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Differentiation Potential of Mesenchymal Stem Cells into Pancreatic β-Cells

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Abstract

Stem cells having the capability to differentiate into other type of cells and renewing themselves, gained so much importance in recent years. Investigations in stem cells revealed that mesenchymal stem cells can successfully differentiate into other type of cells like adipocytes, hepatocytes, osteocytes, neurocytes and chondrocytes. In addition, these cells can also differentiate into insulin-producing beta cells. Insulin is a crucial hormone for glucose balance of the body. Insufficiency or unavailability of insulin is called diabetes. External insulin intake, as well as pancreas or islet transplantation, is the most basic treatment of diabetes. *In vivo* and *in vitro* studies demonstrate that stem cell therapy is also used in the cure of diabetes. Differentiation process of stem cells into beta cells releasing insulin is quite complicated. There are many different reports for the differentiation of stem cells in the literature. The success of differentiation of stem cells into beta cells depends on several factors like the source of stem cells, chemicals added into the differentiation medium and the duration of differentiation protocol. Distinct studies for the differentiation of stem cells into insulin-secreting cells are available in the literature. Moreover, thanks to the superior differentiation capacity of stem cells, they are being preferred in clinical studies. Stem cells were clinically used to heal diabetic ulcer, to increase c-peptide level and insulin secretion in both type 1 and type 2 diabetes. Mesenchymal stem cells having high differentiation potential to insulin-secreting cells are encouraging vehicles for both *in vivo* and *in vitro* studies together with clinical trials for diabetes mellitus.

Keywords

Mesenchymal · Stem cells · Diabetes · Differentiation · Pancreatic β cells · Stem cells

Abbreviations

ASCs	Adult stem cells
bHLH	Basic helix-loop-helix
BME	β-mercaptoethanol
DAPT	N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
DE	Definitive endoderm
DEX	Dexamethasone
Dhh	Desert hedgehog
Dmso	Dimethyl sulfoxide
Dvl	Dishevelled
ESCs	Embryonic stem cells
Fox	Forkhead box
FoxA2	Forkhead box A2

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Fzd	Frizzled
Gck	Glucokinase
GLP	Glucagon-like peptide
Glut-2	Glucose transporter 2
HDACs	Histone deacetylases
Hes1	Hairy and enhancer of split-1
HGF	Hepatocyte Growth Factor
HSCs	Hematopoietic stem cells
IAPP	Islet amyloid poly peptide
Ihh	Indian
iPSCs	Induced pluripotent stem cells
Isl1	Islet-1
LEF/TCF	T-cell 13 factor/lymphoid enhancer factor
MSCs	Mesenchymal stem cells
Ngn3	Neurogenin 3
Pdx-1	Pancreatic and duodenal homeobox 1
Ptc1	Patched1
Ptc2	Patched2
PTF1a	Pancreatic Transcription Factor 1 a
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
Smo	Smoothened
Sonic	Shh
TFs	Transcription factors
Wnt	Wingless-related integration site

1 Introduction

Cells having self-renewal ability and multilineage differentiation capacity are described as stem cells (Bacakova et al. 2018). Responsibilities of stem cells are to provide homeostasis, to repair of damage to the body and differentiation into new cells if needed (Suchánek et al. 2007). According to their sources, stem cells are basically categorized into three groups; adult stem cells (ASCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Lakshmipathy and Verfaillie 2005; Takahashi et al. 2007; Takahashi et al. 2007). As known, inner cell mass of the blostocyte is the source of ESCs. These cells have the capacity of pluripotency and can create all embryonic cell types (Ilic and Ogilvie 2017). Reprogramming of somatic cells into embryonic-like cells having pluripotency is called induced pluripotent stem cells (iPSCs). iPSCs obtained by transferring transcription factors like Sox2, Klf4, Oct3/4 and c-Myc into somatic cells were firstly achieved in 2006 by Yamanaka (Takahashi and Yamanaka 2006). The main cell type to get iPSCs was Mouse fibroblast. However, studies indicated that cells obtained from dental tissues are also used in the generation of iPSCs (Demirci et al. 2016). Mesenchymal stem cells (MSCs), neural stem cells and hematopoietic stem cells (HSCs) can be given in the group of adult stem cells (Yalvac et al. 2010). The primary role of ASCs is to provide renewal of cells in adult tissues. MSCs are obtained from distinct sources like bone marrow, skin, synovial membrane, liver, adipose, nerve and dental tissues (Doğan et al. 2015). MSCs are firstly isolated by Friedenstein in 1970 (Friedenstein et al. 1970). It is very easy to maintain MSCs in cell culture conditions. MSCs express surface antigens like CD 29, CD105, CD90 and CD73 which are mesenchymal stem cell markers as they express no hematopoietic stem cell markers such as CD14, CD45 and CD133 (Aydin et al. 2016). MSCs have high differentiation capacity. It was demonstrated that MSCs can be turned into adipocytes, chondrocytes and osteoblasts (Al-Nbaheen et al. 2013). Moreover, neurocytes and hepatocytes are successfully obtained by differentiation of MSCs *in vitro* (Ullah et al. 2015). It was indicated that human bone marrow stem cells included in MSCs are able to successfully be differentiated into islet-like cell (Chen et al. 2004; Zanini et al. 2011; Milanesi et al. 2012).

There are other crucial features of MSCs except their differentiation ability. It was shown that MSCs have the capacity of secrete various growth factors, chemokines and cytokines (Squillaro et al. 2016). At present, it was indicated that these released agents from MSCs may protect peripheral cells from apoptosis and induce their proliferation which accelerates regeneration of injured tissues (Wang et al. 2011). Apart from their differentiation potential, MSCs possess immunomodulatory features both *in vitro* and *in vivo* (Gebler et al. 2012). MSCs can regulate

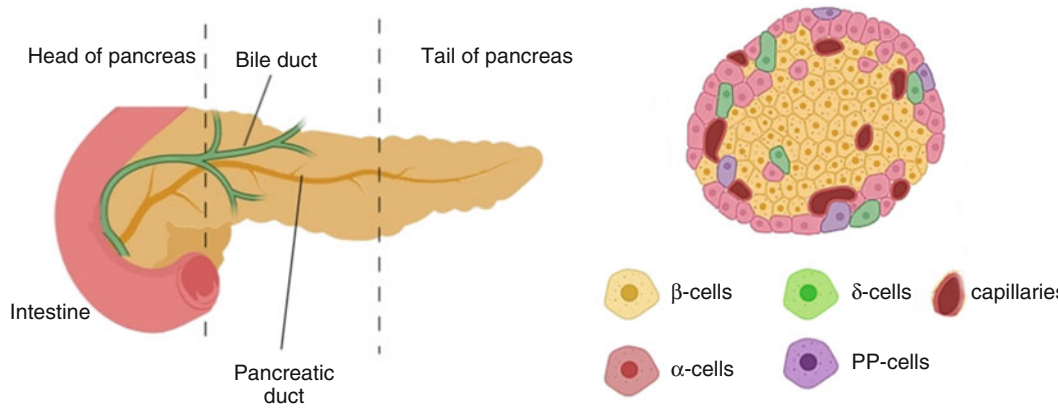

Fig. 1 Composition of distinct cell types in Pancreas

various factors such as NK cells (Spaggiari et al. 2008), macrophages (Singer and Caplan 2011), B cells (Corcione et al. 2006) and regulatory T cells (Maccario et al. 2005) involved in the immune systems. MSCs are preferred in cell therapy applications due to their useful features.

