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Review Article

Manipulation of Sonic Hedgehog Signaling Pathway in Maintenance, Differentiation, and Endocrine Activity of Insulin-Producing Cells: A Systematic Review

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Abstract

Background: Some studies have evaluated the manipulation of the sonic hedgehog (Shh) signaling pathway to generate more efficient insulin-producing cells (IPCs). In a systematic review, we evaluated *in vitro* and *in vivo* studies on the effect of inhibition or activation of the Shh pathway on the production, differentiation, maintenance, and endocrine activity of IPCs.

Methods: A systematic review was conducted using all available experimental studies published between January 2000 and November 2022. The review aimed at determining the effect of Shh manipulation on the differentiation of stem cells (SCs) into IPCs. Keywords and phrases using medical subject headings were extracted, and a complete search was performed in Web of Science, Embase, ProQuest, PubMed, Scopus, and Cochrane Library databases. The inclusion criteria were manipulation of Shh in SCs, SCs differentiation into IPCs, and endocrine activity of mature IPCs. Articles with incomplete data and duplications were excluded.

Results: A total of 208 articles were initially identified, out of which 11 articles were included in the study. The effect of Shh inhibition in the definitive endoderm stage to produce functional IPCs were confirmed. Some studies showed the importance of Shh re-activation at late-stage differentiation for the generation of efficient IPCs. It is proposed that baseline concentrations of Shh in mature pancreatic β-cells affect insulin secretion and endocrine activities of the cells. However, Shh overexpression in pancreatic β-cells ultimately leads to improper endocrine function and inadequate glucose-sensing insulin secretion.

Conclusion: Accurate manipulation of the Shh signaling pathway can be an effective approach in the production and maintenance of functional IPCs.

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Keywords ● Hedgehog proteins ● Hedgehog ● Pancreas ● Insulin ● Stem cells

What's Known

- The Sonic hedgehog (Shh) pathway can potentially affect the activity of insulin-producing cells (IPCs).
- Shh pathway is an essential determinant of IPCs activity.

What's New

- Shh can be considered a therapeutic target for diabetes.
- Accurate manipulation of Shh can be a suitable treatment strategy for diabetes.

Introduction

Diabetes is a severe metabolic disease affecting 422 million adults worldwide.[1] The World Health Organization has projected 1.6 million deaths annually by 2025 due to diabetes.[2] Diabetes causes poorly

controlled hyperglycemia that may lead to serious complications such as cerebrovascular accident, retinopathy, cardiovascular diseases, renal diseases, and neuropathies.[3] Insulin therapy is the most common option to manage hyperglycemia in diabetic patients.[4] However, the main challenges are multiple daily insulin injections and poor regulation of blood glucose concentrations, which may lead to side effects such as hypoglycemia, increased heart rate, and blurry vision.

In recent years, stem cell-based therapy has emerged as a promising treatment option for diabetes.[5] Stem cells (SCs) have the unique property of self-renewal and cell differentiation.[6] Despite the widespread use of SCs in regenerative medicine, there are certain unsolved issues for their clinical application, including tumorigenesis, immune rejection of the transplanted cells, the impact of *in vitro* culture affecting the phenotype of cells, and potentially significant impairment of *in vivo* cell proliferation and differentiation.[7, 8] The main challenge associated with the application of SCs in treating diabetes is the ability to produce a large number of homogeneous and efficient insulin-producing cells (IPCs).[9] Therefore, the optimization of differentiation protocols has been the main focus of researchers worldwide.[10] Sonic hedgehog (Shh) is a small molecule that plays a major role in tissue patterning during early embryogenesis.[11] Some studies found that Shh signaling increases the number of adult SCs.[12-14] These findings are in line with other studies reporting that Shh signaling potentially intervenes in the regeneration of new tissues.[13, 15] Evidence shows that Shh expression inhibits cell differentiation and tissue morphogenesis during the initial steps of pancreas formation.[14, 16] On the other hand, Shh is needed for late-stage pancreatic development, maintenance of mature β-cells, and the ability of β-cells to secrete hormone.[15, 17] Given the importance of Shh signaling in pancreatic development,[16, 18] some researchers have attempted to manipulate the Shh signaling pathway to produce efficient IPCs *in vitro*. However, there is no consensus on the exact role of the Shh signaling pathway in differentiating SCs into functional IPCs.[17-29] Moreover, it has yet to be confirmed whether the Shh signaling of mature β-cells remains active. The present study hence aimed to review *in vitro* and *in vivo* studies on the effect of inhibition or activation of Shh signaling pathway on the production, maintenance, and activity of IPCs.

