with mitomycin prior to the scratch application. After 1hour incubation, a scratch was applied to the cell layer in the plate using a 100 µl pipette tip. The old medium was removed, and the cell layer was washed twice with PBS to remove loose cells from the scratch margins. The plate was filled with fresh medium with VPA or aquadest. At regular intervals for every 10 minutes until 24 hours images were taken from the locations of the scratch applied with a Nikon coolpix digital camera on phase contrast inverted microscope with scattered light illumination.

2.2.7. Transcriptomic analysis (RNA sequencing (RNA-seq)

For RNA-seq analysis, total RNA was isolated from Panc-1 VPA treated and control (wild type) using the RNeasy mini Kit (Qiagen) as mentioned in (2.2.2) was used. For exclusion of genomic DNA contamination, all samples were treated by DNase I (Qiagen). Total RNA and library integrity were verified with LabChip Gx Touch 24 (Perkin Elmer). 1µg of total RNA was used as input for SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian (Clontech) following standard instructions. Sequencing was performed on the NextSeq500 instrument (Illumina) using v2 chemistry, resulting in average of 115M reads per library with 1x75bp single end setup. The resulting raw reads were assessed for quality, adapter content and duplication rates with FastQC (Andrews S. 2010, FastQC: a quality control tool for high throughput sequence data. Available online at: www.bioinformatics.babraham.ac.uk/project/fastqc. Trimmomatic version 0.33 was employed to trim reads after a quality drop below a mean of Q20 in a window of nucleotides [138]. Only

reads between 30 and 150 nucleotides were cleared for further analyses. Trimmed and filtered reads were aligned versus the Ensembl human genome version hg38 (GRCh38) using STAR 2.5.3a with the parameter "--outFilterMismatchNoverLmax 0.1" to increase the maximum ratio of mismatches to mapped length to 10% [139]. The number of reads aligning to genes was counted with featureCounts 1.4.5-p1 tool from the Subread package [140]. Only reads mapping at least partially inside exons were admitted and aggregated per gene. Reads overlapping multiple genes or aligning to multiple regions were excluded. Differentially expressed genes were identified using DESeq2 version 1.62 [141]. Only genes with a minimum fold change of +- 1.5 (log2 +-0.59), a maximum Benjamini-Hochberg corrected p-value of 0.05, and a minimum combined mean of 5 reads were deemed to be significantly differentially expressed. The Ensemble annotation was enriched with UniProt data (release 06.06.2014) based on Ensembl gene identifiers (Activities at the Universal Protein Resource (UniProt)).

3. Results

3.1. VPA increased acetylation of histones in Panc-1 cells

To find out whether the mechanism of action of VPA in Panc-1 cells is through inhibition of HDAC or not, the cells were treated with VPA 0 and 6.0 mM VPA for four days. VPA is added every 24 hours along with fresh medium. On day five cells were harvested, protein was isolated, and further analyzed for acetyl-histone H3 (Lys9), histone H3 and acetyl-histone H4 (Lys8) by Western blot. Untreated cells were used as negative controls. Thus, VPA treatment resulted in increased expression of acetylated H3 and acetylated H4 (Figure 3.1A, B). H3 and H4 which are targets of HDACs showed a lower expression of H3 (Figure 3.1A). Results from this experiment confirmed that VPA acts through acetylation of histones in Panc-1 cells.

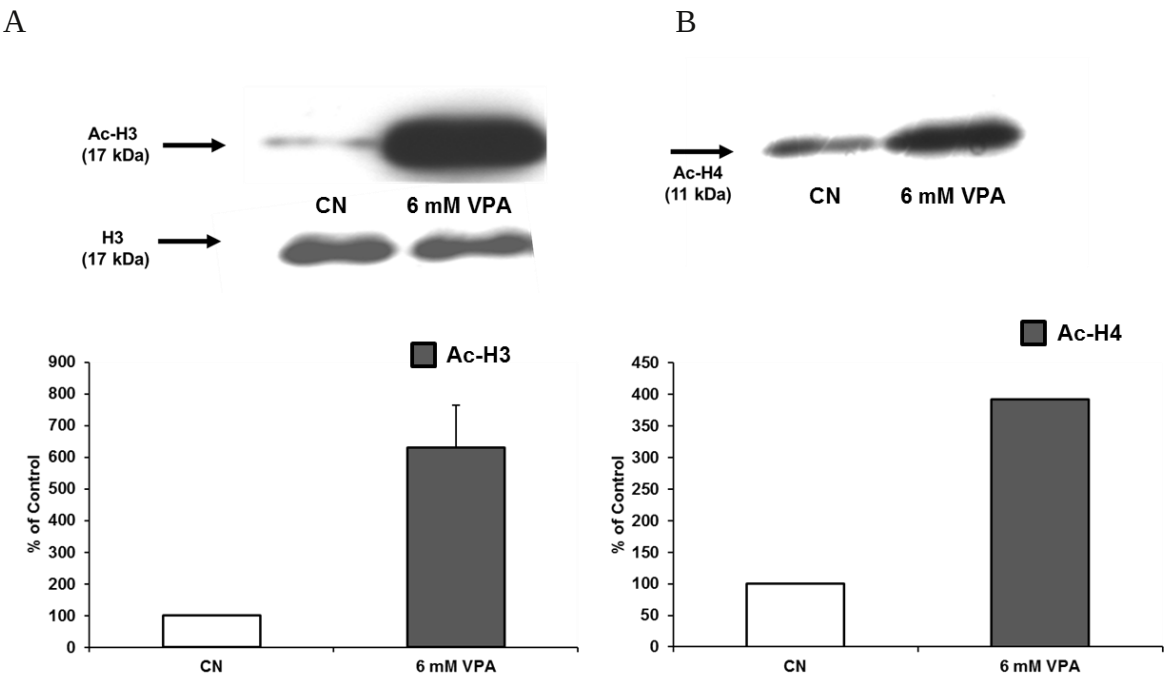


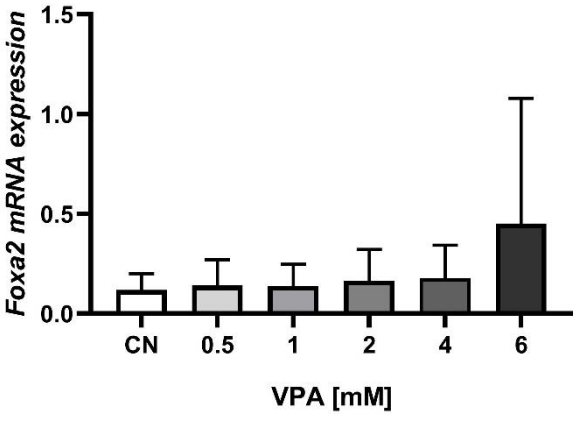
Figure 3.1: (A) Western blot analysis of acetylated histone (Ac-H3) and H3, (B) expression of acetylated histone (Ac-H4) in VPA-treated Panc-1 cells. Relative fold increase was determined and normalized to β-actin. VPA treatment increased acetylation of histones H3 and H4 and result in less expression of H3, n=2 experiments.

3.2. Effect of VPA on expression of key transcription factors for pancreatic lineage

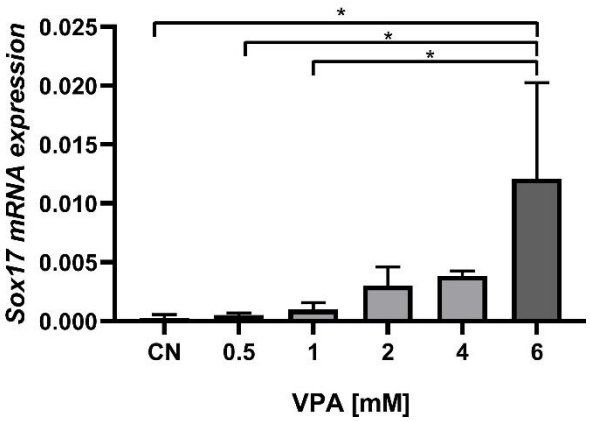
As the process of endocrine differentiation involves several transcription factors that work in a precise and sequential manner and has a highly cell specific expression pattern (1.1.3) it was important to know whether VPA treatment would trigger the expression of these transcription factors. For this purpose the Panc-1 cells were cultured in DMEM medium and were treated with different concentrations of VPA (0.5, 1, 2, 4, 6 mM) for three to five days. VPA was added every 24 hours along with fresh medium. On day five cells were harvested, mRNA was isolated as described in materials and methods and gene expression was analyzed.

mRNA level expression of pancreatic development marker genes Foxa2 and Sox17, which are found to be expressed during pancreatic development as described earlier in section 1.1.3, were analyzed by qRT-PCR. Figure 3.2 shows an increasing trend in the mRNA expression of Foxa2 (A) and Sox17 (B). The upregulation of Sox17 was clearly significant at the highest concentration of VPA (6mM). Pdx1 is considered as master regulator of pancreatic development and beta-cell differentiation (see section 1.1.3). Its mRNA level expression was analyzed by qRT-PCR and the protein expression quantified by Western blot. Expression of Pdx1 transcripts (Figure 3.2 C) was found to be enhanced at low concentration of VPA- 1 mM and a significant upregulation was seen at 6mM.

A



B



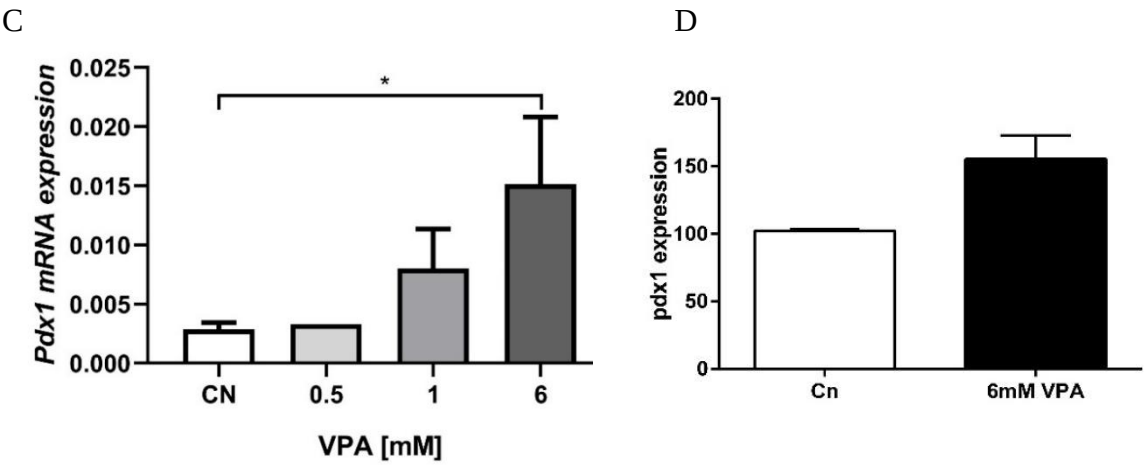


Figure 3.2: qRT-PCR assessment of Foxa2 (A), Sox17 (B) and Pdx1 (C) mRNA levels, after four days of VPA treatment with indicated concentrations. Gene expression was normalized to HPRT (housekeeping gene) and compared to control. Pdx1 protein expression in control (CN) and 6 mM VPA was determined by Western blotting (D). VPA treatment significantly increased the expression of Sox17 and Pdx1 at mRNA level. Data were expressed as mean ± SEM. *P≤ 0.05 by one-way ANOVA followed by Bonferroni´s multiple comparisons test. n = 2-4 experiments.

