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INS-gene mutations: From genetics and beta cell biology to clinical disease

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Abstract

A growing list of insulin gene mutations causing a new form of monogenic diabetes has drawn increasing attention over the past seven years. The mutations have been identified in the untranslated regions of the insulin gene as well as the coding sequence of preproinsulin including within the signal peptide, insulin B-chain, C-peptide, insulin A-chain, and the proteolytic cleavage sites both for signal peptidase and the prohormone convertases. These mutations affect a variety of different steps of insulin biosynthesis in pancreatic beta cells. Importantly, although many of these mutations cause proinsulin misfolding with early onset autosomal dominant diabetes, some of the mutant alleles appear to engage different cellular and molecular mechanisms that underlie beta cell failure and diabetes. In this article, we review the most recent advances in the field and discuss challenges as well as potential strategies to prevent/delay the development and progression of autosomal dominant diabetes caused by *INS*-gene mutations. It is worth noting that although diabetes caused by *INS* gene mutations is rare, increasing evidence suggests that defects in the pathway of insulin biosynthesis may also be involved in the progression of more common types of diabetes. Collectively, the (pre)proinsulin mutants provide insightful molecular models to better understand the pathogenesis of all forms of diabetes in which preproinsulin processing defects, proinsulin misfolding, and ER stress are involved.

Keywords

Diabetes; Pancreatic beta cell; Insulin biosynthesis; Insulin gene mutation; Endoplasmic reticulum stress; Proinsulin misfolding

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1. Introduction

Secreted insulin in the circulation regulates body metabolism to homeostatically maintain blood glucose. In pancreatic beta cells, three major steps lasting 30–150 minutes are needed to synthesize mature bioactive insulin: 1) The insulin precursor, preproinsulin, is translated in the cytoplasm, targeted to and translocated across the endoplasmic reticulum (ER) membrane, and proteolytically processed by signal peptidase on the luminal side of the ER membrane, forming proinsulin. 2) In the oxidizing ER environment, proinsulin undergoes oxidative folding, forming three evolutionarily conserved disulfide bonds (B7-A7, B19-A20, and A6-A11), which allow proinsulin to achieve transport-competence for exit from the ER. 3) Intracellular trafficking of proinsulin through the Golgi apparatus to secretory granules allows proinsulin to be proteolytically processed by prohormone convertases (PC1/3 and PC2) and carboxypeptidase E (CPE) to form C-peptide and mature insulin that are stored in insulin secretory granules and released upon stimulation (Fig. 1) (Alarcon et al., 1995; Dodson and Steiner, 1998; Liu et al., 2014; Steiner, 2011).

Following the discovery of proinsulin in 1967 (Steiner and Oyer, 1967; Steiner et al., 1967), many studies have focused on proinsulin intracellular trafficking and processing as well as the formation of granules that lead to insulin storage and secretion (Guest et al., 1991; Huang and Arvan, 1994; Kuliawat and Arvan, 1992; Orci et al., 1985, 1986; Quinn et al., 1991; Rhodes and Halban, 1987; Steiner et al., 1980). As the ER has been increasingly recognized as a central regulator of secretory pathway dynamics (Barlowe and Miller, 2013), the early events of insulin biosynthesis occurring in the ER have drawn increasing attention. These studies have gained greater urgency since 2007 (see below), when several groups have reported new insulin gene mutations that cause permanent neonatal diabetes in humans (Colombo et al., 2008; Edghill et al., 2008; Polak et al., 2008; Stoy et al., 2007).

The concept of insulinopathies was originally introduced to describe rare monogenic causes of adult onset diabetes associated with insulin gene mutations (Gabbay, 1980; Steiner et al., 1990). Those mutations are located in the insulin moiety or at the dibasic cleavage sites of proinsulin (Gabbay et al., 1976; Oohashi et al., 1993; Shoelson et al., 1983b; Tager, 1984). The mutations underlying these classical insulinopathies impair insulin binding to its receptor (Sakura et al., 1986; Vinik and Bell, 1988) or result in alteration of proinsulin trafficking for storage in insulin secretory granules (Carroll et al., 1988) or failure of endoproteolytic processing to insulin (Barbetti et al., 1990; Tager, 1984; Yano et al., 1992).

