REVIEW

Generating pancreatic β -cells from embryonic stem cells by manipulating signaling pathways

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Abstract

Type 1 diabetes results from an insufficiency of insulin production as a result of autoimmune destruction of the insulin-secreting pancreatic β -cells. It can be treated by transplantation of islets of Langerhans from human donors, but widespread application of this therapy is restricted by the scarcity of donor tissue. Generation of functional β -cells from embryonic stem (ES) cells *in vitro* could provide a source of an alternative graft material. Several ES cell differentiation protocols have reported the production of insulin-producing cells by mimicking the *in vivo* developmental stages of pancreatic organogenesis in which cells are transitioned through mesendoderm, definitive endoderm, foregut

endoderm, pancreatic endoderm, and the endocrine precursor stage, until mature β -cells are obtained. These studies provide proof of concept that recapitulating pancreatic development *in vitro* offers a useful strategy for generating β -cells, but current differentiation protocols employ a bewildering variety of growth factors, mitogens, and pharmacological agents. In this review, we will attempt to clarify the functions of these agents in *in vitro* differentiation strategies by focusing on the intracellular signaling pathways through which they operate – phosphatidylinositol 3-kinase, transforming growth factor β , Wnt/ β -catenin, Hedgehog, and Notch.

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Introduction

Type 1 (insulin-dependent) diabetes mellitus is a T cell-mediated autoimmune disease resulting in the destruction of insulin-secreting β -cells which are found exclusively in the pancreatic islets of Langerhans (Eiselein *et al.* 2004). It is a complex disorder whose pathogenesis involves B and T lymphocytes, macrophages, dendritic cells, and β -cell autoantigens, as well as multiple environmental and genetic components (Mandrup-Poulsen 2003, Yoon & Jun 2005). β -Cell destruction is thought to be induced by cytokines produced by activated β -cell-specific autoreactive T-cells (Eiselein *et al.* 2004). The ability of the endocrine pancreas to produce insulin decreases until insulin production is no longer sufficient to maintain whole-body glucose homeostasis, at which point the patient presents with the clinical symptoms of type 1 diabetes. In brief, reduced circulating insulin causes decreased cellular uptake of glucose by peripheral tissues as well as hepatic overproduction of glucose, both of which result in hyperglycemia and the associated glycosuria, polyuria, and polydipsia. In the UK, type 1 diabetes affects ~15–20 per 100 000 individuals a year with a relative

increase in incidence around 3% yearly. Worldwide, the increase in incidence ranges around the same levels, indicating that it will be 40% higher in 2010 compared to 1998 (Onkamo *et al.* 1999).

A substantial amount of research has focused on providing a permanent cure for diabetes by replacing the lost β -cells. At present, the most promising new therapies are based on replenishing the β -cell mass. The development of the Edmonton protocol for pancreatic islet transplantation proved that cadaveric human islets can be grafted to diabetic patients using a minimal, glucocorticoid-free immunosuppressive regimen and provide insulin independence, if only for a limited period of time (Shapiro *et al.* 2000, Ryan *et al.* 2005). However, the shortage of cadaver pancreata for islet isolation has resulted in the search for alternative sources of insulin-producing cells.

A variety of pluripotent cell types have been suggested as potential starting material from which to generate an unlimited supply of insulin-secreting cells (see Seaberg *et al.* 2004, Suzuki *et al.* 2004, Hori *et al.* 2005), but this review will focus on the progress that has been made using embryonic stem (ES) cells (Evans & Kaufman 1981,

Thomson *et al.* 1998). This area of research was initiated around a decade ago by proof-of-concept studies demonstrating the generation of insulin-expressing cells from mouse ES (mES) cells (Soria *et al.* 2000, Lumelsky *et al.* 2001). Lumelsky *et al.* (2001) described the first culture-based protocol for the *in vitro* generation of pancreatic cells from mESCs using a five-step protocol that was reported to generate insulin-expressing cells by selecting for nestin-positive progenitor cells. The reliability of this strategy was subsequently questioned when it was suggested that the insulin expression reflected uptake of insulin from the culture medium rather than *de novo* synthesis associated with insulin gene expression (Rajagopal *et al.* 2003, Hansson *et al.* 2004, Sipione *et al.* 2004, Paek *et al.* 2005). Despite this, several other groups modified the initial Lumelsky protocol and reported improved β -cell differentiation, although these protocols should be treated with some caution. For example, Hori *et al.* (2002) treated mESCs with an inhibitor of phosphatidylinositol 3-kinase (PI3K) and reported improved results over the original Lumelsky protocol. In a similar approach, Ku *et al.* (2004) increased the number of insulin-positive cells by the inclusion of activin β B, extendin-4, and nicotinamide to the culture medium.

Other groups tried to direct the differentiation of the mES cells by overexpressing transcription factors critical for β -cell development, such as *Pdx1* (Blyszczuk *et al.* 2003, Miyazaki *et al.* 2004), *Pax4* (Blyszczuk *et al.* 2003), or *Nkx2-2* (Shiroy *et al.* 2005), and reported the generation of insulin-expressing cells. This approach seemed to be the most promising strategy until recently, when safety concerns over therapeutic applications of genetically modified cells (Strulovici *et al.* 2007) led to a shift towards inducing differentiation solely by exposure to extracellular factors. D'Amour *et al.* at Novocell developed an *in vitro* differentiation protocol (Novocell protocol) using human ES cells (hESCs) that mimicked the *in vivo* developmental stages of pancreatic organogenesis. Thus, the aim was to provide sufficient cues to enable cells to transition through mesendoderm, definitive endoderm (DE), gut tube endoderm, pancreatic endoderm, and endocrine precursor stages, resulting in insulin-expressing cells with a reported insulin content approaching that of adult islets (D'Amour *et al.* 2006). Since then, several other groups devised similar differentiation protocols addressing the main developmental stages of the endocrine pancreas, especially definitive and pancreatic endoderm (Schroeder *et al.* 2006, Blyszczuk & Wobus 2007, Jiang *et al.* 2007a,b). More recently, the Novocell group has applied their protocol to generate endocrine precursors *in vitro*, and then grafted these progenitors into diabetic mice to promote their full maturation (Kroon *et al.* 2008), generating cells that secreted insulin at levels comparable to mature β -cells.

These proof-of-concept studies demonstrate that recapitulating *in vitro* the signals controlling the development of the endocrine pancreas *in vivo* offers a promising strategy for β -cell generation. To date, published β -cell differentiation protocols have used a bewildering variety of growth factors, mitogens, and pharmacological agents that act by exerting

transcriptional control on the developmental process. Table 1 lists some of these differentiation agents, their cellular targets and, where known, their site of action in the differentiation process. In this review, we will attempt to clarify the use of these media supplements by focusing on the specific signaling pathways through which they operate, predominantly the PI3K, transforming growth factor β (TGF β), Wnt/ β -catenin, Hedgehog, and Notch pathways. Understanding the roles of these pathways in the differentiation of ES cells to functional β -cells will enable the future development of more precisely defined and efficient *in vitro* differentiation protocols.

Modulators of signaling pathways

The PI3K signaling pathway

PI3Ks are a group of lipid kinases that have been implicated in numerous cellular processes controlling proliferation, apoptosis, DNA synthesis, cytoskeletal rearrangements, and cell migration. They are defined in three classes, based on their catalytic subunit. Class I are heterodimers, incorporating a catalytic as well as a regulatory subunit. Class II have a distinct C-terminal C2 domain, whereas Class III are the homologs of the yeast Vps34p protein. Once activated, PI3K generates the second messenger molecules phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol (3,4,5)-triphosphate. These molecules, in turn, transmit the signals downstream through various mediators, such as AKT and glycogen synthase kinase-3 β (GSK-3 β ; reviewed by Vanhaesebroeck *et al.* (2001)).

PI3K signaling and the generation of β -cells The PI3K signaling pathway has been shown to negatively regulate cellular differentiation in human and murine ES cells. Initially, this pathway was shown to be important in maintaining self-renewal of mESC cells through LIF-activated signaling (Paling *et al.* 2004). Consistent with this, it was later shown that activation of Akt, a major downstream component of this pathway, had similar effects in maintaining the pluripotency of murine and primate ES cells (Watanabe *et al.* 2006). More recently, McLean *et al.* (2007) have showed that suppression of PI3Ks facilitated the differentiation of hESCs into mesendoderm and DE under conditions in which activin–Nodal signaling was high. In an earlier study, suppressing the PI3K pathway was reported to promote endocrine differentiation of human fetal pancreatic cells (Ptasznik *et al.* 1997), perhaps suggesting an additional negative regulatory action of PI3K at a later stage in the β -cell differentiation pathway.

Modulators of the PI3K signaling pathway The involvement of PI3K signaling in ES cell differentiation suggests that manipulation of this pathway may enhance ES cell differentiation towards a pancreatic lineage, with PI3K inhibitors being used at an early stage to specify DE and/or at a later stage to promote differentiation of endocrine

Table 1 Biologically active factors that have been reported to influence pancreatic endocrine differentiation in established embryonic stem (ES) cell differentiation protocols, with the reported mode of action (if known)