3.3. Effect of VPA on expression of key transcription factors for endocrine pancreatic lineage

To further investigate VPA - induced Ngn3 expression, a key transcription factor required for endocrine differentiation (described in 1.1.3), mRNA and protein levels in control and VPA treatment cells were quantified. Additional transcription factors like Pax6 and Isl1 that are required for further endocrine specification (described in section 1.1.3) were analyzed at qRT-PCR level.

Results from qRT-PCR and Western blot analysis showed an enhanced expression of Ngn3 with VPA treatment. Pax6, expression was found to be increased at 6 mM VPA concentration. Induced Isl1 expression was observed at higher concentrations, 4 and 6 mM VPA but, a significant expression was not achieved in this gene. Since, enhanced expression was observed in these pancreatic genes that are required for induction of endocrine differentiation, further analysis was continued to analyze endocrine specific genes.

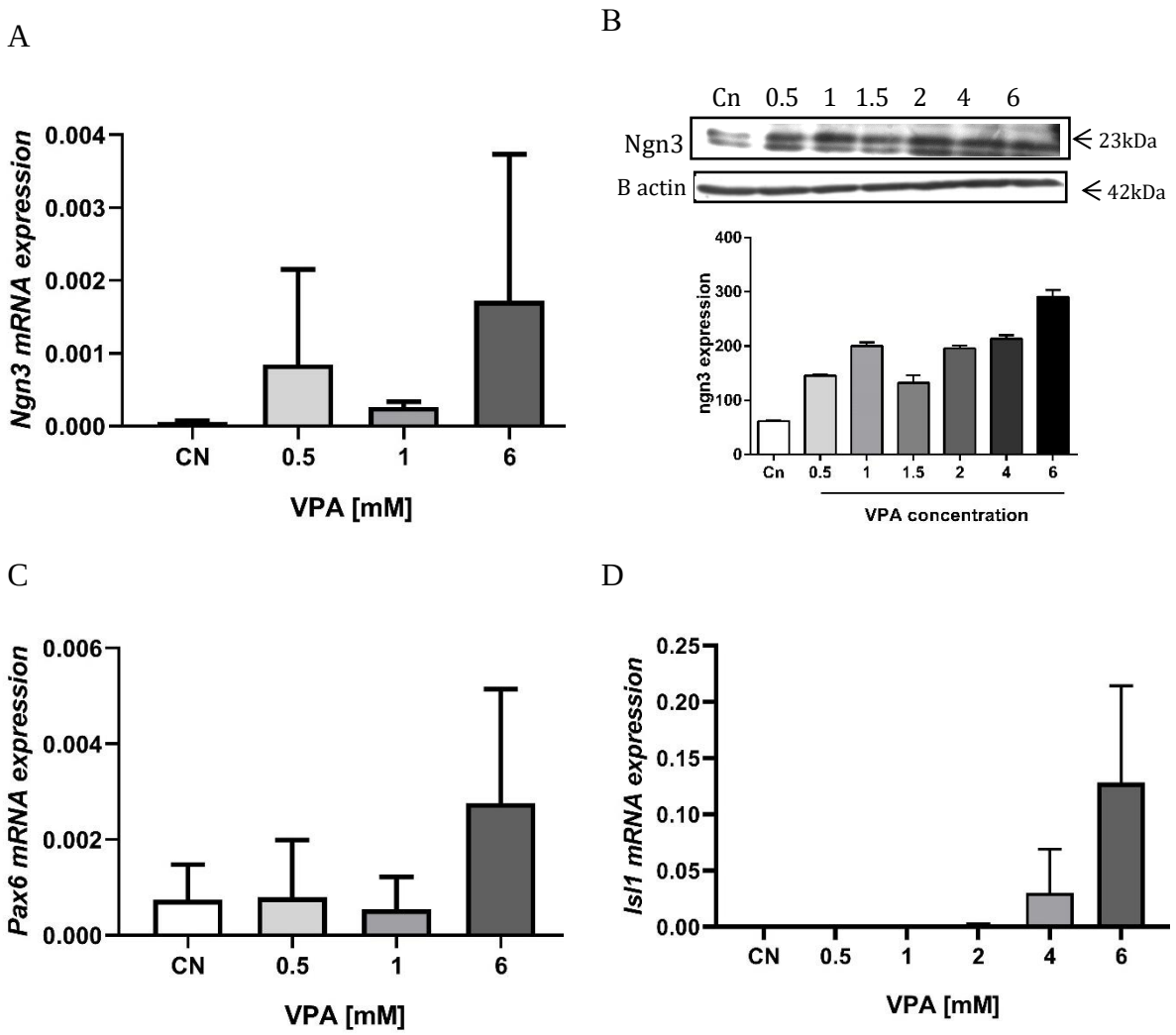


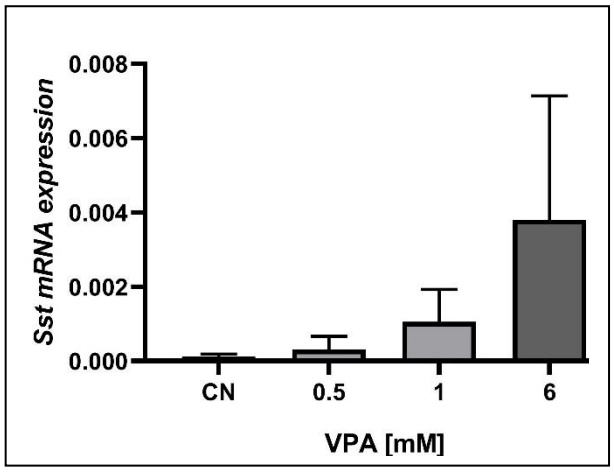
Figure 3.3: Analysis of expression of Ngn3 at mRNA level by qRT-PCR (A) and at protein level by Western blot (B). Quantification of Pax6 (C) and Isl1 (D) mRNA expression by qRT-PCR. Gene expression was determined and normalized to internal endogenous control HPRT. VPA treatment increased the expression of Ngn3, Pax6 and Isl1 at 6 mM concentration. Mean ± SEM. one-way ANOVA followed by Bonferroni´s multiple comparisons test. n=2-4 experiments. Ngn3 protein expression showed a noticeable up-regulation determined by Western blotting, n = 2 experiments.

3.4. Effects of VPA on expression of glucagon in Panc-1 cells

In order to determine whether VPA treatment induced endocrine specific genes in Panc-1 cells, qRT-PCR analysis for insulin, somatostatin and glucagon genes was carried out. Low basal expression of glucagon was detected in control conditions without VPA and a significant

glucagon induction was detected in the presence of VPA. This was verified at protein level by immunohistochemistry and Western blot analysis. Glucagon concentration in lysates was further confirmed by ELISA. Expression of other endocrine markers, insulin and somatostatin, was analyzed at mRNA level. A trend in the expression of somatostatin was observed with increasing concentrations of VPA and highest expression at 6 mM, but statistical significance was not achieved. Likewise, insulin transcription showed no difference with VPA treatment. These results demonstrate that the HDAC inhibitor VPA induced glucagon expression of both gene and protein level in human ductal cell line Panc-1.

A



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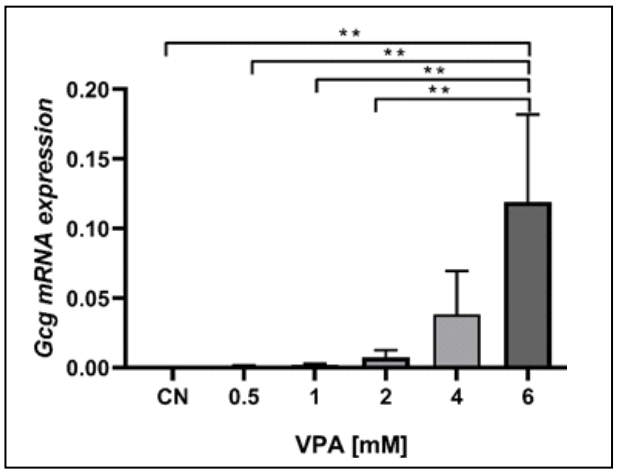

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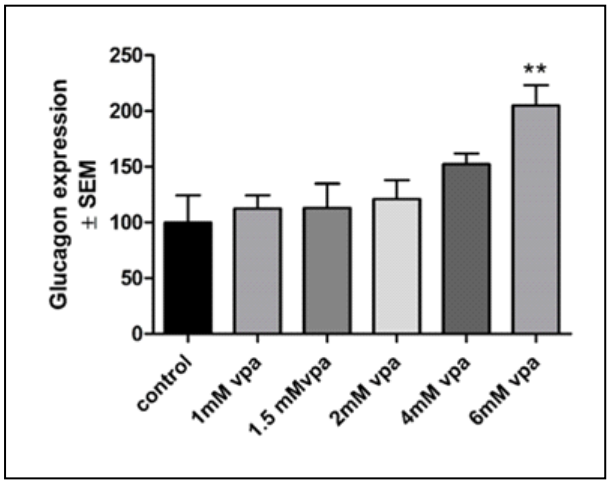

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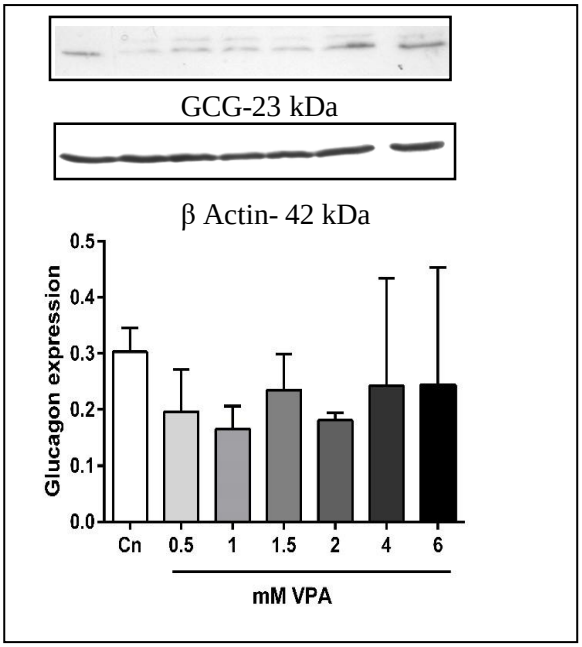

