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Human β-Cell Proliferation and Intracellular Signaling

Driving in the Dark Without a Road Map

Rohit N. Kulkarni,[1,2] Ernesto-Bernal Mizrachi,[3] Adolfo Garcia Ocana,[4] and Andrew F. Stewart[4]

A major goal in diabetes research is to find ways to enhance the mass and function of insulin secreting β-cells in the endocrine pancreas to prevent and/or delay the onset or even reverse overt diabetes. In this *Perspectives in Diabetes* article, we highlight the contrast between the relatively large body of information that is available in regard to signaling pathways, proteins, and mechanisms that together provide a road map for efforts to regenerate β-cells in rodents versus the scant information in human β-cells. To reverse the state of ignorance regarding human β-cell signaling, we suggest a series of questions for consideration by the scientific community to construct a human β-cell proliferation road map. The hope is that the knowledge from the new studies will allow the community to move faster towards developing therapeutic approaches to enhance human β-cell mass in the long-term goal of preventing and/or curing type 1 and type 2 diabetes. ***Diabetes* 61:2205–2213, 2012**

THE CHALLENGE OF INDUCING HUMAN β-CELL REPLICATION

A principal goal of the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases, Juvenile Diabetes Research Foundation, American Diabetes Association, and their European and Asian equivalents is to develop viable therapeutic approaches to induce adult human β-cell replication/expansion for regeneration therapies in patients with type 1 diabetes and/or type 2 diabetes mellitus (T2DM). Unfortunately, all observers of adult human β-cell replication find that it is very limited (~0.2% of β-cells/24 h) and poorly responsive or unresponsive to the many mitogens, growth factors, and nutrients that have been shown to induce expansion in rodent models. For example, glucagon-like peptide-1 (GLP-1) and its analog, exendin-4, hepatocyte growth factor (HGF), lactogens, insulin, IGF-I, and many other molecules have been shown to increase rat and mouse β-cell proliferation and mass expansion, but have shown limited effects in human β-cells.

From [1]Islet Cell Biology and Regenerative Medicine, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts; the [2]Department of Medicine, Harvard Medical School, Boston, Massachusetts; the [3]Division of Metabolism, Endocrinology, and Diabetes, University of Michigan, Ann Arbor, Michigan; and the [4]Division of Endocrinology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.
Corresponding authors: Rohit N. Kulkarni, rohit.kulkarni@joslin.harvard.edu, and Andrew F. Stewart, stewarta@pitt.edu.
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SPECIES- AND AGE-RELATED FAILURE OF β-CELL REPLICATIVE CAPACITY

Some of this refractoriness to proliferation in both rodents and humans is age-related. For example, whereas β-cell replication is easy to induce using exendin-4 or partial pancreatectomy in young mice, it is markedly attenuated in older mice (1). Similarly, whereas β-cell replication has been difficult to induce in adult human β-cells, reports of human embryonic and neonatal β-cell proliferation do indicate that proliferation can occur in juvenile human β-cells (2,3). But even in this study, the proliferation is very limited (~2–4% in embryonic human β-cells by Ki-67 staining) as compared with other fetal and adult tissues (e.g., spleen, bone marrow, gastrointestinal crypts, basal keratinocytes) where proliferation is often 10-fold higher.

In contrast, there are also clear species differences, because rodent β-cell models can display relatively large proliferative responses (e.g., 10–15%), whereas this type of proliferation is never seen in human β-cells under physiological conditions, even in embryonic life. Perhaps more germane to human diabetes, whatever the underlying reasons, these issues are a major hurdle to driving therapeutic human β-cell expansion, because the major source of human β-cells is adult cadaveric donors, and the major therapeutic target for expansion of endogenous human β-cells, at least initially, will be adults with type 1 diabetes mellitus. Thus, there is an urgent need to understand why adult β-cells are refractory to replication, and, at the end of this *Perspectives in Diabetes* article, we suggest a series of questions to be addressed by the scientific community to reverse this state of ignorance.

THE RODENT β-CELL RECEPTOR–NUTRIENT–SIGNALING–CELL-CYCLE PATHWAY ROAD MAP

In rodent β-cells, we have an impressive and expanding intracellular signaling road map, or "wiring diagram," that reveals how proliferation normally occurs, how it intersects with downstream cell-cycle machinery, and how it can be manipulated for therapeutic purposes. Space prevents a detailed discussion of every pathway, but several oversimplified examples are shown in Figs. 1–3 and described briefly below.