Compound	Target	Species	Action	References
Activin A	TGF β	Human, mouse	ME and DE inducers	Kubo <i>et al.</i> (2004), D'Amour <i>et al.</i> (2005), Tada <i>et al.</i> (2005), Yasunaga <i>et al.</i> (2005), Jiang <i>et al.</i> (2007a,b), McLean <i>et al.</i> (2007), Phillips <i>et al.</i> (2007), Cho <i>et al.</i> (2008) and Zhang <i>et al.</i> (2009)
LY294002	PI3K	Human, mouse	Acts as late stage maturation factor and/or ME and DE inducers	Hori <i>et al.</i> (2002) and McLean <i>et al.</i> (2007)
Wortmannin	PI3K	Human, mouse	Acts as late stage maturation factor and/or DE inducer	Hori <i>et al.</i> (2002) and Zhang <i>et al.</i> (2009)
AKT1-II	PI3K	Human	ME and DE inducers	McLean <i>et al.</i> (2007)
TGF β 2	TGF β	Mouse	Increases percentage of <i>Pdx1</i> ⁺ cells	Shiraki <i>et al.</i> (2005)
IDE1, IDE2	TGF β	Human, mouse	DE inducers	Borowiak <i>et al.</i> (2009)
Wnt3 α	Wnt	Human	ME inducer	D'Amour <i>et al.</i> (2006), Cho <i>et al.</i> (2008) and Kroon <i>et al.</i> (2008)
Activin β B	Hedgehog	Mouse, human	Increases percentage of <i>Pdx1</i> ⁺ and <i>insulin</i> ⁺ cells	Ku <i>et al.</i> (2004) and Frandsen <i>et al.</i> (2007)
FGF2 (bFGF)	Hedgehog	Mouse	Acts to expand pancreatic progenitors and/or promote a pancreatic fate	Lumelsky <i>et al.</i> (2001), Miyazaki <i>et al.</i> (2004), Segev <i>et al.</i> (2004), Shi <i>et al.</i> (2005) and Jiang <i>et al.</i> (2007a,b)
HIP	Hedgehog	Mouse	Increases <i>Pdx1</i> and insulin expression	Mfopou <i>et al.</i> (2007)
Cyclopamine, KAAD-cyclopamine	Hedgehog	Human, mouse	Increases <i>Pdx1</i> and insulin expression	Skoudy <i>et al.</i> (2004), D'Amour <i>et al.</i> (2006), Serafimidis <i>et al.</i> (2008) and Cho <i>et al.</i> (2008)
DAPT	Notch	Human	Slight increase in <i>Ngn3</i> expression	D'Amour <i>et al.</i> (2006), Phillips <i>et al.</i> (2007) and Cho <i>et al.</i> (2008)
GLP-1, exendin	PI3K, Hedgehog, PKA	Mouse, rhesus monkey, human	Increases insulin and <i>Pdx1</i> expression and insulin ⁺ cells	Ku <i>et al.</i> (2004), Lester <i>et al.</i> (2004), Bai <i>et al.</i> (2005), Yue <i>et al.</i> (2006), Cho <i>et al.</i> (2008), Hui <i>et al.</i> (2009) and Zhang <i>et al.</i> (2009)
Retinoic acid	Retinoic acid signaling	Human, mouse	Induces <i>Pdx1</i> -expressing foregut endoderm	Micallef <i>et al.</i> (2005), D'Amour <i>et al.</i> (2006), Jiang <i>et al.</i> (2007b), Nakanishi <i>et al.</i> (2007), Shim <i>et al.</i> (2007), Cho <i>et al.</i> (2008), Kroon <i>et al.</i> (2008), Johannesson <i>et al.</i> (2009) and Zhang <i>et al.</i> (2009)
IGF2	IGF	Human	Acts as a late stage maturation factor	Jiang <i>et al.</i> (2007a) and Phillips <i>et al.</i> (2007)
Indolactam V	PKC	Human, mouse	Induces <i>Pdx1</i> expression	Chen <i>et al.</i> (2009)
Nicotinamide	poly(ADP-ribose)	Mouse, human	Acts as a late stage 'maturation' factor	Hori <i>et al.</i> (2002), Vaca <i>et al.</i> (2003), Ku <i>et al.</i> (2004), Jiang <i>et al.</i> (2007a,b), Cho <i>et al.</i> (2008), Naujok <i>et al.</i> (2008a,b) and Zhang <i>et al.</i> (2009)
Sodium butyrate	HDAC	Mouse, human	ME and DE inducers	Goicoa <i>et al.</i> (2006), Jiang <i>et al.</i> (2007a,b) and Haumaitre <i>et al.</i> (2008)
β -Cellulin	EGF	Human	Sustains <i>Pdx1</i> expression and induces islet maturation	Cho <i>et al.</i> (2008)

precursors. Consistent with this, Hori *et al.* (2002) were the first to show that two direct pan inhibitors of the PI3K enzymes, LY294002 and wortmannin, enhanced endocrine differentiation of mESCs when used at the final stage of the differentiation protocol. More recently, Zhang *et al.* (2009) have used wortmannin to specify DE from hESCs, while in another study, an isoform-specific AKT inhibitor (AKTI-II), as well as LY294002, was able to drive differentiation of hESCs into DE (McLean *et al.* 2007).

A number of other PI3K pan inhibitors have not yet been used in pancreatic differentiation protocols, including ZSTK474, PX-866, and SF1126, nor have a number of isoform-specific PI3K inhibitors, including imidazopyridine-related compounds, morpholino-substituted pyridopyrimidine, quinolone and benzopyranone derivatives, quinazolinones and methylxanthines (Ward *et al.* 2003, Ito *et al.* 2007). The PI3K pathway can also be targeted at different sites. Compounds such as AKTI-1, AKTI-1,2, and staurosporine have been shown to inhibit the AKT mediator (Barnett *et al.* 2005). Other agents such as rapamycin and its derivatives can target MTOR, which is a downstream component of AKT (Tsang *et al.* 2007), although targeting downstream pathway components may be less effective than direct inhibition of PI3K.

The TGF β signaling pathway

The TGF β signaling pathway is complex, involving almost 30 different growth and differentiation factors. The signaling molecules belonging to the TGF β superfamily have been subdivided into several subgroups, such as the bone morphogenetic protein, the Nodal, and the TGF β subgroups. This pathway has been implicated in numerous cellular processes including proliferation, differentiation, apoptosis, cell migration, and adhesion (Massagué & Chen 2000). TGF β signaling is mediated through two types of transmembrane serine/threonine kinase receptors, namely type I and type II. Binding of TGF β family members to type II receptors results in phosphorylation and activation of type I receptors. Once activated, type I receptors phosphorylate specific intracellular mediators known as SMAD proteins, which act as signal transducers and translocate to the nucleus, where they regulate gene transcription (Massagué 1998).

TGF β signaling and the generation of β -cells The TGF β pathway is central to the generation of mesendoderm and DE. Nodal ligands, which signal through the Actr1b (listed as *Acvr1b* in the MGI Database) and ALK7 type I receptors as well as the Actr1Ia (*Acvr2a*) and ActRIIB type II receptors (Schier & Shen 2000), are thought to act as mesendoderm inducers. Thereafter, mesendoderm commitment to mesoderm or DE is thought to depend on the level of Nodal signaling, with high levels promoting an endodermal fate (Tremblay *et al.* 2000, Vincent *et al.* 2003). However, it is important to note that Nodal signaling can specify DE only when PI3K signaling is low, as described in section 'PI3K

signaling and the generation of β -cells' (D'Amour *et al.* 2005, McLean *et al.* 2007). The Nodal pathway can also be activated by activin, which is known to signal through the same receptors (Chen *et al.* 2006). Consistent with this, several studies have shown that exogenous administration of activin A promotes DE differentiation of both mouse (Kubo *et al.* 2004, Tada *et al.* 2005, Yasunaga *et al.* 2005) and hESCs (McLean *et al.* 2007). As a result, this compound has been incorporated into several pancreatic differentiation protocols and, in conjunction with low PI3K signaling, has served as a first step to drive pluripotent cells towards DE formation (Shi *et al.* 2005, D'Amour *et al.* 2006, Jiang *et al.* 2007a,b, Phillips *et al.* 2007, Cho *et al.* 2008, Zhang *et al.* 2009).

Little is known about the possible roles of other TGF β family members in promoting pancreatic differentiation. It was reported some time ago that TGF β 1 (TGFB1) enhanced the development of endocrine cells from mouse pancreatic fetal cells, with a more pronounced increase in β - and PP-cells (Sanvito *et al.* 1994), and this effect was subsequently reproduced by Tei *et al.* (2005). Another study showed that TGF β 2 (TGFB2) induced differentiation of mESCs into *Pdx1*-expressing endodermal cells (Shiraki *et al.* 2005), but several other growth factors from this family, including TGF α and TGF β 1, did not have any significant effect on promoting *Pdx1* expression. In addition, the *Pdx1*-expressing cells induced by TGF β 2 expressed endodermal markers, but did not express insulin or glucagon, suggesting that TGF β 2 may be useful to promote an endodermal fate at an early stage in a differentiation protocol, whereas TGF β 1 may be useful after the specification of pancreatic or endocrine progenitors to enhance development of endocrine cells.

The importance of TGF β signaling in defining DE was supported by a recent study in which two small molecules, named IDE1 and IDE2, were shown to direct differentiation towards the endodermal lineage with very high efficiency (Borowiak *et al.* 2009). Both molecules act by activation of the TGF β signaling pathway, through SMAD2 phosphorylation, to drive ~80% of mESCs and 60% of hESCs into DE, a higher efficiency than that achieved by activin A.

The Wnt/ β -catenin signaling pathway

The Wnt/ β -catenin pathway (Fig. 1) plays a key role in the development of many organs such as brain (McMahon & Bradley 1990, McMahon *et al.* 1992), kidney (Kispert *et al.* 1998, Itäranta *et al.* 2006), and pancreas (Wells *et al.* 2007). This pathway is activated when Wnt ligands bind to specific cell surface receptors, called frizzled (Fzd), leading to activation of the intracellular Ca^{2+} , the planar cell polarity, or the β -catenin/canonical branch of the pathway. In the latter case, Fzd activation induces phosphorylation of intracellular proteins called dishevelled (Dvl), which, in turn, block the action of the GSK-3 β enzyme which normally phosphorylates β -catenin. As a result of this, unphosphorylated β -catenin accumulates in the cytoplasm and is translocated into the nucleus, where it forms a complex

Figure 1 The Wnt signaling pathway. Wnt ligands bind to the frizzled receptor (Fzd) and activate the cytoplasmic protein dishevelled (Dvl) to inhibit the activity of glycogen synthase kinase-3 β (GSK3 β) or GSK3B. Gsk3 β phosphorylates cytoplasmic β -catenin, targeting it for degradation at the proteasome. Inhibition of GSK3 β activity therefore stabilizes the cytoplasmic pool of β -catenin, some of which then translocates to the nucleus to interact with members of the T-cell factor (TCF) family of transcription factors, leading to changes in the transcription of Wnt target genes. A variety of growth factors (GF), such as insulin, insulin-like growth factor 1, and epidermal growth factor, bind to specific receptors (GFR) and activate phosphatidylinositol 3-kinases (PI3K), which can interact with the Wnt signaling pathway at the level of GSK3 β . However, it is unclear whether the same subcellular pool of GSK3 β can be targeted by both PI3K and Wnt. The schematic also shows pharmacological agents that influence developmental processes through actions at specific stages of the Wnt signaling pathway (see main text for details).

with T-cell factor (Tcf)/lymphoid enhancer factor (Lef) proteins (such as TCF1, LEF, TCF3, and TCF4), as well as co-activators, to activate transcription of target genes (Peifer & Polakis 2000, Logan & Nusse 2004).

GSK3 β is also a component of the PI3K pathway, although it is unclear whether the same subcellular pool of GSK3 β is targeted by both PI3K and Wnt. Several studies indicate that these two pathways crosstalk at the GSK3 β level, suggesting that there is a single pool of the enzyme (Fukumoto *et al.* 2001, Almeida *et al.* 2005, He *et al.* 2007). However, a recent study has showed that GSK-3 β could only be phosphorylated by PI3K when it is not bound to axin, whereas axin-bound GSK3 β was dedicated to Wnt signaling (Ng *et al.* 2009).