In 2007, the first study reporting 10 new insulin gene mutants causing neonatal diabetes (Stoy et al., 2007) set off a wave of new discovery about insulin biosynthesis and mutations that perturb insulin production. Over the past seven years, the list of insulin gene mutations associated with human diabetes has significantly expanded and is likely to grow further as genetic analysis becomes increasingly available. At the time of this writing, 51 *INS*-gene mutations have been identified to cause monogenic diabetes (Table 1). These mutations have been identified in the untranslated regions of the *INS* gene, the sequence encoding the preproinsulin signal peptide, the proteolytic cleavage sites of signal peptidase (SPase), the insulin B-chain, C-peptide, insulin A-chain, and the cleavage sites for prohormone convertases PC1/3 and PC2. Diabetogenic *INS*-gene mutations have a broad spectrum of

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clinical presentations ranging from severe neonatal onset to mild adult onset, suggesting that the product of different mutant *INS* alleles behave differently and utilize distinct mechanisms to cause diabetes. Accumulating evidence suggests that clinical severity of diabetogenic *INS*-gene mutations is linked to the nature of the particular mutation and the steps in the pathway of insulin biosynthesis affected by these mutants (Fig. 1 and Table 1) (Liu et al., 2010b, 2014; Støy et al., 2010). Herein, we review the most recent advances in this field, and discuss challenges and potential strategies to prevent/delay the development and progression of this form of monogenic diabetes.

2. Diabetogenic *INS*-gene mutations: recessive *vs.* dominant and early-onset *vs.* late-onset

Based on their pathological consequences, the insulin gene mutations can be divided into two major groups: recessive and dominant. Recessive *INS* gene mutations are “loss-of-function” mutations, which affect insulin biosynthesis at transcriptional and/or translational levels through different mechanisms including insulin gene deletion or truncation with failure of insulin transcription; instability of insulin mRNA; or defective translational initiation due to loss of the natural start codon (Garin et al., 2010; Raile et al., 2011). Such mutations may result in a >80% decrease of insulin production from the mutant allele. The fact that these mutations are inherited in a recessive mode indicates that the remaining normal *INS* gene allele is sufficient to maintain normoglycemia. This conclusion is consistent with earlier studies of insulin gene knockout mice (Leroux et al., 2001). Rodents have two functional insulin genes: *Ins1* and *Ins2* (Deltour et al., 1993). It has been shown that homozygous deletion of *Ins1* or *Ins2* plus heterozygous deletion of the second *Ins* gene does not lead to diabetes, supporting the notion that the product of even one functional insulin gene allele is ordinarily sufficient to maintain blood glucose in the normal range (Leroux et al., 2001) [although loss of a functional insulin allele may predispose individuals to a higher risk of developing diabetes in adulthood (Raile et al., 2011)].

About 80% (39 of 51) of insulin gene mutations are inherited in an autosomal dominant fashion. Most of them (30 of 39) cause the syndrome called Mutant *INS*-gene-induced Diabetes of Youth (MIDY) (Liu et al., 2010b). Since insulin haploinsufficiency cannot itself account for early-onset insulin-deficient diabetes, the development and progression of MIDY are attributed to a gain-of-toxic function from the mutant gene product. Indeed, studies using *Akita* mice, which carry the C96Y mutation in one of two *Ins2* alleles, demonstrate that expressing mutant proinsulin leads to ER stress and ultimately, beta cell death (Oyadomari et al., 2002; Ron, 2002; Wang et al., 1999; Yoshioka et al., 1997). Beta cell death culminating from the expression of other MIDY mutants was also evident (Colombo et al., 2008). In addition to cytotoxicity, increasing evidence has suggested that the gain-of-toxic function of MIDY mutants (leading to insulin deficiency in MIDY patients) may be initiated by abnormal interactions between co-expressed mutant and wild-type proinsulin molecules in the ER of pancreatic beta cells. Indeed, the expression of MIDY mutants blocks co-expressed wild-type proinsulin exit from the ER, and therefore decreases insulin production from the wild-type *INS* allele (Liu et al., 2010a, 2012; Park et al., 2010; Wright et al., 2013b) prior to any decrease in beta cell mass, suggesting that in the