2 Pancreatic Development

The pancreas originates from foregut derived from definitive endoderm in the fifth month of pregnancy. Definitive endoderm folds into a primitive gut tube with the help of growth factors secreted from adjacent tissues. The epithelial tissue extending toward the mesoderm forms the ventral and the dorsal pancreatic buds. (Can 2014). Pancreas consists of two parts; ventral and dorsal buds. Along the developmental process, these buds grow, branch and the ventral bud switches to the other side. Therefore, two buds combine to create the head of pancreas. A whole pancreas is consisted of the head, body and tail sections.

Definitive endoderm differentiates into several cell types like ductal, acinar, and pancreatic multipotent cells. Since it composes of many cell types, the pancreas has different characteristics. The pancreas contains 90% exocrine cells called ductal and acinar cells and 10% endocrine cells which are pancreatic multipotent cells. While exocrine cells are responsible for providing the secretion of enzymes for digestion to the duodenum, endocrine cells secrete hormones into the blood (Fig. 1).

When looking at the development of pancreas closely, the multipotent progenitor cells can transform into many types of pancreatic cells like stated before. Each cell group has a different role on body homeostasis. As Alpha (α) cells produce glucagon, while Delta (δ) cells are important for the secretion of somatostatin. Beta (β) and Gamma (γ) cells produce insulin and pancreas related polypeptides, respectively. There is another group of cells found in the pancreas. Epsilon (ε) cells are responsible for secreting *ghrelin* hormone of the pancreas with the stomach and small intestine.

Dorsal pancreatic buds release insulin more than ventral buds because of the difference between beta cell composition (Kim et al. 2001). These differences based on the development during gestation. The destiny of a cell is determined by silencing the genes necessary for differentiation or absence of signaling pathways important for the maturation of pancreatic cells. In the presence of Neurogenin3 and Isl1 and absence of Notch signaling pathway, the progenitor cells transform to the endocrine precursor cells. Although cells are different from each other, they co-express the same transcriptional factors like Pdx-1 and Nkx6.1. The origin of Alpha and

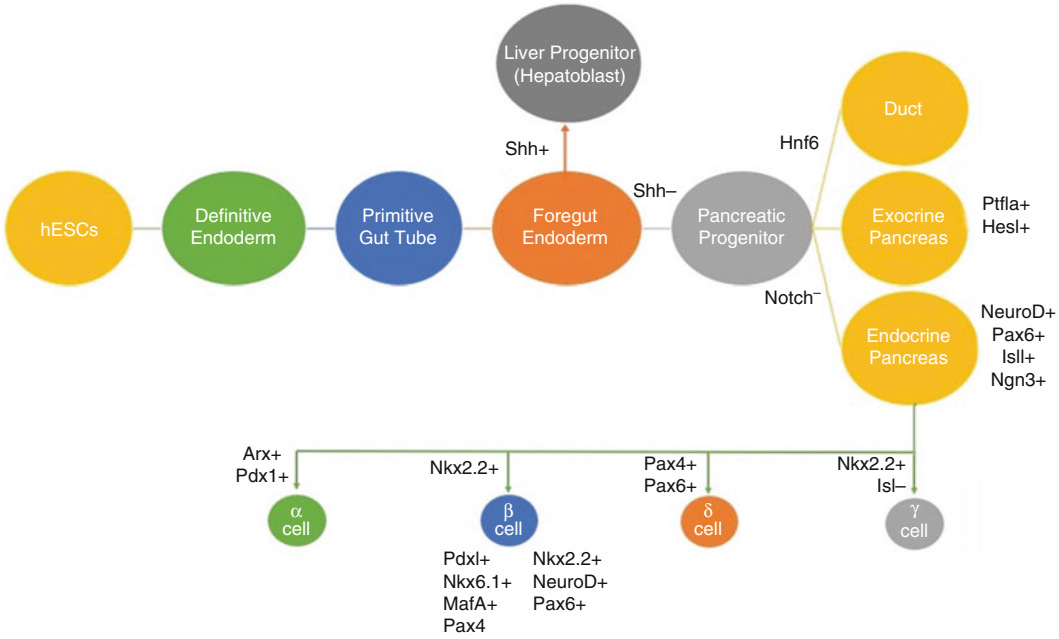

Fig. 2 Pancreatic Linage. (hESCs, human embryonic stem cells)

gamma cells is slightly different as well as delta and beta cells. In other words, the cells expressing Neurogenin-3 gene are divided into two groups Pax4 expressing and Arx expressing groups. The Pax4 precursors form the Beta and Delta cells. The differences are based on the expression of different genes. For example, to produce beta cells, the Nkx2.2, Nkx6.1 and MafA genes must be expressed, while Isl1 and Pax6 are required to produce Delta cells. The same situation is also present in Arx precursors. To achieve alpha cells, Arx precursors express Brn4, MafB, and Pax6 and they express Nkx2.2 for delta cells (Fig. 2). Scientists working with mice recommend that the transformation of endocrine cells continue after birth (Brennand et al. 2007; Dor et al. 2004).

3 Transcription Factors

Transcription factors (TFs) are important signal transduction molecules in whole cellular actions by binding promoter and enhancer regions. They help to transfer signals to the cytoplasm or nucleus or vice versa. Transcription factors regulate gene levels by repressing or promoting them.

3.1 Pancreatic and Duodenal Homeobox 1 (Pdx-1)

Pancreatic and duodenal homeobox 1 (Pdx; well-known as Insulin Promoting Factor 1 (Ipf1)) is a transcription factor that contains homeobox sequences. Pdx gene is required for the differentiation of progenitor cells into the β-cell phenotype and the whole pancreatic development. The expression of Pdx-1 gene is seen the pancreas, intestine and stomach. (Fukuda et al. 2006; Jørgensen et al. 2007). Pdx-1 is regulated as a response to the amount of glucose by phosphorylation and translocation of the Pdx1 protein into the nucleus (MacFarlane et al. 1994; Rafiq et al. 1998). Level of Pdx-1 expression changes between stages of pancreatic development. It decreases during early stages but then the