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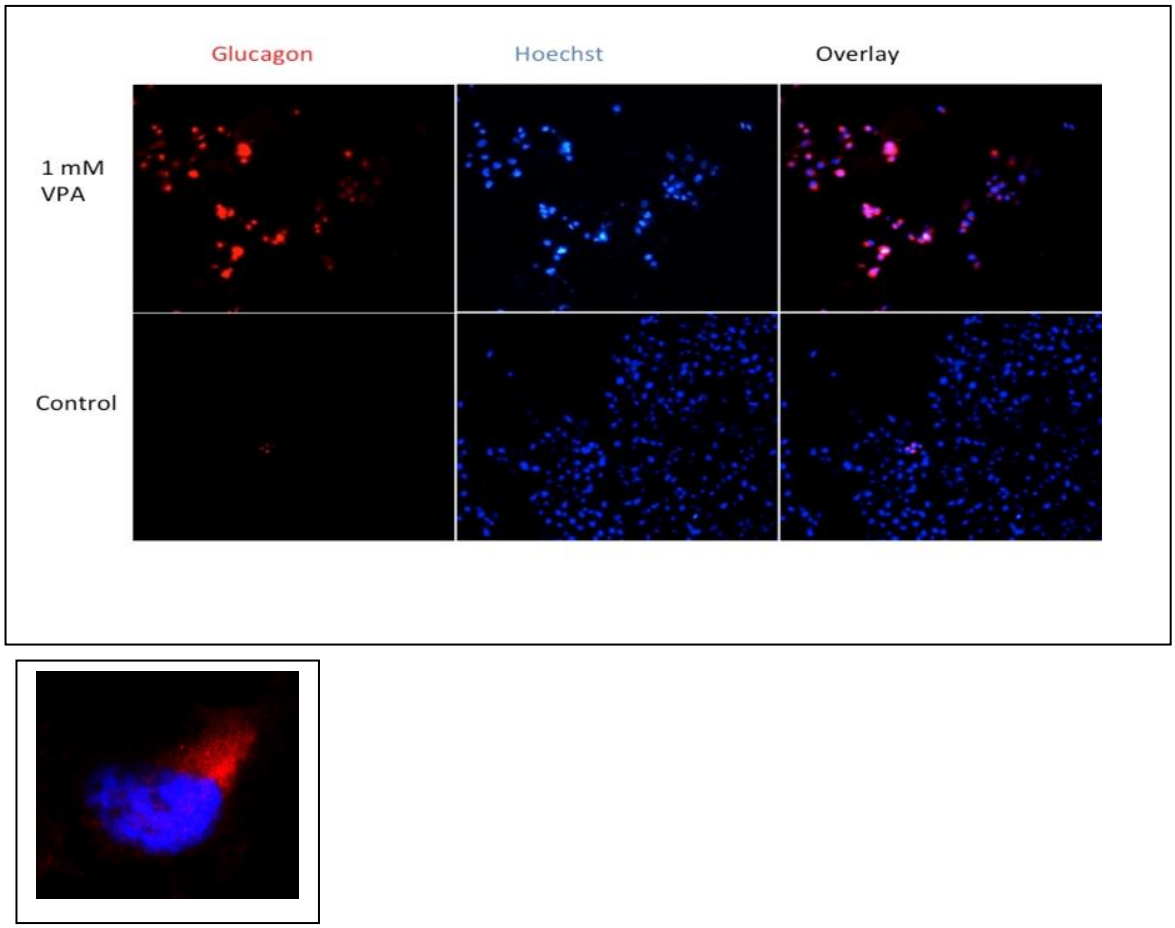

Glucagon
Hoechst
Overlay
1 mM VPA
Control

F

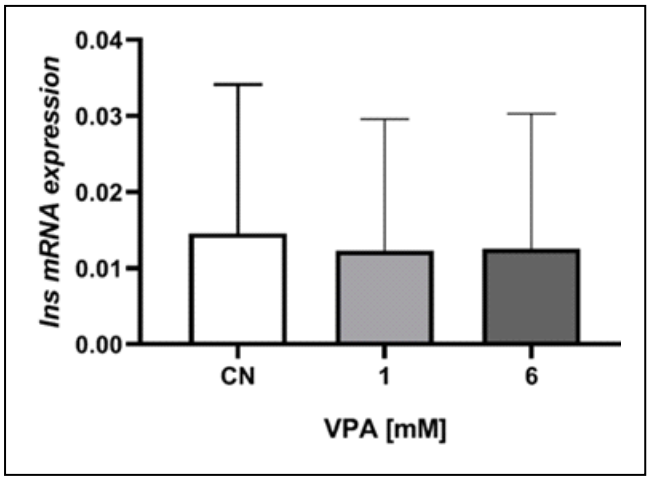
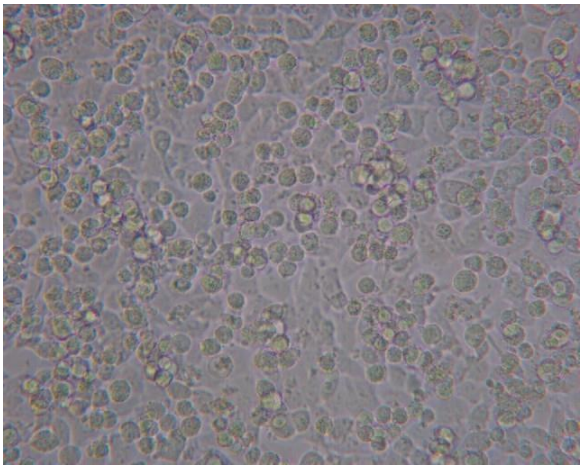

Ins mRNA expression
0.04
0.03
0.02
0.01
0.00
CN
1
6
VPA [mM]

Figure 3.4: Treatment of Panc-1 cells with increasing concentrations of VPA showed a trend augmentation in the pancreatic endocrine somatostatin expression and a significantly enhanced glucagon expression at 6 mM VPA determined by qRT-PCR (A, B). Control=CN. Mean ± SEM, n=3-4 experiments. Glucagon content in cell lysates was analyzed by ELISA, n=5 experiments (C). A significant up-regulated glucagon protein expression is observed. Data represent the mean ± SEM; **p< 0.01, by one-way ANOVA followed by Bonferroni´s multiple comparisons test. Glucagon expression at protein level was quantified by Western blot mean ± SEM, n = 3 (D). Immunocytochemical analysis of glucagon (red) after four days of VPA treatment. Nuclei were stained with Hoechst (blue). Images were taken at 10x magnification. Single cell with granular structures in cytoplasm positive for glucagon (red) at 63x magnification (E). Insulin mRNA level expression showed no regulation with VPA treatment, assessed by qRT-PCR (F).

3.5. Treatment with VPA induced morphologic changes in Panc-1 cells

During treatment with different concentrations of VPA, cells displayed morphological changes. To characterize these morphological changes cells were further examined under light microscopy. Cells in untreated control were rounded and confluent with defined borders and with frequent cell to cell contact. Cells treated with higher concentration of VPA (6 mM) showed morphological changes and reduced cell density. They acquired a flatter, spindle shaped conformation with more distinct cellular borders and decreased points of cell-to-cell contact between cells.

A



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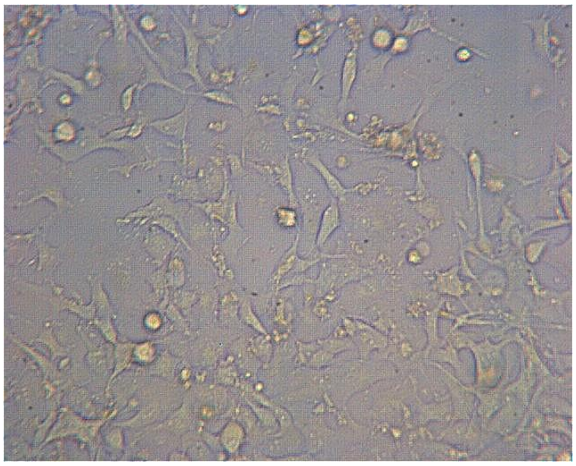
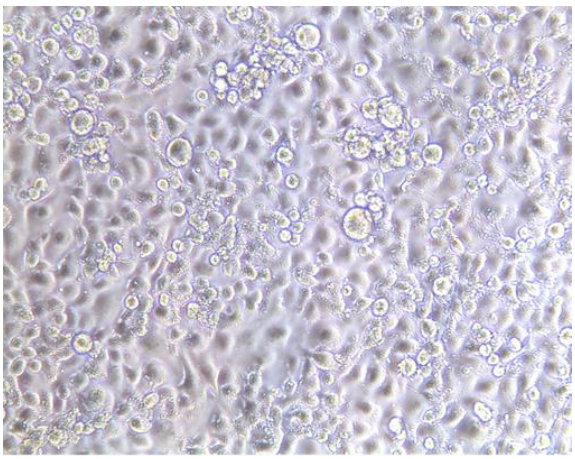


Figure 3.5. Changes in morphology of Panc-1 cells with VPA treatment detected by phase contrast microscope: Images are representative for two separate experiments. (A) Cells without VPA- control for four days look round, dense, and epithelial like. (B) Panc-1 cells treated with 6 mM VPA for four days exhibited more extensions and reduced cellular density.

Based on these observations, further studies were performed to investigate if the impact of 6 mM concentration of VPA on the morphology of cells changing to spindle shape remained even after removal of VPA.

For this purpose, cells treated with 6 mM VPA for four days were further cultured in the same plate for four more days without VPA treatment. At removal of VPA the cells turned back to their original round shape with defined cellular margins and close contacts. Cells in continuous VPA treatment for eight days, appeared less dense and more spindle shaped. This revealed that morphological changes, induced in Panc-1 cells with high concentration of HDACi VPA was reversible upon its removal.