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MIDY syndrome, blockade of wild-type proinsulin by mutant proinsulin is a triggering event in the onset of insulin deficiency (Gupta et al., 2010; Hodish et al., 2010; Renner et al., 2013).

In addition to mutations causing MIDY, a few autosomal dominant insulin gene mutations (9 out of 39) are associated with late-onset diabetes. These include the original and now classic insulinopathies plus two new preproinsulin signal peptide mutations (Boesgaard et al., 2010; Edghill et al., 2008; Meur et al., 2010; Molven et al., 2008). Unlike the MIDY mutants, the mutations associated with late-onset diabetes do not appear to be linked to proinsulin folding events that occur in the ER. Herein, we describe the distinct groups of insulin gene mutations that are clustered based largely on experimentally confirmed defects in the cell biology of insulin biosynthesis. The mutations that have not been experimentally tested are integrated into these groups based on predicted defects associated with these mutations (Fig. 1 and Table 1).

3. Lessons from *INS*-gene mutations: the links between biological defects and diabetes phenotypes

Diabetogenic *INS* gene mutations present a broad spectrum of diabetes severity ranging from neonatal onset severe insulin deficient diabetes to late-onset mild diabetes. One example of this comes from a family carrying proinsulin-C43G mutation. The mutation disrupts one of critical interchain disulfide bonds, leading to proinsulin misfolding in the ER, and the proband carrying this mutation developed very severe diabetes at 43 weeks of age. However, his father who carries the identical mutation was diagnosed with mild type 2 diabetes at 30 years of age (Stoy et al., 2007). Thus, heterogeneity of clinical presentation depends both on the biological behavior of the *INS* mutants themselves and other genetic and environmental factors.

3.1. Insulin gene mutations affecting insulin gene transcription and translation

In pancreatic beta cells, insulin biosynthesis is tightly regulated at both transcriptional and translational levels (Hay and Docherty, 2006; Itoh and Okamoto, 1980; Leibiger et al., 2000; Sharma and Stein, 1994). Upon glucose stimulation, while general protein synthesis increases 2-fold, preproinsulin biosynthesis can increase as much as 30-fold in one hour, indicating specific control mechanisms (Alarcón et al., 1993; German et al., 1995; Guest et al., 1991; Wicksteed et al., 2001). The physiological and pathological importance of regulatory elements within untranslated region of the *INS* gene is genetically demonstrated by the newly discovered insulin gene mutations that affect insulin gene transcription/translation (Garin et al., 2010). Twelve such mutations are inherited in a recessive manner (Garin et al., 2010; Raile et al., 2011). Among these mutations, 5 are located in the insulin promoter region, resulting in either a deletion of the promoter region regulated by MAFA and NEUROD1, or disruption of binding sites for additional DNA binding proteins. Those mutations that result in 90% reduction of promoter activity provide the first human genetic evidence that discrete *INS* cis regulatory elements are essential for regulating insulin biosynthesis (Garin et al., 2010). Another interesting mutation, c.*59 A>G, is found in the polyadenylation signal of 3′ untranslated region (3′-UTR) of insulin mRNA. This mutation

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results in severe instability of insulin mRNA, supporting previous findings that the 3′-UTR of insulin mRNA plays an essential role in regulating insulin biosynthesis through modulating insulin mRNA stability (Fred and Welsh, 2009; Tillmar et al., 2002). Two mutations (c. 3G>T and c.3G>A) found at the 3rd nucleotide of preproinsulin start codon abolish the native translational initiation site for the preproinsulin. Although there is an ATG encoding another methionine at the 5th residue of preproinsulin that may potentially function as an alternative start codon, the nucleotide sequence upstream of this ATG does not favor translational initiation resulting in an ~80% decrease of insulin production (Garin et al., 2010).