Materials and Methods

A systematic review was conducted using all available experimental studies published between January 2000 and November 2022. The review was performed according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) and MOOSE (Meta-analyses of Observational Studies in Epidemiology) guidelines.[28, 29]

Search Strategy

A complete search was performed in Scopus, MEDLINE/PubMed Central, Embase, Web of Science (ISI), and Cochrane Library. The search keywords and phrases using medical subject headings (MeSh) were Sonic hedgehog protein (MeSh) OR *sonic hedgehog signaling* OR *SHH* OR "Hedgehog Signaling Regulation" OR "Sonic Hedgehog" OR "sonic hedgehog" OR "Sonic hedgehog pathway" OR "Shh" AND "Pancreatic B Cells (MeSh)" OR "Pancreatic Polypeptide-Secreting Cells" "Pancreatic beta Cells" OR "Insulin Secreting Cells" OR "Pancreatic B Cells" OR "Pancreatic beta Cells" AND "Stem Cells" OR "Embryonic Stem Cells" OR "Mesenchymal Stem Cells" OR "Pluripotent Stem Cells" OR "Induced Pluripotent Stem Cells" OR "IPS Cells". No language restriction was applied. In case of eligibility for a non-English language study, the required information was obtained through the translation of the article by a translation agency. To prevent missing data, the reference list of eligible articles was manually searched for additional information.

Given the high heterogeneity in the protocol and results of studies in this field, studies with similar objectives were included in the study to better understand the effect of manipulating the Shh pathway on IPCs. The inclusion criteria were manipulation of Shh in SCs, SCs differentiation into IPCs, and endocrine activity of mature IPCs. No restriction was applied in terms of used cell type or study design. Articles with incomplete data were excluded.

Data Extraction

Two of the authors (ME and DD) independently reviewed and screened the title and abstract of all selected articles. The extracted information included the name of the first author, country, sample size, type of study, type of cells, type of gene, intervention protocol and duration, shh inhibitor and activator, methods of evaluating the efficacy of Shh pathway manipulation, glucose response element activation following intervention, expression of pancreatic specific genes following an intervention, expression of pancreatic specific proteins following an intervention, and main findings (table 1). In case of disagreement between reviewers, a third reviewer was consulted to resolve the issue until a consensus was reached.

Table 1: Summary of the studies selected for the systematic review

Authors	Country	Type of study	Hh inhibitor	Hh activator	Type of cells	Methods	Protocol of intervention	Results
Thomas et al.[17]	USA	*In vitro*	Cyclopamine	pShh	INS-1 β-cells, MIN6 β-cells (ADC)	Plasmid construction, cell culture, immunohistochemistry, transfections, RT-PCR, southern blots, insulin assays, northern RNA blots, immunoprecipitations, and western immunoblots	Both clonal INS-1 and MIN6 β-cells were exposed to rat insulin I promoter-reporter constructs and pShh with or without cyclopamine.	Hh signaling regulates differentiated pancreatic β-cell endocrine activity, insulin production, and insulin secretion.
Thomas et al.[18]	USA	*In vitro*	Cyclopamine	pShh	INS-1 β-cells (ADC)	Plasmid construction, cell culture, transfections, nuclear extract preparation, western blot, DNA-binding assays, northern RNA blot	The Hh-responsive regions were located within the rat insulin I promoter that included the glucose-response elements Far (E2) and Flat (A2/A3).	Hh signals are required in differentiated β-cells of the endocrine pancreas for proper insulin production.
Mfopou et al.[19]	Belgium	*In vitro*	ES-CM removal	ES-CM Shh-N peptide	Mouse ES cells	ES cell culture, endoderm Induction, explants isolation, culture, RNA extraction, RT-PCR, immunocytochemistry, dot blotting, western blot, ELISA	ES cells (mouse 14 ES cell line) were exposed to EB-CM or Shh-N peptide with or without Hip.	Hh Signals are implicated in the inhibition of pancreas formation. However, EBs-secreted factors (Shh) promote the differentiation of hepatic progenitors.
Hui et al.[20]	USA	*In vitro*	Cyclopamine	NE	HUES1 (human ES cells)	Cell culture, transcriptional activity, RT-PCR, RIA	HUES1 cells were differentiated into IPCs using GLP1 (as differentiation inducer) with or without cyclopamine.	Co-treatment of ES cells with GLP1 and cyclopamine disrupted the effect of GLP1 in differentiation into IPCs.
Landsman et al.[21]	USA	*In vivo*	NE	Pdx1 overexpression	Pancreatic B-cells (ADC)	qPCR, ELISA, immunofluorescence, western blot	The Tamoxifen-treated Pdx1-CreER, CLEG2 male mice were studied for the evaluation of Hh overexpression in mature pancreatic β-cell endocrine function.	The pancreatic β-cells of Tamoxifen-treated Pdx1-CreER, CLEG2 mice showed impaired glucose-stimulated insulin secretion with a decreased level of specific pancreatic β-cell genes due to Hh overexpression.

