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REVIEW

Maturation of beta cells: lessons from in vivo and in vitro models

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Abstract

The ability to maintain normoglycaemia, through glucose-sensitive insulin release, is a key aspect of postnatal beta cell function. However, terminally differentiated beta cell identity does not necessarily imply functional maturity. Beta cell maturation is therefore a continuation of beta cell development, albeit a process that occurs postnatally in mammals. Although many important features have been identified in the study of beta cell maturation, as of yet no unified mechanistic model of beta cell functional maturity exists. Here, we review recent findings about the underlying mechanisms of beta cell functional maturation. These findings include systemic hormonal and nutritional triggers that operate through energy-sensing machinery shifts within beta cells, resulting in primed metabolic states that allow for appropriate glucose trafficking and, ultimately, insulin release. We also draw attention to the expansive synergistic nature of these pathways and emphasise that beta cell maturation is dependent on overlapping regulatory and metabolic networks.

Keywords AMPK · Beta cells · Circadian · Differentiation · Islets · Maturation · Metabolism · mTOR · Review · Stem cells

Abbreviations

AICAR	5-Aminoimidazole-4-carboxamide riboside
ALK5	Transforming growth factor β receptor 1
AMPK	AMP-activated protein kinase
BMAL1	Aryl hydrocarbon receptor nuclear translocator like
BMP4	Bone morphogenetic protein 4
DHAP	Dihydroxyacetone phosphate
GSIS	Glucose-sensitive insulin release
mTOR	Mechanistic target of rapamycin
mTORC	mTOR complex
OGC	Solute carrier family 25 member 11
OxPhos	Oxidative phosphorylation
PEP	Phosphoenolpyruvate
PPP	Pentose phosphate pathway
SC-islets	Stem-cell-derived islets
SIX2	SIX homeobox 2
SIX3	SIX homeobox 3
SLC25A1	Solute carrier family 25 member 1
TCA	Tricarboxylic acid
UCN3	Urocortin 3

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Introduction

Understanding the mechanisms of beta cell differentiation and maturation is integral to studies of diabetes pathophysiology, regenerative therapies and stem-cell-derived models of beta cell dysfunction and replacement. While the developmental processes that lead to a stable beta cell identity are relatively well known, the mechanisms underlying the functional maturation of beta cells are less clear. In simple terms, beta cell 'differentiation' defines the acquisition of a terminally differentiated insulin-positive cell identity throughout in utero development or in vitro stem-cell-based protocols. Conversely, beta cell 'maturation' is a measurement of the phenotypic properties of beta cells and their ability to respond to, and control, blood glucose levels through glucose-sensitive insulin secretion (GSIS). Acquisition of this function occurs postnatally in rodents [1–3] and humans [4], and therefore is a property of beta cell development, but one that occurs beyond the attainment of fetal beta cell identity. This divide in cell identity and

mature functionality is exemplified in early stem-cell-based differentiation studies that generated beta-like cells with high expression patterns of canonical beta cell markers but with limited functional activity [5, 6]. The issue of defining what constitutes a mature beta cell can be therefore quite problematic if only certain aspects of beta cell biology are investigated, as has been recently reported [7]. Some hallmark features of beta cell 'identity' and 'maturity' are outlined in the Text box and will be elaborated upon further throughout this review.

The current understanding of beta cell maturation is that such a process is a spectrum rather than a binary state [8–10]. Mature functionality may itself also be a dynamic process, whereby beta cells flux from active to inactive states, or be dependent on the interplay of functionally heterogeneous beta cell pools [11–16]. Functional maturation can be a reversible process, as beta cell dedifferentiation and senescence, with resultant functional deterioration, are known to be associated with the onset of diabetes [17–19]. Therefore, the study of functional maturation of beta cells is necessary to understand the underlying mechanisms of dysfunction eventually leading to diabetes, as well as to improve the efficacy of therapeutic interventions and stem-cell-based islet replacement therapies.

This review will primarily focus on the reported multifaceted mechanisms that drive and maintain beta cell functional maturation within in vivo and in vitro models.

Extrinsic triggers and circadian modulation of beta cell maturation

Nutrient exposure The functional maturation of mammalian beta cells is known to occur postnatally, and continues to develop post-weaning [1, 2]. There are many potential drivers of this response, the most critical ostensibly being the neonate's need to adjust to shifting patterns of nutrient consumption and composition. Mouse studies have implicated the change from high amino-acid-based nutrient availability in utero (and the high-fat milk diet of newborns) to pulsatile carbohydrate-based diet post-weaning as a stimulating factor in postnatal beta cell maturation [10, 20, 21]. This change in nutrient type induces a shift in the relative activity of the energy-sensing pathways of mechanistic target of rapamycin (mTOR) complex (mTORC) 1 and AMP-activated protein kinase (AMPK), with functional maturation favouring a basal activity of AMPK signalling. Conversely, the

Characteristic features of beta cell identity and maturity

Beta cell identity

- Monohormonal insulin expression, production and retention
- Expression of beta cell-enriched transcription factors (e.g. *PDX1*, *NKX6.1*, *NKX2.2*, *NEUROD1*)
- Expression of voltage-dependent ion channels and membrane polarisation machinery associated with triggering of insulin release