The Wnt/ β -catenin pathway and the generation of β -cells Defining a precise role for Wnt signaling in the development of the endocrine pancreas is confounded by the complexity of the Wnt signaling pathway (Fig. 1), and the possibility of crosstalk between the canonical and non-canonical pathways. However, Wnt signaling has been implicated in both mesendoderm and foregut endoderm specification. Bakre *et al.* (2007) showed that activation of the pathway in mouse and hESCs causes mesendoderm-specific differentiation and generation of mesendodermal progenitor cells that can differentiate along various lineages. Similarly,

Marikawa *et al.* (2008) reported that Wnt/ β -catenin signaling was sufficient to induce the formation of mesendoderm in mouse embryonic carcinoma cells. In addition, a study by McLin *et al.* (2007) implicated Wnt signaling in foregut endoderm specification in *Xenopus* embryos. Thus, repressing Wnt signaling in the anterior endoderm was required for maintaining foregut fate, whereas high levels of Wnt signaling in the posterior endoderm enhanced intestinal development. They also postulated that Wnt factors from mesoderm can signal to the posterior endoderm to activate specific gene programs that inhibit foregut development, but cannot signal to the anterior endoderm, which is protected by secreting several Wnt antagonists. No similar studies have yet been carried out in other vertebrates, but several lines of evidence suggest that this is also the case in mice. For example, Wnt overexpression in the *Pdx1* domain severely affects subsequent pancreatic development (Heller *et al.* 2002). In addition, deletion of *Tcf1* (listed as *Hnf1a* in the MGI Database) and *Tcf4* causes defects in hindgut development (Gregorieff *et al.* 2004), whereas expression of *Tcf3* can be detected in the posterior but not the anterior DE (Merrill *et al.* 2004).

Modulators of the Wnt/ β -catenin pathway Given its role in mesendoderm and possibly in foregut endoderm specification, Wnt signaling should be activated at an early stage in an ES cell differentiation protocol and inhibited at a later stage, whereas a sustained activation of the pathway would be expected to enhance the formation of mesendoderm. This could be accomplished by using purified natural or recombinant Wnt-secreted proteins. So far, only three studies have employed Wnt agonism to generate mesendoderm from hESCs, and all of them have used Wnt3a (D'Amour *et al.* 2006, Cho *et al.* 2008, Kroon *et al.* 2008). However, more than 20 Wnt-secreted proteins have been identified, but their mechanisms of action are not well understood and their effects can be cell type and tissue specific (Naylor *et al.* 2000). Proteins which signal through β -catenin, such as WNT1, WNT2, WNT3, and WNT3a (van Gijn *et al.* 2002), would be predicted to be useful in endocrine pancreas differentiation protocols. Moreover, small-molecule Wnt agonists could also prove useful for this purpose. A large number of GSK3 inhibitors have been reported. Examples include maleimides such as SB-216763 (Coghlan *et al.* 2000) and bis-7-azaindolymaleimide (Kuo *et al.* 2003), indirubins such as indirubin-3'-monoxime (Leclerc *et al.* 2001) and 6-bromoindirubin-3'-oxime (Meijer *et al.* 2003), pyrazolopyrimidine and benzimidazole-pyrazolopyrimidine derivatives (Peat *et al.* 2004a,b) as well as lithium ions (Stambolic *et al.* 1996), AR-A014418 (Bhat *et al.* 2003) and CHIR 98014 (Ring *et al.* 2003). However, these are not specific to GSK3 β and can also inhibit GSK3 α and other kinases, so more specific Wnt inhibition may be achievable by targeting other pathway components. For example, WAY-316606 specifically inhibits the Wnt antagonist sFRP-1 (Bodine *et al.* 2009), whereas QS11 synergizes with Wnt3a by binding to and inhibiting an ARFGAP protein, possibly

resulting in increased β -catenin translocation (Zhang *et al.* 2007). Another compound, named compound 1, has been shown to induce Wnt signaling in a GSK3 β -independent manner (Liu *et al.* 2005).

Although the available evidence also suggests an important role for Wnt suppression in the formation of posterior foregut endoderm, inhibition of the Wnt pathway has not yet been employed in *in vitro* differentiation protocols designed to generate endocrine pancreas. There is a long list of compounds that can inhibit this pathway at different levels. Some are endogenous secreted Wnt antagonists, including Dickkopfs (Dkks), Wnt inhibitory factor-1 (WIF-1), and secreted Fzd-related proteins (sFRPs). The most promising candidates include Dkk-1 and 4, which exert their actions by binding to a component of the Wnt receptor and preventing its activation, as well as sFRP2, sFRP3, and WIF-1, which bind to Wnt ligands (Kawano & Kypta 2003). Several natural and synthetic small-molecule inhibitors have also been identified, primarily because of their potential use as anti-cancer agents. For example, a high-throughput screening assay identified six natural compounds which prevented the formation of the Tcf/ β -catenin complex, and the efficacy of these compounds was subsequently confirmed both *in vitro* and *in vivo* (Lepourcelet *et al.* 2004). Another Tcf/ β -catenin complex inhibitor, PNU-74654, was identified by virtual screening of 17 700 synthetic compounds and shown to be active *in vitro* (Trosset *et al.* 2006). Another synthetic Wnt inhibitor, ICG-001, was reported to reduce Wnt signaling via binding to a transcriptional co-activator of β -catenin (Emami *et al.* 2004), while two other compounds, named NSC668036 and FJ9, were shown to disrupt the interaction between Fzd receptors and the Dvl PDZ domain (Shan *et al.* 2005, Fujii *et al.* 2007). The abundance of pharmacological modifiers of the Wnt signaling pathway (see Fig. 1) makes this an attractive target for inclusion in future *in vitro* differentiation protocols, as shown in Fig. 2.

The Hedgehog signaling pathway

The *Hedgehog* gene was identified almost 30 years ago and named after its ability to induce a hedgehog-like appearance in *Drosophila* (Nusslein-Volhard & Wieschaus 1980). Subsequent studies led to the identification of the Hedgehog homologs in other species, including mouse and human (Marigo *et al.* 1995). In vertebrates, three secreted hedgehog

proteins have been identified – Desert, Indian, and Sonic Hedgehog (Echelard *et al.* 1993) – and it is now known that these proteins play vital roles in directing cell differentiation during embryonic development and organ formation. Hedgehog signaling is initiated when one of these ligands binds to specific cell surface receptors called Patched (Ptc). This leads to activation of Smoothened (Smo), a G-protein coupled-like receptor. Thereafter, signaling is mediated through a multiprotein complex, the Hedgehog signaling complex, which modifies the activity of Gli transcription factors in order to regulate expression of target genes (Villavicencio *et al.* 2000).

Hedgehog signaling and the generation of β -cells

The Hedgehog pathway has an important role to play in early pancreas development. Repression of Sonic hedgehog expression in the foregut region is essential for specification of the pancreatic domain. This repression occurs through activin β B and FGF2 signals, which are secreted by the notochord, thereby permitting expression of pancreatic transcription factors, such as *Pdx1*. Consistent with this, isolated notochord or purified activin β B and FGF2 have similar effects when applied to foregut endoderm cultures *ex vivo* (Hebrok *et al.* 1998). In accordance with this, increased Hedgehog activity during development impairs pancreas formation (Apelqvist *et al.* 1997, Kawahira *et al.* 2005), while inhibition of Hedgehog signaling causes an enlargement in islet and pancreas mass (Kim & Melton 1998, Hebrok *et al.* 2000). Hedgehog signaling may also be involved in the maintenance of pancreatic endocrine function. Thus, Thomas *et al.* (2000) used the INS-1 β -cell line to demonstrate that downregulation of hedgehog signaling decreased insulin secretion as a result of reduced insulin gene transcription. In a subsequent study, it was also shown that *Pdx1*, a key inducer of insulin expression, has Hedgehog-responsive regions within its promoter, and its expression is downregulated in the absence of Hedgehog. Conversely, ectopic Hedgehog expression was reported to increase both insulin and *Pdx1* expression (Thomas *et al.* 2001).

Modulators of the Hedgehog signaling pathway The developmental evidence suggests a dual role for Hedgehog signaling in a differentiation protocol for the generation of endocrine pancreas, with an initial inhibition of the pathway

Figure 2 A proposed differentiation protocol for the generation of β -cells from embryonic stem cells. Manipulating the signaling cascades as shown is predicted to drive differentiation along the sequence of ES, embryonic stem cells; ME, mesendoderm; DE, definitive endoderm; PF, posterior foregut; PE, pancreatic endoderm; IP, islet precursors; BC, insulin-expressing β -cells. The role of PI3 and Shh (sonic hedgehog pathway) at the final stage of differentiation is not well understood (see main text for details).

at an early stage of ES differentiation, followed by reactivation at a later stage.

The optimal timing for Hedgehog inhibition would be after DE has formed and Wnt signaling has been down-regulated. In accordance with this, Shh is highly upregulated during embryoid body formation and DE induction, leading to repression of *Pdx1* expression (Mfopou *et al.* 2005, 2007), implying that Hedgehog inhibitors would be beneficial at this stage. However, it is not immediately apparent which inhibitors would be most effective. Thus, when the Hedgehog inhibitor activin β B was applied during a mESC differentiation protocol at a late stage, it was reported to increase the percentage of insulin-expressing cells (Ku *et al.* 2004). In a later study, the same compound was shown to induce expression of the *Pdx1* gene in cultured hESCs, but at the same time increased Shh expression (Frandsen *et al.* 2007). These unexpected results might indicate that the effects of activin β B are cell and tissue specific, whereas its differentiation-promoting activity might be Hedgehog-independent. In contrast, FGF2 (or bFGF) has been used extensively in differentiation protocols to expand pancreas progenitors and promote a pancreatic fate (Lumelsky *et al.* 2001, Miyazaki *et al.* 2004, Segev *et al.* 2004, Shi *et al.* 2005, Jiang *et al.* 2007a,b). It should be noted, however, that the effects of FGF2 seem to be concentration-dependent. In chicks, low concentrations of FGF2 increased *Pdx1* and insulin expression and downregulated Shh expression, whereas high concentrations had the opposite effects (Hebrok *et al.* 1998). Other Hedgehog repressors that have been used in differentiation studies include Hedgehog-interacting protein (HIP) and cyclopamine. HIP is an endogenous hedgehog modulator which reduces Hedgehog signaling by direct binding to Hedgehog proteins (Chuang & McMahon 1999) and which has been reported to be efficient in increasing the percentage of insulin-positive cells in mESC cultures (Mfopou *et al.* 2007). Cyclopamine and its more potent derivative KAAD-cyclopamine are steroidal alkaloids known to block Smo, thereby preventing downstream signaling of the pathway (Taipale *et al.* 2000). Their efficiency has been demonstrated in both mouse (Skoudy *et al.* 2004, Serafimidis *et al.* 2008) and human pancreatic differentiation protocols (D'Amour *et al.* 2006, Cho *et al.* 2008). Several small-molecule inhibitors of the Hedgehog pathway could also prove useful in the derivation of β -cells from ES cells, including steroidal alkaloid inhibitors such as jervine, AY9944, triparanol, and U18666A (Cooper *et al.* 1998, Incardona *et al.* 2000). Chen *et al.* (2002) identified four naturally occurring small molecules (SANT-1 to 4) that were shown to directly inhibit Smo activity, as does a synthetic aminoproline named CUR61414 (Williams *et al.* 2003). Other agents, such as forskolin, 3-isobutyl-1-methylxanthine, and dibutyryl-cAMP (db-cAMP), act by increasing the activity of protein kinase A, which is known to antagonize Hedgehog signaling (Frank-Kamenetsky *et al.* 2002). Hedgehog downregulation was also shown to be promoted by JK184, an inhibitor of alcohol dehydrogenase

7 (ADH7), which is assumed to be correlated with Hedgehog signaling (Lee *et al.* 2007).