SIGNALING PATHWAYS

Insulin receptor substrate/phosphatidylinositol-3 kinase/Akt signaling. Insulin and IGF-I constitute two primary members of the growth factor family for which receptors are expressed ubiquitously and mediate the growth and metabolic effects of the hormones in virtually all mammalian tissues. Insulin and IGF-I classically bind to

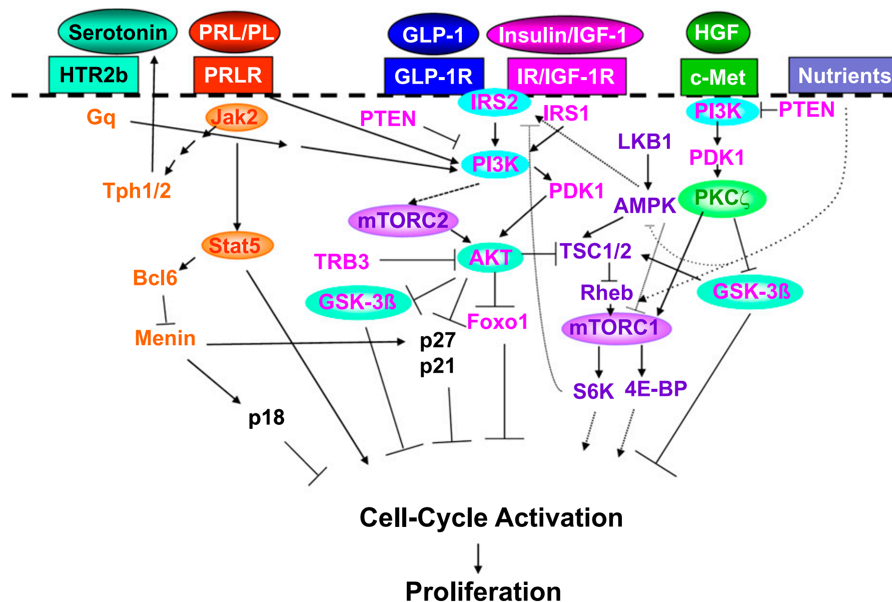

FIG. 1. A working model depicting some of the multiple signaling pathways that have been reported to modulate cell-cycle activation and/or proliferation in rodent β-cells. Note that although this model is reasonably robust, it is nonetheless preliminary and will require amendment and addition over time. The growth factors (insulin, IGF-I, HGF) and the incretin hormone GLP-1 are linked to the IRS/PI3K pathway, which in turn signals via PDK-1 to modulate Akt and FoxO1. Nutrients have also been reported to modulate PI3K, block AMP-activated protein kinase, and modulate mTORC1. PI3K activation following HGF binding to its tyrosine kinase receptor, c-Met, results in the production of phosphatidylinositol trisphosphate that directly binds to the pleckstrin homology domain of PDK-1. PDK-1 then attaches to the hydrophobic motif of PKCζ, phosphorylates Thr410, and exposes the kinase domain to autophosphorylation of Thr560, a phosphorylation required for full catalytic activity. PKCζ activation leads to inactivation of GSK3β and increased phosphorylation and activation of mTORC1. Activation of mTORC1 is required for PKCζ-mediated induction of rodent β-cell proliferation. Lactogen signaling is activated by the prolactin receptor and acts via Jak2/Stat5 and/or Jak2/Bcl6/menin and/or tryptophan hydroxylases, which generate serotonin. Serotonin is assumed to act via the *HTR2b* receptor to modulate intracellular calcium and PKC or PI3K members. The FoxO proteins, downstream targets of Akt, are reported to regulate the cell cycle, although it is unclear how this directly impacts β-cell cycle control. Transcriptional repression of cyclin D is required for FoxO-mediated inhibition of cell-cycle progression and transformation, and FoxO proteins have been reported to upregulate the transcription of p27Kip1. The phosphorylation of FoxO1 by Akt is reported to prevent the transcription factor Foxa2 from driving the expression of Pdx1, which mediates the proliferation/differentiation of β-cells in response to FoxO1 (90). Akt can also downregulate p21Cip1 activity indirectly through MDM2/p53. Insulin and IGF-I can also promote proliferation independently of the PI3K pathway by acting through proteins that act by extracellular signal–related kinase-dependent and -independent (Raf1) pathways. (A high-quality color representation of this figure is available in the online issue.)

their own receptors, but can also cross-react and activate common downstream proteins. Receptor activation transmits signals by phosphorylating insulin receptor substrates (IRS), including the four IRS proteins—Shc, Gab-1, focal adhesion kinase, and Cbl—and others, leading to activation of phosphatidylinositol-3 kinase (PI3K)/Akt (see below). Several recent reviews provide an excellent resource for interested readers (4,5). Mouse β-cells express both the insulin and IGF-I receptors, and most components of their signaling pathways. Recent studies on insulin receptor (IR) signaling in β-cells have provided cumulative evidence for an autocrine role of insulin on its own receptor. Two early mouse models that provided direct genetic evidence for a role for insulin/IGF-I signaling in the regulation of β-cell biology include the β-cell–specific knockout of the IR (βIRKO) (6) and the global knockout of IRS-2 (7). Both βIRKO and IRS2KO mice failed to maintain their β-cell mass and manifested a phenotype most resembling human T2DM. Following these two studies, multiple laboratories have reported the creation and characterization of transgenics/KOs complemented by in vitro and ex vivo approaches to indicate the significance of proteins in the insulin/IGF-I cascade for the regulation of β-cells (5).