Beta cell maturity

- Glucose-sensitive insulin release (GSIS) at physiologically relevant glucose concentrations
- Insensitivity to pyruvate/lactate-stimulated insulin release
- Absence of 'disallowed' genes (e.g. *SLC16A1*, *LDHA*, *HK1*)
- Glucose-responsive mitochondrial oxidative metabolism
- K_{ATP} channel-independent metabolic amplification pathways for GSIS modulation
- Appropriate balance between energy-sensing mTOR/AMPK pathways in basal and stimulatory conditions
- Ability to respond to and maintain circadian entrainment
- Expression of transcription factors/transcriptional regulators associated with advanced maturation (e.g. *MAFA*, *SIX2*, *SIX3*, *BMAL1/ARNTL*, *NR1D1*)
- Epigenetic and microRNA signatures associated with mature functionality and 'disallowed' gene repression

activation of mTORC1 signalling becomes more restricted to periods of glucose stimulation. Intriguingly, the maintenance of a high-fat diet into adulthood retains a more functionally immature beta cell phenotype [21], while the transient inhibition of mTOR in stem-cell-derived islets (SC-islets) improves functional outcomes [20], suggesting that these pathway shifts are causative and not simply incidental to beta cell maturation.

The concept of beneficial mTOR signalling in beta cell differentiation and function is well documented [22]. However, the specific mechanisms driving beta cell maturation through mTOR signalling modulation are still vague. Beta cell-specific overexpression of a kinase-dead mTOR is detrimental to function in mice [23], as is beta cell-specific mTOR knockout [24]. Specific functions of the mTORC1 and -2 complexes have also been implicated in different aspects of beta cell maturation and function. The raptor subunit of the mTORC1 complex is necessary for the regulation of beta cell function, autophagy and repression of disallowed genes [24–26] whereas mTORC2 complexes, which function through the presence of the rictor subunit, have been implicated in the mediation of beta cell mass and proliferation, islet cytoarchitecture and modulation of GSIS through activation of protein kinase Cα [24, 27, 28]. Postnatal rearrangement of islet cytoarchitecture is another key process in mature functionality seen in vivo [29, 30] as well as in recent models of SC-islet maturation in vitro [31]. Modulation of these processes through mTORC2-mediated signalling may indeed be responsible for islet reorganisation, prior to downregulation of mTOR signalling, and the onset of functional maturity.

Another intriguing concept is the coupling of beta cell glucose sensing to mTOR activity. It has recently been shown that acute glucose stimulation of mTORC1 activity in beta cells is only partially dependent on mitochondria-derived glucose metabolism [32]. In line with this, a study reported that glycolytically derived dihydroxyacetone phosphate (DHAP) may signal glucose availability directly to the mTORC1 complex (albeit in human embryonic kidney cells) [33]. Metabolic tracing studies by us and others have shown that SC-islets show strong functional profiles despite limited mitochondrial metabolism of glucose; furthermore, a glycolytic bottleneck beyond the glyceraldehyde-3-phosphate (GA3P)/DHAP enzymatic step is present in SC-islets [34, 35]. It is tempting to speculate that such a direct interplay of glycolytic DHAP generation and mTOR activity may in part be responsible for strong in vitro SC-islet function, without the canonical mitochondrial coupling seen in mature adult islets.

Circadian clock In concert with post-weaning feeding cycle and nutrient composition changes, the entrainment of systemic and intrinsic islet circadian clocks has an active role in beta cell maturation in mammals [36–38]. The core circadian clock transcription factors clock circadian regulator (CLOCK) and aryl hydrocarbon receptor nuclear translocator like (BMAL1) are known to cyclically drive the oscillating expression of many beta cell genes necessary for secretory function and regulation of insulin release [39], correlated with the acquisition of GSIS [36]. Although this review focuses primarily on beta cell biology, it is worth noting that the core components of the circadian clock regulate cell-type-specific gene networks within each endocrine population [40]. *BMAL1* (also known as *ARNTL*) and/or *CLOCK* deletion within pancreatic lineages (or beta cells specifically) disrupts the functionality of beta cells, resulting in an oxidative-stress-induced state [41, 42]. Conversely, the overexpression of *Bmal1* was able to increase the amplitude of circadian oscillations and protect against obesity-induced glucose intolerance in mice [43].

The ability of the circadian clock to rhythmically induce genes that enhance the glucose-sensitive function of mature beta cells may also share some overlap with key components of metabolic energy-sensing pathways [44, 45] (Fig. 1). Indeed, the kinase activity of AMPK is an integral constituent of the clock-cycling mechanism, possibly linking the activity of mTOR/AMPK signalling with clock activity [46–48], and BMAL1 itself is a reported target of the mTOR-effector kinase S6K1 [49]. In a recent study wherein rhythmic circadian clock expression patterns were induced effectively in SC-islets, circadian entrainment as a mechanism for beta cell maturation was shown in principle [50]. Stimulation indices, calcium fluxes and cyclical oxygen consumption were all increased following entrainment. In agreement with this, following SC-islet implantation and maturation in vivo, core clock components *BMAL1*, *RORA* and *BHLHE41* were all upregulated, showing that enhanced beta cell maturation correlates with enhanced expression of core clock components [35]. Perhaps most intriguingly, the recent finding that circadian clock cycling may regulate the alternative splicing of subsets of target genes within beta cells adds a new dimension to the concept of circadian control of beta cell maturation and transcriptional regulation [51].