The reactivation of the Hedgehog pathway at a relatively late stage in the differentiation process may enhance insulin production after the endocrine progenitor stage. Hedgehog agonism can be achieved using purified recombinant SHH, IHH, or DHH proteins, all of which have been used *in vitro* (Dyer *et al.* 2001, Mfopou *et al.* 2007). Synthetic small-molecule agonists have also been identified, including SAG and Hh-Ag 1:1–1:5. SAG is a chloroben-zothiophene, which acts as an agonist at low concentrations but as an antagonist at higher concentrations (Chen *et al.* 2002). Hh-Ag 1:1 was identified by screening a library of 140 000 synthetic compounds and was reported to promote Hh signaling by stabilizing Smo. Subsequently, several other Hh-Ag 1:1 derivatives were synthesized. Hh-Ag 1:2 was shown to be the most stable, whereas Hh-Ag 1:3 showed the lowest toxicity in embryonic tissue cultures (Frank-Kamenetsky *et al.* 2002). In addition, purmorphamine, a 2,6,9-trisubstituted purine molecule, is a Hedgehog agonist which is thought to act by activating Smo or another upstream protein (Wu *et al.* 2004).

The Notch signaling pathway

The notch signaling pathway is a critical regulator of tissue development and cell fate decisions during embryogenesis. This pathway acts by either inhibiting or inducing the spread of cellular differentiation, known as lateral inhibition or induction respectively. Notch signaling is also used in an iterative way to regulate consecutive cell fate decisions required for the formation of specialized tissues. Signaling is initiated when transmembrane Notch ligands, belonging to the Delta–Serrate–Lag2 family, bind to Notch receptors. Upon stimulation, the receptor is proteolytically processed by the sequential actions of two enzymes: tumor necrosis factor converting enzyme and γ -secretase, leading to the release of the Notch intracellular domain (NICD), which translocates to the nucleus and interacts with CBF1/SuH/Lag1 (CSL) transcription factors to initiate expression of target genes, including the basic helix-loop-helix Hes and Hey genes (Bray 2006).

Notch signaling and the generation of β -cells Notch signaling is important for regulating the expression of neurogenin 3 (*Ngn3* or *Neurog3*), a critical transcription factor for the formation of pancreatic endocrine cells. *Ngn3*-expressing cells are considered to be islet progenitors (Apelqvist *et al.* 1999, Gu *et al.* 2002), and lack of *Ngn3* expression leads to complete ablation of the endocrine component in developing mice (Gradwohl *et al.* 2000). Notch signaling represses transcription of *Ngn3* through Hes activation to prevent premature endocrine differentiation. This step serves to expand the pool of pancreatic progenitors before differentiation is initiated and notch inhibition results in *Ngn3* expression, and further differentiation towards the

endocrine fate (Apelqvist *et al.* 1999, Jensen *et al.* 2000). Consistent with this, mouse embryos overexpressing *Ngn3* or lacking the CSL or Delta-like1 mediators or both *Hes1* alleles show an accelerated endocrine differentiation coupled with pancreatic hypoplasia (Apelqvist *et al.* 1999, Jensen *et al.* 2000). Conversely, sustained notch activity strongly represses endocrine differentiation (Hald *et al.* 2003, Murtaugh *et al.* 2003).

Modulators of the Notch signaling pathway Given its role in pancreas development, repressing the notch pathway *in vitro* would be expected to enhance differentiation towards a β -cell fate. Notch repressors could be employed after Hedgehog downregulation and subsequent *Pdx1* activation, in accordance with the known developmental process *in vivo*. However, in three studies employing hESCs that incorporated notch inhibition in their differentiation protocols (D'Amour *et al.* 2006, Phillips *et al.* 2007, Cho *et al.* 2008), this inhibition had only a slight impact on promoting endocrine differentiation. Both of these studies used *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), which was added after *Pdx1* activation. DAPT belongs to a class of compounds called γ -secretase inhibitors, which, as the name suggests, inhibit notch signaling by preventing activation of the γ -secretase enzyme which, in turn, blocks NICD release (De Strooper *et al.* 1999). However, not all γ -secretase inhibitors specifically affect notch signaling (Lundkvist & Näslund 2007), and γ -secretase inhibitors other than DAPT may prove to be more efficient within the context of β -cell differentiation. The first γ -secretase inhibitors, including the peptidyl aldehydes MG-132 and MDL-28170, were identified more than a decade ago (Higaki *et al.* 1995, Kopan *et al.* 1996). Soon thereafter, a difluoroketone chemical, named MW167 (sometimes referred to as compound 1), was also shown to inhibit γ -secretase activity (Wolfe *et al.* 1998). All of these compounds also tested positive for Notch inhibition, either *in vivo* (Taniguchi *et al.* 2002) or *in vitro* (Beck & Slack 2002). More recent inhibitors that are also known to modulate Notch include a hydroxyethylene peptidomimetic named L-685,458 (Shearman *et al.* 2000, Sernee *et al.* 2003) together with its homologous counterpart III-31-C (Li *et al.* 2000, Esler *et al.* 2002), the structurally related compounds D and E (Seiffert *et al.* 2000, Beher *et al.* 2001), CBAP (Beher *et al.* 2001), LY411575 (Dovey *et al.* 2001, Wong *et al.* 2004), a dibenzazepine and benzodiazepine (Milano *et al.* 2004), and a group of epoxides consisting of compounds IL, ILX, and KILX (Piper *et al.* 2003). The abundance of pharmacological inhibitors of Notch signaling makes this another attractive target for inclusion in future *in vitro* differentiation protocols, as shown in Fig. 1.

Other modulators

A number of biologically active compounds that have been used in published endocrine pancreas differentiation

protocols cannot be classified under the 'signaling pathway' scheme described in this review, either because they operate through alternative pathways or because their mode of action is uncertain.

Glucagon-like peptide-1 (GLP-1) agonists are thought to promote β -cell differentiation by acting through several intracellular pathways such as the PI3K, the Hedgehog, the Ras/MAPK, the PKA, and the MEK/ERK pathway to modify the expression of β -cell transcription factors, including *Pdx1* (Buteau *et al.* 1999, Montrose-Rafizadeh *et al.* 1999, Wang *et al.* 1999, Zhou *et al.* 2002, Hui *et al.* 2009). Recent studies have shown that the GLP-1 peptide and exendin (a potent GLP-1 agonist) can increase the percentage of insulin-expressing cells in mouse (Ku *et al.* 2004, Bai *et al.* 2005), rhesus monkey (Lester *et al.* 2004), and hESC pancreatic differentiation protocols (Yue *et al.* 2006, Cho *et al.* 2008, Hui *et al.* 2009, Zhang *et al.* 2009). Insulin-like growth factor 2 also has a known role in β -cell neogenesis and islet hyperplasia (Petrik *et al.* 1999, Calderari *et al.* 2007), and has been used at late stages of the differentiation process in the studies of β -cell generation from hESCs (Jiang *et al.* 2007a, Phillips *et al.* 2007).

Retinoic acid is essential for pancreatic development through the induction of *Pdx1* expression (Martin *et al.* 2005, Molotkov *et al.* 2005) and promoting the generation of *Ngn3*⁺ endocrine progenitors (Öström *et al.* 2008). Consistent with this, mouse (Micallef *et al.* 2005, Nakanishi *et al.* 2007) and human (D'Amour *et al.* 2006, Jiang *et al.* 2007b, Shim *et al.* 2007, Kroon *et al.* 2008, Johannesson *et al.* 2009, Zhang *et al.* 2009) ES cell pancreatic differentiation protocols that incorporate retinoic acid result in the induction of a pancreatic endoderm-like stage.

Some biologically active molecules have been used in ES differentiation protocols with little insight on their mechanisms of action, including indolactam V, nicotinamide, sodium butyrate, and β -cellulin. Indolactam V is thought to promote differentiation at least, in part, by activating protein kinase C signaling. It was reported to induce expression of *Pdx1* from mouse and hESCs, and this effect was enhanced in the presence of FGF10 (Chen *et al.* 2009). Nicotinamide has been reported to promote endocrine differentiation in fetal pancreatic cells (Otonkoski *et al.* 1993), and has been widely used in *in vitro* differentiation protocols, either to induce pancreatic differentiation in undifferentiated mESCs (Chen *et al.* 2008, Vaca *et al.* 2008) or, more commonly, as a 'maturation factor' in the later stages of mouse (Hori *et al.* 2002, Vaca *et al.* 2003, Ku *et al.* 2004, Naujok *et al.* 2008a,b) and hESC pancreatic differentiation protocols (Jiang *et al.* 2007a,b, Cho *et al.* 2008, Zhang *et al.* 2009). The precise mode of action of nicotinamide as a morphogen is uncertain, but some of its effects may be mediated through the inhibition of poly(ADP-ribose) synthase which is thought to cause chromatin rearrangements and changes in gene transcription (Otonkoski *et al.* 1993). β -Cellulin has been used in a study by Cho *et al.* (2008), where it was reported to act synergistically with nicotinamide to sustain *Pdx1* expression and induce

pancreatic endoderm and islet maturation. Its effects might be due to activation of the epidermal growth factor pathway (Ishiyama *et al.* 1998).