C D



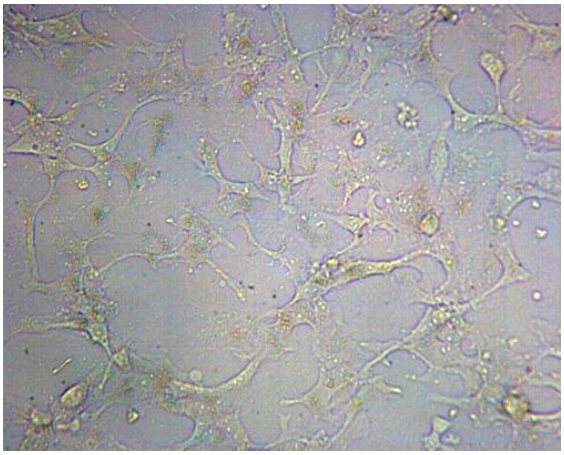


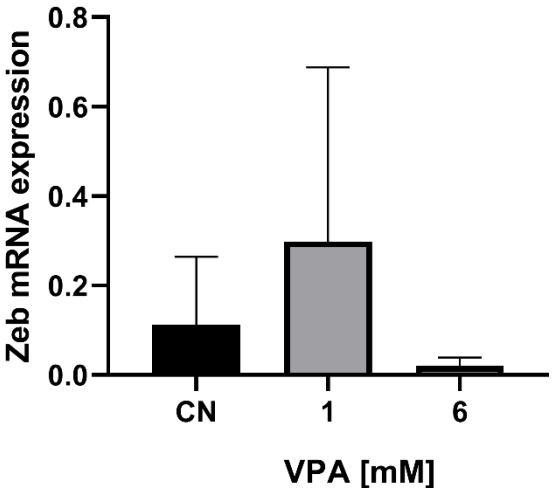
Figure 3.5: Removal of VPA treatment retained epithelial morphology. (C) Cells restored their epithelial phenotype treated with 6 mM VPA for four days and followed by four days without VPA treatment revealing that the effect of VPA was reversible. (D) More spindle shaped cells emerged with continuous treatment of 6 mM VPA for eight days.

3.6. Evaluation of the effect of VPA on EMT associated markers

Panc-1 cells treated with higher concentration of VPA showed morphological changes, with loss of cell–cell contact, depriving epithelial and acquiring more spindle (mesenchymal) shape with less cell density which are found to be the characteristics of epithelial mesenchymal transition (EMT) as shown in figure 1.4. The question that now arose was if VPA was inducing EMT in these cells which are cancerous by nature. To find out this, further experiments were carried out.

m-RNA expression levels of EMT markers were analysed in control and VPA treated samples. Expression of E-cadherin (E-cad), N-cadherin (N-cad) and Notch was observed to be slightly increased and transcripts of Snail, Zeb were found to be decreased. Expression of Vimentin, N-cadherin, E-cadherin were analysed at protein level but again no regulation was observed. These results indicated that there was no clear induction of EMT with VPA treatment. Intrestingly, expression of Slug, which plays an important role in regulating EMT, was found to be enhanced in VPA treated cells compared to control.

A



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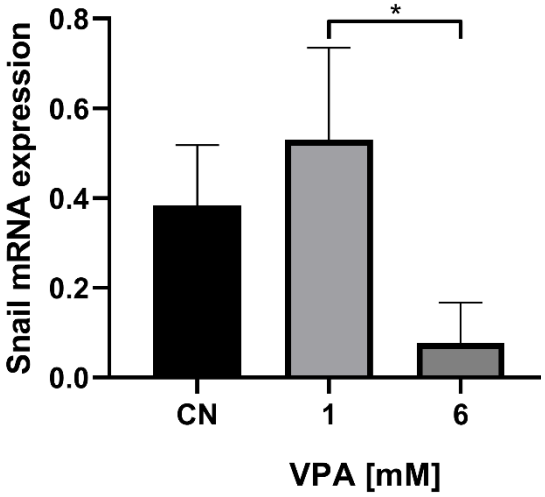


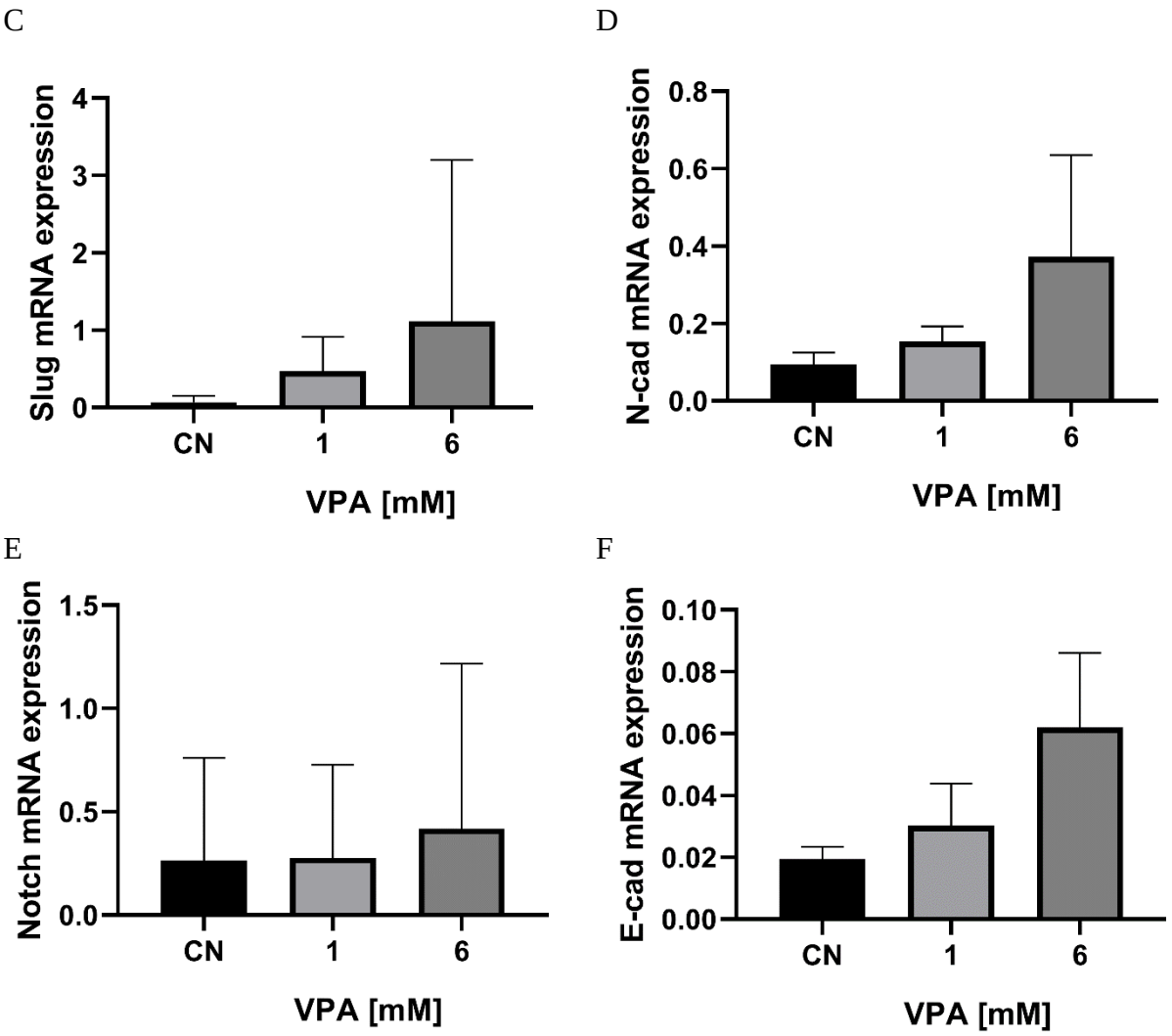
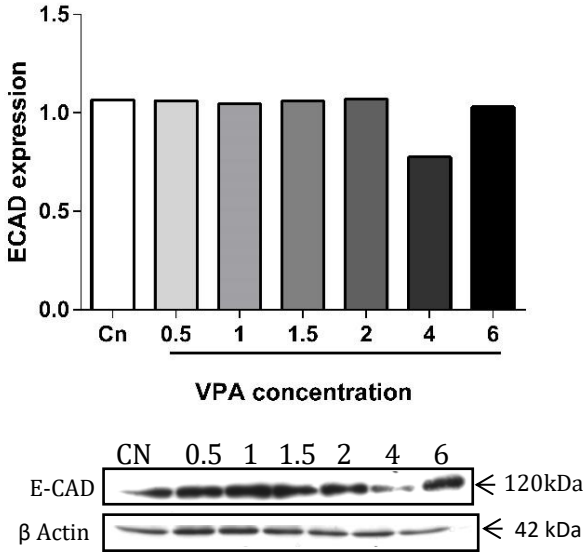


Figure 3.6: Gene expression analysis of epithelial mesenchymal transition (EMT) associated markers by qRT-PCR. Zeb (A), Snail (B), Slug (C), N-cad (D), Notch (E), and E-cad (F) are analyzed at mRNA levels. Relative gene expression was normalized to HPRT. A significant decrease in expression of Snail was observed in 6 mM when compared to 1 mM *$p<0.05$. one-way ANOVA followed by Bonferroni´s multiple comparisons test. A slight increase in expression of N-cad, Notch and E-cad mRNA was observed. An enhanced expression of Slug, one of the markers of EMT, was seen in 6 mM VPA treated cells. Data represent mean ± SEM, n = 2-5.

A



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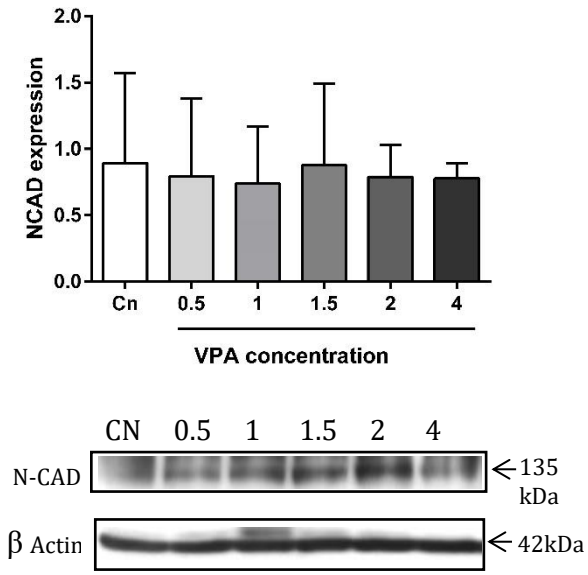

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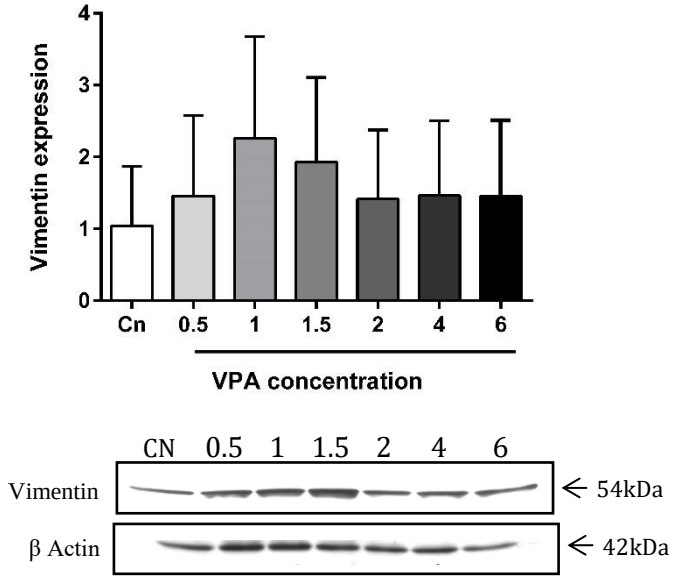
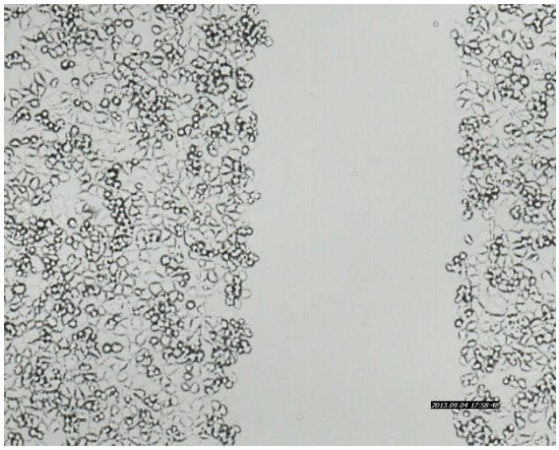


Figure 3.6-1: Analysis of expression of N-cad, E-cad and Vimentin expression by Western blot. Beta-actin was used as a loading control. No clear regulation was observed in in N-cad, E-cad, and Vimentin expression in VPA treated cells leading to search further reasons. Mean ± SEM, n = 2.