expression increases in murine pancreatic β-cells (Bernardo et al. 2008). On e8.5, Pdx-1 expression is firstly observed in murine foregut endoderm. The expression of Pdx-1 is observed in both dorsal and ventral pancreatic buds on e9.5 (Offield et al. 1996). Between e11.5 and e13.5, the expression of Pdx-1 is seen along the developing ductal formation. While the exocrine pancreas comes out and the islets start to create cells producing hormone (e14–e15), the expression of Pdx-1 shifts to the endocrine compartment. When the exocrine portion of pancreas shows up and islets start to create cells which produce hormone on e14 and e15, Pdx-1 is expressed in the endocrine portion. On e16.5, Pdx-1 expression markedly diminishes and it is almost unseen in the exocrine pancreas of adult mice. On e18.5 (in later stages), Pdx-1 expression is limited to endocrine pancreas (Habener et al. 2005). Pdx-1 is initially indicated as confined to β and δ cells of islets in the mature pancreas (Stoffers et al. 1997), but in some cases such as pancreatic injury it is re-expressed in acinar cells. Since Pdx-1 is determined as gamma and beta cell-specific regulator for the expression of somatostatin and insulin genes like stated before. But glucagon producing alpha cells rarely express the Pdx-1 gene (Habener et al. 2005). Pdx-1 gene is also found to control the gene expression of other islet-specific factors, including islet amyloid poly peptide (IAPP), glucokinase (Gck) and glucose transporter 2 (Glut-2). (Ahlgren et al. 1998; Al-Khawaga et al. 2017). Pdx-1 expression begins at week 4 and becomes confined to the beta cells in human pancreas (Lyttle et al. 2008; Jennings et al. 2013). Interestingly, Pdx-1 expression is detected in islet cells, exocrine cells of adult pancreas in both murine and humans (Pan and Wright 2011; Rooman et al. 2000; Castaing et al. 2005). It is reported that the development of the pancreas from the gut by budding can be seen without branching and expansion in Pdx null mice. Hence, it can be said that the activity of the Pdx-1 gene is vital for differentiation of the pancreas. (Offield et al. 1996; Jonsson et al. 1994).

Heterozygous Pdx-1 (Pdx-1+/−) mice develop normally, but the insulin deficiency increases in time because of the genetic mutation. This suggests that Pdx-1 should be expressed at a high rate for insulin production. Pdx-1 gene mutations are connected to the development of Diabetes mellitus type 2 in humans (Hani et al. 1999). MODY4, which is portrayed by agenesis of pancreas, is known to occur as a consequence of homozygous mutations in the Pdx-1 gene (Stoffers et al. 1997).

3.2 Pancreatic Transcription Factor 1A (PTF1a)

Pancreatic Transcription Factor 1a (PTF1a; also known as p48) is a crucial gene for the development of the acinar composition. In the pancreas, the knockout of the Ptf1a gene pioneers to an absence of acinar cells. In mice, deletion of Ptf1a causes the death of the animal after birth. In the literature, it was shown that Ptf1a is found in the ductal, exocrine, and endocrine progenitors, whereas inactivation of the gene indicated that these progenitors changed to intestinal epithelial progenitors (Kawaguchi et al. 2002).

3.3 Islet-1(Isl1)

Islet 1 (Isl1) included in LIM/homeodomain transcription factors family found in the developing pancreas and some neural cells during embryogenesis (Karlsson et al. 1990). Isl-1 expression is firstly detected in the mesenchyme, and then in all hormone secreting pancreatic islet cells. Interestingly, it is rarely expressed in insulin-producing beta cells due to the regulation of insulin secretion. Knocking out of Isl-1 gene causes the blocking the development of pancreas at e9.5 (Thor et al. 1991). Especially, the development of dorsal pancreas is not observed and endocrine cells are not present. However, the progression of

ventral pancreatic epithelium and mesenchyme continues to develop normally in the Isl null mice (Habener et al. 2005). These outcomes indicate that Isl1 is necessary for the progression of the dorsal pancreatic bud and it also necessary for the differentiation of the dorsal pancreatic epithelium to the endocrine cells.

3.4 Neurogenin 3 (Ngn3)

Neurogenin-3 is a TF encoded by the NEUROG3 gene in humans and classified to the basic helix-loop-helix transcription factors (bHLH) family. The fate of endocrine cells starts with Ngn3 protein. Induction of Ngn3 causes the expression of other transcription factors such as Pax4, Isl, Arx and Nkx family proteins (Gouzi et al. 2011). Ngn3 is one of the leading markers that initiates the differentiation of cell in the primary endocrine direction. In humans, Ngn3 positive cells appear from 9th week of pregnancy and the appearance continues until the week 17. Then this amount decreases rapidly (Jeon et al. 2009). The expression of Ngn3 begins at e9.5, reaches the highest point during endocrine cell production at e15.5, and is reduced with birth, in mice. Ngn3 is almost non-existent in the adult pancreas (Rukstalis and Habener 2009). Timing of the expression of Ngn3 is very important during embryonic development. The expression at later phases causes formation of β, γ, and δ cells; while it causes formation of glucagon-expressing cells at the early phase of expression (Al-Khawaga et al. 2017).

Studies with mice showed that neither endocrine cells nor endocrine progenitor cells are formed during development in the Ngn3 null group, whereas the exocrine section remains intact (Gradwohl et al. 2000). Thus, it was confirmed that Ngn3 own a vital role in the pancreatic improvement. Hes1 (hairy and enhancer of split-1) is another TF that involves in Notch pathway. It works antagonistically with Ngn3 for the protection of pancreatic precursor cell pool. (Jensen et al. 2000). In another study with mice indicated that Ngn3-expressing cells were able to secrete insulin and other hormones after injection into the pancreas of Ngn3 defective mice. However, mice in the control group did not secrete any hormones (Xu et al. 2008).

3.5 SRY (Sex Determining Region Y)-Box (Sox)

The SRY-box gene family is largely protected during development. Two of them, Sox9 and Sox17 own a role especially in the progression of the pancreas. Sox17 is associated with the early stages of development. According to studies on xenopus and zebrafish, the expression of Sox17 is vital for endodermal growth.

Sox9 is related to proliferation and maintenance of progenitor cells and expressed with Pdx-1 between e9-e12.5. However, the accumulation of Sox9 is limited with Pdx-1 expressing cells in epithelial cord. Sox9 knockout studies show that Pdx-Cre-mediated deletion increased glucose level of the blood and caused death on postnatal day 4. This is the evidence of the importance of Sox9 expression for progenitor cells (Seymour et al. 2007).

In adults, Sox9 is accumulated in the acinar cells and duct and targeted deletion of the gene is caused cysts. This situation also demonstrates the role of Sox9 in ductal cell maintenance by reducing the expression of ductal markers (Delous et al. 2012; Magenheim et al. 2011). Conversely, Sox9 initiates the expression of Neurogenin3 gene (Lynn et al. 2007). Scientists discovered that haplo-insufficiency of Sox9 invoke aberrant acinar phenotype and the formation of endocrine pancreas in humans (Piper et al. 2002). Current evidences demonstrate the considerable role of Sox9 in controlling and maintaining progenitor missions of the pancreas.

3.6 Homeobox Protein Nkx Family (Nkx2.2, Nkx 6.1)

Homeobox protein Nk-gene family is expressed during pancreatic progression. Henseleit and colleagues proved that mutation in Nkx6.1 gene causes defect in only β cells, while mutation in Nkx6.2 gene provokes no certain pancreatic

deformities in mice. Remarkably, when mutation occurred in both genes, pancreatic development continued with diminished amounts of α and β cells (Henseleit et al. 2005). Thus, it is suggested that both genes are vital for the development of islet cells; however, the solo mutation of Nkx6.2 is compensated by Nkx6.1. The Nkx6.1 expression starts at embryonic day 9.5 and continues until at e13 in beta cells. The Nkx6.1 transcription factor is involved in crucial events such as beta cell development or insulin production. According to studies in adult mice, the presence of a mutation in Nkx6.1 did not show any effect in other cell groups, but the mutation reduced the number and the growth of beta cells (Sander et al. 2000).