Contrary to traditional thought, we and others have used genetic approaches to directly demonstrate that the insulin/IGF-1 signaling pathway is not critical for early development of β-cells (5,8). However, whereas both βIRKO and β-cell–specific IGF-1 receptor KO mice exhibit impaired glucose tolerance and secretory defects, only βIRKO mice show an age-dependent decrease in β-cell mass and an increased susceptibility to develop overt diabetes, suggesting a dominant role for insulin signaling in the regulation of adult β-cell mass (9). It has been observed for several decades that mouse models of diabetes and obesity exhibit a remarkable ability to compensate for the increase in insulin demand in response to insulin resistance. One such model, the liver-specific IR KO mouse, develops severe insulin resistance and glucose intolerance, but the mice do not become overtly diabetic due, in part, to an ~30-fold increase in β-cell mass as a compensatory mechanism to counter the ambient insulin resistance (9,10).

The IRSs and Akt, important downstream signaling molecules in the IR/IGF-I receptor signaling pathway, have been reported to play a dominant role in β-cell growth. Indeed, global KO of IRS-1 in mice leads to postnatal growth retardation and hyperplastic and dysfunctional islets, but the mice do not develop overt diabetes due to β-cell compensation (11–13). In contrast, IRS-2 global KOs develop mild growth retardation and, depending on their genetic background, develop either mild glucose intolerance or β-cell hypoplasia and overt diabetes (7,14). β-cell–specific deletion of IRS-2 also leads to mild diabetes (15). Importantly, IRS-2 mediates the effects of the incretin hormone, GLP-1, to promote survival and/or proliferation of rodent β-cells (16). Thus, IRS-2 appears to be a positive regulator of β-cell compensation, whereas IRS-1 predominantly regulates insulin secretion.

A similar scenario is observed in the context of the Akt isoforms. Thus, global KOs for Akt2 develop overt diabetes largely due to insulin resistance in peripheral tissues and β-cell failure, despite islet hyperplasia and hyperinsulinemia (17,18). Transgenic mice expressing a kinase-dead mutant of Akt1 under control of the rat insulin I promoter showed increased susceptibility to diabetes following fat feeding (19). Islet hyperplasia, β-cell hypertrophy, and hyperinsulinemia are observed in β-cell–specific transgenic mice expressing constitutively active Akt1 (20,21). Akt regulates proliferation by modulation of multiple downstream targets including glycogen synthase kinase-3 (GSK3) (see below), FoxO1, and tuberous sclerosis proteins (TSC)/mammalian target of rapamycin (mTOR) among others. Recent experiments have also linked the cyclin/cyclin-dependent kinase (CDK) 4 complex to Akt in β-cell proliferation, showing that Akt1 upregulates cyclins D1 and D2, p21Cip1 levels (but not p27Kip1), and CDK4 activity (22). Collectively, these in vivo data suggest a dual role for the Akts in the regulation of β-cell mass. In addition to regulating proliferation, class Ia PI3K also modulates β-cell function by multiple mechanisms (23).

GSK3 and liver kinase B1 signaling. GSK3 is a ubiquitously expressed serine/threonine protein kinase originally identified as a regulator of glycogen metabolism. It is now well-established that GSK3 acts as a downstream regulatory switch for numerous signaling pathways and is involved in cell-cycle regulation and cell proliferation. There are two mammalian GSK3 isoforms encoded by distinct genes: GSK3α and GSK3β. Activated Akt phosphorylates and inactivates GSK3. GSK3 phosphorylation was observed in mice overexpressing a constitutively active form of Akt1 in β-cells (22), which correlated with an increase in cyclin D1 levels in islets, suggesting that GSK3 is an important negative regulator of β-cell cycle progression in mice. Indeed, a decrease in GSK3β expression can correct diabetes in mouse models of insulin resistance (24,25). More recently, the GSK3β/β-catenin/T-cell factor pathway has been shown to act in concert with cAMP-responsive element–binding protein (CREB) to downregulate cyclin D2 expression by phosphatase and tensin homolog (PTEN), suggesting a convergence of the PI3K/PTEN and Wnt pathways. Furthermore, phosphorylation and activation of TSC2 by GSK3β inhibits mTOR signaling (26). However, whether this occurs in β-cells is unknown.

Another kinase that physically associates in vivo with GSK3β is the tumor suppressor liver kinase B1 (LKB1). Loss of LKB1 in adult β-cells increases β-cell proliferation, size, and mass and enhances glucose tolerance in mice (27,28), suggesting that this kinase is a regulator of β-cell growth in vivo in rodents. LKB1 is also known to associate with protein kinase Cζ (PKCζ), as described below. Whether there is any interaction among these three different kinases in the β-cell is currently unclear.

TSC, mTOR, S6 kinase, and 4E-BP1 signaling. mTOR is essential for cell growth and proliferation and is part of two mTOR complexes (mTORC), mTORC1 and 2 [recently reviewed (29)]. mTORC1 activity is negatively regulated by TSC1, TSC2, and the small G protein Rheb. TSC2 phosphorylation and inactivation by Akt and extracellular signal–related kinase, among other kinases, releases the inhibition of Rheb, leading to activation of mTOR (29). In contrast, phosphorylation and activation of TSC2 by several kinases inhibits mTOR signaling (29). mTORC1 constitutes the rapamycin-sensitive arm of mTOR signaling and contains at least three proteins: raptor, mLst/GβL, and PRAS40 (29). This pathway integrates signals from growth factors and nutrients and controls growth (cell size), proliferation (cell number), and metabolism by directly modulating 4E-BP and S6 kinases (S6K) and by indirectly attenuating Akt signaling via an mTORC1/S6K-mediated negative-feedback loop on IRS signaling. S6K phosphorylates downstream substrates, such as ribosomal S6 protein and eukaryotic translation initiation factor 4B, to promote mRNA translation and synthesis of ribosomes. Phosphorylation of 4E-BPs triggers their release from eukaryotic translation initiation factor 4E and initiates cap-dependent translation. Thus, mTORC1 substrates regulate cell growth, proliferation, and mRNA translation initiation and progression, thereby controlling the rate of protein synthesis.