In aggregate, the triggering of beta cell maturation through the entrainment of circadian clock machinery and the balancing of energy-sensing pathways following postnatal development are key aspects of beta cell functional acquisition. The underlying metabolic shifts that allow for enhanced glucose sensitivity beyond these signals are discussed further below.

Metabolic control of glucose sensitivity during beta cell maturation

The purpose of this review is not to outline an exhaustive list of all known bioenergetic pathways that couple glucose metabolism to insulin secretion, as many excellent resources

already exist for many aspects of beta cell function [52–55]. Instead, our aim is to highlight the interplay between some of these metabolic pathways and their development during beta cell maturation.

Oxidative phosphorylation Mitochondrial metabolism, particularly oxidative phosphorylation (OxPhos), is essential for beta cell function [56, 57]. Classically this is encompassed by the 'triggering pathway' model of GSIS, whereby mitochondrially generated ATP/ADP ratio shifts inhibit plasma membrane localised K_{ATP} channels, resulting in depolarisation and insulin release [58, 59] (Fig. 1). This acquisition of heightened OxPhos activity during maturation is mirrored in SC-islet studies that demonstrate increased abundance of OxPhos-related genes following enhanced in vitro culture conditions and maturation during murine engraftment [35, 60, 61]. Proteomics, transcriptomics and metabolomics studies within rat islets, as they transition from juvenile-to-adult states, also display enhanced OxPhos gene network signatures [62, 63].

Tricarboxylic acid cycle-derived metabolites In parallel with the core OxPhos-mediated triggering pathway model, the cytosolic cycling of numerous mitochondrial metabolites has also been implicated in the generation and maintenance of beta cell function [64, 65]. These proposed metabolite-coupling factors include the malate–aspartate shuttle [66], the pyruvate–malate cycle [67], the pyruvate–citrate cycle