Li et al.[22]	China	*In vitro*	shShh	NE	bmMSCs	Cell culture, flow cytometry, plasmid construct, qRT-PCR	The bmMSCs were differentiated from IPCs by concomitant repression of Rest/Nrsf and overexpression of Pdx1.	The reprogrammed bmMSCs expressed both genes and proteins specific to islet cells. These converted cells were capable of releasing insulin in a glucose-responsive manner.
Dayer et al.[23]	Iran	*In vitro*	Cyclopamine and b-FGF	Shh-N peptide	ADMSCs	Cell culture, RT-PCR, ELISA, RNA extraction	The ADMASCs were differentiated from IPCs through a three-step protocol utilizing Nicotinamide and ITS. The Shh pathway was inhibited on day three and reactivated on day 11 of the differentiation protocol.	IPCs obtained after Shh manipulation secreted higher amounts of insulin *in vitro*. This phenotype was accompanied by increased expression of both genes critical for β-cell function and transcription factors associated with their mature phenotype.
Hashemi Tabar et al.[24]	Iran	*In vitro* and *in vivo*	Cyclopamine and b-FGF	Shh-N peptide	ADMSCs	Cell culture, RT-PCR, RNA extraction, flow cytometry, DTZ staining, ELISA	The ADMASCs were differentiated from IPCs through a three-step protocol utilizing Nicotinamide and ITS. The Shh pathway was inhibited on day three and reactivated on day 11 of the differentiation protocol. The cells were transfected by pCDNA-Pdx1 at day 10 of differentiation.	The rats that received manipulated IPCs exhibited a higher ability to normalize blood glucose and insulin secretion when compared to controls. However, blood glucose concentration did not reach the normal level.
Wang et al.[25]	China	*In vitro*	Shh siRNA	NE	hADMSCs, hAMSCs	Cell culture, qRT-PCR, RNA extraction, flow cytometry, staining, ELISA, immunofluorescence, western blot, MTT	hADSCs and hAMSCs were differentiated into IPCs using NRSF siRNA and Shh siRNA.	The differentiated cells produced and released insulin in a glucose-responsive manner.

Yung et al.[26]	Canada	*In vitro*	NE	Sufu and Spop knockout	B-cells (ADC) Pancreatic organoids	Histology, immunohistochemistry, immunofluorescence, flow cytometry, qPCR, pancreatic organoid culture, hESC culture, and differentiation, glucose-stimulated insulin secretion assay, RNA sequencing	The Sufu and Spop were knocked out in pancreatic β-cells.	The Sufu and Spop from the mesenchymal gut niche inhibit Hh expression. Sufu and Spop down-regulate the Gli, Hh, and Wnt expressions. Sufu and Spop are required for pancreatic progenitor growth and pancreatic β-cells development.
Lee et al.[27]	Korea	*In vitro*	SANT1	NE	iPSC (reprogramed SCs)	Cell culture, teratoma analysis, immunostaining, qRT-PCR, insulin release assay, flow cytometry	The iPCs were differentiated through a three-stage protocol using some small molecules with or without SANT into IPCs.	iPCs formed spheroids from day five and continuously secreted insulin.