As relates to β-cells, evidence from mouse genetic models demonstrates that mice with conditional deletion of TSC2 (leading to mTORC1 activation) in β-cells exhibit increases in β-cell mass, proliferation, and cell size (30). Separate studies demonstrated that conditional deletion of TSC2 or TSC1 in β-cells causes a similar phenotype, but these mice developed diabetes and β-cell failure after 40 weeks (31,32). Further confirmation of the effect of mTORC1 activation to increase β-cell mass comes from mice overexpressing Rheb in β-cells (33). However, proliferation in β-cells with mTORC1 activation has not been demonstrated in all studies: thus, precisely how mTORC1 acting upon 4E-BPs and S6K modulates β-cell mass, proliferation, and function is unclear. Evidence for a role of S6K in β-cell proliferation was demonstrated in mice with activation of Akt signaling in an S6K1-deficient background (34). However, S6K1 gain of function by transgenic overexpression failed to induce proliferation, in part due to negative feedback on IRS-1 and -2 signaling (35). The importance of 4E-BPs on regulation of β-cell proliferation is hard to decipher at present because of alterations in insulin sensitivity in global KO models.

Further evidence for a role of mTORC1 in β-cell proliferation comes from studies with its inhibitor, rapamycin. These studies demonstrate that: *1*) rapamycin treatment blocks β-cell expansion, cell size, and proliferation induced by activation of Akt in β-cells (36). The inhibition of β-cell proliferation by rapamycin results from decreases in cyclin D2 and D3 and in cyclin-dependent kinase (cdk) 4 activity. *2*) Rapamycin treatment results in reduced proliferation in β-cells in pregnant mice and causes antiproliferative effects on transplanted rat β-cells in vivo (37,38). *3*) Rapamycin also attenuates β-cell expansion in a model of insulin resistance and β-cell regeneration (39,40) and reduces proliferation of rodent islets in vitro (41). These studies support the concept that mTORC1 pays an important role in the regulation of β-cell mass and cell cycle and can mediate adaptation of β-cells to insulin resistance.

The mTORC2 complex, containing rictor, mSin, and protor, is insensitive to rapamycin. This complex phosphorylates Akt on Ser473, suggesting that this pathway indirectly could be linked to proliferation. However, the only evidence for a role for mTORC2 in β-cells comes from mice with conditional deletion of rictor in β-cells (42). These mice exhibit mild hyperglycemia resulting, in part, from decreased β-cell proliferation, mass, and insulin secretion.

Other pathways: PKCζ signaling. PKCζ is a member of the atypical PKC subfamily activated by PI3K/3′-PI-dependent protein kinase-1 (PDK-1). Multiple studies

during the 1990s showed that PKCζ is a critical kinase for mitogenic signal transduction in a variety of cell types, including fibroblasts, brown adipocytes, endothelial cells, and oocytes (43). More recently, the importance of PKCζ for β-cell replication has also been elucidated. We and others have shown that growth factors such as GLP-1, parathyroid hormone-related protein, HGF, and nutrients such as glucose increase PKCζ phosphorylation and activity in insulinoma cells and rodent islets (44–46). Interestingly, activation of PKCζ using a constitutively active form of this kinase (CA-PKCζ) markedly increases β-cell proliferation in insulinoma and primary mouse β-cells in vitro, suggesting that PKCζ activation could be involved in growth factor-induced rodent β-cell proliferation (45,46). Indeed, small interfering RNA-based downregulation of PKCζ or the use of a dominant-negative form of PKCζ completely abolished growth factor-induced β-cell proliferation (45,46). Conversely, transgenic mice with expression of CA-PKCζ in β-cells display increased β-cell proliferation, size, and mass with a concomitant enhancement in insulin secretion and improved glucose tolerance (47). Downstream, activation of mTOR and upregulation of cyclin Ds and A are essential for PKCζ-mediated mitogenic effects in rodent β-cells (47). Collectively, these results indicate that PKCζ is a critical kinase for mitogenic signal transduction in rodent β-cells in vitro and in vivo.

PKCζ activation in rodent β-cells also leads to phosphorylation and inactivation of GSK3β (47), suggesting that PKCζ-mediated β-cell mitogenic effects might also be favored by GSK3β inactivation. GSK3β is one of the kinases known to phosphorylate D-cyclins and to regulate their degradation (48). Because PKCζ activation results in increased GSK3β phosphorylation/inactivation and increased expression of D-cyclins, it is possible that PKCζ increases the accumulation of D-cyclins in β-cells through inactivation of GSK3β.