Nkx2.2 is another homeobox TF basic for pancreatic progenitor cell pattern (Doyle and Sussel 2007). As in Nkx6.1, expression of Nkx2.2 begins early in multipotent pancreatic progenitor cells at e9.5 and keeps on in α, β, and γ cells (Al-Khawaga et al. 2018). Nkx2.2 is expressed with Pdx-1 during the pancreatic progenitor cells; however, its expression becomes controlled to the Ngn3 endocrine progenitors (Doyle and Sussel 2007). When the transcription factor is silenced, the formation of various cell types is not observed (Prado et al. 2004). Papizan et al. revealed that Nkx2.2 is vital for the maintenance of beta cell in adults (Papizan et al. 2011).

3.7 Forkhead Box Factors (Fox)

Fox-O is another key transcriptional factor essential for the maintenance and beta cell differentiation during the early improving of the pancreas (Wang et al. 2010). Insulin negatively regulates working mechanism of Fox proteins. The absence of growth factors or insulin results in nuclear translocation of Fox-O proteins and increased expression of Fox-O activated genes (Brunet et al. 1999). Fox-O1 is the most abundant form of Fox-O genes in the adult pancreas. In mice, Fox-O1 rivaled with Forkhead box A2 (FoxA2) in the case of binding on the Pdx1 promoter. Thus, it acts as a negatory regulator for the expression of the gene. Through murine pancreatic development, Fox-O1 and Pdx-1 have identical generation design. Between e9.5-e14.5 they are thoroughly produced in the pancreatic epithelium. These discoveries recommend that Fox-O1 is involved in different steps of the pancreatic development in mice (Kitamura and Ido Kitamura 2007). FoxA1-KO mice own phenotypically normal appearance but with abnormal function of beta cell (Vatamaniuk et al. 2006).

4 Signaling Pathways

Signaling pathways conduct and regulate all cellular communications. It has been documented that the related-cells use different pathways throughout the embryonic development of the pancreas. The main signaling pathways underlying the development process are in Table 1.

4.1 Hedgehog Signaling Pathway

The hedgehog pathway is a negative regulator of pancreatic development and owns a vital role for tissue homeostasis in adult, embryonic development, and taking part in the division and differentiation of cells. Three hedgehog genes have been discovered in mammals, Indian (Ihh), Desert hedgehog (Dhh) and Sonic (Shh) which are well expressed throughout embryogenesis and also important for the growth of several organs (Chuang and Kornberg 2000). Shh is the most expressed form of genes in embryonic and adult mammalian tissues. However, Shh is kept out from pancreatic epithelium throughout development (Apelqvist et al. 1997; Hebrok et al. 1998).

Hedgehog proteins are ligand of Patched2 (Ptc2) and Patched1 (Ptc1) transmembrane receptors found in cells contiguous to hedgehog

Table 1 Signal pathways

1	Hedgehog
2	Notch
3	Wnt/β-catenin
4	Retinoic acid
5	TGF-β superfamily

producing cells (Motoyama et al. 1998). When Shh binds to the receptors, it activates the pathway. The absence of ligands, Ptch blocks another receptor-like protein named as Smoothened (Smo) (Motoyama et al. 1998; Stone et al. 1996). Patched receptors inhibit the binding of Smo agonist, an endogenous intracellular small molecule, to Smo and carries this molecule out of the cell. In the presence of hedgehod ligands, Patched receptors become inactivated by binding to ligands and the suppressive effect on smo is eliminated. Thus, Smo is activated and the hh signal is transferred to the cytoplasm. In cytoplasm, active-Smo separates and activates the Gli molecule from SuFu-Gli complex. The active Gli transcriptional factors bind to the nucleus and stimulate target genes which will respond hedgehod ligands (Fig. 3).

It was indicated that inhibition of sonic hedgehog gene is crucial for initialization of early pancreatic development (Hebrok et al. 1998; Martí et al. 1995). Furthermore, in the early pancreatic development, abnormal activation of the hedgehog pathway leads to loss of pancreatic marker genes and pancreatic tissue. On the other hand, the abnormal activation increases duodenal properties (Fendrich et al. 2008). Activation of the pathway is limited to beta cells of endocrine pancreas in the regulation of insulin production in the adult pancreas. However, it is necessary for regeneration of exocrine pancreas in case of injury or disease (Algül et al. 2002). Also, the abnormal activation of the Hh pathway in human pancreatic tissue has been documented that it causes the pancreatic cancer (He et al. 2016).

4.2 Notch Signaling Pathway

Notch signaling is tightly conserved pathway that determines cellular fate through embryonic development. It is crucial for the regulation of survival/apoptosis, cell differentiation and the cell cycle in adults and is also good example of the direct cell to cell contact. On the other hand, ongoing investigations in mice state that Notch pathway controls endocrine and exocrine fate of pancreatic progenitors (Habener et al. 2005).

Notch is a large protein which has a transmembrane region. It co-acts as a receptor in signal transduction with other transmembrane proteins (ligands) located on the surface adjacent cells.