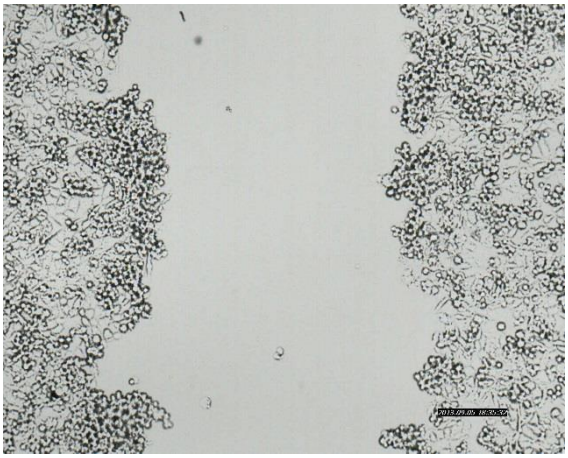
3.7. VPA enhances migration of Panc-1 cells detected by wound healing assay

From the above experiments, it is observed that VPA treatment induced phenotypic changes in the cells and increased the expression of Slug, which plays an important role in regulating EMT. From the literature it is known that EMT is associated with increased cell migration [135]. In knowledge of the literature and from current results, the mobility of these cells was further investigated by scratch assay. Panc-1 cells were incubated with and without 2 mM VPA and a scratch wound was made in treated and untreated cells. Wound was monitored under microscope for up to 24 hours. Pictures were taken by time-lapse microscope for every ten minutes interval. As depicted the wound in treated cells has been closed to 75% due to increased cell migration. In untreated control this closure has been less, and the scratch is clearly visible. These results suggest that VPA treatment has enhanced the migration of Panc-1 cells.

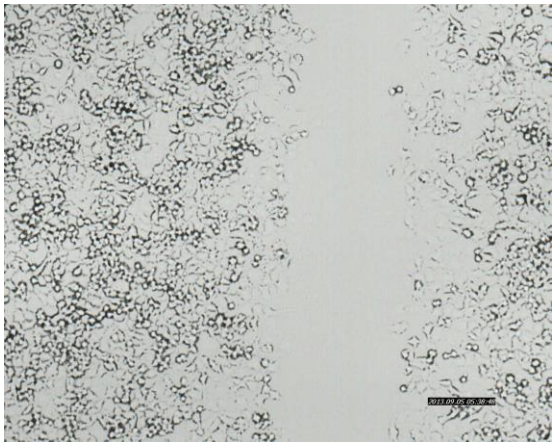
A Control 0hr



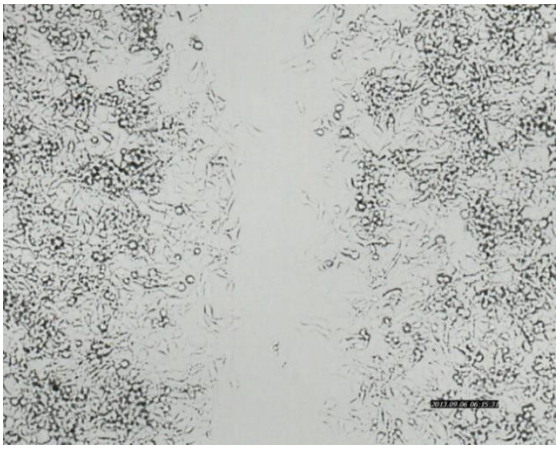
B VPA treated 0hr



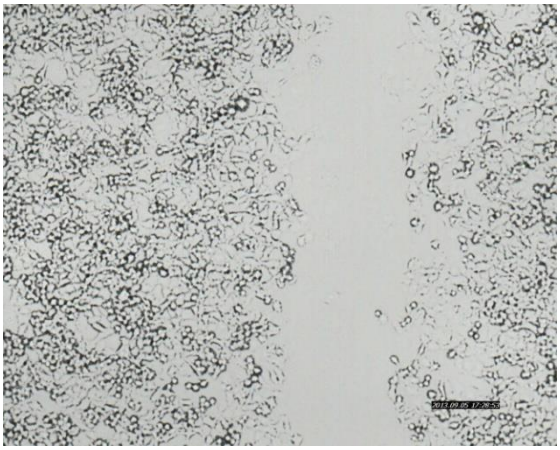
C Control 12hr



D VPA treated 12hr



E Control 24hr



F VPA treated 24hr

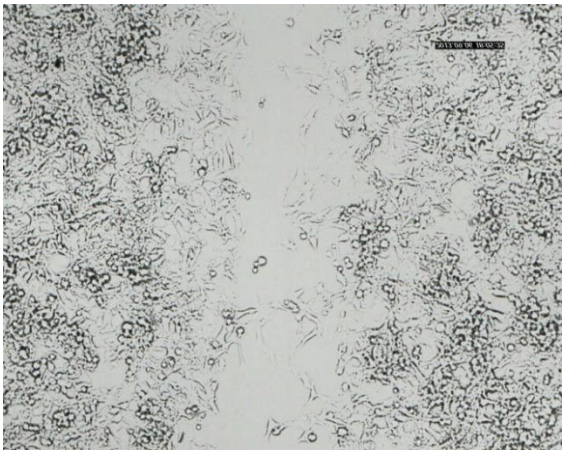


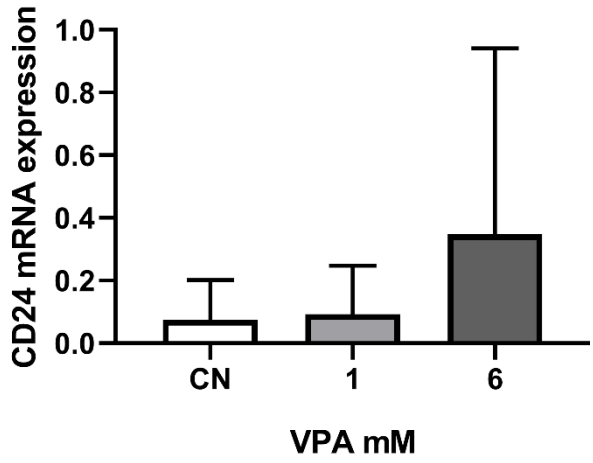
Figure 3.7: Wound-healing assay was used to detect migration of Panc-1 cells. In this assay cells were treated with and without VPA, and a scratch wound was made in both the plates. Representative pictures were taken by time lapse microscopy from both treated and untreated cells at 0h (Figure 3.7: A; B), 12h (Figure 3.7: C; D) and 24hr (Figure 3.7: E; F). The scratch was about 75% closed in the VPA treated cells while still clearly visible in non-VPA treated (control) cells indicating reduced movement of the latter (n=3).

3.8. Expression of cancer stem cell markers in VPA treated cells.

It was observed in above experiment that presence of VPA enhanced migration ability of Panc-1 cells. Since EMT is associated with cancer stem cells and further implicates tumor cell

migration [135], the expression levels of pancreatic cancer stem cell markers CD24, CD44, CD133, Esa, Oct4, Cxcr4, and Abcg2, known from literature (mentioned in section 1.4.3), were quantified by qRT-PCR in control and VPA treated cells. Concentrations of transcripts from CD133, CD24 and Oct4 were enhanced in cells treated with 6 mM VPA, however none of them with statistical significance.

A



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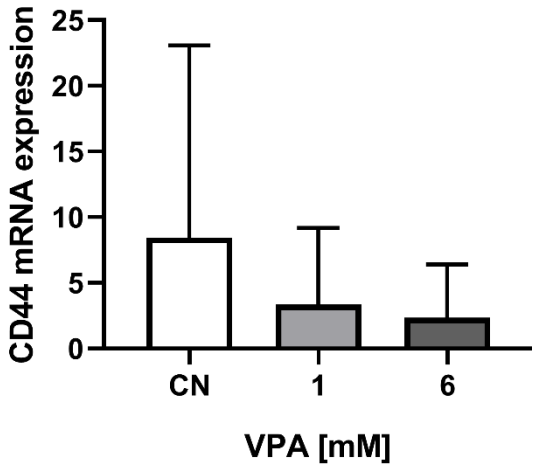

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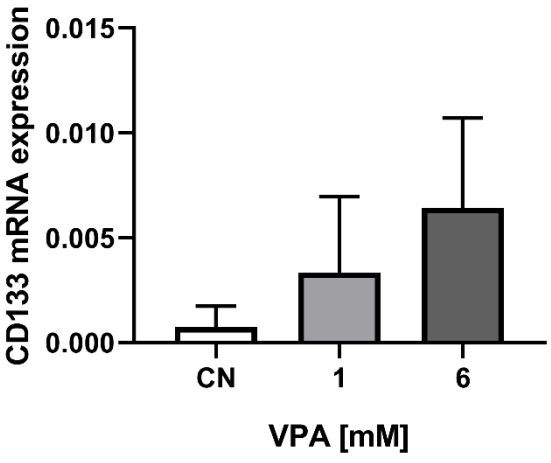

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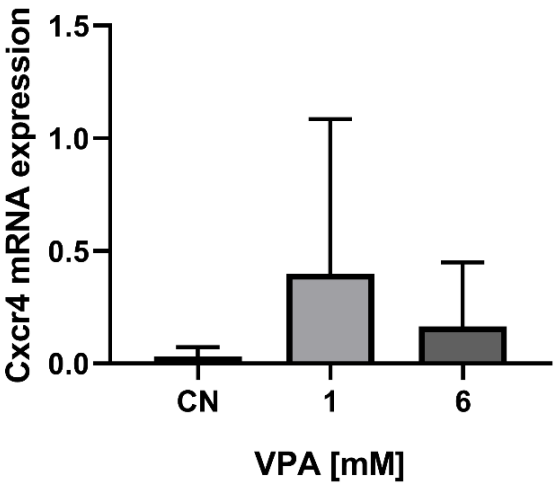