Lactogen and Janus kinase–signal transducer and activation of transcription signaling. In rodent β-cells, pregnancy-associated lactogens bind to the prolactin receptor, which then activates the Janus kinase JAK2, which results in phosphorylation/activation of the transcription factor signal transducer and activation of transcription 5 (STAT5) and its nuclear translocation. Phospho-STAT5 dimers bind to the promoter of the cyclin D2 gene and upregulate transcription of cyclin D2 mRNA, with resultant induction of β-cell proliferation. This pathway was demonstrated to exist in rodent β-cells (49). More recently, two reports have added depth and definition to this pathway. First, Kim and colleagues (50) has demonstrated that lactogenic signaling during pregnancy upregulates the transcription factor Bcl6, with resulting repression of the epigenetic histone methylase complex, trithorax component, menin. Through the trithorax complex, menin acts as a transcriptional repressor of the CDK inhibitors p18INK4 and p27cip, and their repression is therefore permissive for β-cell replication in pregnancy. Most recently, the German group (Kim et al.) (51) has demonstrated that lactogenic signaling during pregnancy also is associated with a dramatic, 1,000×, increase in serotonin production by β-cells, a reflection of the induction of β-cell tryptophan hydroxylases by lactogens. Unanswered questions still remain, such as which signaling pathways does 5HT2b activate in the β-cell? How exactly do these downstream signaling molecules affect the cell-cycle symphony?

Additional growth factor signaling pathways. In this brief *Perspectives in Diabetes*, only a few rodent β-cell signaling pathways can be described. It should be clear, however, that multiple other signaling pathways linking growth factors to rodent β-cell replication exist. Examples include: a glucose–glut2–ChREBP–cMyc pathway (52); a glucose–AMP-activated protein kinase–LKB1–calcineurin–nuclear factor of activated T-cell pathway (27,28,53); an epidermal growth factor (EGF) ligand family (EGF, betacellulin, heparin-binding EGF, amphiregulin, and trefoil factor family 3)–EGF receptor–ras/raf–mitogen-activated protein kinase (MAPK)–cdk–cyclin pathway (54,55); a cAMP–cAMP-dependent protein kinase–CREB–cAMP response element modulator–cyclin A pathway (56,57); parathyroid hormone-related protein induction of proliferation (5); a leptin–IRS-2 pathway (58); a transforming growth factor-β activin family–SMAD pathway (59); sex hormone signaling pathways (60); cannabinoid receptors (CB1) that cross-talk with insulin receptors and IRS-2 (61) and calpains (62); and recently, a platelet-derived growth factor (PDGF) receptor linked to MAPK signaling pathway, driving the polycomb repressive complex family member Ezh2, with repression of p16INK4, thereby permitting activation of downstream cell-cycle molecules such as the pRb family pocket proteins and E2Fs (63).

CELL-CYCLE CONTROL OF PROLIFERATION IN THE RODENT β-CELL

This is an important and rapidly moving area. It is complex and well beyond the scope of a brief review, but detailed reviews are available (64–66). Briefly, downstream of every developmental program and nutrient- or growth factor-driven signaling pathway that is able to drive rodent β-cell proliferation, intracellular signaling pathways converge on the molecules that control the G_1/S checkpoint. This checkpoint is shown schematically in Fig. 2, and examples of interactions with upstream signaling pathways are shown in Fig. 3. Briefly, there are a number of cell cycle–activating molecules: the D-cyclins and their cognate cdks

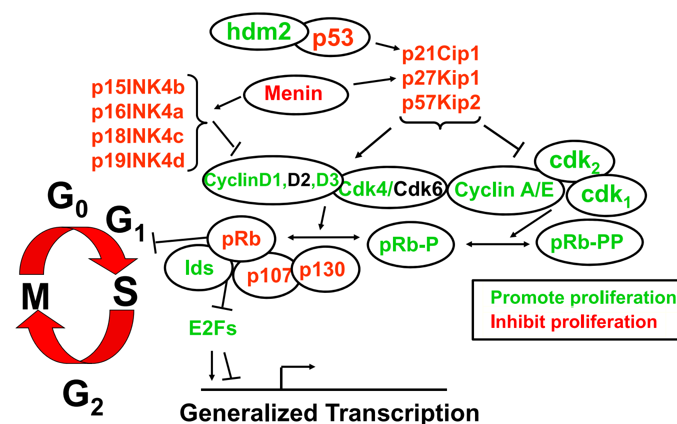

FIG. 2. The rodent and human β-cell G_1/S molecule road map. These are the ultimate targets of upstream mitogenic signaling molecules. Those that activate cell-cycle progression (cyclins and cyclin-dependent kinases) are shown in green, and those that inhibit cell-cycle progression (the pocket proteins, INK4s, and the CIP/KIP families of cell-cycle inhibitors) are shown in red. The two wiring diagrams in rodents and human β-cells appear to be similar, with two exceptions (cyclin D2 and cdk6), indicated in black: *1*) human islets have little cyclin D2, whereas this is abundant and critical in rodent β-cells; and *2*) human islets contain abundant cdk6, a cdk that is absent in rodent β-cells. See text and references (64–66,71) for details. Modified from Cozar-Castellano et al. (66). (A high-quality color representation of this figure is available in the online issue.)