3.2. Insulin gene mutations affecting preproinsulin ER targeting and translocation

The first step of insulin biosynthesis involves the targeting and translocation of newly synthesized preproinsulin from the cytosol into the ER. This process is led by the signal peptide of preproinsulin at its N-terminus. Preproinsulin has a 24 residue signal peptide, which comprises three regions: a positive charged n-region; a central core hydrophobic h-region; and a polar c-region containing a cleavage site of the SPase (for reviews, see Liu et al., 2014; Rapoport, 2007). To date, four new mutations located in the preproinsulin signal peptide have been reported to cause diabetes (Boesgaard et al., 2010; Hussain et al., 2013; Meur et al., 2010; Stoy et al., 2007). Mutations are found in all three regions of the signal peptide (Fig. 2). Interestingly, the clinical diabetes phenotypes associated with these mutants range from severe neonatal-onset insulin-deficient diabetes caused by L13R or A24D, to mild adult onset diabetes associated with R6C or R6H, suggesting the possibility that different cellular defects or molecular mechanisms may underlie the onset and development of diabetes in these patients. Indeed, we have recently shown that the mutation at the site of signal peptide cleavage, A24D, leads not only to inefficient signal peptide cleavage, but the cleavage occurs at an alternative site producing an abnormal proinsulin. Thus, although the proinsulin moiety of preproinsulin-A24D has the same predicted sequence as that of wild-type proinsulin, the combination of uncleaved and improperly cleaved preproinsulin-A24D causes misfolding of both species within the ER. This highlights the physiological and pathological significance of the coordination of signal peptide cleavage and downstream proinsulin folding (for detail, see Liu et al., 2012; Liu et al., 2014). In the next paragraphs, we focus on the most recent findings revealing preproinsulin targeting and translocation defects caused by R6C and R6H.

In mammalian cells, most newly synthesized secretory proteins undergo co-translational targeting and translocation into the ER. In this process, when the signal peptide of the nascent polypeptide chain emerges from the ribosome, it is first recognized and bound by the signal recognition particle (SRP), forming the SRP-ribosome-nascent polypeptide complex. The complex is then targeted to the ER membrane via the docking of SRP to the SRP receptor on the ER membrane. Upon targeting to the ER membrane, the signal peptide interacts with the Sec61 translocon, through which the signal sequences of secretory proteins (or signal anchors of membrane proteins) are positioned and oriented for subsequent translocation of the remaining polypeptide (Pilon et al., 1998; Plath et al., 1998; Zimmermann et al., 2011). At least three factors play roles in determining the orientation of the signal sequence within the Sec61 translocon: the length of the h-region; the charge

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gradient of signal sequence flanking the h-region; and the charge gradient of the translocon spanning the ER membrane (Goder et al., 2004; Hartmann et al., 1989; Parks and Lamb, 1991; von Heijne, 1986; Wahlberg and Spiess, 1997). Since the Sec61 translocon possesses a conserved charge gradient with a positive charge toward the ER lumen (Goder et al., 2004), a signal sequence with a positive charge in the n-region tends to make a "loop", orienting the N-terminus of the signal sequence towards the cytosol and the C-terminus oriented towards the ER lumen, referred as the "positive-inside" rule (Iino et al., 1987; Peterson et al., 2003; Sasaki et al., 1990; von Heijne, 1989). However, although this "positive-inside" rule is considered as a general principle for orienting the signal sequence during preprotein translocation across the ER membrane, the potential physiological or pathological significance of the n-region positive charge in the signal peptide of secretory and membrane preproteins has only recently been examined.