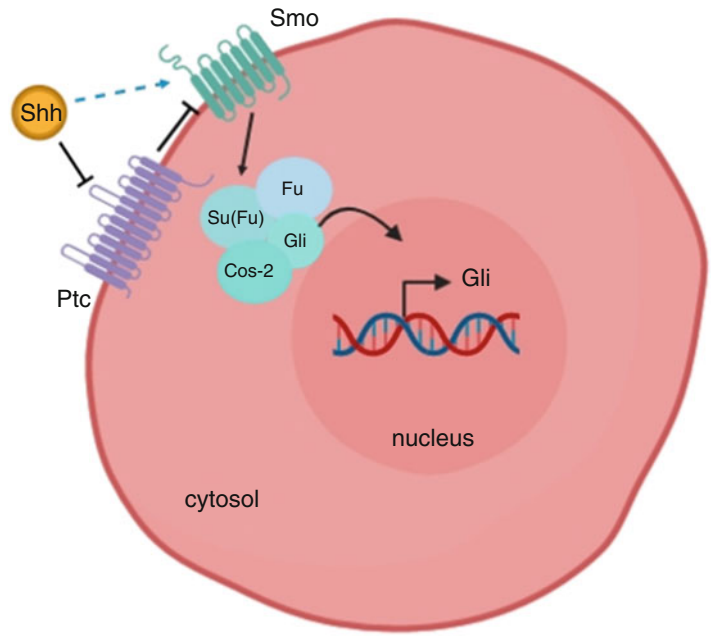

Fig. 3 Hedgehog signaling pathway

Notch signaling pathway has two main classes of ligands in mammals; Delta ligands (Delta-4, Delta-3 and Delta-1) and Jagged ligands (Jagged-2 and Jagged-1). When the ligand attached to the receptor, it causes proteolytic cleavage of Notch protein. Thus, intracellular part of Notch (ICDN) passes to the nucleus and it connects with the transcriptional factor (Suppressor of Hairless, Su (H), in Drosophila and CBF1 in mammals,). So, the genes that determine cell fate are expressed. Various targets of Notch signaling pathway are well-known. One of them in mammals and Drosophila is the Hes (hairy/enhancer of split) repressors belonging to bHLH (Iso et al. 2003; Kim et al. 2010). Hes repressor negatively regulates the gene expression by hiding the transcriptional activators. In addition, ICDN directly promotes the expression of transcription factors, growth factor receptors and cell cycle regulators. This complicated situation shows that Notch signaling is included in various cellular events. The necessary activation of Notch signaling in pancreatic progenitors inhibits their differentiation into the exocrine or endocrine cell lineage (Li et al. 2016; Ahnfelt-Ronne et al. 2007). Interestingly, the activation of the pathway induces untimely differentiation of the progenitor cells to endocrine cells (Nakhai et al. 2008). In pancreatic development, pro-endocrine factor neurogenin3 (Ngn3)-expressing cells have to become endocrine cells, and these cells have higher expression of Notch ligands Serrated, Delta (Dll), and Jagged. Likewise, some researchers have suggested that the Notch pathway refers to pancreatic progenitors that differentiate into the endocrine lineage or the inactivation of Notch pathway supports the differentiation of acinar cells (Afelik et al. 2012; Hosokawa et al. 2015) (Fig. 4).

As mentioned before, Notch signaling leads to the transformation of progenitor cells into endocrine cell types with two possible mechanisms (Field et al. 2003). The first one is "lateral inhibition" blocking contiguous cells from having the identical phenotype. Lateral inhibition is necessary for many types of cell fate determination. The second one is "suppressive maintenance" which clarifies the function of the Notch signals in pancreatic differentiation (Jensen 2004).

Studies on animal pancreatic regeneration models and diseases have demonstrated that Notch signals play a role in controlling the plasticity of fully differentiated adult pancreatic cells (Li et al. 2016).

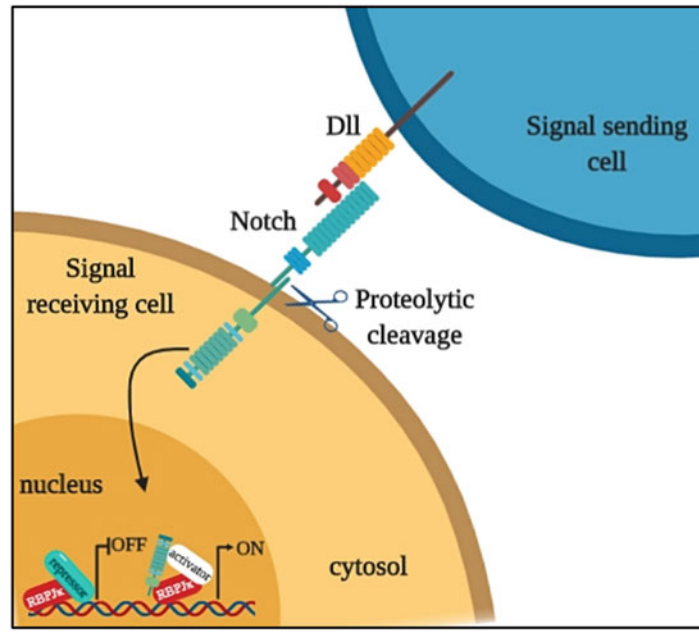

Fig. 4 Notch signaling pathway

4.3 Wnt/β-Catenin Pathway

As Hedgehog and Notch signaling pathways, Wnt (Wingless-related integration site)/β-catenin pathway is also evolutionary conserved for cell-fate and cell proliferation during embryonic development (McMahon and Bradley 1990; Pin and Fenech 2017). Wnt signaling contains various signaling pathways; β-catenin-mediated canonical pathway and two non-canonical pathways which are planar cell polarity and Wnt/calcium. In pancreatic development, the canonical form of the pathway controls the expression of key transcriptional factors (114, 163), whereas the non-canonical forms of the pathway only regulate the cellular shape and calcium uptake (Wells et al. 2007; Pin and Fenech 2017). Some Wnt ligands such as WNT2b, WNT4, WNT5a, and WNT7b were announced for the process of pancreatic development (Heller et al. 2002).

In the presence of Wnt protein, the protein binds to the Frizzled (Fzd) receptors and they induce Dishevelled (Dvl) in the canonical form. Through the cascade, Wnt pathway is activated. Then, APC/Axin/GSK3β complex become inactivated. Thus β-catenin remains intact and accumulates in the cytoplasm. The accumulated proteins relocate to the core and bind to the LEF/TCF (T-cell 13 factor/lymphoid enhancer factor) proteins which bind to the DNA, fundamentally. However, in the absence of Wnt protein, the Fzd-Dvl complex does not occur and APC/Axin/GSK3β/CK1 cleavage complex phosphorylates and digests the β-catenin by ubiquination. Therefore, β-catenin cannot pass to the nucleus and the signal is blocked (Behrens et al. 1996).

Before taking the final version of the pancreas, enforced expression of the WNT signal prevents the development of the liver and pancreas (Heller et al. 2002). Direct erasure of β-catenin exhibits the loss of exocrine pancreatic tissue and increases the tubular forms. Recent investigations demonstrate that β-catenin is primarily necessary for the development of pancreatic epithelium prior to differentiation. Besides loss of β -catenin in pancreatic progenitors which are Pdx-1-positive prevent creation of acinar cells (Wells et al. 2007) (Fig. 5).

4.4 Retinoic Acid Pathway

Retinoic acid (RA) is a metabolite of vitamin A and assumes a role in assigning the polarity of the body during embryonic development with the help of retinoic acid signaling molecules. This pathway owns a vital role in the creation of gut endoderm. In order to produce a continuous RA signal in pancreatic progenitors, Pdx-1 expression must be initiated (Serup 2012). In the pathway, the enzyme called Retinaldehyde dehydrogenase type II is responsible for generation of various retinoic acid derivatives (Niederreither and Dollé 2008). The enzyme is present until

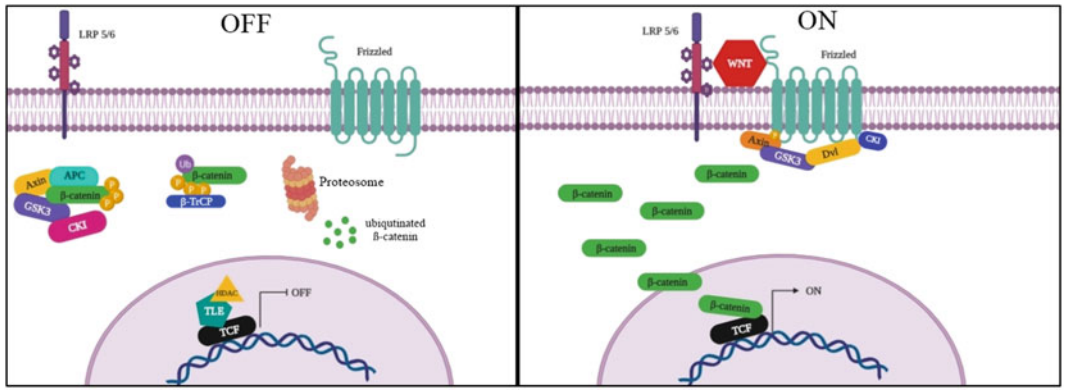

Fig. 5 Schematic representation of Wnt/β-Catenin Pathway

embryonic day 12.5 in the primitive pancreas of mice. Thus, the RA is necessary for the development of pancreas at an early stage (Martín et al. 2005). Tulachan and Shen separately showed that the retinoids support the differentiation of beta cells (Tulachan et al. 2003; Shen et al. 2007).