INS-1 β-cells: Insulinoma cell line; MIN6 β-cells: Insulinoma cell line; pShh: Plasmid containing sonic hedgehog gene; Hh: Hedgehog; Ptc: Patched; Smo: Smoothened; Ihh: Indian hedgehog; Dhh: Desert hedgehog; IDx1: Islet/duodenum homeobox-1; EB-CM: Embryoid body conventional medium; Shh-N peptide: N-terminus recombinant sonic hedgehog protein; ES: Embryonic SCs; IPCs: Insulin producing cells; GLP1: Glucagon like peptide 1; RT-PCR: Real-time PCR; ELISA: Enzyme-linked immune sorbent assay; Pdx1: Pancreatic and duodenal homeobox 1; Ngn3: Neurogenin 3; Isl1: Islet-1, Ptf1a: Pancreas transcription factor 1 alpha; Mist1: Muscle, intestine, and stomach expression-1; qPCR: quantitative real-time PCR; Gli1: Glioma-associated oncogene homologue 1; Sox9: SRY-box transcription factor 9; Hes1: Hairy and enhancer of split-1; MafA: Musculoaponeurotic fibrosarcoma oncogene homolog A; NeuroD1: Neurogenic differentiation factor 1; NKx6.1: NK6 homeobox1; Sox2: SRY-box transcription factor 2; Glut2: Glucose transporter 2; bmMSC: Bone marrow-derived mesenchymal stem cell; Rest: RE1 silencing transcription factor; NRSF: Neuron-restrictive silencer factor; shShh: Small hairpin RNA of Shh; Salc2a: Solute carrier 2a; Pax4: Paired box 4; Gcg: Proglucagon; Sst: Somatostatin; ADMSC: Adipose-derived mesenchymal stem cell; ITS: Insulin transferrin selenium; b-FGF: Fibroblast growth factor-2; NKX2.2: NK2 homeobox2; PEI@Fe3O4 NPN: polyethyleneimine coated Fe3O4 nanoparticles; SiRNA: Small interfering RNA; Shh: Sonic hedgehog; Wnt: Wingless-related integration site; Sufu: Suppressor of fused; Spop: Speckle type BTB/POZ protein; Axin: Axis inhibition protein 1; Ccnd2: Cyclin D2; Tcf4: Transcription factor 4; SANT1: Cell-permeable antagonist; iPCs: Induced pluripotent SCs; ADC: Adult pancreatic cells; SCs: Stem cells; NE: Not evaluated

The level of agreement between the two reviewers was assessed using Cohen's Kappa statistic.

Results

Literature Search: A total of 208 articles were identified, 53 through MEDLINE/PubMed Central, 70 through Scopus, 65 through ISI, 10 through ProQuest, and 10 through other sources (figure 1). Out of these, 11 articles were found to be eligible for the systematic review.

Type of Study: Except for two studies (18.18%),[21, 24] all other included studies were only conducted *in vitro*. All selected studies (100%) were case-control.

Type of Cells: Four studies (36.36%)[17, 18, 21, 26] evaluated mature pancreatic β-cells, and seven (63.63%)[19, 20, 22, 25] evaluated IPCs obtained from the differentiation of stem cells (table 1).

Protocols Used for Shh Inhibition: Nine studies (81.81%) included Shh inhibition in the study design. Shh suppression was done by the use of cyclopamine in five studies (45.45%),[17, 18, 20, 23, 24] cyclopamine plus fibroblast growth factor-2 (b-FGF) in two studies (18.18%),[23, 24] cell-permeable antagonist (SANT) in one study (9.1%),[27] Shh antibody in one study (9.1%),[19] hedgehog-interacting protein (Hip) in one study (9.1%),[19] Forskolin in one study (9.1%),[19] small hairpin RNA of Shh (Shh siRNA) in one study (9.1%),[22] embryonic stem cell-conditioned medium (ES-CM) removal in one study (9.1%),[19] and Shh small interfering RNA (SiRNA) in one study (9.1%)[25] (table 2).

Protocols Used for Shh Reactivation: Shh reactivation was performed using Shh plasmids in two studies (18.18%),[17, 18] 50 ng/mL Shh recombinant protein in one study (9.1%),[19] pancreatic and duodenal homeobox 1 (Pdx1) overexpression in one study (9.1%),[21]