Preproinsulin-R6C or R6H mutates the highly conserved positively charged residue in the n-region of the signal peptide that is thought to be important in targeting/translocation of secretory and membrane proteins (Fujita et al., 2010; Goder and Spiess, 2001; Parks and Lamb, 1991; Sakaguchi et al., 1992; Sasaki et al., 1990). Surprisingly, using a GFP-tagged preproinsulin construct, one study reported no defect of preproinsulin-R6C/R6H in targeting to insulin granules (Meur et al., 2010). We also examined the translocation efficiency of preproinsulin-R6C/H with a large GFP-tag (248 amino acids), and compared that to preproinsulin-R6C/H with a small Myc-tag (10 amino acids) or without any tag. We confirmed that GFP-tagged preproinsulin-R6C/H has no detectable defect in ER targeting and translocation (Guo et al., 2014). By contrast, at least 50% of newly synthesized Myc-tagged or untagged preproinsulin-R6C/H failed to be efficiently translocated after initial targeting of the mutant preproinsulin to the ER membrane (Guo et al., 2014). The results suggest that larger secretory proteins may be less sensitive than short secretory polypeptides to the effect of loss of positive charge in the n-region of the signal peptide. The underlying mechanism of this length dependence – and whether this behavior is specific to preproinsulin – remains unknown. A recent study shows that polypeptide length is an important factor for determining whether secretory polypeptides undergo co-translational translocation or post-translational translocation (Lakkaraju et al., 2012). Importantly, because of the short time window for signal peptide recognition by SRP, the signal peptide of small secretory proteins may not be accessible for co-translational recognition and targeting by SRP before the completion of synthesis of the full-length precursor. Thus, post-translational modes of targeting and translocation may provide an important backup mechanism to enhance overall translocation efficiency for small secretory proteins including preproinsulin (Johnson et al., 2012, 2013; Shao and Hegde, 2011).

Preproinsulin-L13R mutation was identified in a baby girl with *in utero* growth retardation who developed severe diabetes on the second day after birth (Hussain et al., 2013). The mutation is located in the h-region of preproinsulin signal peptide. An arginine (charged residue) substitution of the hydrophobic leucine disrupts the characteristic hydrophobic core of the h-region that normally plays a critical role in SRP recognition, ER targeting and translocation of secretory proteins (Bird et al., 1990; Haeuptle et al., 1989; Janda et al., 2010). Therefore, the L13R mutation is expected to affect insulin biosynthesis at the earliest

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step of entry of newly synthesized preproinsulin into the ER. However, the actual cellular defect underlying beta cell failure and diabetes caused by preproinsulin-L13R remains to be experimentally determined.

3.3. Insulin gene mutations affecting proinsulin folding in the ER

Among all autosomal dominant insulin gene mutations, more than 70% (30 out of 39) are predicted to affect the normal folding pathway of proinsulin in the ER (Table 1). To date, about half of these mutants have been experimentally confirmed to cause proinsulin misfolding in the ER (Colombo et al., 2008; Liu et al., 2010a, 2012; Park et al., 2010). The most studied insulin gene mutation of this type is preproinsulin-C96Y mutation. It was found first in the *Akita* mouse (thus, C96Y mutation is often referred as "the *Akita* mutation"), which carries a spontaneous C96Y mutation in one of two *Ins2* alleles. The mouse develops diabetes shortly after weaning (Wang et al., 1999; Yoshioka et al., 1997). The C96Y mutates one of the six conserved Cys residues of proinsulin, disrupting the formation of the B7-A7 disulfide bond, and creating an unpaired B7 cysteine. Both *in vivo* and *in vitro* studies demonstrate that the C96Y mutation causes proinsulin misfolding in the ER, induces ER stress and leads ultimately to beta cell death (Hodish et al., 2010; Izumi et al., 2003; Liu et al., 2007, 2010a; Park et al., 2010; Wang et al., 1999). Another diabetic mouse line, the Munich mouse expressing preproinsulin-C95S, was generated by chemical (*N*-ethyl-*N*-nitrosourea) mutagenesis (Herbach et al., 2007). Importantly, both C95S and C96Y mutations are found in humans and cause the MIDY syndrome (Colombo et al., 2008; Stoy et al., 2007). Therefore, the two mouse lines are appropriate animal models in which to study cellular defects and stress caused by misfolded proinsulin *in vivo*. Besides C95S and C96Y mutations, 16 additional mutations also generate novel unpaired Cys residues – either because of mutations at the site of native cysteines, or because the mutations create new Cys residues (Table 1). Since native disulfide bond formation among the cysteine residues is the most well recognized event in the proinsulin folding pathway in the ER, all of the mutations with unpaired cysteines are likely to seriously interfere with disulfide maturation, leading to proinsulin misfolding (Liu et al., 2005, 2010a, 2012; Rajpal et al., 2012).