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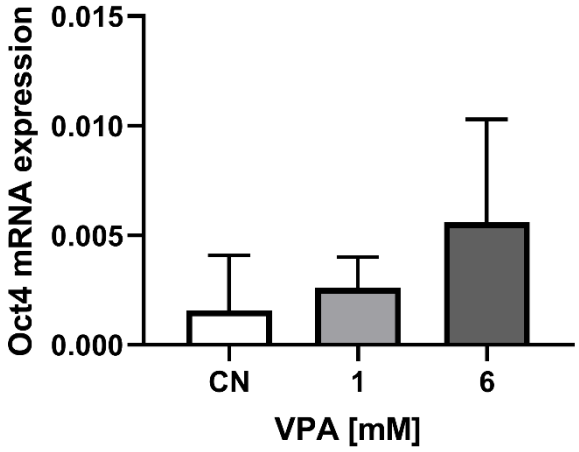

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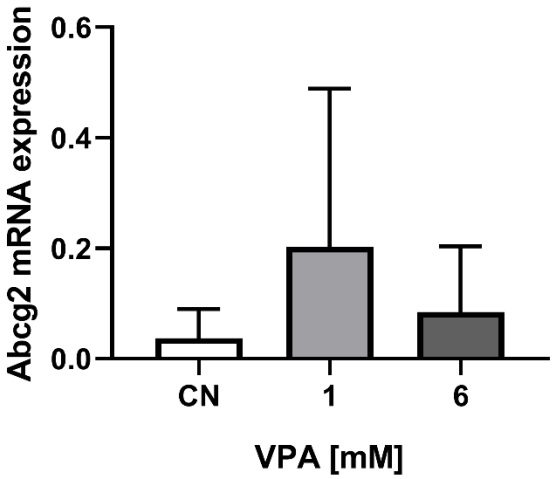

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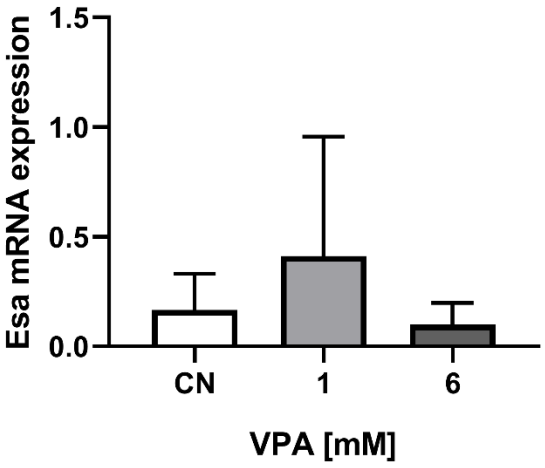
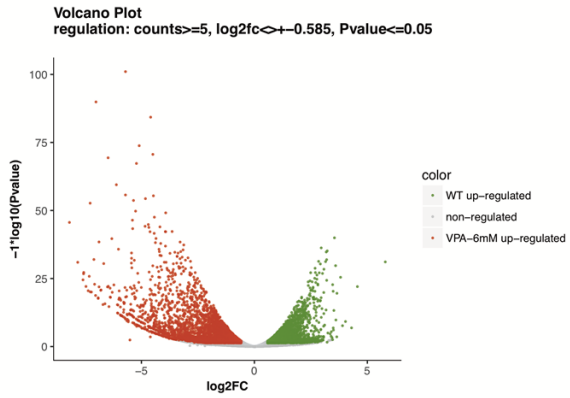


Figure 3.8: Gene expression analysis of cancer stem cell associated markers. Cells cultured in DMEM medium for 4 days with or without VPA were finally lysed. mRNA level expression for cancer stem cell associated marker genes CD24 (A), CD44 (B), CD133 (C), Cxcr4 (D), Oct4 (E), Abcg2 (F), and Esa (G) was analyzed by qRT-PCR. An increase in expression of CD133 cancer stem cell marker was observed in VPA treated samples. Mean ± SEM, one-way ANOVA followed by Bonferroni´s multiple comparisons test. n = 2-4.

3.9. Panc-1 cell gene expression profiling after VPA treatment

To analyse the effect of VPA exposure on Panc-1 cells, RNA sequencing (RNA-Seq) of Panc-1cells was performed. Cells without VPA (Wild Type- WT) vs 1 mM VPA-treatment, wild type vs 6 mM VPA- treatment, 1 mM VPA-treatment vs. 6 mM VPA-treatment. RNA-Seq data showed that VPA upregulated multiple genes in Panc-1 cells, which was found to be concentration dependent with the highest seen in 6 mM VPA treated.

A



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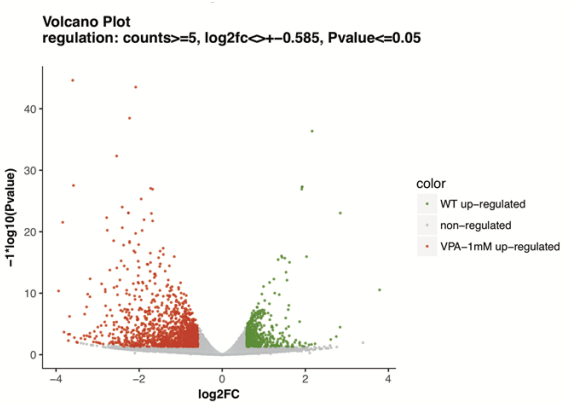

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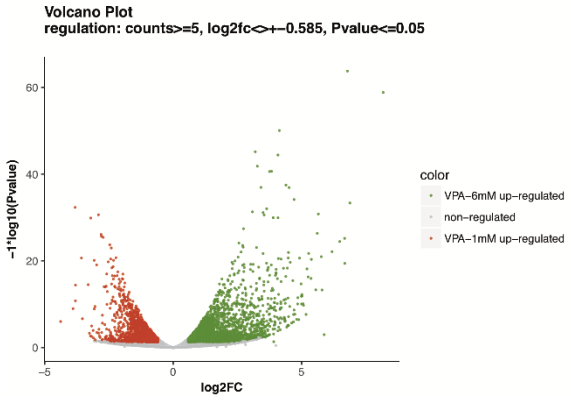
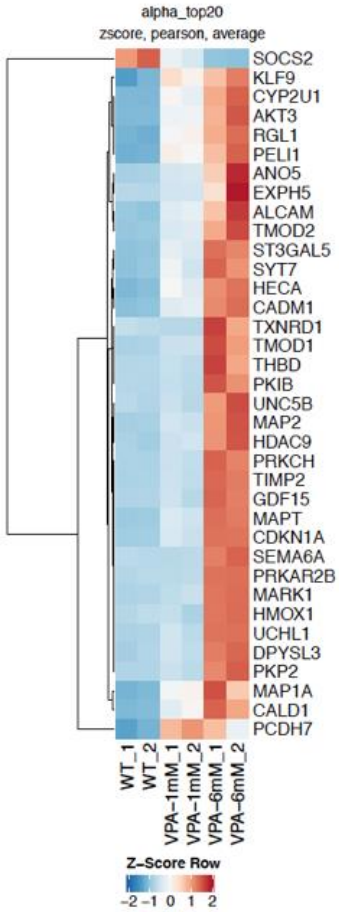


Figure 3.9: Volcano plot shows gene regulation pattern between control (WT) and VPA treated cells. (A) Grey dots represent genes that are non-regulated; red and green dots indicate genes upregulated in 6 mM VPA and control (WT). (B) Red and green dots indicate upregulated genes in 1 mM VPA and WT and grey indicate non-regulated. (C) Red dots represents genes upregulated with 1mM and green dots indicate genes upregulated in 6 mM VPA and grey non-regulated.

X axis indicates log2FC –log2fold change, y axis indicates $-\log_{10}$ (P-value). The analysis shows multiple genes have been upregulated in Panc-1 cells with VPA treatment which is

clearly evident in figures 1 A; B (red dots) and this regulation is concentration dependant. Highest gene upregulation was observed in 6 mM VPA treated cells.

A



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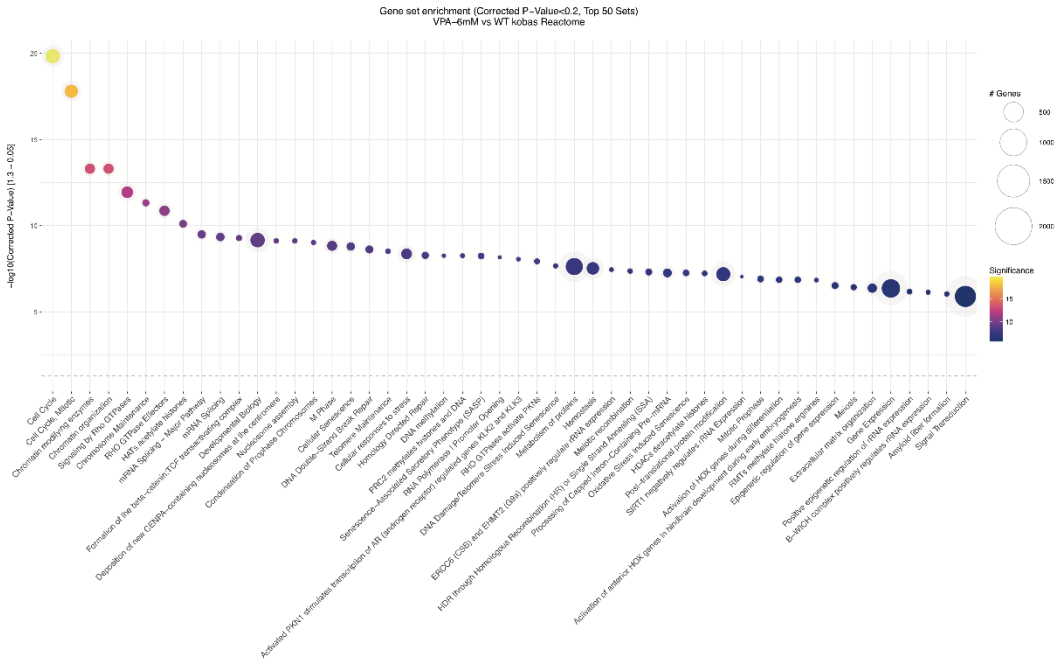


Figure 3.9.1: (A) Gene ontology (GO) analysis of genes differentially expressed in Panc-1 cells based on [142, 143]. Heat map of top 20 alpha-cell enriched related GO terms in Panc-1. Rows are genes and columns are ordered as control (WT), 1 mM and 6 mM VPA. Red colour shows upregulated genes, the majority of them was seen in 6 mM VPA. The blue colour shows the downregulation of the genes which is mostly seen in control (WT).
(B) Analysis of gene enrichment of biological process of differentially expressed genes: By using RNA-sequence analysis we observed that VPA treatment altered the expression of thousands of genes in Panc-1 cells.