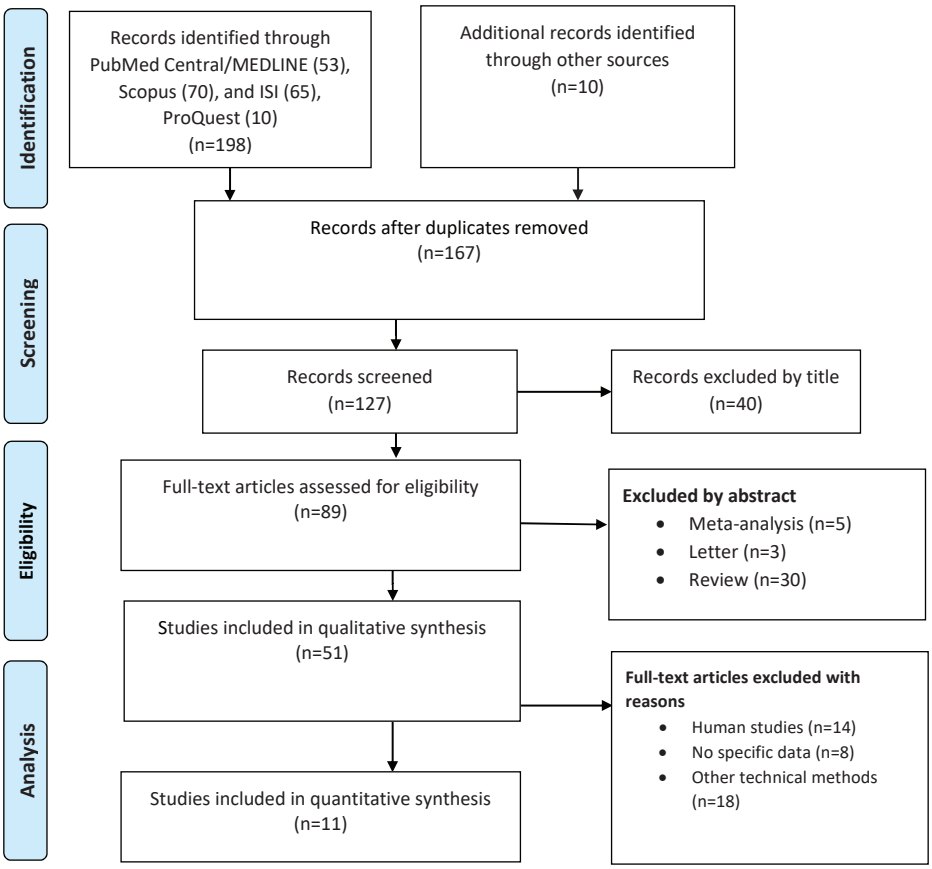

Figure 1: The flow diagram shows the study selection strategy according to PRISMA guidelines.

Table 2: The effect of an intervention on glucose response element activation, pancreatic-specific genes, and proteins

Author	Intervention duration (day)	The effect of intervention on glucose response element activation	The effect of intervention on pancreatic specific Genes	The effect of intervention on pancreatic-specific proteins	Main findings
Thomas et al.[17]	1	● Hh signaling increases Insulin1 glucose response element activity ● Cyclopamine decreases Insulin1 glucose response element activity	● Ptc, Smo, Ihh, and Dhh were expressed in INS-1 and pancreatic islets ● pShh increased IG expression ● Cyclopamine reduced IG expression	NE	Hh signaling defective in the pancreas should be considered in the pathogenesis of type 2 diabetes.
Thomas et al.[18]	1	● The multimerized Far/Flat enhancer-reporter construct was activated following the Hh expression.	● Significant increase in IDX1 gene expression following pShh utilization ● Significant decrease in IDX1 gene expression following pShh and cyclopamine administration	● Significant increase in IDX1 protein expression following pShh utilization ● Significant decrease in IDX1 protein expression following pShh and cyclopamine administration	Hh signaling regulates IDX-1 expression in the endocrine pancreas.
Mfopou et al.[19]	28	NE	● Exposing ES cells to EB-CM or Shh-N peptide reduced the expression of Ptf1a, Mist1, Insulin, and Amylase.	● Significant increase in Shh protein expression ● Significant elevation of Gli1 gene expression following exposure to Eb-CM	Hh production in EBs limits pancreatic fate acquisition and forms a major obstacle in the specification of pancreatic cells from ES-derived definitive endoderm.