In addition to mutations that create unpaired cysteines, there are 12 mutations that are also expected to cause proinsulin misfolding in the ER. Eleven of these have been found within the insulin B-chain while one has been located at the preproinsulin signal peptide cleavage site (none has yet been reported within the A-chain; see Table 1). More work is still needed to understand how these point mutations affect proinsulin folding in the ER. However, it is worth noting that nearly all of these mutations are located at highly conserved residues in the preproinsulin molecule, some of which have already been shown to be important for local folding of the insulin moiety, which may help to align the B- and A-chains in order to facilitate native proinsulin disulfide pairing (Hua et al., 2006b; Liu et al., 2009, 2010c; Sohma et al., 2010; Weiss, 2009). Thus, even these mutations that do not directly alter proinsulin Cys residues are likely to perturb normal disulfide pairing of proinsulin. Indeed, all experimentally tested mutants, including A24D, H29D, L30P, G32R, L35P, R46Q, and G47V, have been shown to exhibit defective disulfide maturation of newly synthesized proinsulin, leading to an increase of mispaired disulfide isomers (Liu et al., 2010a, 2012, 2014). Studies focusing on the pathway of native proinsulin folding and disulfide maturation

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in the ER are still needed in order to understand how these processes are affected by this subset of insulin gene mutations.

3.4. Insulin gene mutations affecting trafficking and processing of proinsulin

Following oxidative folding and dimerization within the ER, properly folded proinsulin exits from the ER for delivery to the Golgi apparatus. As the Zn^{2+} concentration begins to rise in the lumen of the trans-Golgi network (TGN) (Dunn, 2005), proinsulin can initiate zinc-dependent hexamerization (Dodson and Steiner, 1998; Huang and Arvan, 1995; Orci et al., 1986). Upon entry into immature secretory granules, proinsulin hexamers undergo endoproteolytic cleavage via the actions of PC1/3 and PC2 in concert with carboxypeptidase E (CPE) to process proinsulin into mature insulin and C-peptide (Alarcón et al., 1993; Bailyes et al., 1992; Davidson and Hutton, 1987; Fricker et al., 1986; Steiner, 1998; for detailed reviews, please see Dodson and Steiner, 1998; Liu et al., 2014. Five insulin gene mutations are located at the junctions between the proinsulin B-chain and C-peptide, or between the C-peptide and A-chain, corresponding to PC1/3-preferred and PC2-preferred cleavage sites, respectively. The clinical phenotypes caused by mutations at the cleavage sites vary greatly depending upon the different substitutions by particular residues. Specifically, substitutions of arginine with either leucine, histidine, or proline at C-A junction results in proinsulin processing defects that are accompanied by relatively asymptomatic hyperproinsulinemia (Gabbay et al., 1979; Steiner et al., 1990), borderline glucose intolerance (Gruppuso et al., 1984), or late-onset mild diabetes (Oohashi et al., 1993; Yano et al., 1992). In contrast, substitution of arginine with cysteine at either B-C junction or C-A junction causes severe early onset insulin-deficient diabetes (Colombo et al., 2008; Edghill et al., 2008; Rubio-Cabezas et al., 2009; Stoy et al., 2007). Direct side-by-side comparison indicates that while the leucine substitution R89L is efficiently secreted from the cells, the cysteine substitution R89C causes proinsulin misfolding and retention in the ER (Colombo et al., 2008). Thus, although R89C mutates the PC2 cleavage site, the underling mechanism of diabetes caused by this mutation is not due to a defect of PC2-mediated proinsulin processing, but rather due to proinsulin misfolding and ER stress. This further highlights severe pathological consequences caused by proinsulin misfolding in the ER.