4.5 TGF-β Superfamily Signaling Pathway

TGF- β superfamily is a class of various transcriptional factors including TGF-β factors, Activins, Nodal and Bone morphogenetic proteins (BMPs). The pathway plays vital role in most of cellular process during the whole life (Kashima and Hata et al. 2018).

4.5.1 TGF- β

The expression of distinct TGF-β proteins is seen in the pancreatic epithelium till E12.5 and they are limited to acinar cells over time (Crisera et al. 2000). A pilot research demonstrated that the mutation on TGF-β1, TGF-β2, TGF-β3 and TGF receptor type 2 are resulted in death in the embryonic period (Serup 2012).

4.5.2 Activins & NODAL

Activins are included in various biological processes during the life of the individual (Namwanje and Brown 2016). The expression of activins is seen in gut endoderm and the early pancreas (Serup 2012). Two different types of Activin (Activin A and B) are actually closely related peptides which show 63% identity and are almost similar in their domains (Namwanje and Brown 2016). Activin A and B are limited to progressing glucagon-positive cells. Hebrok et al. drew attention to that the Activin B can block the sonic hedgehog pathway in the pancreatic endoderm at the early stage (Hebrok et al. 1998). Furthermore, presence of Activin A inhibits ductal and acinar differentiation (Ritvos et al. 1995). Studies on transgenic mice exhibited that the activin receptors type II A and B knocking mice did not show any pancreatic developmental abnormalities in type IIA mutated group whereas type IIB mutated group shows phenotypically defective pancreas (Kim et al. 2000). Serup et al. reported that this might be related to the failure of Hh signaling (Serup 2012). It seems that Activin assumes a role by repressing the expression of a vital transcriptional factor for alpha cell differentiation and improves the expression of Neurogenin3 (Zhang et al. 2001). These points determine the importance of activins in early pancreatic development by provoking endocrine differentiation and blocking ductal and acinar differentiation (Al-Khawaga et al. 2017).

Nodal protein is included in TGF- β superfamily and effector for downstream signaling. It plays a role in cellular transformation in early embryogenesis. The absence of Nodal can not initiate the primitive streak, the line in the blastula.

5 Methods for β Cell Differentiation

As mentioned before, beta cells are very important group of cells for body homeostasis and digestion processes. Defective or dysfunctional beta cells cause many diseases, especially diabetes. There are several approaches to cure this situation. Pancreatic or beta-cell transplantation is important in type 1 diabetes. However, transplanted pancreas or beta cells might not be accepted by the immune system. In such cases, the differentiation of stem cells form the patient into beta cells *in vitro* and subsequent injection to the dysfunctional area are considered as an important issue for today's physiologists and researchers. The first study related to pancreatic beta cell differentiation was done by using human pluripotent stem cells in 2001 (Assady et al. 2001) and the number of studies about differentiation of stem cells into beta cells significantly increased in recent years.

The most important factor in the differentiation period is that proliferation does not prevent differentiation. During differentiation, cells need to use the source for the differentiation instead of the proliferation. For this situation, scientists have used proliferation reducing agents. Therefore, toxic substances such as dimethyl sulfoxide (DMSO) or β-mercaptoethanol (BME) are added

to the differentiating medium. Reducing the oxygen level or nutrients can be given as examples to stop proliferation of stem cells.

In the literature, various differentiation methods have been published. (Table 2). But the main idea is based on direct differentiation of stem cells from any source, into early pancreatic development stages *in vitro*. Some studies have successfully established insulin-secreting β cells, but these studies have established that hormone expressing cells are not only β cells, they determined that differentiation only reaches until the primitive-fetal pancreatic period.

Protocols for producing insulin-secreting cells *in vitro* modify depending on the cell line, but there are plenty of mutual subjects among them. Table 2 summarizes stem cell differentiation from several sources into into β cells capable of secreting insulin using diverse chemicals at different time points.

5.1 Extrinsic Factors

The beta cell differentiation process is completed in 5 stages, induction to definitive endoderm, primitive gut tube formation, development of foregut, pancreatic progenitor cell development and maturation of β cell, respectively.

5.1.1 Induction to Definitive Endoderm

Definitive endoderm (DE) is the first station of differentiation. Definitive endoderm is one of the three germ layers of an embryo. It forms gastrointestinal organs and gut tube which is origin of primitive pancreas. Several components have been used to back definitive endoderm, experimentally.

Activin A is necessary for stem cell transformation for the creation of definitive endoderm (Kumar et al., Bose et al). Activin A is used with another substance, Wnt3a and they have found that Activin a and wnt3a triggered the meso-endoderm transition (D'Amour et al.; Jiang et al). Activin A blocks the ductal and acinar differentiation of DE cells. For this purpose, Pagliuca et al. used Activin A through the next stage of differentiation (Pagliuca et al. 2014). Kumar et al., have demonstrated that activin A, with sodium butyrate, initiates differentiation of hESCs into pancreatic cells during the first a few stages of endoderm development (Kumar et al. 2014).

Wnt3a is the co-worker of Activin A to form definitive endoderm through the differentiation. According to Table 2, D'Amour et al. added Wnt3a in induction medium for 2 days. Then it was withdrawn after 2 days. This alteration augments the effect of mesoendoderm formation and transition to DE.

Wortmannin is a chemical substance that inhibits PI3K pathway directly, so it promotes pancreatic development by initiating Nodal and TGFb pathways throughout DE formation (Kumar et al. 2014).

Histone deacetylases (HDACs) are inhibited by sodium butyrate (SB) which is a short chain fatty acid. SB also blocks the dedifferentiation of DE cells. It was demonstrated that sodium butyrate aids to trigger the early stage of β cell differentiation with Activin A in hESCs. Moreover, it also enhances the secretion of insulin and glucagon at high level (Kumar et al. 2014). As known, sodium butyrate stimulates the transition of stem cells into DE, too. Studies have shown that removal of sodium butyrate from inducing media reduces PDX1 gene expression (Goicoa, et al. 2006).

5.1.2 Primitive Gut Tube Formation and Development of Foregut

The second station of beta cell differentiation is the creation of the foregut endoderm. Foregut endoderm is the period in which the cell fate is determined in the direction of the liver or pancreas. Various activities of some transcriptional factors lead the differentiation into pancreatic direction *in vivo*. However, different stimulators have been used to mimic the same way *in vitro* studies.