Hui et al.[20]	21	NE	NE	● Cyclopamine reduces the Insulin protein expression.	GLP-1 directs human ES cell differentiation into IPCs via Hh, cyclic insulin adenosine monophosphate, and PI3K.
Landsman et al.[21]	28	NE	● Tamoxifen elevated the Hh, Gli1, Ptc2, Sox9, and Hes1 expression in pancreatic β-cells ● Tamoxifen reduced the Insulin, Pdx1, MafA, NeuroD1, Ngn3, NKX6.1 genes expression.	● Tamoxifen increased the expression of Sox2 protein in pancreatic β-cells ● Tamoxifen decreased expression of Glut2, Pdx1, and Insulin proteins	Deregulation of the Hh pathway impairs β-cell function by interfering with the mature β-cell differentiation state.
Li et al.[22]	1	NE	● Significant increase in Insulin gene expression in shShh+ Pdx1, shShh+sh Rest/ Nrsf and shShh+ sh Rest/Nrsf+ Pdx1 ● Significant elevated expression of Ngn3-NKX6.1, MafA, NeuroD1, Slac2a, Pax4, Gcg, Sst in shShh+ sh Rest/Nrsf+ Pdx1 group	● Significant elevation in insulin protein secretion in shShh+ sh Rest/Nrsf+ Pdx1 group	bmMSCs may ultimately be reprogrammed into functional insulin-secreting cells.
Dayer et al.[23]	14	NE	● Manipulated IPCs increased expression of MafA, Nkx2.2, Nkx6.1, Ngn3, insulin, and Isl1 genes	● Significantly elevated insulin protein expression by manipulated cells *in vitro*	Early inhibition and late reactivation of the Shh signaling pathway during the differentiation of ATDMSCs improved the functional properties of IPCs *in vitro*.
Hashemi Tabar et al.[24]	14	NE	● Manipulated IPCs exhibited significantly higher expression of MafA, Nkx2.2, Nkx6.1, Ngn3, insulin, and Isl1 genes	● Significantly elevated insulin protein expression by manipulated cells both *in vitro* and *in vivo*	Early inhibition and late reactivation of the Shh signaling pathway accompanied by Pdx1 improved functional properties of the required IPCs *in vivo*.
Wang et al.[25]	14	NE	● Significant increase in Pdx1, Ngn3, Pax4, and IG expression both in IPCs that differentiated from hADMSCs, hAMSCs	● Significant increase in insulin protein expression in IPCs that differentiated from hAMSCs	hADSCs and hAMSCs may be induced to differentiate into iPCs via PEI@Fe3O4 NP-mediated NRSF and SHH silencing.
Yung et al.[26]	23	NE	● Significant increase in Gli1 and Ptc1 expression following Sufu and Spop knockout ● Significant increase in Wnt2, Wnt2b, Wnt4, Wnt5a, Wnt9a, Axin2, Ccnd2, Ccnd1, and Tcf4 genes expression following Sufu and Spop knockout	NE	Organoid and human stem cell cultures demand Wnt and Shh pathways inhibition.

Lee et al.[27]	16	NE	● The higher expression of Pdx1, Ngn3, Sox9, NeuroD, NKX6.1. insulin, Glu genes following SANT1 administration	● Significant insulin protein expression following SANT1 administration	A simple differentiation method using small molecules that produced functional IPCs that responded to glucose stimulation within a relatively short period was developed.

INS-1 β-cells: Insulinoma cell line; MIN6 β-cells: Insulinoma cell line; pShh: Plasmid containing sonic hedgehog gene; Hh: Hedgehog; Ptc: Patched; Smo: Smoothened; Ihh: Indian hedgehog; Dhh: Desert hedgehog; IDx1: Islet/duodenum homeobox-1; EB-CM: Embryoid body conventional medium; Shh-N peptide: N-terminus recombinant sonic hedgehog protein; ES: Embryonic stem cells; IPCs: Insulin producing cells; GLP1: Glucagon-like peptide 1; RT-PCR: Real-time PCR; ELISA: Enzyme-linked immunosorbent assay; Pdx1: Pancreatic and duodenal homeobox 1; Ngn3: Neurogenin 3; Isl1: islet-1; Ptf1a: Pancreas transcription factor 1 alpha; Mist1: Muscle intestine and stomach expression-1; qPCR: quantitative real-time PCR; Gli1: Glioma-associated oncogene homologue 1; Sox9: SRY-box transcription factor 9; Hes1: Hairy and enhancer of split-1; MafA: Musculoaponeurotic fibrosarcoma oncogene homolog A; NeuroD1: Neurogenic differentiation factor 1; NKx6.1: NK6 homeobox1; Sox2: SRY-box transcription factor 2; Glut2: Glucose transporter 2; bmMSC: Bone marrow-derived mesenchymal stem cell; Rest: RE1 silencing transcription factor; NRSF: Neuron-restrictive silencer factor; shShh: Small hairpin RNA of Shh; Salc2a: Solute carrier 2a; Pax4: Paired box 4; Gcg: Proglucagon; Sst: Somatostatin; ADMSC: Adipose derived mesenchymal stem cell; ITS: Insulin transferrin selenium; b-FGF: Fibroblast growth factor-2; NKX2.2: NK2 homeobox2; PEI@Fe3O4 NPN: Polyethyleneimine coated Fe3O4 nanoparticles; SiRNA: Small interfering RNA; Shh: Sonic hedgehog; Wnt: Wingless-related integration site; Sufu: Suppressor of fused; Spop: Speckle type BTB/POZ protein; Axin: Axis inhibition protein 1; Ccnd2: Cyclin D2; Tcf4: Transcription factor 4; SANT1: Cell-permeable antagonist; iPCs: Induced pluripotent stem cells; NE: Not evaluated; IG: Insulin gene; PI3K: Phosphatidylinositol-3-kinase pathways