Surprisingly, another mutation that causes hyperproinsulinemia is located at neither the PC1/3 or PC2 cleavage site, but rather stems from a substitution of the conserved histidine with aspartic acid at position 34, H34D, corresponding to the 10th residue of the proinsulin B chain (Chan et al., 1987; Gruppuso et al., 1984). Studies using a transgenic mouse line expressing this mutation revealed that more than 50% of proinsulin-H34D is normally transferred to the secretory granules and processed to insulin. However, a significant proportion of the newly synthesized mutant proinsulin (~15%) is secreted from the cells through an unregulated or constitutive pathway, and ~20% is rapidly degraded within beta cells (Burgess and Kelly, 1987; Carroll et al., 1988). A similar result is also obtained in At-T20 cells transiently expressing proinsulin-H34D (Gross et al., 1989). In beta cells, almost all of the newly synthesized proinsulin that exits the ER is ultimately packaged into the regulated secretory granules where proinsulin processing occurs (Liu et al., 2007; Rhodes and Halban, 1987; Steiner et al., 1990). The sorting defects of proinsulin-H34D mutant

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suggest that proinsulin has structural information that plays a role in its intracellular sorting, trafficking and storage in pancreatic beta cells.

3.5. Mutations affecting insulin binding to the insulin receptor

Three of the classical insulinopathies, F48S, F49L, and V92L [corresponding to F(B24)S, F(B25)L, and V(A3)L], identified in the early 1980s (Given et al., 1980; Sakura et al., 1986; Shoelson et al., 1983a, 1983b; Tager et al., 1979), are mutations located in regions thought to be important for insulin binding to its receptor (Hua et al., 1993; Menting et al., 2013; Xu et al., 2004). Indeed, the binding efficiency of those mutant insulins to the insulin receptor ranges from about 14% to 0.2% of that of wild-type insulin (Assoian et al., 1982; Sakura et al., 1986; Shoelson et al., 1983a, 1983b; Steiner et al., 1990). Defective binding of these secreted mutant insulins to insulin receptors leads to a significant impairment of insulin clearance – resulting in an increased half-life of the mutant insulins in the circulation, leading to an elevated ratio of circulating insulin to C-peptide (Shoelson et al., 1984). These findings provide direct evidence that receptor-mediated uptake leading to physiological degradation of insulin is a major pathway of insulin clearance *in vivo* (Steiner et al., 1990).

In addition to defective binding to the insulin receptor, a recent study reveals surprising intracellular defects of proinsulin-F48S, corresponding to F(B24)S. Compared to proinsulin-V92L or the mutants causing MIDY, F48S presents an intermediate secretory phenotype, including a moderate defect in oxidative folding in the ER, a limited decrease in secretion, a modest activation of unfolded protein response (UPR), and a partial dominant-negative effect on co-expressed proinsulin wild-type (Liu et al., 2010a). Studies have shown that phenylalanine at the 24th residue of the insulin B-chain plays an important role in stabilizing the native-like cluster of hydrophobic side chains, increasing the efficiency of B19-A20 disulfide pairing (Hua et al., 2006a). Computer modeling from NMR spectroscopic data indicates F48S mutation results in surprising variation (ie, instability) in the structure of residues B20-B30, consistent with perturbation of B19-A20 disulfide pairing. By contrast, the structure of another classical mutation insulin-V92L is essentially identical to wild-type insulin and causes only trivial decrements in thermodynamic stability (Fig. 3, Liu et al., 2010a).