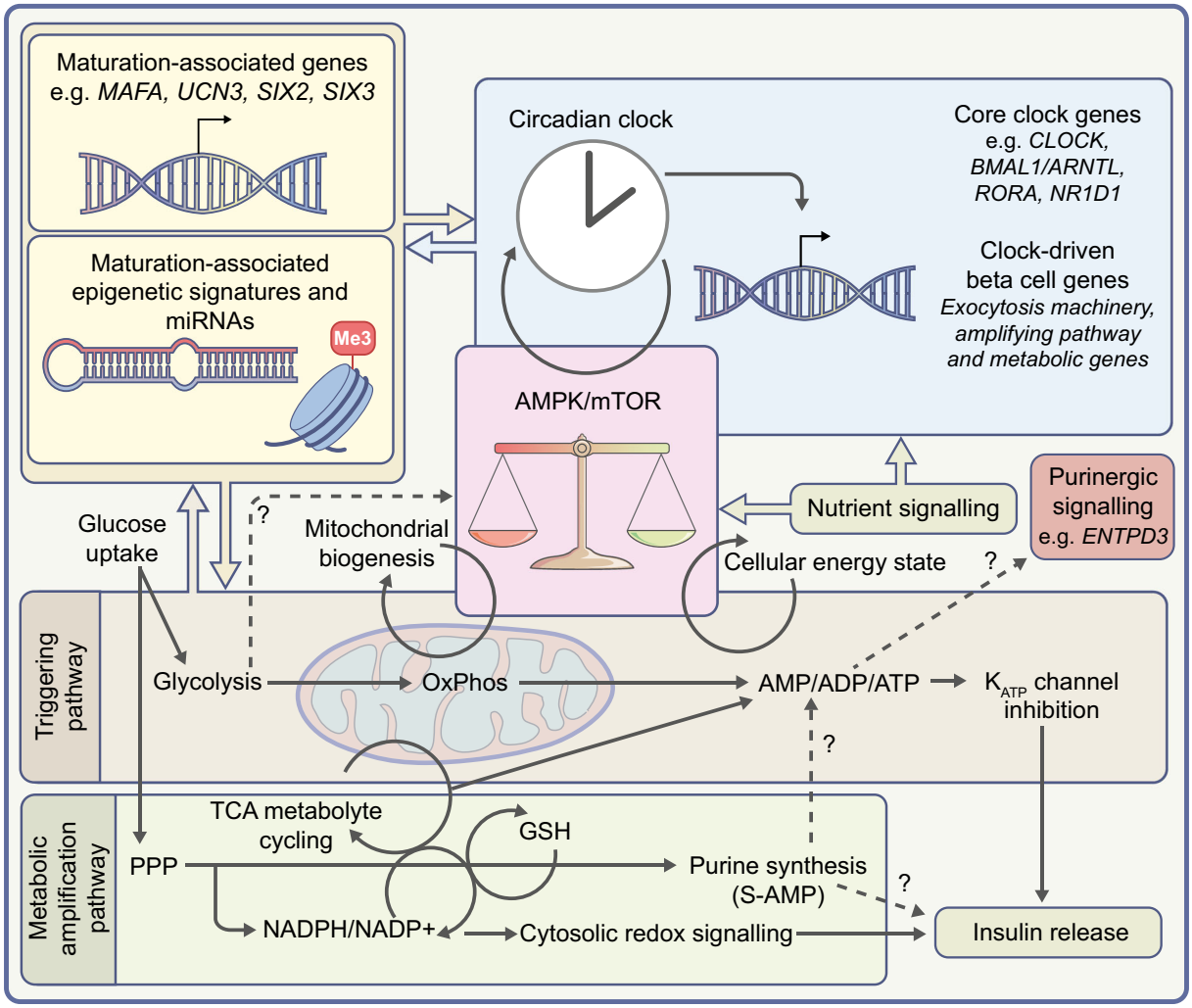

Fig. 1 Overlapping transcriptomic, metabolic and energy-sensing machinery that enables the functional maturation of beta cells. The ability of beta cells to derive GSIS is dependent on the synergistic interplay of many metabolic and regulatory features. The post-weaning maturation of beta cells is characterised by the re-balancing of the AMPK/mTOR energy-sensing pathways and their interaction with circadian clock entrainment. Both of these elements further interact with the canonical triggering and metabolic amplification pathways of GSIS involving NADP-mediated glutathione redox cycling. The feedback between these metabolic and nutrient-sensitive control points also trigger/respond to transcriptional shifts of maturation-associated genes, microRNA regulation and epigenetic signatures in beta cells. Dotted arrows with '?' symbols denote indirect or mechanistically unknown pathways of regulation. GSH, glutathione; S-AMP, adenylosuccinate. This figure is available as part of a downloadable slideset

[68], the pyruvate–isocitrate cycle [69–71], the phosphoenolpyruvate (PEP) cycle [72, 73] and the glycerolipid/NEFA cycle [74, 75] (Fig. 2). The common thread within most of these cycles is the export and metabolism of tricarboxylic acid (TCA) cycle intermediates that are coupled to GSIS without direct inclusion into OxPhos pathway reactions. However, the extent and importance of each of these cycles in their contribution to beta cell functionality is highly contentious. For instance, within pyruvate–malate and pyruvate–citrate cycling, the activity of the cytosolic malic enzyme (ME1) and the ATP-citrate lyase enzyme (ACLY) are key enzymatic steps. Nevertheless, the genetic reduction of either of these enzymes in beta cell models has been shown to be detrimental to GSIS or to have no detectable effect [67, 68, 76, 77]. A recent proteomic analysis of juvenile-to-adult islet maturation did demonstrate an upregulation in both of these genes, correlating with the acquisition of GSIS functionality [62]. In either case, the unifying concept of malate cycling pathways is the generation of cytosolic NADPH as the coupling factor that augments the insulin release response, ostensibly through the glutathione/redox-mediated modulation of SUMO specific peptidase 1 (SENP1) activity and its interaction with insulin granule release machinery [70] (Fig. 1).

Another TCA-derived pathway, the pyruvate–isocitrate cycle, generates cytosolic NADPH through the activity of the cytosolic isocitrate dehydrogenase enzyme (IDH1). The presence and activity of this enzyme are necessary for beta cell function [69–71]. However, in keeping with the duality of metabolic reports in beta cell models, opposite findings have been reported [78]. It is unlikely that each metabolic cycle acts