150 ng/mL Shh-N recombinant protein in two studies (18.18%),[23, 24] and suppressor of fused/speckle-type POZ protein (Sufu/Spop) inhibition in one study (9.1%)[26] (table 2).

Discussion

The results showed that Shh manipulation can be a useful treatment option for diabetes. Cyclopamine and tamoxifen were the key agents that effectively inhibited glycogenolysis. Cyclopamine inhibited the gluconeogenic pathway by reducing the expression of insulin and IDX1 genes. Similarly, tamoxifen inhibited the pathway by reducing the expression of Pdx1, insulin, MafA, NeuroD, Ngn3, and Knx1 genes.

The success of stem cell-based therapy in treating diabetes depends on the exact determination of signaling pathways that promote pancreatic development during embryogenesis.[30] In a previous study, primary protocols for differentiating embryonic stem cells into β-like cells were discarded to produce cells that could not go beyond the definitive endoderm stage.[31] It was reported that the inhibitory effect of some signaling pathways during the differentiation protocol has been the reason for the inability to produce IPCs.[32, 33] It is suggested that the Hedgehog pathway significantly inhibits IPCs generation.[14] The results of our study showed the benefits of manipulating the Shh signaling pathway in the production, maintenance, proliferation, and endocrine activity of IPCs. A previous study showed the importance of notochord signals in patterning the pancreatic endoderm.[34] Shh is described as a potent protein patterning many systems in humans. During early-stage embryogenesis, specific signals from the notochord inhibit Shh expression that leads to the formation of the pancreas.[35] Activin-βB and FGF2 are the well-known signals from the notochord that repress Shh and allow the formation of the pancreas.[36] Inhibition of Shh expression in the definitive endoderm stage results in pancreatic development.[37] A previous study showed the presence of active Shh in embryoid bodies (EB) extracts.[38] Embryonic bodies exposed to EB extracts have shown significantly reduced Amylase, Ptf1a, and Mist1, commonly expressed by fully differentiated pancreatic cells.[38] Mfopou and colleagues reported the presence of 200 pg/mL of Shh in EB conditional medium (EB-CM).[19] However, the concentration of recombinant Shh used to block EB differentiation to β-cells was equal to 2.5 µg/mL. The difference could be due to the lipid modification of Shh-N or the presence of IHH in EB-CM. Mfopou and colleagues also reported that EBs treated with Activin A overexpressed Shh and *Gli1*, whereas the expression of Pdx1 decreased. They concluded that the inhibition of hedgehog pathway is needed to differentiate ES-derived definitive endoderm cells from β-cells.[19]

Li and colleagues reported that bone marrow-derived stem cells efficiently differentiated into IPCs by simultaneous inhibition of RE1 silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) and Shh with and without overexpressing Pdx1.[22] In contrast to early-stage differentiation into β-cells that

require Shh inhibition, mature glucose-sensitive IPCs require Shh expression at late-stage differentiation.[23, 24] Thomas and colleagues showed that ectopic expression of Shh leads to the activation of glucose-sensing modules on the insulin promoter. However, Shh inhibition with cyclopamine decreased the activity of Far/Flat element (FAR-linked AT-rich).[17] In another study, Thomas and colleagues reported that Shh directly affects insulin I promoter. They showed a direct relationship between Shh activation and Pdx1 expression in mature β-cells. They proposed a direct effect of Shh on insulin production via direct activation of Pdx1 in pancreatic β-cells.[17, 18]