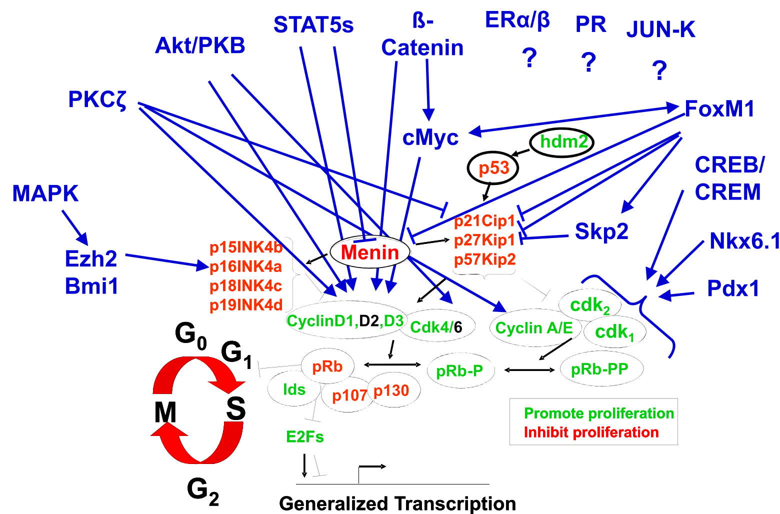

FIG. 3. Examples of reported upstream signaling pathways that activate cell-cycle progression in the rodent β-cell. This figure illustrates that there are many different ways in which a growth factor or nutrient can activate cell-cycle progression. Please note that the figure is oversimplified for clarity and is also incompletely defined. The Akt/PKB, PKCζ STAT5s, and β-catenin activate one or more cyclins and cdk proteins. MAPK has been reported to modulate Ezh2 to act via p16. CREB/cAMP response element modulator, Nkx6.1 and Pdx1 have been reported to act via the "late" cdks and cyclins, cdk1/2 and cyclins A/E. FoxM1 (91) has been reported to inhibit the Cip/Kip proteins, and menin, activate cMyc, and regulate Skp2 (92,93). PKCζ activation in rodent β-cells leads to upregulation of cyclin Ds and A and diminished expression of p21. Modulation of one or more of these cell-cycle proteins is critical for phosphorylation and inactivation of pRb so that it can release the E2F transcription factors that are required for cell-cycle progression. Several additional β-cell mitogenic signaling pathways likely exist, including those activated by estrogen/progesterone receptors, and JUN kinase (depicted as "?"). Green indicates proteins that promote proliferation, whereas red indicates proteins that inhibit proliferation. (A high-quality color representation of this figure is available in the online issue.)

and the E/A-cyclins and their cognate cdks, cdk1 and -2. Overexpression of all of these in various combinations is able to drive rodent β-cell proliferation, either when delivered adenovirally (67) or transgenically, and in some cases (but not all), disruption of their genes leads to β-cell hypoplasia, hypoinsulinemia, and diabetes (64–66). Thus, these cell-cycle activators are key targets of upstream pathways, and they have been shown to be regulated by these signaling pathways, growth factors (as described above), and glucose and glucokinase activators (68).

These cell-cycle activators are balanced by a series of cell-cycle inhibitors, which include the so-called "pocket proteins" (pRb, p107, and p130), the INK4 family (p15, p16, p18, and p19), the CIP/KIP family (p21, p27, and p57), menin, and p53. The details of their actions and interactions with the cyclins and cdks are too complex for a brief discussion, but detailed reviews are available. The key points in the current context are that they, too, are essential regulators of β-cell cycle progression (e.g., p21 and p27 are regulated by mitogens and p16 and p18 by free fatty acids), and genetic disruption of the genes encoding some of these molecules leads to β-cell hyperplasia and hyperfunction (69,70).

In this context, it is important to emphasize that these G_1/S molecules have short half-lives and frequently, if not most often, are regulated at the level of protein stability. This is important because changes seen at the mRNA level frequently correlate poorly with those observed at the protein level.

Thus, we know an enormous amount about cell-cycle control in the rodent β-cell and how key signaling pathways regulate these molecules.

CELL-CYCLE CONTROL IN THE HUMAN β-CELL

As in the rodent β-cell, the G_1/S molecules controlling human β-cell proliferation have also been studied, and much is now known. For example, we have a relatively complete understanding of which of the ~30 G_1/S molecules are present in the human islet, and they can be arranged in a working wiring diagram or road map, which looks very similar to the rodent version in Fig. 2 (71). In addition, we know that adenoviral overexpression of certain cell-cycle regulatory molecules (e.g., cMyc, the cdks, or the cyclins D or E) in the human β-cell activates proliferation robustly. For example, with adenoviral overexpression of cell-cycle molecules, as many as 10–15% of adult human β-cells are able to replicate as assessed using Ki-67 immunohistochemistry or bromodeoxyuridine incorporation (71–74).