FGF Family (FGF2, FGF7, FGF10): Fibroblast growth factor family members are widely used in cellular events, especially differentiation. These members participate in the developmental connection between the pancreas and other gastroenteric organs. FGF2 controls the transition

Table 2 Different approaches in order to generate pancreatic β cells

Author	Source	Differentiation Approach					
Moshtagh et al. (2013)	Human Adipose stem cells (2×10^5 per well of 6 well plate)	Three stages	**Stage1**	**Stage2**	**Stage3**	**Stage 4**	**Stage 5**
			To induce differentiation (definitive endoderm)	**Primitive gut tube**	**Foregut endoderm**	**Pancreatic progenitor**	**Mature insulin producing cells**
			DMEM-low glucose +5% FBS + nicotinamide **For 2 days**	DMEM-high glucose +2.5% FBS + BME + NA **For 10 days**			DMEM-high glucose +1.5% FBS + BME + nicotinamide + Exendin-4 **For 14 days**
Govindasamy et al. (2011)	Human dental pulp stem cells (1×10^6 cells/cm^2)	Three stages	DMEM-KO + BSA + ITS + Activin A, sodium butyrate + BME **For 2 days**	SFM B: BSA + DMEM-KO + taurine + ITS **For 2 days**			SFM C: Taurine + DMEM-KO + ITS + BSA + GLP-1 + nicotinamide, + NEAA **For 2 days**
				SFM B and SFM C change for 5 days, alternating every 2 days			
Chandra et al. (2009)	Mouse Adipose stem cells (1×10^6 cells/cm^2)	Three stages	DMEM/F12 (1:1) + BSA + additive glucose + ITS + sodium butyrate + BME + Activin	SFM B: DMEM/F12(1:1) + additive glucose + BSA + ITS + taurine			SFM C: DMEM/F12(1:1) + additive glucose + taurine + BSA + ITS + nicotinamide + GLP-1 + NEAA
			For 2 days	**For 2 days**			**For 2 days**
				SFM B and SFM C change for 5 days, alternating every 2 days			
Zhang et al. (2009)	Human induced pluripotent stem cells and	Four stages	DMEM/F12 (1:1) + N2 + B27 + BSA + Wortmannin + Activin A	DMEM/F12 / IMDM + RA + BSA + B27 + ITS + KGF + noggin	DMEM-high + BSA + ITS + N2 + EGF		DMEM/F12(1:1) + ITS + BMP4 + bFGF + Exendin-4 + nicotinamide

(continued)

Table 2 (continued)

Author	Source	Differentiation Approach							
	human embryonic stem cells		**For 4 days**	**For 4 days**	**For 5 days**		**For 7–9 days**		
Jiang et al. (2007)	Human embryonic stem cells	Four stages	RPMI 1640 + glucose + B27 (RPMI 1640/ B27) + Sodium Butyrate + Activin A	RPMI1640/ B27 + EGF + bFGF + noggin	RPMI1640/B27 + EGF + noggin		RPMI 1640 + BSA + Nicotinamide + IGF-II		
			For a day After; RPMI 1640/B27 + Sodium Butyrate + Activin A	**For 14 days**	**For 7 days**		**For 5 days** w/o IGF-II another 2 days		
			For 6 days						
D'Amour et al. (2006)	Human embryonic stem cells	Five stages	RPMI + Activin A + Wnt3a **For 2 days**	RPMI + FGF10 + Cyclopamine +2% FBS	DMEM + B27 + RA + Cyclopamine + FGF10	DMEM + B27 + DAPT + Exendin-4	CMRL + B27 + Exendin-4 + IGF1 + HGF		
Pagliuca et al. (2014)	Human embryonic stem cells and human induced pluripotent stem cells (6×10^5 cells/ml)	Five stages	w/o Wnt another 2 days S1 + Activin A + Chir99021	**For 2–4 days** S1 + Activin A	**For 2–4 days** S2 + KGF	**For 2–3 days** S3 + KGF + RA + SANT1 + LDN193189 (for a day) + PdBU	**For 3+ days** S5 + Alk5i + RA + T3 + xxi + SANT1 + Betacellulin	S6+ Alk5i II+ T3	
			For a day	**For 3 days**	**For 3 days**	**For 2 days** w/o LDN193189 + PdbU another 5 days	**For 7 days** w/o Sant1 for 4 days	**For 7–14 days**	
Rezania et al. (2014)	Human embryonic stem cells (1.3–1.5 ×	Seven stages	MCDB 131 + BSA + GDF8 + MCX-928	MCDB 131 + BSA + FGF7 + ascorbic acid	BLAR medium* + BSA + ascorbic acid + FGF7 + SANT-1 + RA +	BLAR + BSA + ascorbic acid + FGF7 + RA + SANT-1 +	BLAR medium + BSA + SANT-1 + RA + LDN193189 + ITS + T3 + ALK5	BLAR + BSA + LDN193189 + ITS-X + T3 + ALK5 inhibitor	BLAR + BSA + ITS + T3 + ALK5i II + zinc Sulphate +

	10^5 cells/ cm^2)				LDN193189 + ITS + TPB	LDN193189 + ITS + TPB	Inh II + zinc Sulphate + heparin	II + zinc sulfate + xxi	N-Cys + Trolox + R428 + heparin
			For 2 days On day 3 MCX-928 replaced by CHIR-99021	**For 2 days**	**For 2 days**	**For 3 days**	**For 3 days**	**For 7 days** With heparin another 7 days	**For 7–15 days**
Chao et al. (2008)	Human Umblical cord stem cells	Four stages	10% FBS + DMEM	10% FBS + DMEM + AraC	DMEM/F12 + 2% FBS + B27 + NA	DMEM/F12 + 2% FBS + glutamine+ NA			
			For 3–6 days	**For 7 days**	**For 7 days**	**For 14 days**			

NA Nicotinamide, *BME* Beta Mercaptoethanol, *BSA* bovine serum albumin, *ITS* insulin-transferrin-selenium, *DMEM-KO* DMEM Knock out, *GLP-1* glucagon-like peptide 1, *NEAA* Non-essential amino acid, *KGF* keratinocyte growth factor, *RA* retinoic acid, *EGF* epithelial growth factor, *bFGF2* basic fibroblast growth factor 2, *BMP-4* bone morphogenic protein 4, *IGF* insulin growth factor, *HGF* hepatocyte growth factor, *DAPT* γ secretase inhibitor, *Chir99021* glycogen synthase kinase 3b inhibitor, *SANT1* sonic hedgehog pathway inhibitor, *LDN193189* inhibitor of the BMP pathway, *PdBU* protein kinase C activator, *Alk5i* inhibitor of TGF-β, *Xxi* γ-secretase inhibitor, *MCX-928* ALK inhibitor

of DE cells into various foregut lineages in time dependent manner. Furthermore, Jiang et al. indicated that addition of FGF2 into induction medium with noggin resulted in the termination of liver formation (Mfopou et al. 2010). According to Cai et al., PDX1 gene expression has enhanced in hESCs more than 70%, when FGF7 is used in differentiation procedure (Kumar et al. 2014; Cai et al. 2010). Both FGF2 and FGF7 help the formation of islet-like clusters. FGF10 owns a major impact on the creation of pancreatic epithelium from MSCs. FGF10 is necessary during pancreatic development together with KAAD-cyclopamine which is hedgehod-signaling inhibitor (Kumar et al. 2014; D'Amour et al. 2006). In another study, it was displayed that addition of FGF10 with KAAD-Cylopamine caused 160-fold increase of Insulin mRNA compared with activin removal alone (D'Amour et al. 2006).