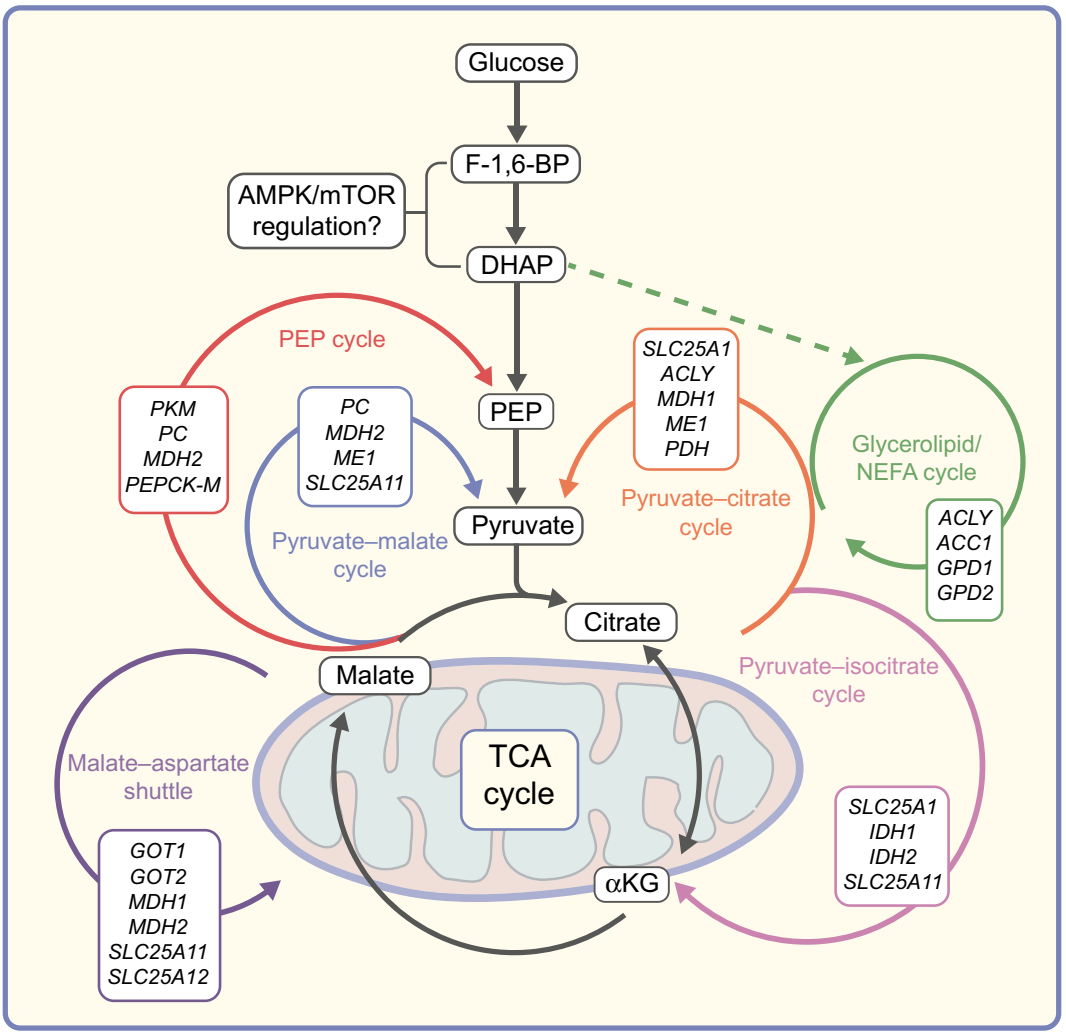

Fig. 2 Proposed glucose-sensitive metabolic cycles in functionally mature beta cells. The metabolic processing of glucose into TCA cycle intermediates with the resultant oxidative phosphorylation pathway is a core component of canonical GSIS. However, the processing of TCA-derived metabolites throughout a multitude of mitochondrial–cytosolic cycling reactions have also been shown to be a component of mature beta cell function. Genes that form core components of each cycle are shown in boxes outlined in the colour of the relevant cycle. Glycolytic intermediates may also act in the regulation of glucose-sensitive metabolism, together with interactions with elements of cellular energy-sensing machinery. F-1,6-BP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; αKG, α-ketoglutarate. This figure is available as part of a downloadable slideset

in isolation. Indeed, many of the TCA metabolite cycles/shuttles rely on overlapping enzymes and mitochondrial carriers (Fig. 2). Mitochondrial citrate export through solute carrier family 25 member 1 (SLC25A1) and α-ketoglutarate import through solute carrier family 25 member 11 (SLC25A11, also known as 2-oxoglutarate carrier [OGC]) are both reliant on malate anti-port. All of which fits with the importance of malate trafficking as a mature metabolic feature of beta cell function. This trafficking may occur through either the pyruvate cycling pathways or the malate–aspartate shuttle function, which ties more directly to OxPhos activity. Indeed, malate import into mitochondria is important for beta cell function (perhaps more important than the cycling kinetics alone) [79]. Appropriate mitochondrial channel expression patterns must therefore be key to glucose-sensitive beta cell function, as repression of either the SLC25A1 or OGC transporters results in the inhibition of GSIS [80, 81]. Intriguingly, direct chemical inhibition of SLC25A1 recapitulates the gene knockdown findings and reduces GSIS [68] but direct inhibition of OGC does not [82].

Cytosolic redox regulation Regardless of the relative strengths of each element within these cycles, the core concept relates to that of cytosolic redox regulation through glutathione cycling as a key metabolic coupling factor of glucose metabolism (Fig. 1). Clues relating to this acquisition of glutathione-based redox signalling are seen in human SC-islet models of maturation whereby a multitude of glutathione-related genes, as well as de novo production of glutathione itself, are increased following extended maturation periods in engrafted mice and primary islets [35]. This pattern is also true of aspartate and glutamate, possibly demonstrating an underdeveloped function of malate–aspartate shuttle activity within immature SC-islets as well as limited intracellular glutamate signalling, which has also been implicated as a triggering and amplifying messenger within GSIS [83, 84]. Interestingly, metabolic signatures of the pyruvate–isocitrate cycle are present within SC-islets without extended maturation periods and despite low glucose-responsive OxPhos metabolism [35]. Conversely, the presence of the PEP cycle as a mechanism of GSIS appeared underdeveloped in at least one SC-islet study [34]. It is tempting to conclude, therefore, that a subset of these metabolic cycling pathways may be able to compensate for SC-islet functionality without extended maturation periods, and that each of these pathway cycles may appear independently and throughout beta cell maturation, as is seen in rat islet maturation studies [62, 63, 85].