4. Discussion

Diabetes mellitus is a metabolic disorder characterized by loss or dysfunction of insulin producing beta-cells. Thus beta-cell replacement from renewable sources was suggested as a desirable aim of translational research. Investigators have focused their efforts on gene technology to generate beta-cells from stem cells and other pancreatic cells by inducing key transcription factors, and by using small molecules known to regulate beta-cell development. Studies have stressed the role of epigenetic mechanisms in determining the fate of cells during development of the pancreas [76, 89].

Identifying compounds that modulate the cell cycle and promote differentiation into beta cell phenotype would provide insights for future diabetic therapies. In the present study the ability of the HDAC inhibitor VPA to induce endocrine lineage from the human ductal Panc-1 cell line was investigated. This cell line was established as a useful in vitro model for understanding beta-cell development [63, 144].

4.1. Effect of VPA on transcriptional hierarchy directing PANC-1 cell differentiation

Activation of transcription factors determines differentiation programs of individual cell lineages. Often expression of the same key transcription factor is present during early cellular development and later in maintaining the phenotype of finally differentiated cells. Several factors became known to be critical regulators for endocrine cell development [3,5].
Expression pattern of some of the genes encoding the transcription factors Pdx1, Foxa2, Sox17, Ngn3, Isl1, and Pax6 that regulate endocrine cell development as well as genes encoding markers characteristics of endocrine cells such as glucagon, insulin and somatostatin were examined in the current study.

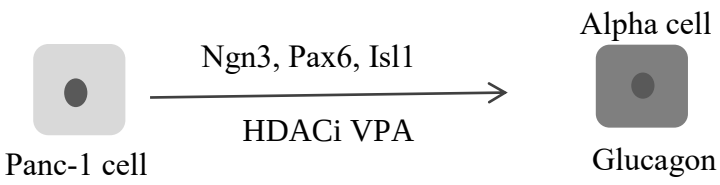


Fig 4.1: Treatment of Panc-1 cells with VPA enhanced the expression of pancreatic genes Ngn3, Pax6, Isl1 and promoted endocrine (glucagon expressing alpha-cell) differentiation.

Pdx1, the homeodomain transcription factor is a master regulator of pancreas development and beta-cell formation. As shown in (Figure 1.1) Pdx1+ progenitor cells further differentiate into Ngn3 expressing pre-endocrine cells [145]. So primarily, the effect of VPA treatment on Pdx1 expression, which is constitutively expressed by Panc-1 cells as discussed earlier in results chapter, was studied. The present data showed a significant upregulation of Pdx1 transcripts. During fetal development the level of Pdx1 was described to define pancreatic cell lineage differentiation [17, 19]. But from our data enhanced Pdx1 and also upregulated glucagon expression was observed.

Previous gene mutation studies and recent proteomic and promoter region studies have shown that Foxa2 acts upstream of Pdx1 in regulatory hierarchy. Foxa2 regulates the expression of Pdx1 during early stages of pancreatic development. In later stages its expression is required for beta-cell formation and glucagon expression [10, 11, 146, 147]. It was indicated that during pancreatic development Foxa2 expression is not required during delineation of first wave of glucagon positive cells and is required at a late stage of alpha-cell differentiation [9]. In the present study a trend increase in the expression of Foxa2 was observed, but significant upregulation was not found out. Thus, based on present data, this kind of differentiation of ductal origin Panc-1 cells into glucagon positive cells by VPA may involve Foxa2 which has to be confirmed by additional studies.

4.2. Increased Ngn3 expression with VPA treatment

The basic helix loop helix factor Ngn3 is a pancreatic endocrine cell - specifying gene. Lineage studies showed that Ngn3 expressing cells are islet progenitor cells, so it is interesting to study the expression of Ngn3 with VPA treatment [1]. In the present study, data from qRT-PCR and protein analyses show that VPA treatment increased the expression of Ngn3 in Panc-1 cells. It is known that ectopic expression of Ngn3 alone or in combination with other genes is enough to induce endocrine differentiation in different cell lines and pancreatic duct primary cells [24, 58, 148]. Recent studies showed that a reactivation of Ngn3 appears to be critical for sustained beta-cell regeneration in vivo [148, 149]. These reports have emphasized the crucial role of Ngn3 in endocrine development and regeneration. In the present study, we observed an increase in the expression of Ngn3 with 6 mM VPA treatment, which potentially facilitated expression of glucagon.

Initiation of endocrine differentiation is followed by specification of endocrine cells by expression of additional genes; these genes include Arx, Pax4, Pax6, MafA, Nkx2.2, Nkx6.1, and Isl1 [5]. Their expression directs Ngn3 positive cells towards the four mature endocrine cell fates. It is an unsolved issue whether expression of Ngn3 is enhanced with or without activation of its downstream genes involved in induction of endocrine differentiation. For this reason, expression of Isl1 and Pax6 was analyzed. Both transcription factors are essential for endocrine cell development. Studies in mutant mice showed that the pancreas from Isl1 (-/-) and Pax6 (-/-) mice did not generate insulin, glucagon and somatostatin positive cells [26, 29]. Pax6 is an important regulator of pancreatic endocrine cell development, especially in generating alpha-cells. Further its expression is critical for alpha-cell function by coordinating glucagon gene expression as well as its synthesis and secretion [27]. Results from qRT-PCR showed a trend increase in the expression of Pax6 and Isl1 with VPA treatment. Hence, it is assumed that the mechanism by which Ngn3 induces differentiation in these cells involves further activation of its downstream genes Pax6 and Isl1, which are essential for the cells to adopt the endocrine phenotype.

4.3. VPA treatment promoted endocrine differentiation/differentiation into glucagon positive cells

The initial aim in this study was to reach a stage of insulin producing cells. As discussed earlier though enhanced expression of beta-cell marker Pdx1 was observed, still a stage of insulin expression could not be attained. But interestingly, endocrine expression was still observed in these cells showing enhanced somatostatin and significant glucagon expression by VPA treatment. Data from qRT-PCR and ELISA demonstrated a significant upregulation in mRNA and protein expression of glucagon at 6 mM VPA concentration. This finding was further confirmed with immunocytochemical staining and Western blot analysis as glucagon expression was increased in VPA treated compared to control cells. In the data, always some basal expression of glucagon was observed in the control cells. This was not surprising, since it was shown earlier that in 1-2% of Panc-1 cells, when grown under serum containing medium, endocrine expression was seen [62, 144]. It is obvious that Panc-1 cells rarely exhibit endocrine cell characteristics. Studies in the past showed that, when differentiating pluripotent cells or reprogramming adult exocrine cells, formation of glucagon secreting alpha-cells is the default pathway [150, 151]. This could be the possible reason that differentiation protocols end

up in glucagon positive cells. Together with these studies the results of the present study confirmed that VPA treatment promoted the human ductal Panc-1 cells to glucagon producing cells.

It is also well known from the development studies that the earliest endocrine cells detected, express glucagon and in next developmental step so called polyhormonal cells co-express glucagon, insulin and pancreatic polypeptide before they further divide into alpha and beta cell lineages [152]. Earlier, it was demonstrated that treatment of islets with Adox (a histone methyltransferase inhibitor) resulted in partial reprogramming from alpha- to beta-cell like cells with co-expression of Pdx1 and glucagon and with co-localization of insulin and glucagon [76].

Taken together, from these observations it is hypothesized that Panc-1 cells under VPA treatment were in an immature stage with increase in expression of Pdx1, Ngn3, Pax6, Isl1, and glucagon coupled with partial augmentation in expression of somatostatin.

4.4. Role of alpha-cells and glucagon in beta-cell regeneration and Diabetes mellitus

Our understanding of the pathogenesis of diabetes mellitus is mainly revolved around insulin deficiency or insulin resistance due to either the loss of pancreatic beta-cells in type I diabetes or loss of insulin sensitivity in type II diabetes. Investigations started appreciating the contribution of other pancreatic cells types in regulating blood glucose levels and their role in the pathogenesis of diabetes mellitus [73, 153]. Nearly 40% of the total number of cells in the islet comprises of glucagon secreting alpha cells, which controls blood glucose levels during fasting. It was observed that dysregulation of alpha-cells and impaired glucagon secretion predisposes people to diabetes mellitus [153, 154]. Though the mechanisms contributing to alpha-cell dysfunction were studied to some extent and agents regulating glucagon secretion were proposed, further studies that address mechanism of alpha-cell dysfunction and therapeutic approaches for restoring function are required. In order to understand such complex phenomenon developing suitable disease model systems is important. In the present study reprogramming of Panc-1 cells into glucagon secreting alpha-cells with VPA was detected. Panc-1 cells may be used as tool to understand complex mechanisms contributing to hyperglucagonemia.

VPA was used for treatment of neurological and psychiatric disorders including epilepsy, bipolar disorder, and major depression [100]. Interestingly, enhanced insulin release and

weight gain was observed in epileptic patients consuming valproic acid compared to those with other antiepileptic drugs [155]. According to the results of this work non-endocrine pancreatic progenitor cells could switch to an increased glucagon release, highlighting unexpected effects of VPA on the pancreas. Regular monitoring of glucagon in patients consuming VPA may be warranted.

Patients with type 1 diabetes on intensive insulin treatment experience an increased risk for hypoglycemic episodes. Regarding this, reports from studies show that bi-hormonal delivery of insulin along with glucagon reduce the risk of hypoglycaemia when compared with delivery of insulin alone [156]. Although bi-hormonal therapy appears to be beneficial for type-1 diabetic patients, the underlying disease modifying molecular mechanisms contributing at the level of pancreatic cell types are sparse. For this purpose, the reprogrammed human Panc-1 cells by VPA might serve as a tool for further understanding of the complex interplay between insulin and glucagon signalling pathways. This may eventually pave the way for the development of better therapeutic options.

Studies have emphasized the importance of HDAC inhibitors in pancreatic differentiation, beta cell regeneration, stimulation of beta cell proliferation and regulation of insulin release [94, 109, 157]. Therefore, they are considered as possible anti-diabetic drugs [99, 109, 158]. By contrast, a group of HDACis inhibited beta cell marker gene expression showing that chromatin modification with small molecules does not always cause changes in the transcriptome [159]. Gene expression of Pdx1 and MafA as well as insulin secretion of pancreatic beta cell line was preferably regulated by a high dose of HDACis treatment [160]. VPA exposure of pregnant rats during organogenesis disturbed pancreas development, insulin synthesis and secretion of the offspring [161]. Hence a better understanding of the effect of HDACis is essential before using them as therapeutic tools.