Finally, a number of biologically active molecules have been shown to influence pancreatic differentiation but have not yet been incorporated into protocols to drive ES cells to an endocrine pancreas phenotype. One example is stauprimide, which has been shown to promote a DE fate in both mouse and hESCs by interacting with the NME2 protein, causing c-Myc downregulation (Zhu *et al.* 2009). Another example is conophylline, a vinca alkaloid (Umezawa *et al.* 1994) that has been reported to promote differentiation of murine bone marrow mesenchymal cells (Hisinaga *et al.* 2008) as well as rat pancreatic acinar carcinoma and pancreatic precursor cells (Umezawa *et al.* 2003, Ogata *et al.* 2004, Kitamura *et al.* 2007) into insulin-producing cells. Its effects are possibly due to induction of the *Ngn3* transcription factor by a p38-dependent mechanism (Umezawa *et al.* 2003). Another compound, trichostatin A (TSA), which is a hydroxamic acid histone deacetylase inhibitor (HDACi), has been shown to promote differentiation in human endometrial adenocarcinoma cells (Uchida *et al.* 2005) and promyelocytic leukemia cells (Kitamura *et al.* 2000), and to induce insulin expression in mouse bone marrow stem cells (Tayaramma *et al.* 2006). Similarly, other hydroxamic acid HDACis, such as SAHA and pyroxamide, have been shown to induce terminal differentiation in various cell lines including murine erythroleukemia cells (Butler *et al.* 2001), human endometrial adenocarcinoma cells (Uchida *et al.* 2005), and human breast cancer cells (Munster *et al.* 2001). Sodium butyrate has HDACi activity (Rada-Iglesias *et al.* 2007) which may account for its reported effects to induce DE formation from mouse and hESCs respectively (Goicoa *et al.* 2006, Jiang *et al.* 2007b). HDACis have also been reported to upregulate gelsolin, a protein that is a major building block for actin filament formation and which may be associated with the pro-differentiating effects of this class of compound (Mielnicki *et al.* 1999, Huang & Pardee 2000, Rombouts *et al.* 2002, Glaser *et al.* 2003). Histone deacetylases have been implicated recently in the regulation of expression of key pancreatic transcription factors (Haumaitre *et al.* 2008), and HDACis, such as TSA and sodium butyrate, have been shown to influence the timing and determination of pancreatic cell fate (Haumaitre *et al.* 2008), suggesting that this class of compounds may be useful in *in vitro* differentiation protocols.

Summary and conclusions

A number of recent studies have demonstrated that recapitulating *in vitro* the signals controlling the development of the endocrine pancreas *in vivo* offers a promising strategy for generating insulin-expressing β -cells from pluripotent ES cells. However, current protocols are not yet optimized for a number of reasons, including the pleiotropic effects induced

by individual morphogens, the complexity of the signaling pathways involved, and the likelihood of crosstalk between pathways. We suggest that the establishment of efficient and reproducible protocols will be facilitated by focusing on the intracellular signaling pathways that regulate and direct the developmental transitions. Figure 1 shows a proposed differentiation protocol based on known developmental processes and on currently available pharmacological modulators of the signaling systems. Initially, ES cells can be driven to differentiate into mesendoderm by suppressing the PI3K pathway and keeping Nodal and Wnt signaling sufficiently high. DE can then be generated by maintaining suppression of the PI3K pathway while activating the Nodal pathway activation. Thereafter, developmental evidence is in favor of suppressing the Wnt pathway for generating posterior foregut endoderm, followed by repression of Hedgehog signaling to allow for specification of the pancreatic domain, while inhibiting Notch signaling to promote further differentiation towards the endocrine fate. Finally, suppressing PI3K and activating Hedgehog signaling might further enhance the differentiation towards the much desired functionally mature β -cells.

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References

- Almeida M, Han L, Bellido T, Manolagas SC & Kousteni S 2005 Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. *Journal of Biological Chemistry* **280** 41342–41351.
- Apelqvist E, Ahlgren U & Edlund H 1997 Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Current Biology* **7** 801–804.
- Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, de Angelis MH, Lendahl U & Edlund H 1999 Notch signalling controls pancreatic cell differentiation. *Nature* **400** 877–881.
- Bai L, Meredith G & Tuch BE 2005 Glucagon-like peptide-1 enhances production of insulin in insulin-producing cells derived from mouse embryonic stem cells. *Journal of Endocrinology* **186** 343–352.
- Bakre MM, Hoi A, Mong JC, Koh YY, Wong KY & Stanton LW 2007 Generation of multipotential mesendodermal progenitors from mouse embryonic stem cells via sustained Wnt pathway activation. *Journal of Biological Chemistry* **282** 31703–31712.
- Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, Kahana JA, Kral AM, Leander K, Lee LL *et al.* 2005 Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. *Biochemical Journal* **385** 399–408.

- Beck CW & Slack JMW 2002 Notch is required for outgrowth of the *Xenopus* tail bud. *International Journal of Developmental Biology* **46** 255–258.
- Behr D, Wrigley JD, Nadin A, Evin G, Masters CL, Harrison T, Castro JL & Shearman MS 2001 Pharmacological knock-down of the presenilin 1 heterodimer by a novel gamma-secretase inhibitor: implications for presenilin biology. *Journal of Biological Chemistry* **276** 45394–45402.
- Bhat R, Xue Y, Berg S, Hellberg S, Ormö M, Nilsson Y, Radesäter AC, Jerning E, Markgren PO, Borgegård T *et al.* 2003 Structural insights and biological effects of glycogen synthase kinase 3-specific inhibitor AR-A014418. *Journal of Biological Chemistry* **278** 45937–45945.
- Blyszczuk P & Wobus AM 2007 *In vitro* differentiation of embryonic stem cells into the pancreatic lineage. *Methods in Molecular Biology* **330** 373–385.
- Blyszczuk P, Czyz J, Kania G, Wagner M, Röll U, St-Onge L & Wobus AM 2003 Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *PNAS* **100** 998–1003.
- Bodine PVN, Stauffer B, Ponce-de-Leon H, Bhat RA, Mangine A, Seestaller-Wehr LM, Moran RA, Billiard J, Fukayama S, Komm BS *et al.* 2009 A small molecule inhibitor of the Wnt antagonist secreted frizzled-related protein-1 stimulates bone formation. *Bone* **44** 1063–1068.
- Borowiak M, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL & Melton DA 2009 Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. *Cell Stem Cell* **4** 348–358.
- Bray SJ 2006 Notch signalling: a simple pathway becomes complex. *Nature Reviews. Molecular Cell Biology* **7** 678–689.
- Buteau J, Roduit R, Susini S & Prentki M 1999 Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. *Diabetologia* **42** 856–864.
- Butler LM, Webb Y, Agus DB, Higgins B, Tolentino TR, Kutko MC, LaQuaglia MP, Drobniak M, Cordon-Cardo C, Scher HI *et al.* 2001 Inhibition of transformed cell growth and induction of cellular differentiation by pyroxamide, an inhibitor of histone deacetylase. *Clinical Cancer Research* **7** 962–970.
- Calderari S, Gangnerau MN, Thibault M, Meile MJ, Kassir N, Alvarez C, Portha B & Serradas P 2007 Defective IGF2 and IGF1R protein production in embryonic pancreas precedes beta cell mass anomaly in the Goto-Kakizaki rat model of type 2 diabetes. *Diabetologia* **50** 1463–1471.
- Chen JK, Taipale J, Young KE, Maiti T & Beachy PA 2002 Small molecule modulation of Smoothened activity. *PNAS* **99** 14071–14076.
- Chen YG, Wang Q, Lin SL, Chang CD, Chung J & Ying SY 2006 Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. *Experimental Biology and Medicine* **231** 534–544.
- Chen C, Zhang Y, Sheng X, Huang C & Zang YQ 2008 Differentiation of embryonic stem cells towards pancreatic progenitor cells and their transplantation into streptozotocin-induced diabetic mice. *Cell Biology International* **32** 456–461.
- Chen S, Borowiak M, Fox JL, Maehr R, Osafune K, Davidow L, Lam K, Peng LF, Schreiber SL, Rubin LL *et al.* 2009 A small molecule that directs differentiation of human ESCs into the pancreatic lineage. *Nature Chemical Biology* **5** 258–265.
- Cho YM, Lim JM, Yoo DH, Kim JH, Chung SS, Park SG, Kim TH, Oh SK, Choi YM, Moon SY *et al.* 2008 Betacellulin and nicotinamide sustain PDX1 expression and induce pancreatic beta-cell differentiation in human embryonic stem cells. *Biochemical and Biophysical Research Communications* **366** 129–134.
- Chuang PT & McMahon AP 1999 Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397** 617–621.
- Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L *et al.* 2000 Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chemistry & Biology* **7** 793–803.
- Cooper MK, Porter JA, Young KE & Beachy PA 1998 Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280** 1603–1607.
- D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E & Baetge EE 2005 Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nature Biotechnology* **23** 1534–1541.
- D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK & Baetge EE 2006 Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nature Biotechnology* **24** 1392–1401.
- De Strooper B, Annaert W, Cupers P, Saftig P, Craessaerts K, Mumm JS, Schroeter EH, Schrijvers V, Wolfe MS, Ray WJ *et al.* 1999 A presenilin-1-dependent [gamma]-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398** 518–522.
- Dovey HF, John V, Anderson JP, Chen LZ, de Saint Andrieu P, Fang LY, Freedman SB, Folmer B, Goldbach E, Holsztyńska EJ *et al.* 2001 Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *Journal of Neurochemistry* **76** 173–181.
- Dyer MA, Farrington SM, Mohn D, Munday JR & Baron MH 2001 Indian Hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neuroectodermal cell fate in the mouse embryo. *Development* **128** 1717–1730.
- Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA & McMahon AP 1993 Sonic Hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75** 1417–1430.
- Eiselein L, Schwartz HJ & Rutledge JC 2004 The challenge of type 1 diabetes mellitus. *ILAR* **45** 231–236.
- Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, Moon RT, Teo JL, Oh SW, Kim HY *et al.* 2004 A small molecule inhibitor of beta-catenin/CREB-binding protein transcription. *PNAS* **101** 12682–12687.
- Esler WP, Kimberly WT, Ostaszewski BL, Ye W, Diehl TS, Selkoe DJ & Wolfe MS 2002 Activity-dependent isolation of the presenilin- gamma -secretase complex reveals nicastrin and a gamma substrate. *PNAS* **99** 2720–2725.
- Evans MJ & Kaufman MH 1981 Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292** 154–156.
- Frandsen U, Porneki AD, Floridon C, Abdallah BM & Kassem M 2007 Activin B mediated induction of *Pdx1* in human embryonic stem cell derived embryoid bodies. *Biochemical and Biophysical Research Communications* **362** 568–574.
- Frank-Kamenetsky M, Zhang X, Bottega S, Guicherit O, Wichterle H, Dudek H, Bumcrot D, Wang F, Jones S, Shulok J *et al.* 2002 Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *Journal of Biology* **1** 10.
- Fujii N, You L, Xu Z, Uematsu K, Shan J, He B, Mikami I, Edmondson LR, Neale G, Zheng J *et al.* 2007 An antagonist of dishevelled protein-protein interaction suppresses beta-catenin-dependent tumor cell growth. *Cancer Research* **67** 573–579.
- Fukumoto S, Hsieh CM, Maemura K, Layne MD, Yet SF, Lee KH, Matsui T, Rosenzweig A, Taylor WG, Rubin JS *et al.* 2001 Akt participation in the Wnt signaling pathway through dishevelled. *Journal of Biological Chemistry* **276** 17479–17483.
- van Gijn ME, Daemen MJAP, Smits JFM & Blankesteijn WM 2002 The wnt-frizzled cascade in cardiovascular disease. *Cardiovascular Research* **55** 16–24.
- Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG & Davidsen SK 2003 Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Molecular Cancer Therapeutics* **2** 151–163.
- Goicoa S, Alvarez S, Ricordi C, Invernardi L & Dominguez-Bendala J 2006 Sodium butyrate activates genes of early pancreatic development in embryonic stem cells. *Cloning and Stem Cells* **8** 140–149.
- Gradwohl G, Dierich A, LeMeur M & Guillemot F 2000 Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *PNAS* **97** 1607–1611.
- Gregorieff A, Grosschedl R & Clevers H 2004 Hindgut defects and transformation of the gastro-intestinal tract in Tcf4^{-/-}/Tcf1^{-/-} embryos. *EMBO Journal* **23** 1825–1833.