Pentose phosphate pathway The pentose phosphate pathway (PPP) is involved in the coupling of glucose metabolism to insulin release through two complementary mechanisms. The first relates to the cytosolic formation of NADPH from the initial glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGDH) reactions, which may act to fuel the NADPH-dependent cytosolic redox signalling pathway as outlined above [86] (Fig. 1). The second is through the direct formation of adenylosuccinate (S-AMP) and other intermediates within the purine synthesis pathway, downstream from the PPP [87, 88]. The mechanism of coupling of these intermediates to insulin release is still poorly understood, although it is hypothesised that these pre-AMP intermediates may activate AMPK, and therefore modify the mTOR/AMPK energy-sensing axis. This is an intriguing possibility, as the AMP mimetic 5-aminoimidazole-4-carboxamide riboside (AICAR), also an intermediate within the purine synthesis pathway, has been shown to have both positive and negative GSIS modulating properties under acute treatment [89]. AMPK signalling in beta cells has far-reaching implications for many aspects of metabolic network formation and glucose coupling during beta cell maturation [90, 91]. Such signalling may act to modulate the extent of certain metabolic cycling pathways, such as the pyruvate–citrate cycle and glycerolipid/NEFA cycle, through the AMPK-driven inhibition of acetyl-CoA carboxylase 1 (ACC1) [92, 93]. Basal AMPK activation under non-stimulatory glucose concentrations was found to drive the upregulation of mitochondrial OxPhos-related genes in a model of neonatal maturation [21], in line with the concept of enhanced mitochondrial development as an integral factor of mature beta cell functionality. Nevertheless, chronic activation of AMPK has also been shown to have detrimental effects on beta cell functionality [94]. It remains unclear how these glucose-responsive purine pathway intermediates would have beneficial effects on acute GSIS through AMPK activation, if we assume that this activation suppresses mTOR signalling, supposedly the dominant signalling cascade during GSIS [20]. Of course, purine pathway intermediates might not act through AMPK at all; in one metabolic study, an increase in glucose-stimulated 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP) (a phosphorylated form of AICAR) did not result in detectable changes in AMPK activation [88]. Additionally, direct glucose-sensing and reactive oxygen species-sensing capability of AMPK, independent of the cellular energy state (AMP/ADP/ATP ratio), may help to explain this discrepancy and might suggest that glucose-coupled purine synthesis pathways alter GSIS through alternative mechanisms [95, 96].

In summary, the underlying metabolic networks that derive beta cell function are multi-faceted and form progressively throughout beta cell maturation. The specific interplay between energy-sensing machinery, mitochondrial metabolism and metabolite trafficking networks are highly coupled. Therefore, we should be mindful that modifying one aspect of this symphony will have many far-reaching consequences across the biology of the beta cell. However, the generation and stability of these metabolic networks are dependent on the acquisition of appropriate transcriptomic profiles. Candidate

pathways and genes that mediate these changes during beta cell maturation are discussed next.

Signalling pathways and gene markers of beta cell maturation

The extent of beta cell differentiation is generally evaluated, in vitro and in vivo, through the upregulation and maintenance of a set of known beta cell marker genes (including *INS*, *PDX1* [97], *NKX6.1* [98], *NEUROD1* [99], *MAFA* [100] and *UCN3*) [3]. However, the presence of these genes is not necessarily an indication of mature beta cell functionality [10, 101]. Indeed, upregulation of urocortin 3 (UCN3) occurs during the postnatal maturation of beta cells [3, 10] but UCN3 itself appears to be functionally redundant in driving this maturation process [102]. It is therefore important to understand the difference between genes that are critical for maintaining beta cell identity and those that further determine the functional properties of beta cells, and of course, the intrinsic overlap between these two groups. The direct transcriptional regulation of beta cell-specific transcription factors and the influence on metabolic gene regulation is largely unknown. However, certain regulatory patterns have been discovered [103]. For instance, MafA may help repress 'disallowed' metabolic genes while maintaining expression of specific glucose transporter genes (*GLUTs*), glucokinase (*GCK*) and *PGC1α* (coding for a regulator of mitochondrial biogenesis and circadian oscillation) within beta cells [104, 105]. A transcriptional network driven by oestrogen-related receptor γ (*ERRγ*) has been shown to regulate multiple OxPhos-related genes, as well as regulating the pyruvate–citrate cycle-related enzyme encoded by *MDH1*, during beta cell maturation [106]. It has also been reported that transcriptional regulation through calcineurin–nuclear factor of activated T cell (NFAT) pathways tailor the expression of *GCK* and *GLUT2* (GLUT2 is the predominant glucose transporter in murine beta cells) [107, 108]. In contrast, the transcription factor activity of regulatory factor X6 (RFX6) is linked to regulation of *GCK* but not *GLUT2* [109]. These overlapping functions in metabolic gene regulation may be due to the web of beta cell-enriched transcription factors directly regulating each other, although there is evidence that the physical interaction of multiple transcription factors is necessary to maintain metabolically mature states, such as the co-binding of neuronal differentiation 1 (NEUROD1) and cAMP responsive element binding protein 1 (CREB1) in beta cell-specific enhancer regions [110].