RA is a precursor of Vitamin A and formed in the mesoderm by retinaldehyde dehydrogenase (RALDH) (Oström et al. 2008). It also regulates pancreatic differentiation of hESCs at early phase by stimulating the PDX1 gene expression. However, in this situation, RA requires distinct compounds such as FGFs or Noggin. Studies have shown that the level of PDX1 gene decreases when RA is used alone (Kumar et al. 2014).

Cyclopamine (or KAAD-Cyclopamine) is a plant derivative that inhibits Hedgehog signaling pathway protein Smoothened, thus also inhibiting that pathway (Chen et al. 2006). As mentioned earlier, enhanced expression of HH pathway proteins allows the formation of liver by cell fate. Therefore, the inhibition of HH pathway triggers pancreatic development. The addition of FGF10, RA and indolactam V in the differentiation medium together with cyclopamine promotes gut formation. This leads to high expression of PDX1, NeuroD1 and Neurogenin3.

Noggin is an antagonist that binds and inactivates the member of TGF-β signaling proteins such as BMP-2, BMP-4, and BMP-7 (Rifas 2007). The inactivation of TGF-β signaling protein encourages pancreatic development at the later stage. Noggin also induces PDX1 gene expression from DE cells (Takeuchi et al. 2014). Noggin works with RA to decrease the formation of liver precursor cells in the foregut development (Kumar et al. 2014).

5.1.3 Pancreatic Progenitor Cell Development

The third station is differentiation into the pancreatic progenitors which are origin of pancreatic cells, duct and acinar cells. *In vivo*, silencing of Notch and expression of Hnf6 transcription factors cause different type and roles of cells. To consist of duct cells, Hnf6 must be expressed or Hes1 and Ptf1a must be shown for exocrine pancreas. To form of endocrine cells, various TFs are expressed such as NeuroD, Pax6, Isl1 or Ngn3. As mentioned before, different chemicals are used to catch same effects *in vitro*.

Exendin-4 is a peptide acting as an agonist of the glucagon-like peptide (GLP) receptor that encourages the secretion of insulin (Ding et al. 2006). In the pancreatic differentiation process, Exendin-4 accelerates the maturation of pancreatic cells and enhances β cell proliferation alone or in combine with Hepatocyte Growth Factor (HGF) and betacellulin (Bose and Shenoy 2015).

EGF is the acronym of epithelial growth factor family and it is vital for the progression of pancreatic progenitors and islet growth by rising the number of cells expressing PDX1 (Zhang et al. 2009).

ALK5 Inhibitor II is another inhibitor of TGF-β type I receptor. Expression of NKX6.1 necessary for β-cell development increased four-fold when Alk5i II and Noggin were added into the medium at the pancreatic endoderm stage Kumar et al. 2014).

5.1.4 Maturation of β Cell

β cell maturation is the terminal stage of endocrine cell differentiation. At this stage, it is important to separate and mature the cell secreting the desired hormone.

Nicotinamide is an inhibitor of poly ADP-ribose synthetase that promotes

proliferation, regeneration and β cell differentiation (Hosoya 2012; Kumar et al. 2014).

Betacellulin belongs to Epithelial Growth Factor (EGF) superfamily. In literature, it is combined with another TGFβ family protein, Activin A and this combination promote the secretion of insulin from islet-like cells, also encourages the growth of differentiated β cells (Bose and Shenoy 2015).

BME is a chemical compound that act as reducing agent *in vitro* systems and used for various cellular processes such as mimicking cell environment, protecting from oxidative stress or differentiating, on the other hand, inducing cells. Pruett and his colleagues discovered that BME promotes survival and cell growth of murine lymphocytes by increasing protein synthesis as two-fold (Inui et al. 1997; Pruett et al. 1989).

Sant1 is a kind of inhibitor of sonic hedgehog pathway proteins which decides the cell fate to the formation of the liver. Therefore, SANT promotes pancreatic endoderm from foregut precursor cells.

DAPT is abbreviation of N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester which is γ secretase inhibitor and blocks the Notch signaling pathway inhibitor (Hosoya 2012; Akinci et al. 2013).

CHIR99021 is a selective glycogen synthase kinase 3b inhibitor that activates the canonical Wnt pathway. It works interactively with Wortmannin to induce through definitive endoderm of hPSCs (Hosoya 2012; Takeuchi et al. 2014).

Ascorbic Acid is a portion of Vitamin C usually used in differentiation process and treatment of various cancers (Chen et al. 2005; Du et al. 2012; Doğan et al. 2015). It is known that Ascorbic Acid plays a role in cell fate and several epigenetic demethylations. In differentiation processes, it was also determined as a supporter for the transformation and maturation of the mouse pancreatic β-cell. (Zhu et al. 2016).

Dexamethasone (DEX) is a synthetic glucocorticoid that is comparable to natural glucocorticoid hydrocortisone. Dexamethasone is an agonist of glucocorticoid receptors (Zhu et al. 2016). It induces reprogramming of hepatocytes from pancreatic cells of murine (Al-Adsani et al. 2010).

Lastly, forskolin is an activator of Adenylyl Cyclase. It synergistically works with DEX and they have been shown to promote maturation of cells that differentiated (Hosoya 2012; Zhu et al. 2016).

6 Clinic Use of Mesenchymal Stem Cells

Based on the official website of US National Institute of Health, it was reported that 759 clinical studies conducted with MSCs. Most of MSCs-based clinical trials were performed in diabetes, immune system-based disease, cardiovascular disease, bone and cartilage-based disease and neurological diseases (Squillaro et al. 2016).

The application of MSCs together with tissue engineering is one of the most commonly used procedures in clinical studies. As known, MSCs may boost cell proliferation and angiogenesis as well as immune regulation. Thanks to these features of MSCs, they can be used in wound healing studies. Autologous MSCs, bone marrow-derived, have efficiently been used to treat a diabetic ulcer in a combination with fibroblasts and Coladerm, which is a biodegradable collagen membrane (Vojtaššák et al. 2006). In a pilot study conducted in 2011, 10 type 2 diabetic patients were given injections of MSCs derived from placenta three times with one-month interval. Results showed that daily insulin intake of patients with type 2 diabetes undergoing placenta-derived MSCs injection decreased while C-peptide level of the patients increased (Jiang et al. 2011). In a study performed in 2015, it was demonstrated that autologous MSCs treatment leads to increased C-peptide response with no side effect in type 1 diabetic patients (Carlsson et al. 2015). It was reported that isolated MSCs from adipose tissue was differentiated into cells which can secrete insulin and these cells were given into the patients having type 1 diabetes. Results indicated that the requirement of exogenous insulin in the patients with type 1 diabetes was

decreased while the c-peptide level was increased (Thakkar et al. 2015).

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