Although the human β-cell G_1/S road map is similar to that in the rodent β-cell, there are two principal differences, indicated by the black numbers in Fig. 2. First, cdk6 is absent in rodent β-cells but abundant in human β-cells (71). This is relevant because genetic loss of cdk4 in mice leads to β-cell hypoplasia and diabetes, presumably because there is no cdk6 to compensate for this loss. Second, although all three D-cyclins are present in the mouse β-cell, genetic loss of cyclin D2 (but not cyclins D1 or D3) in mice leads to β-cell hypoplasia and diabetes, indicating that this is the key D-cyclin in the rodent β-cell (75,76). In contrast, human islets contain little or no cyclin D2 (71), suggesting, paradoxically, either that cyclin D2 is irrelevant in the human β-cell or that it is absolutely essential, with its paucity being precisely the reason that human β-cells do not replicate.

Failure to signal from cell surface to cell-cycle machinery. Most importantly in the current context, because adult human β-cells do not replicate in response to the long list of growth factors, nutrients, and maneuvers that induce rodent β-cells to replicate, but clearly can replicate when cyclins and cdks are overexpressed (71–73), it is clear that failure or inadequacy of the cell-cycle

machinery in human β-cells is not the reason they do not replicate; the machinery is there and waiting to be activated. Thus, the likely missing link in adult human β-cell replication is not failure to express key cell-cycle molecules, but rather a failure to activate them in response to what would appear to be appropriate upstream signals. More specifically, the blockade(s) would appear to be somewhere among the levels of the upstream growth factors, their receptors, signaling pathways that should, but do not, alter these cell-cycle molecules.

THERE IS NO HUMAN β-CELL RECEPTOR–NUTRIENT–SIGNALING–CELL-CYCLE PATHWAY ROAD MAP

If human β-cells possess the requisite G_1/S molecules to drive β-cell replication, and their upregulation can drive human β-cell replication, it is then axiomatic that signaling events that should be reaching the cell-cycle machinery are not. It therefore would be particularly useful to have a human β-cell signaling road map that describes these intracellular signaling pathways, with their upstream ligands and receptors and downstream cell-cycle targets. Unfortunately, in contrast to the rich and complex road map for growth factor and nutrient induction of rodent β-cell replication, no such road map exists for the human β-cell: we remain very much in the dark regarding receptors, nutrient transporters/sensors, their downstream signaling pathways, and their putative connections to the ultimate downstream cell-cycle regulatory molecules. Simply said, the human β-cell signaling road map is almost a blank slate, a tabula rasa, as shown in the light gray lines and text in the upper portion of Fig. 4, which should be contrasted to their counterparts in Fig. 1. The following sections briefly summarize the little that we do know.

IRS-PI3K-Akt signaling. Human islet cells express insulin and IGF-I receptors and components of these signaling pathways (5,77). In functional terms, several studies support the concept of a direct insulin action in the β-cell that promotes a positive-feedback effect in vivo in healthy humans (78) that is blunted in patients with T2DM (79,80). Ex vivo examination of human islets and pancreas sections from T2DM patients (77,81) supports a role for the insulin-signaling network in the regulation of cell-cycle proteins. Although an increase in β-cell volume has been reported in humans with obesity (82), it is unclear whether this occurs as a compensatory response to insulin resistance, as occurs in animal models, or is genetically predetermined. Additional studies that directly address this possibility are warranted.

mTORC1 and regulation of human β-cell proliferation. The expression of some mTOR signaling components has been demonstrated in human islets (83) exemplified by its induction by glucose, amino acids, and insulin (84). Most of our understanding of mTOR signaling in human islets is derived from studies using rapamycin derivatives as immunosuppressants or as treatment of patients with insulinomas and other neuroendocrine tumors. Rapamycin treatment has been shown to inhibit human β-cell proliferation in vitro (41). Moreover, *TSC2* and *PTEN* expression is decreased in insulinomas and pancreatic endocrine tumors, suggesting that activation of mTORC1 and Akt signaling plays a role in the proliferative changes observed in these tumors (85). However, insulinomas are rare in patients with tuberous sclerosis. In contrast, mTORC1 inhibition by everolimus (a rapamycin analog) improves glycemic control in patients with insulinoma and prolongs progression-free survival in patients with pancreatic neuroendocrine tumors (86,87). The extent to which reduced β-cell mass or inhibition of insulin secretion contributes to the responses to mTORC1 inhibition is unclear, but, based on the current evidence, it is likely that inhibition of β-cell proliferation plays a major role.