- Gu G, Dubauskaite J & Melton DA 2002 Direct evidence for the pancreatic lineage: Ngn3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129** 2447–2457.
- Hald J, Hjorth JP, German MS, Madsen OD, Serup P & Jensen J 2003 Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. *Developmental Biology* **260** 426–437.
- Hansson M, Tonning A, Frandsen U, Petri A, Rajagopal J, Englund MCO, Heller RS, Hakansson J, Fleckner J, Skold HN *et al.* 2004 Artfactual insulin release from differentiated embryonic stem cells. *Diabetes* **53** 2603–2609.
- Haumaitre C, Lenoir O & Scharfmann R 2008 Histone deacetylase inhibitors modify pancreatic cell fate determination and amplify endocrine progenitors. *Molecular Cell Biology* **28** 6373–6383.
- He XC, Yin T, Grindley JC, Tian Q, Sato T, Tao WA, Dirisina R, Porter-Westpfahl KS, Hembree M, Johnson T *et al.* 2007 PTEN-deficient intestinal stem cells initiate intestinal polyposis. *Nature Genetics* **39** 189–198.
- Hebrok M, Kim SK & Melton DA 1998 Notochord repression of endodermal Sonic Hedgehog permits pancreas development. *Genes and Development* **12** 1705–1713.
- Hebrok M, Kim SK, St Jacques B, McMahon AP & Melton DA 2000 Regulation of pancreas development by Hedgehog signaling. *Development* **127** 4905–4913.
- Heller RS, Dichmann DS, Jensen J, Miller C, Wong G, Madsen OD & Serup P 2002 Expression patterns of Wnts, Frizzleds, sFRPs, and misexpression in transgenic mice suggesting a role for Wnts in pancreas and foregut pattern formation. *Developmental Dynamics* **225** 260–270.
- Higaki J, Quon D, Zhong Z & Cordell B 1995 Inhibition of beta-amyloid formation identifies proteolytic precursors and subcellular site of catabolism. *Neuron* **14** 651–659.
- Hisinaga E, Park KY, Yamada S, Hashimoto H, Takeuchi T, Mori M, Seno M, Umezawa K, Takei I & Kojima I 2008 A simple method to induce differentiation of murine bone marrow mesenchymal cells to insulin-producing cells using conophylline and betacellulin-delta4. *Endocrine Journal* **55** 535–543.
- Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD & Kim SK 2002 Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *PNAS* **99** 16105–16110.
- Hori Y, Gu X, Xie X & Kim SK 2005 Differentiation of insulin-producing cells from human neural progenitor cells. *PLoS Medicine* **2** e103.
- Huang L & Pardee AB 2000 Suberoylanilide hydroxamic acid as a potential therapeutic agent for human breast cancer treatment. *Molecular Medicine* **6** 849–866.
- Hui H, Tang Y, Zhu L, Khoury N, Hui Z, Wang K, Perfetti R & Go V 2009 Glucagon like peptide-1-directed human embryonic stem cells differentiation into insulin-producing cells via Hedgehog, cAMP, and PI3K pathways. *Pancreas* **39** 315–322.
- Incardona JP, Gaffield W, Lange Y, Cooney A, Pentchev PG, Liu S, Watson JA, Kapur RP & Roelink H 2000 Cyclopamine inhibition of Sonic Hedgehog signal transduction is not mediated through effects on cholesterol transport. *Developmental Biology* **224** 440–452.
- Ishiyama N, Kanzaki M, Seno M, Yamada H, Kobayashi I & Kojima I 1998 Studies on the betacellulin receptor in pancreatic AR42J cells. *Diabetologia* **41** 623–628.
- Itäranta P, Chi L, Seppänen T, Niku M, Tuukkanen J, Peltoketo H & Vainio S 2006 Wnt-4 signaling is involved in the control of smooth muscle cell fate via Bmp-4 in the medullary stroma of the developing kidney. *Developmental Biology* **293** 473–483.
- Ito K, Caramori G & Adcock IM 2007 Therapeutic potential of phosphatidylinositol 3-kinase inhibitors in inflammatory respiratory disease. *Journal of Pharmacology and Experimental Therapeutics* **321** 1–8.
- Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P & Madsen OD 2000 Control of endodermal endocrine development by Hes-1. *Nature Genetics* **24** 36–44.
- Jiang J, Au M, Lu K, Eshpeter A, Korbitt G, Fisk G & Majumdar AS 2007a Generation of insulin-producing islet-like clusters from human embryonic stem cells. *Stem Cells* **25** 1940–1953.
- Jiang W, Shi Y, Zhao D, Chen S, Yong J, Zhang J, Qing T, Sun X, Zhang P, Ding M *et al.* 2007b *In vitro* derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Research* **17** 333–344.
- Johannesson M, Ståhlberg A, Ameri J, Sand FW, Norrman K & Semb H 2009 FGF4 and retinoic acid direct differentiation of hESCs into PDX1-expressing foregut endoderm in a time- and concentration-dependent manner. *PLoS ONE* **4** e4794.
- Kawahira H, Scheel DW, Smith SB, German MS & Hebrok M 2005 Hedgehog signaling regulates expansion of pancreatic epithelial cells. *Developmental Biology* **280** 111–121.
- Kawano Y & Kypta R 2003 Secreted antagonists of the Wnt signalling pathway. *Journal of Cell Science* **116** 2627–2634.
- Kim SK & Melton DA 1998 Pancreas development is promoted by cyclopamine, a Hedgehog signaling inhibitor. *PNAS* **95** 13036–13041.
- Kispert A, Vainio S & McMahon AP 1998 Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. *Development* **125** 4225–4234.
- Kitamura K, Hoshi S, Koike M, Kiyoi H, Saito H & Naoe T 2000 Histone deacetylase inhibitor but not arsenic trioxide differentiates acute promyelocytic leukaemia cells with t(11;17) in combination with all-trans retinoic acid. *British Journal of Haematology* **108** 696–702.
- Kitamura RI, Ogata T, Tanaka Y, Motoyoshi K, Seno M, Takei I, Umezawa K & Kojima I 2007 Conophylline and betacellulin-delta4: an effective combination of differentiation factors for pancreatic beta cells. *Endocrine Journal* **54** 255–264.
- Kopan R, Schroeter EH, Weintraub H & Nye JS 1996 Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *PNAS* **93** 1683–1688.
- Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazar S, Young H, Richardson M, Smart NG, Cunningham J *et al.* 2008 Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells *in vivo*. *Nature Biotechnology* **26** 443–452.
- Ku HT, Zhang N, Kubo A, O'Connor R, Mao M, Keller G & Bromberg JS 2004 Committing embryonic stem cells to early endocrine pancreas *in vitro*. *Stem Cells* **22** 1205–1217.
- Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ & Keller G 2004 Development of definitive endoderm from embryonic stem cells in culture. *Development* **131** 1651–1662.
- Kuo GH, Prouty C, DeAngelis A, Shen L, O'Neill DJ, Shah C, Connolly PJ, Murray WV, Conway BR, Cheung P *et al.* 2003 Synthesis and discovery of macrocyclic polyoxygenated bis-7-azaindolylmaleimides as a novel series of potent and highly selective glycogen synthase kinase-3beta inhibitors. *Journal of Medicinal Chemistry* **46** 4021–4031.
- Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, Greengard P, Biernat J, Wu YZ, Mandelkow EM *et al.* 2001 Indirubins inhibit glycogen synthase kinase-3beta and CDK5/P25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *Journal of Biological Chemistry* **276** 251–260.
- Lee J, Wu X, di Magliano MP, Peters EC, Wang Y, Hong J, Hebrok M, Ding S, Cho CY & Schultz PG 2007 A small-molecule antagonist of the hedgehog signaling pathway. *Chembiochem* **8** 1916–1919.
- Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, Bruseo C, Wood AW & Shivdasani RA 2004 Small-molecule antagonists of the oncogenic Tcf/[beta]-catenin protein complex. *Cancer Cell* **5** 91–102.
- Lester L, Kuo HC, Andrews L, Nauert B & Wolf D 2004 Directed differentiation of rhesus monkey ES cells into pancreatic cell phenotypes. *Reproductive Biology and Endocrinology* **2** 42.
- Li YM, Xu M, Lai MT, Huang Q, Castro JL, Muzio-Mower J, Harrison T, Lellis C, Nadin A, Neduvilil JG *et al.* 2000 Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature* **405** 689–694.
- Liu J, Wu X, Mitchell B, Kintner C, Ding S & Schultz PG 2005 A small-molecule agonist of the Wnt signaling pathway. *Angewandte Chemie (International ed. in English)* **44** 1987–1990.
- Logan CY & Nusse R 2004 The Wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology* **20** 781.

- Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R & McKay R 2001 Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* **292** 1389–1394.
- Lundkvist J & Näslund J 2007 Gamma-secretase: a complex target for Alzheimer's disease. *Current Opinion in Pharmacology* **7** 112–118.
- Mandrup-Poulsen T 2003 Beta cell death and protection. *Annals of the New York Academy of Sciences* **1005** 32–42.
- Marigo V, Roberts DJ, Lee SMK, Tsukurov O, Levi T, Gastier JM, Epstein DJ, Gilbert DJ, Copeland NG, Seidman CE *et al.* 1995 Cloning, expression, and chromosomal location of SHH and IHH: two human homologues of the *Drosophila* segment polarity gene hedgehog. *Genomics* **28** 44–51.
- Marikawa Y, Tamashiro DAA, Fujita TC & Alarcon VB 2008 Regulation of mesendoderm formation and axial elongation by Wnt signaling in mouse embryonal carcinoma cells. *Biology of Reproduction* **78** 331.
- Martin M, Gallego-Llamas J, Ribes V, Keding M, Niederreither K, Chambon P, Dollé P & Gradwohl G 2005 Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. *Developmental Biology* **284** 399–411.
- Massagué J 1998 TGF- β signal transduction. *Annual Review of Biochemistry* **67** 753.
- Massagué J & Chen YG 2000 Controlling TGF- β signaling. *Genes and Development* **14** 627–644.
- McLean AB, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, Sheppard AM, Liu H, Xu Y, Baetge EE *et al.* 2007 Activin A efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. *Stem Cells* **25** 29–38.
- McLin VA, Rankin SA & Zorn AM 2007 Repression of Wnt/ β -catenin signaling in the anterior endoderm is essential for liver and pancreas development. *Development* **134** 2207–2217.
- McMahon AP & Bradley A 1990 The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62** 1073–1085.
- McMahon AP, Joyner AL, Bradley A & McMahon JA 1992 The midbrain–hindbrain phenotype of Wnt-1^{−/−} mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69** 581–595.
- Meijer L, Skaltsounis AL, Magiatis P, Polychronopoulos P, Knockaert M, Leost M, Ryan XP, Vonica CA, Brivanlou A, Dajani R *et al.* 2003 GSK-3-selective inhibitors derived from tyrian purple indirubins. *Chemistry & Biology* **10** 1255–1266.
- Merrill BJ, Pasolli HA, Polak L, Rendl M, Garcia-Garcia MJ, Anderson KV & Fuchs E 2004 Tcf3: a transcriptional regulator of axis induction in the early embryo. *Development* **131** 263–274.
- Mfopou JK, Willems E, Leyns L & Bouwens L 2005 Expression of regulatory genes for pancreas development during murine embryonic stem cell differentiation. *International Journal of Developmental Biology* **49** 915–922.
- Mfopou JK, De Groote V, Xu X, Heimberg H & Bouwens L 2007 Sonic Hedgehog and other soluble factors from differentiating embryoid bodies inhibit pancreas development. *Stem Cells* **25** 1156–1165.
- Micallef SJ, Janes ME, Knezevic K, Davis RP, Elefánty AG & Stanley EG 2005 Retinoic acid induces Pdx1-positive endoderm in differentiating mouse embryonic stem cells. *Diabetes* **54** 301–305.
- Mielnicki LM, Ying AM, Head KL, Asch HL & Asch B 1999 Epigenetic regulation of gelsolin expression in human breast cancer cells. *Experimental Cell Research* **249** 161–176.
- Milano J, McKay J, Dagenais C, Foster-Brown L, Pognan F, Gadiet R, Jacobs RT, Zacco A, Greenberg B & Ciaccio PJ 2004 Modulation of Notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicological Sciences* **82** 341–358.
- Miyazaki S, Yamato E & Miyazaki JI 2004 Regulated expression of pdx-1 promotes *in vitro* differentiation of insulin-producing cells from embryonic stem cells. *Diabetes* **53** 1030–1037.
- Molotkov A, Molotkova N & Duester G 2005 Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. *Developmental Dynamics* **232** 950–957.
- Montrose-Rafizadeh C, Avdonin P, Garant MJ, Rodgers BD, Kole S, Yang H, Levine MA, Schwindinger W & Bernier M 1999 Pancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells. *Endocrinology* **140** 1132–1140.
- Munster PN, Troso-Sandoval T, Rosen N, Rifkind R, Marks PA & Richon VM 2001 The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. *Cancer Research* **61** 8492–8497.
- Murtaugh LC, Stanger BZ, Kwan KM & Melton DA 2003 Notch signaling controls multiple steps of pancreatic differentiation. *PNAS* **100** 14920–14925.
- Nakanishi M, Hamazaki TS, Komazaki S, Okochi H & Asashima M 2007 Pancreatic tissue formation from murine embryonic stem cells *in vitro*. *Differentiation* **75** 1–11.
- Naujok O, Francini F, Jörns A & Lenzen S 2008a An efficient experimental strategy for mouse embryonic stem cell differentiation and separation of a cytokeratin-19-positive population of insulin-producing cells. *Cell Proliferation* **41** 607–624.
- Naujok O, Francini F, Picton S, Jörns A, Bailey CJ & Lenzen S 2008b A new experimental protocol for preferential differentiation of mouse embryonic stem cells into insulin-producing cells. *Cell Transplantation* **17** 1231–1242.
- Naylor S, Smalley MJ, Robertson D, Gusterson BA, Edwards PA & Dale TC 2000 Retroviral expression of Wnt-1 and Wnt-7b produces different effects in mouse mammary epithelium. *Journal of Cell Science* **113** 2129–2138.
- Ng SS, Mahmoudi T, Danenberg E, Bejaoui I, de Lau W, Korswagen HC, Schutte M & Clevers H 2009 Phosphatidylinositol 3-kinase (PI3K) signaling does not activate the Wnt cascade. *Journal of Biological Chemistry* **284** 35308–35313.
- Nusslein-Volhard C & Wieschaus E 1980 Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287** 795–801.
- Ogata T, Li L, Yamada S, Yamamoto Y, Tanaka Y, Takei I, Umezawa K & Kojima I 2004 Promotion of beta-cell differentiation by conophylline in fetal and neonatal rat pancreas. *Diabetes* **53** 2596–2602.
- Onkamo P, Väänänen S, Karvonen M & Tuomilehto J 1999 Worldwide increase in incidence of type I diabetes – the analysis of the data on published incidence trends. *Diabetologia* **42** 1395–1403.
- Öström M, Loffler KA, Edfalk S, Selander L, Dahl U, Ricordi C, Jeon J, Correa-Medina M, Diez J & Edlund H 2008 Retinoic acid promotes the generation of pancreatic endocrine progenitor cells and their further differentiation into beta-cells. *PLoS ONE* **3** e2841.
- Otonkoski T, Beattie GM, Mally MI, Ricordi C & Hayek A 1993 Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *Journal of Clinical Investigation* **92** 1459–1466.
- Paek HJ, Morgan JR & Lysaght MJ 2005 Sequestration and synthesis: the source of insulin in cell clusters differentiated from murine embryonic stem cells. *Stem Cells* **23** 862–867.
- Paling NRD, Wheadon H, Bone HK & Welham MJ 2004 Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. *Journal of Biological Chemistry* **279** 48063–48070.
- Peat AJ, Boucheron JA, Dickerson SH, Garrido D, Mills W, Peckham J, Preugschat F, Smalley T, Schweiker SL, Wilson JR *et al.* 2004a Novel pyrazolopyrimidine derivatives as GSK-3 inhibitors. *Bioorganic & Medicinal Chemistry Letters* **14** 2121–2125.
- Peat AJ, Garrido D, Boucheron JA, Schweiker SL, Dickerson SH, Wilson JR, Wang TY & Thomson SA 2004b Novel GSK-3 inhibitors with improved cellular activity. *Bioorganic & Medicinal Chemistry Letters* **14** 2127–2130.
- Peifer M & Polakis P 2000 Wnt signaling in oncogenesis and embryogenesis – a look outside the nucleus. *Science* **287** 1606–1609.
- Petrik J, Pell JM, Arany E, McDonald TJ, Dean WL, Reik W & Hill DJ 1999 Overexpression of insulin-like growth factor-II in transgenic mice is associated with pancreatic islet cell hyperplasia. *Endocrinology* **140** 2353–2363.
- Phillips BW, Hentze H, Rust WL, Chen QP, Chipperfield H, Tan EK, Abraham S, Sadasivam A, Soong PL, Wang ST *et al.* 2007 Directed differentiation of human embryonic stem cells into the pancreatic endocrine lineage. *Stem Cells and Development* **16** 561–578.