Some studies reported the central role of Pdx1 in insulin gene expression and alteration in pancreatic β-cell mass.[39-41] Pdx1 promotes normal pancreatic function by regulating some specific pancreatic gene expression. Pdx1 regulates the expression of insulin, somatostatin, islet amyloid polypeptide, glucose transporter type 2, and glucokinase by pancreatic β-cells.[42] Given the direct effect of Shh on altering Pdx1 activity, preservation of the exact concentration of Shh is essential in both differentiation of stem cells into β-cells and the maintenance of mature β-cells activity.[18-21] Hui and colleagues showed that insulin expression inhibited Shh expression, however, the expression of IHH was not affected.[20] Previous studies have reported that Shh transcripts were elevated on day six of EBs and gradually increased till day 18 of differentiation.[19, 20] Another study reported that the expression of Shh receptor (PTC) began at day 10 of differentiation into IPCs.[20] Using recombinant Shh at day 11 (of 14 days duration) of differentiation, Dayer and colleagues concluded that Shh reactivation at late-stage differentiation produced more efficient IPCs.[23] Another study further verified the positive synergistic effect of Pdx1 and Shh expression on the efficiency of IPCs at late-stage differentiation.[24] These findings confirm the importance of Shh signaling activation at late-stage differentiation into IPCs. However, the role of Shh in mature IPCs is disputed. An *in vitro* study of mature β-cells showed a direct effect of Shh on insulin I promoter that induced Pdx1 expression.[18] However, another *in vivo* study showed that elevated Hh signaling led to insufficient insulin secretion, glucose intolerance, and decreased functionality of mature β-cells in mice.[22] Furthermore, it was reported that Shh overexpression induced Sox9 and Hes1 expression by mature β-cells.[21] However, these two genes are expressed by precursors of β-cells, which are usually expressed by β-cell progenitors. Transgenic β-cells revealed reduced musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), neurogenic differentiation factor 1 (NeuroD), and neurogenin 3 (Ngn3), which are commonly involved in β-cells with normal and efficient activity.[21] The difference between Shh reactivation *in vitro* and *in vivo* may stem from the difference in the functionality of pancreatic β-cells and β-cell lines, and the different methods of genetic manipulation.[21]

The results of all included studies indicated that the production of normal β-cells with maximum activity depended on the exact adjustment of Shh concentration.[17-19] On the other hand, the production of mature β-cells depended on the inhibition of the Shh pathway at early-stage differentiation and the presence of Shh activation at late-stage differentiation.[23] However, after the expression of insulin by mature β-cells, Shh concentration decreased to baseline level and remained constant throughout the life span of β-cells.[21] Maintaining basal concentrations of Shh must be considered an essential factor for immortalization and functionality of mature β-cells.[17, 18] Therefore, the elevation of Shh within a specific range for optimal β-cells activity results in a higher insulin secretion capacity by pancreatic β-cells.[23] The Shh pathway activity is significantly reduced in diabetic patients.[40] In these patients, impairment of the AKT/GSK3β signaling pathway leads to insufficient Shh activity.[40, 41] Generally, Shh activates PI3K/AKT pathway which in turn regulates the Shh pathway through the GSK3β pathway.[42] It was reported that alteration in Shh pathway activity alleviates diabetic complications.[43] Qin and colleagues reported that administrating the Shh pathway receptor agonist improved neovascularization in diabetes.[44] Overall, the results of the included studies indicated that regulation of the Shh pathway is an important factor in early-stage pancreatic development. However, initiation and persistent endocrine activity of the pancreas requires continual basal concentrations of Shh.

As the main limitation of the present study, we could not assess the quality of the included studies since different versions of the same method had been used. This may have ultimately undermined the quality of the extracted data.

Conclusion

Given the effect of the Shh pathway on IPCs activity, accurate manipulation of the Shh signaling pathway can be an effective treatment option for diabetes. More information is required on the association between Shh and other signaling pathways in order to accurately

determine Shh concentrations at multiple stages during pancreatic formation, development, maturation, and endocrine activity. Further studies are required to better understand factors disrupting the Shh signaling pathway.

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Authors' Contribution

D.D: Study design, the search of electronic databases, data collection, and analysis, writing the draft manuscript. V.B: Data analysis, and review of the manuscript. M.E: Search of electronic databases, data collection, review of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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