PKCζ and GSK3 signaling in the human β-cell. Pharmacological inhibition of GSK3β has been shown to enhance (approximately fourfold) glucose-mediated human

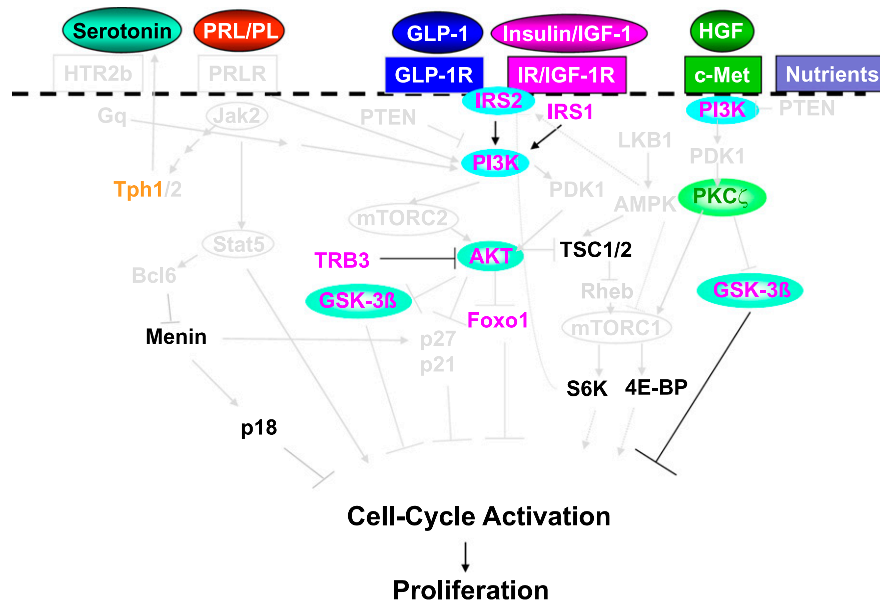

FIG. 4. The rudimentary human β-cell mitogenic signaling road map. Contrast this figure to Fig. 1. The gray lines are molecules and pathways that are known to exist in rodents but are unstudied in human β-cells. The human β-cell signaling road map is underdeveloped: Although the human G_1/S molecules are reasonably well-characterized in the human β-cell (66), only a handful of growth factor receptors (e.g., IR, IGF-1R, and c-Met) and signaling molecules (IRS-1, IRS-2, PI3K, Akt, PKCζ, and GSK3β) are known to be present in human β-cell, and the majority of the proteins involved in signaling have not been studied.

β-cell proliferation (although from very low to low basal rates) (41), and adenoviral transduction of human islets with CA-PKCζ in vitro enhances (approximately fourfold) human β-cell proliferation (45). However, whether PKCζ-induced human β-cell proliferation is mediated or potentiated by GSK3β inactivation is unknown.

In summary, it is important to emphasize that at present, although many examples exist in rodent β-cells, we do not have a single example of a complete pathway in the human β-cell that links a growth factor or nutrient, via a cell-surface receptor through a complete signaling pathway, to the downstream cell-cycle machinery that translates to proliferation. In this regard, the recent report of a PDGF–PDGF receptor–MAPK–Ezh2–p16 pathway (63) is exciting and comes the closest to filling this void.

WHY WE NEED A HUMAN β-CELL MITOGENIC SIGNALING PATHWAY ROAD MAP

Comparing Figs. 1 and 4 makes it clear that informed efforts to drive therapeutic human β-cell proliferation are severely hampered by the essentially complete lack of a human β-cell road map. Further, the fact that direct cell-cycle activation by cyclin/cdk overexpression can markedly activate human β-cell proliferation places at least one key obstacle to human β-cell proliferation within these upstream signaling pathways, about which we know so little. As noted below, it would be informative to investigate the upstream signaling pathways and identify proteins that link the cell-surface receptors with key molecules in the cell-cycle machinery in human β-cells.

Examples of key questions about which we have no answers are: *1*) do the gray molecules and lines in Fig. 4 even exist in adult human β-cells?; *2*) are there key signaling pathways in human islets that we have completely overlooked? [e.g., erythropoietin, insulin, serotonin, and osteocalcin have all been proposed as physiologic drivers of rodent β-cell replication (51,88,89), but have not been comprehensively tested in human β-cells]; *3*) do adult human β-cells require engagement of receptors or nutrients distinct from those on rodent β-cells; *4*) what are the intracellular signaling networks and cell-cycle targets regulated by the kinases in Fig. 4 that *are* present in human β-cells?; *5*) are these kinases activated by growth factors and nutrients, and, if not, what is preventing them from being activated?; *6*) can coordinate activation of combinations of pathways synergize to enhance proliferation (e.g., if overexpression of PKCζ and inhibition of GSK3β can increase human β-cell replication from 0.2 to 1.0%, would coordinate activation of multiple pathways further increase replication?)?; *7*) are the cell-cycle inhibitors that are so abundant in human β-cells restraining proliferation, and how are they regulated?; *8*) are key cell-cycle regulatory elements epigenetically regulated (in this study, for example, the epigenetic methylases and demethylases menin, Ezh2, and Bmi1 have all been shown to participate in restraining rodent β-cell replication)?; *9*) in this era of high-throughput screening, which are the optimal small-molecule targets in the human β-cell to drive proliferation?; and *10*) why and how do β-cells in young humans proliferate at a higher rate compared with those from older individuals?

The answers to these critical questions are simply that we know very little: we have no road map from which to hypothesize, model, and integrate data from multiple systems. We believe that obtaining the information required to create such a road map is essential. Without this information, we are driving at night without headlights, without a road map, and without global positioning system navigation. It is time to develop such a high-content signaling road map that connects the cell surface to cell-cycle machinery in the human β-cell.

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