Prior studies [72, 73, 75, 152] showed the importance of transdifferentiation of alpha-cells for beta cell regeneration. Glucagon is the major secretory product and active marker of alpha-cells. It was proposed that alpha-cells are an endogenous reservoir of potential new beta-cells [162]. Epigenome analysis in human pancreatic islets showed that differentiated alpha-cells expressed bivalent genes carrying both active and repressive marks suggesting that alpha- to beta- reprogramming could be promoted in these cells [76]. A recent lineage study revealed that long term administration of γ-aminobutyric acid (GABA) induced alpha-cell mediated

beta-cell- like neogenesis [163]. VPA when given to epilepsy patients raised GABA disposability by inhibiting GABA transaminase and thereby controlled epilepsy [100]. More studies are warranted to confirm the hypothesis that GABA supports conversion of alpha-cells and Panc-1 cells could serve as a platform to investigate active compound in this process.

4.5. Acetylation of histones

The levels of histone acetylation contribute to the control of gene expression by altering chromatin structure. By administration of HDACi, acetylation of lysine in histone tails is induced which relaxes the chromatin structure so that it is more accessible for transcription complexes and promotes gene transcription [81, 164]. In fact, the present data showed that the action of VPA on pancreatic differentiation occurred through the acetylation of H3 and H4 histones in Panc-1 cells.

4.6. Changes in cell morphology and triggered migration of cells

Panc-1 cells treated with increasing concentrations of VPA changed from epithelial to more spindle- like shape with reduced cell density. Similar morphological alterations were reported in small cell lung cancer cells when treated with up to 10mM of VPA [165]. This kind of transformation posed the question whether treatment with HDAC inhibitor VPA enhanced EMT, heightened invasiveness and increased properties of cancer stem cells. To further study phenotypic changes in continuation with the light microscopic observations, structural proteins like E-cadherin, N-cadherin, and vimentin that are involved in morphology of cells were looked at protein level by Western blot.

In addition, genes that are known as markers for epithelial to mesenchymal transitions (EMT) from earlier studies, were also studied [128, 166]. qRT-PCR in present study was performed and analysed the genes involved in EMT showing a significant decrease in expression of Snail. However, a strong increase in expression of Slug, another EMT associated transcription factor gene, was observed. Though HDAC inhibitors were approved as anticancer drugs, mechanisms of anticancer effects of HDACi´s are not uniform. They may be depend on the type of cancer, the specificity of the HDAC inhibitor and its dose which suggests that more clinical trials are required to define patients who may benefit from such therapy [119].

HDACis were reported to suppress EMT transition in cancer cells [121, 167], but there are other studies indicating that in some cancer types treatment with HDACis induced EMT

transition, impacted cancer stem cells, and further triggered invasion and metastasis [124, 125, 168]. For example treatment of colorectal cancer cells with VPA triggered EMT via up regulation of Snail [169]. Another recent study revealed that superoylanilide hydroxamic acid one of the most advanced HDACi anti-cancer agents promoted EMT in triple negative breast cancer cells [170].

As a connection between EMT and cancer stem cells was proposed, pancreatic cancer stem cells associated marker genes reported in the literature were examined in the present study [129] [132, 134]. The results from qRT-PCR showed no significant change in such transcripts except an increased expression of cancer stem cell marker CD133. CD133 expression with VPA treatment was also upregulated in human neuroblastoma cell lines [171]. Further a scratch assay was made to measure the migration rate of cells. The results from scratch assay indicated that the wound made in VPA treated culture plate closed by 75% at 24 hr, while it was still visible in control cells which showed little movement. The results from present study were in agreement with other studies demonstrating the importance of the selection of suitable drug and dose condition for different tumours [160-162].

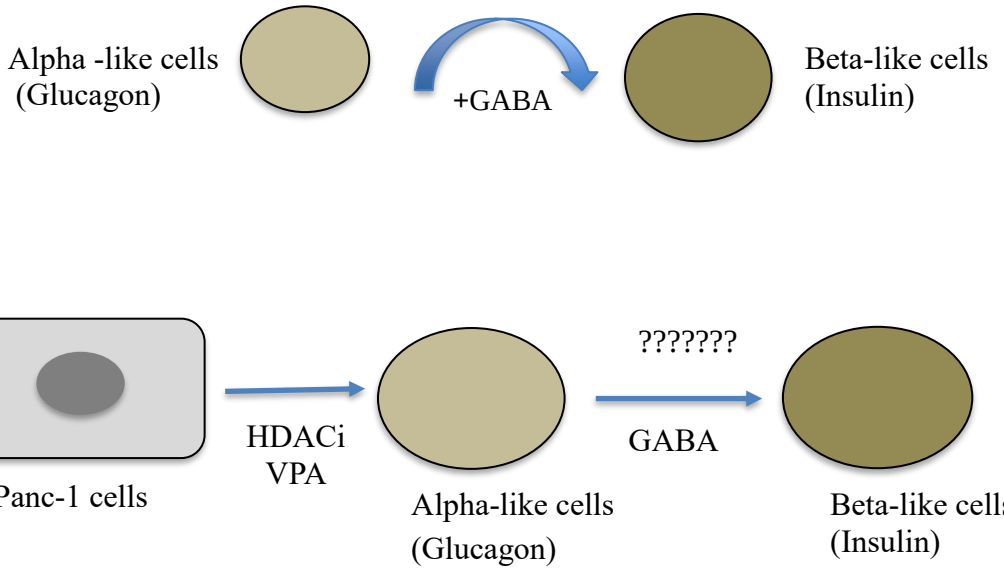
4.7. Future perspectives

Specific epigenomics of VPA contributing to lineage conversion from ductal origin-to-endocrine fate may be studied in future. It is known that Arx plays an important role in alpha cell differentiation [32]. Therefore, it will be also interesting to study the effect of VPA on Arx expression. Although VPA treatment enhanced expression of endocrine gene transcripts, insulin as an important marker for beta cell differentiation could not be detected. Intrinsic differences between the differentiations programs that Panc-1cells adopt need to be further researched. For example, the glucose concentration in the medium is known to be important for cell physiology and cell survival in culture. In the present experiments high glucose medium was used, but it would be also interesting in future to examine whether changes in glucose concentrations in medium would affect glucagon secretion from these cells by VPA treatment.

4.8. Limitations

Although enhanced pancreatic gene and endocrine expression was found in VPA treated cells, the level of expression between the experiments was quite variable. This variability could be

dependent on passage number, the variations in time of VPA exposure in culture and the sample collection. This situation was tried to overcome by minimising the impact of above stated conditions but still the variability could not be eliminated completely. Although Panc-1 cells resisted the highest VPA concentration 6 mM, there were dead cells that could not be eliminated entirely from the culture dish and seen floating in the medium.



5. Conclusion

In conclusion, the results of the present study demonstrated that VPA treatment of non-endocrine Panc-1 cells converted them into glucagon expressing endocrine-like cells. The mechanism is not entirely clear, but indicates that this took place along with enhanced expression of pancreatic genes Pdx1, Ngn3, Isl1, and Pax6. Panc-1 cells may serve as starting material for studies that are focussed on identification of compounds that promote reprogramming of alpha- to beta-cells and in studies looking for mechanisms underlying glucagon dysregulation. In addition, current results also demonstrated that VPA enhanced migration potential of cells, an increase in expression of one of the EMT associated gene Slug and cancer stem cell gene CD133, revealing the unexpected dual effect of VPA in these cells.

Abbreviations

ABCG2	ATP binding cassette subfamily G member 2
APS	Ammonium persulfate
BSA	Bovine serum albumin
cDNA	Complementary DNA
CD	Cluster of differentiation
CSCs	Cancer stem cells
CXCR4	Chemokine receptor type 4
DNase	Deoxyribonuclease
dNTPs	2´-deoxynucleoside-5´-triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
E-cad	E-cadherin
EMT	Epithelial to Mesenchymal transition
ESA	Epithelia-specific antigen
FCS	Foetal calf serum
Foxa2	Winged-helix/forkhead member A2
GABA	γ-aminobutyric acid
Gcg	Glucagon
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HPRT	HypoxanthineGuanine phosporibosyl transferase
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
Ins	Insulin
Isl1	Islet factor 1
mM	Millimolar
mg	Milligram
ml	Milliliter
NGN3	Neurogenein3

NP-40	Nonidet P-40
PAGE	Polyacrylamide gel electrophoresis
PANC1	Pancreatic adenocarcinoma cells
Pax6	Paired homeodomain factor 6
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDX-1	Pancreatic homeoduodenal box 1
RNase	Ribonuclease
SDS	Sodiumdodecylsulphate
Sox17	Sex determining region Y- box-17
Sst	Somatostatin
TEMED	N,N,N,N-Tetra-methyl-ethlenediamine
TSA	Trichostatin A
VPA	Valproic acid

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Acknowledgments

First and foremost, I would like to thank my Mother Mrs. Chandra Kumari Kandula for her unconditional love and support in my life. Today whatever I am it is just because of my mother and I dedicate this dissertation to her. I would like to thank my father too for his support in my life.

I want to express my sincere gratitude to my supervisor Prof. Dr. Thomas Linn. If I remember, even there are times where I felt that I couldn't make it at all, but today it was become possible only because of him, I couldn't forget his entire support throughout my very long work who, motivated me constantly to finish it. I really appreciate him for his kind co-operation and patience. I am glad that I got a chance to work with you and learned from your experience & knowledge.

I would also like to thank Prof Dr. Anand, University of Sydney, from whom I learned technique like CHIP during my lab rotation which is very useful for my scientific career. Special thanks for Dr. Anand & Mugda for their kind hostage, where I have learned useful for both my personal and professional level.

My special thanks to our team of lab technicians Doris Erb, Gundula Hertl and Birte Husmann for their support especially Doris for her technical support and invaluable help in several aspects in day to day life in Germany.

I would like to thank Manju Padmasekhar for teaching me the cell culture and for her support and guidance during my initial days of lab.

My heartfelt thanks to Nadine Rekittke and her husband Ingo for their support during my thesis writing and at times of difficulties, especially I couldn't get the grant without their inputs. Your suggestions and support helped me throughout my writing of thesis.

I thank my lab members Divya, Rahul and Sebastian at Third Medical Department, Giessen Germany for their help and support whenever I needed.

I would like to thank, inequality office for their support with the grant, which supported me well during the critical stage of my thesis writing.

I would like to extend my sincere thanks to the “Giessen Graduate school of Life Sciences” (GGL), where I had the opportunity to acquire scientific knowledge and got the grant for lab rotation in university of Sydney, Australia. Especially Dr. Lueck for her support during my thesis writing when I need.

I would like to extend my heartfelt thanks to my husband Kishore Nallapati, for his support and care. I would like to thank my little angel Aarohi who much inspired me to finish my thesis.

Last but not the least I would like to thank all my friends Rajshekar reddy, Padmaja, Ranjith and my extended family especially my sister Naga Lakshmi, has been my best friend all my life, gave me lot of moral strength in my up and downs, my brother (Hari Krishna Gullapalli), brother in laws (Srinivas and Mallikarjun), and my in laws.

Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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