Transcriptomics studies comparing healthy and diabetic beta cell pools offer many clues to the subsets of genes that may be necessary for maintaining beta cell function, either through candidate transcription factors or through direct regulation of beta cell metabolism. One example of the latter is the higher expression of glucose-6-phosphatase catalytic subunit 2 (*G6PC2*) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (*PFKFB2*) within healthy beta cell samples [111–116] and the upregulation of these genes during maturation of SC-islet beta cells [35, 61]. Both genes encode glycolytically linked enzymes that have been shown to have direct regulatory control over the glucokinase-mediated step of glycolysis [117–120]. The functional maturation of beta cells therefore correlates with heightened control over this initial step of glycolysis, which may regulate the pattern of downstream metabolism and glucose trafficking. The governing mechanisms of glycolytic flux within mature beta cells may also encompass the regulatory effect of cytosolic citrate and PEP (as products of the TCA metabolite cycles outlined previously) on phosphofructokinase 1 (PFK1) activity [112], with the generation of fructose-1,6-bisphosphate as a possible direct modulator of glucose-sensitive AMPK/mTOR activity [95] (Fig. 2). Even the oligomerisation state of the glycolytic enzyme GAPDH (rather than expression level) has been associated with beta cell functional maturation [34]. Some recent findings have also identified the SIX homeobox 2 (SIX2) and SIX homeobox 3 (SIX3) transcription factors as regulators of beta cell functional maturation [121, 122]; this has been demonstrated in SC-islet knockdown models of SIX2, wherein GSIS function was strongly impaired [123]. Interestingly, although SIX2 is necessary for SC-islet functional acquisition in vitro, SIX3 expression appears to be important for advanced maturation events and is not detected in SC-islets in vitro or after extended murine engraftment [35, 123].

The regulation and temporal sequence of genes within this context must at some level be run through transcription factor networks that are responsive to cell lineage signalling and systemic nutritional cues [101]. The 'holy grail' within the field of SC-islet generation is an optimised cocktail of signalling and patterning factors that would trigger in vitro beta cell maturation to the same level that is seen post-engraftment. Therefore, SC-islet generation protocols represent fertile ground to test candidate maturation signalling molecules, while simultaneously providing information on processes occurring during postnatal maturation [9, 124, 125].

A recent study found that non-canonical Wnt signalling, through Wnt4, may be one such signalling pathway that triggers maturation events within SC-islet beta cells [126]. It has long been established that Wnt signalling has a variety of important functions throughout islet organogenesis that are spatially and temporally controlled [127]. Furthermore, in SC-islets at earlier stages of differentiation, Wnt signalling affects the balance and penetrance of pancreatic progenitor formation [128, 129]. The exogenous application of Wnt4 to SC-islets increases an assortment of beta cell marker genes as well as mitochondrial OxPhos responsiveness to glucose [126], a pattern that is also seen when Wnt4 is added to human islet and beta cell lines [13]. *WNT4* expression has also been

observed in neonatal rat islets, suggesting a role in postnatal functional maturation [85]. However, another SC-islet study was unable to detect any discernible improvement in beta cell maturation following Wnt4 treatment, and conversely found that canonical Wnt signalling inhibition improved SC-islet maturation [130]. Regardless, the interplay of Wnt signalling in different aspects of beta cell differentiation and maturation is well founded. Tantalising evidence of Wnt signalling-derived AMPK/mTOR pathway changes in the regulation of the *Tcf7l2* gene in beta cell proliferation in mice is a clear demonstration of the holistic interactivity of cell signalling, energy-sensing machinery and mature beta cell functionality [131].

Many members of the TGF-β family have also been connected with beta cell maturation, although again with some inconsistent findings between research groups. Inhibition of the transforming growth factor β receptor 1 (TGFBR1, also known as ALK5) during SC-islet maturation has been shown to increase many beta cell marker genes, including *MAFA* [5]. However, more recent studies have shown either a marked improvement of SC-islet beta maturation in the absence of ALK5 inhibitors [132] or, in contrast, an increase in insulin expression in the presence of ALK5 inhibition [31]. Another member of the superfamily, bone morphogenetic protein 4 (BMP4), may also aid in the postnatal maturation of beta cell function following temporally controlled release from islet pericytes [133]. However, one study found that BMP4 treatment inhibited GSIS through the reduction of calcium currents [134], again indicating that particular cellular milieus and developmental timings elicit strong control over specific signalling outcomes. The thyroid hormone triiodothyronine (T3) has also been shown to accelerate the postnatal maturation of beta cells and boost *MAFA* expression in SC-islet models [105, 135], demonstrating that beta cell maturation is affected by systemic hormonal exposure.