- Piper SC, Amtul Z, Galipanes-Garcia L, Howard VG, Ziani-Cherif C, McLendon C, Rochette MJ, Fauq A, Golde TE & Paul Murphy M 2003 Peptide-based, irreversible inhibitors of gamma-secretase activity. *Biochemical and Biophysical Research Communications* **305** 529–533.
- Ptasznik A, Beattie GM, Mally MI, Cirulli V, Lopez A & Hayek A 1997 Phosphatidylinositol 3-kinase is a negative regulator of cellular differentiation. *Journal of Cell Biology* **137** 1127–1136.
- Rada-Iglesias A, Enroth S, Ameer A, Koch CM, Clelland GK, Respuela-Alonso P, Wilcox S, Dovey OM, Ellis PD, Langford CF *et al.* 2007 Butyrate mediates decrease of histone acetylation centered on transcription start sites and down-regulation of associated genes. *Genome Research* **17** 708–719.
- Rajagopal J, Anderson WJ, Kume S, Martinez OI & Melton DA 2003 Insulin staining of ES cell progeny from insulin uptake. *Science* **299** 363.
- Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabick T *et al.* 2003 Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization *in vitro* and *in vivo*. *Diabetes* **52** 588–595.
- Rombouts K, Knittel T, Machesky L, Braet F, Wielant A, Hellemans K, De Bleser P, Gelman I, Ramadori G & Geerts A 2002 Actin filament formation, reorganization and migration are impaired in hepatic stellate cells under influence of trichostatin A, a histone deacetylase inhibitor. *Journal of Hepatology* **37** 788–796.
- Ryan EA, Paty BW, Senior PA, Bigam D, Alfaradhi E, Kneteman NM, Lakey JRT & Shapiro AMJ 2005 Five-year follow-up after clinical islet transplantation. *Diabetes* **54** 2060–2069.
- Sanvito F, Herrera PL, Huarte J, Nichols A, Montesano R, Orci L & Vassalli JD 1994 TGF- β 1 influences the relative development of the exocrine and endocrine pancreas *in vitro*. *Development* **120** 3451–3462.
- Schier AF & Shen MM 2000 Nodal signalling in vertebrate development. *Nature* **403** 385–389.
- Schroeder IS, Rolletschek A, Blyszczuk P, Kania G & Wobus AM 2006 Differentiation of mouse embryonic stem cells to insulin-producing cells. *Nature Protocols* **1** 495–507.
- Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbitt G & van der Kooy D 2004 Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nature Biotechnology* **22** 1115–1124.
- Segev H, Fishman B, Ziskind A, Shulman M & Itskovitz-Eldor J 2004 Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* **22** 265–274.
- Seiffert D, Bradley JD, Rominger CM, Rominger DH, Yang F, Meredith JE Jr, Wang Q, Roach AH, Thompson LA, Spitz SM *et al.* 2000 Presenilin-1 and -2 are molecular targets for gamma-secretase inhibitors. *Journal of Biological Chemistry* **275** 34086–34091.
- Serafimidis I, Rakatzi I, Episkopou V, Gouti M & Gavalas A 2008 Novel effectors of directed and Ngn3-mediated differentiation of mouse embryonic stem cells into endocrine pancreas progenitors. *Stem Cells* **26** 3–16.
- Sernee FM, Evin G, Culvenor JG, Villadangos JA, Beyreuther K, Masters CL & Cappai R 2003 Selecting cells with different Alzheimer's disease gamma-secretase activity using FACS. Differential effect on presenilin exon 9 gamma- and epsilon-cleavage. *European Journal of Biochemistry* **270** 495–506.
- Shan J, Shi D-L, Wang J & Zheng J 2005 Identification of a specific inhibitor of the dishevelled PDZ domain. *Biochemistry* **44** 15495–15503.
- Shapiro AMJ, Lakey JRT, Ryan EA, Korbitt GS, Toth E, Warnock GL, Kneteman NM & Rajotte RV 2000 Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *New England Journal of Medicine* **343** 230–238.
- Shearman MS, Behr D, Clarke EE, Lewis HD, Harrison T, Hunt P, Nadin A, Smith AL, Stevenson G & Castro JL 2000 L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid β -protein precursor gamma-secretase activity. *Biochemistry* **39** 8698–8704.
- Shi Y, Hou L, Tang F, Jiang W, Wang P, Ding M & Deng H 2005 Inducing embryonic stem cells to differentiate into pancreatic β cells by a novel three-step approach with activin A and all-trans retinoic acid. *Stem Cells* **23** 656–662.
- Shim JH, Kim SE, Woo DH, Kim SK, Oh CH, McKay R & Kim JH 2007 Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. *Diabetologia* **50** 1228–1238.
- Shiraki N, Lai CJ, Hishikari Y & Kume S 2005 TGF- β signaling potentiates differentiation of embryonic stem cells to Pdx1 expressing endodermal cells. *Genes to Cells* **10** 503–516.
- Shiroy A, Ueda S, Ojui Y, Moriya K, Sugie Y, Fukui H, Ishizaka S & Yoshikawa M 2005 Differentiation of embryonic stem cells into insulin-producing cells promoted by Nkx2-2 gene transfer. *World Journal of Gastroenterology* **11** 4161–4166.
- Sipione S, Eshpeter A, Lyon JG, Korbitt GS & Bleackley RC 2004 Insulin expressing cells from differentiated embryonic stem cells are not β cells. *Diabetologia* **47** 499–508.
- Skoudy A, Rovira M, Savatier P, Martin F, León-Quinto T, Soria B & Real FX 2004 Transforming growth factor (TGF) β , fibroblast growth factor (FGF) and retinoid signalling pathways promote pancreatic exocrine gene expression in mouse embryonic stem cells. *Biochemical Journal* **379** 749–756.
- Soria B, Roche E, Berná G, León-Quinto T, Reig JA & Martín F 2000 Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* **49** 157–162.
- Stambolic V, Ruel L & Woodgett JR 1996 Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Current Biology* **6** 1664–1669.
- Strulovici Y, Leopold PL, O'Connor TP, Pergolizzi RG & Crystal RG 2007 Human embryonic stem cells and gene therapy. *Molecular Therapy* **15** 850–866.
- Suzuki A, Nakauchi H & Taniguchi H 2004 Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting. *Diabetes* **53** 2143–2152.
- Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M, Nakao K, Chiba T & Nishikawa SI 2005 Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development* **132** 4363–4374.
- Taipale J, Chen JK, Cooper MK, Wang B, Mann RK, Milenkovic L, Scott MP & Beachy PA 2000 Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* **406** 1005–1009.
- Taniguchi Y, Karlstrom H, Lundkvist J, Mizutani T, Otaka A, Vestling M, Bernstein A, Donoviel D, Lendahl U & Honjo T 2002 Notch receptor cleavage depends on but is not directly executed by presenilins. *PNAS* **99** 4014–4019.
- Tayaramma T, Ma B, Rohde M & Mayer H 2006 Chromatin-remodeling factors allow differentiation of bone marrow cells into insulin-producing cells. *Stem Cells* **24** 2858–2867.
- Tei E, Mehta S, Tulachan SS, Yew H, Hembree M, Preuett B, Snyder C, Yamataka A, Miyano T & Gittes G 2005 Synergistic endocrine induction by GLP-1 and TGF- β in the developing pancreas. *Pancreas* **31** 138–141.
- Thomas MK, Rastalsky N, Lee JH & Habener JF 2000 Hedgehog signaling regulation of insulin production by pancreatic β -cells. *Diabetes* **49** 2039–2047.
- Thomas MK, Lee JH, Rastalsky N & Habener JF 2001 Hedgehog signaling regulation of homeodomain protein islet duodenum homeobox-1 expression in pancreatic β -cells. *Endocrinology* **142** 1033–1040.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS & Jones JM 1998 Embryonic stem cell lines derived from human blastocysts. *Science* **282** 1145–1147.
- Tremblay KD, Hoodless PA, Bikoff EK & Robertson EJ 2000 Formation of the definitive endoderm in mouse is a Smad2-dependent process. *Development* **127** 3079–3090.
- Trosset J-Y, Dalvit C, Knapp S, Fasolini M, Veronesi M, Mantegani S, Gianellini LM, Catana C, Sundström M, Stouten PFW *et al.* 2006 Inhibition of protein-protein interactions: the discovery of druglike β -catenin inhibitors by combining virtual and biophysical screening. *Proteins* **64** 60–67.
- Tsang CK, Qi H, Liu LF & Zheng XFS 2007 Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discovery Today* **12** 112–124.

- Uchida H, Maruyama T, Nagashima T, Asada H & Yoshimura Y 2005 Histone deacetylase inhibitors induce differentiation of human endometrial adenocarcinoma cells through up-regulation of glycodelin. *Endocrinology* **146** 5365–5373.
- Umezawa K, Ohse T, Yamamoto T, Koyano T & Takahashi Y 1994 Isolation of a new vinca alkaloid from the leaves of *Ervatamia microphylla* as an inhibitor of ras functions. *Anticancer Research* **14** 2413–2417.
- Umezawa K, Hiroki A, Kawakami M, Naka H, Takei I, Ogata T, Kojima I, Koyano T, Kowithayakorn T, Pang H-S *et al.* 2003 Induction of insulin production in rat pancreatic acinar carcinoma cells by conophylline. *Biomedicine & Pharmacotherapy* **57** 341–350.
- Vaca P, Berná G, Martín F & Soria B 2003 Nicotinamide induces both proliferation and differentiation of embryonic stem cells into insulin-producing cells. *Transplantation Proceedings* **35** 2021–2023.
- Vaca P, Berná G, Araujo R, Carneiro EM, Bedoya FJ, Soria B & Martín F 2008 Nicotinamide induces differentiation of embryonic stem cells into insulin-secreting cells. *Experimental Cell Research* **314** 969–974.
- Vanhaesebroeck B, Leeyers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ & Waterfield MD 2001 Synthesis and function of 3-phosphorylated inositol lipids. *Annual Review of Biochemistry* **70** 535.
- Villavicencio EH, Walterhouse DO & Iannaccone PM 2000 The sonic Hedgehog–Patched–Gli pathway in human development and disease. *American Journal of Human Genetics* **67** 1047–1054.
- Vincent SD, Dunn NR, Hayashi S, Norris DP & Robertson EJ 2003 Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes and Development* **17** 1646–1662.
- Wang X, Cahill CM, Pineyro MA, Zhou J, Doyle ME & Egan JM 1999 Glucagon-like peptide-1 regulates the beta cell transcription factor, PDX-1, in insulinoma cells. *Endocrinology* **140** 4904–4907.
- Ward S, Sotsios Y, Dowden J, Bruce I & Finan P 2003 Therapeutic potential of phosphoinositide 3-kinase inhibitors. *Chemistry & Biology* **10** 207–213.
- Watanabe S, Umehara H, Murayama K, Okabe M, Kimura T & Nakano T 2006 Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. *Oncogene* **25** 2697–2707.
- Wells J, Esni F, Boivin G, Aronow B, Stuart W, Combs C, Sklenka A, Leach S & Lowy A 2007 Wnt/beta-catenin signaling is required for development of the exocrine pancreas. *BMC Developmental Biology* **7** 4.
- Williams JA, Guicherit OM, Zaharian BI, Xu Y, Chai L, Wichterle H, Kon C, Gatchalian C, Porter JA, Rubin LL *et al.* 2003 Identification of a small molecule inhibitor of the Hedgehog signaling pathway: effects on basal cell carcinoma-like lesions. *PNAS* **100** 4616–4621.
- Wolfe MS, Citron M, Diehl TS, Xia W, Donkor IO & Selkoe DJ 1998 A substrate-based difluoro ketone selectively inhibits Alzheimer's gamma-secretase activity. *Journal of Medicinal Chemistry* **41** 6–9.
- Wong GT, Manfra D, Poulet FM, Zhang Q, Josien H, Bara T, Engstrom L, Pinzon-Ortiz M, Fine JS, Lee HJ *et al.* 2004 Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *Journal of Biological Chemistry* **279** 12876–12882.
- Wu X, Walker J, Zhang J, Ding S & Schultz PG 2004 Purmorphamine induces osteogenesis by activation of the Hedgehog signaling pathway. *Chemistry and Biology* **11** 1229–1238.
- Yasunaga M, Tada S, Torikai-Nishikawa S, Nakano Y, Okada M, Jakt LM, Nishikawa S, Chiba T, Era T & Nishikawa SI 2005 Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nature Biotechnology* **23** 1542–1550.
- Yoon J-W & Jun H-S 2005 Autoimmune destruction of pancreatic beta cells. *American Journal of Therapeutics* **12** 580–591.
- Yue F, Cui L, Johkura K, Ogiwara N & Sasaki K 2006 Glucagon-like peptide-1 differentiation of primate embryonic stem cells into insulin-producing cells. *Tissue Engineering* **12** 2105–2116.
- Zhang Q, Major MB, Takanashi S, Camp ND, Nishiya N, Peters EC, Ginsberg MH, Jian X, Randazzo PA, Schultz PG *et al.* 2007 Small-molecule synergist of the Wnt/beta-catenin signaling pathway. *PNAS* **104** 7444–7448.
- Zhang D, Jiang W, Liu M, Sui X, Yin X, Chen S, Shi Y & Deng H 2009 Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. *Cell Research* **19** 429–438.
- Zhou J, Pineyro MA, Wang X, Doyle ME & Egan JM 2002 Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3beta transcription factors. *Journal of Cell Physiology* **192** 304–314.
- Zhu S, Wurdak H, Wang J, Lyssiotis CA, Peters EC, Cho CY, Wu X & Schultz PG 2009 A small molecule primes embryonic stem cells for differentiation. *Cell Stem Cell* **4** 416–426.

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