In parallel with the signalling pathways outlined above, beta cell maturation may also be self-regulated via the modulation of extracellular ATP release and purinergic receptor-based signalling, through the activity of ectonucleoside triphosphate diphosphohydrolase 3 (ENTPD3) [136]. This has been identified in numerous beta cell transcriptomic studies [114, 137] and has also been shown to be a marker of beta cell maturation within SC-islets [31]. An intriguing overlap between these findings and the model of glucose-sensitive purine synthesis within mature beta cells may imply yet another nexus point of cellular energy state (through AMPK/mTOR modulation), metabolic trafficking (ATP production and release) and the regulation of GSIS in mature beta cells [138] (Fig. 1).

Another intriguing feature of beta cell functional maturation is the regulatory influence of microRNAs. Shifting patterns of microRNA expression have been shown to elicit robust regulatory effects on metabolic gene expression and beta cell functionality in a nutrient-sensitive manner, as well as throughout postnatal maturation [139–141]. The upregulation of the miR-129 family in beta cells during postnatal weaning in mice correlated with enhanced glucose-responsive insulin release [139]. This mirrors the postnatal increase in the expression of the miR-29 family, which has also been shown to repress the 'disallowed' genes *REST* [139] and *SLC16A1* [142]. In contrast, downregulation of the miR-181 and miR-17 families during postnatal maturation leads to the upregulation of *GPD2*, *MDH1* and *PFKP* metabolic genes [139]. Other microRNAs such as the miR-223 family (which ostensibly maintains *PDX1* and *NKX6.1* expression through suppression of forkhead box O1 [FOXO1] and SRY-box transcription factor 6 [SOX6] pathways [143]) and the miR-7 family (which reportedly boosts GSIS and *PDX1* levels in SC-islets [144] while suppressing mTOR signalling and proliferation [145]) are all enriched in mature beta cells. However, conclusions about the presence or absence of a particular microRNA family should be assessed in relative terms. For example, the miR-375 family is upregulated during SC-islet maturation [144], yet the forced overexpression of miR-375 in primary islets was reported to blunt GSIS responses and reduce glucose-responsive OxPhos [146]. This drop in functional activity could be explained by the increased expression of *PDK4* and reduced *PC* and *MDH1* expression within the primary islets. The shifting patterns and balance of microRNA family expression is therefore another key component of the onset and maintenance of beta cell maturity.

Finally, epigenetic signatures may help explain particular functional features of mature and immature beta cells. Both DNA methylation and histone modification are mechanisms by which beta cell identity and function are maintained, through tailoring the expression pattern of beta cell-enriched transcription factors, as well as being regulated by the transcription factors themselves [147, 148]. Some relevant examples include DNA methylation through the activity of DNA methyltransferase 3 α (DNMT3A), which has been linked to the repression of beta cell 'disallowed' genes, regulated through the mTORC1 component raptor [26] and through the inhibition of Wnt signalling during SC-islet maturation [130]. Histone methylation involving the activity of the polycomb repressor complex (PRC2) may act in juvenile islets to maintain an immature transcriptomic state together with trithorax group (TrxG) proteins [121, 149]. Evidence for extensive epigenetic shifts throughout beta cell maturation has also been seen in SC-islets [50]. All of these aspects of regulation are intricately tied to the metabolic state of the beta cell, as each form of epigenetic modification is fuelled by specific metabolic inputs [150].

In summary, the concept of tracking beta cell maturation through panels of marker genes is one that should be approached cautiously. While core beta cell identity genes

are no doubt important for many facets of beta cell maturation, the upregulation of one particular gene is not necessarily a strong argument for predicting functional maturation. Indeed, much more needs to be uncovered about how signalling pathways fully trigger beta cell maturity and through which mechanisms they operate. Furthermore, interpreting expression levels of particular genes, especially those within core metabolic pathways, should be done carefully so as not to misconstrue what is necessary for specific beta cell function and what is key for basal cellular metabolism. Additionally, overexpression of particular genes within a pathway may not necessarily trigger systemic maturation events. Full understanding of beta cell maturity clearly needs to go beyond simplistic models of gene and protein expression, and the regulation and maintenance of epigenetic signatures in beta cell function and disease must be considered.

Concluding remarks

Beta cell maturation is a multi-faceted process that takes cues from systemic nutritional and hormonal signals and ultimately results in a primed transcriptomic and metabolic beta cell state conducive to drive GSIS (Fig. 1). Recent advances have uncovered many of the core factors and machinery that are necessary to achieve functional maturity, and have woven together how these gene and metabolic networks form and maintain beta cell function. However, a unified model of the acquisition of beta cell functional maturation has yet to be completed. Even so, the vast interconnectedness and synergistic properties of cellular energy-sensing, signalling pathways, metabolic networks and transcriptional regulation in the generation of this maturation state is clear. We hope that this review has highlighted how each element of reported beta cell function is intricately aligned with many other aspects of beta cell biology, and that phenotypic outcomes of gene-knockout or chemical-intervention studies may elicit robust changes beyond the expression patterns of canonical beta cell markers. The recent application of sequencing, metabolic tracing and proteomic assays to probe beta cell maturation and dysfunction offers an incredible resource with which to better understand the acquisition of beta cell functionality, ultimately aiding in the understanding of diabetic pathologies and in the development of novel therapies.

Supplementary Information The online version contains a slideset of the figures for download, which is available at https://doi.org/10.1007/s00125-022-05672-y.

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