4. Molecular mechanisms underlying pancreatic beta cell failure and diabetes caused by insulin gene mutations

4.1. Cellular response to misfolded proinsulin in the ER

Upon delivery into the ER lumen, preproinsulin is immediately processed into proinsulin, which undergoes rapid oxidative folding. As discussed above, the subset of *INS*-gene mutations that lead to the autosomal dominant syndrome of MIDY cause proinsulin misfolding in the ER. These MIDY mutants can be recognized by the ER quality control system, with the misfolded molecules bound by ER chaperones. The immunoglobulin heavy chain-binding protein (BiP) is one of the major chaperones that has been experimentally confirmed to bind to misfolded proinsulin and proinsulin folding intermediates (Liu et al., 2005; Scheuner et al., 2005; Schmitz et al., 1995). As a member of the heat shock protein 70 (HSP70) superfamily, BiP is a master chaperone that recognizes unfolded and misfolded

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proteins, sensing changes in the ER environment, and maintaining ER homeostasis by contributing indirectly to the activation of three unfolded protein response (UPR) signaling pathways (Hendershot, 2004; Liu and Kaufman, 2003; Volchuk and Ron, 2010; Wang et al., 2009). Those pathways are initiated by three ER transmembrane proteins, including protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (for review, please see Cavener et al., 2010; Walter and Ron, 2011; Zhang and Kaufman, 2008). In normal beta cells, the lumenal domain of all three transmembrane sensor proteins have the potential to be bound by BiP. However, in beta cells expressing MIDY mutants, BiP preferentially relocates its binding to misfolded proinsulin concomitant with the activation of IRE1 (Gardner and Walter, 2011), PERK (Bertolotti et al., 2000) and ATF6 (Shen et al., 2005), triggering the tripartite UPR. Recent studies suggest that, at least for IRE1 pathway, unfolded proteins can also directly bind and activate IRE1 (Bernales et al., 2006; Gardner and Walter, 2011; Kimata et al., 2007). Acute activation of UPR is considered as a defense mechanism that could potentially alleviate ER stress by attenuating protein translation, selectively upregulating ER chaperones, and accelerating retrotranslocation of misfolded protein for proteasomal degradation, a process called ER-associated degradation (ERAD) (Smith et al., 2011). However, in *Akita* mice (and likely in MIDY patients), continuous expression of misfolded mutant proinsulin in the ER results in an enlarged ER and prolonged activation of the UPR, which could disrupt ER protein homeostasis, leading to chronic ER stress and activation of apoptosis signaling, and promoting beta cell death (Hartley et al., 2010; Izumi et al., 2003). ER stress-related apoptosis appears to be mediated by transcriptional activation of the C/EBP homologus protein (CHOP: gene name GADD153). Targeted disruption of this gene decreases the rate of beta cell death and delays the onset of diabetes in heterozygous *Akita* mice by 8–10 weeks (Oyadomari et al., 2002).

4.2. Bystander effects of misfolded proinsulin on co-expressed wild-type proinsulin

It has been a commonly held view that diabetes *in Akita* mice is caused solely by ER stress-mediated beta cell death. However, given the fact that decreased beta cell death (by disrupting GADD153) in *Akita* mice delays but does not prevent diabetes onset, factors other than decreased beta cell mass are likely to be involved in the development of diabetes caused by misfolded proinsulin. Using a compound heterozygous mouse line co-expressing *Akita* mutant proinsulin and proinsulin fused with GFP in beta cells, several recent studies find that there is no decrease in beta cell mass in newborn *Akita* mice (Hodish et al., 2010) or even at the time of initial presentation of hyperglycemia – indeed, Gupta et al. argued that the pancreatic islets of *Akita* mice may even exhibit beta cell hyperplasia at the time of initial onset of diabetes (Gupta et al., 2010). More recently, a transgenic pig model expressing *Akita* mutant proinsulin in beta cells has been established. Hyperglycemia is evident within 24 hours after birth in the transgenic pigs, a direct sign of insulin deficiency. Yet the transgenic pigs exhibit virtually no difference of beta cell mass with that of littermate controls more than one week after birth (Renner et al., 2013). Altogether, these studies indicate that insulin deficiency precedes the decrease in beta cell mass during diabetes onset as a consequence of the expression of misfolded MIDY mutant proinsulin – and this is likely to be true in humans as well.

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As noted in section 2 above, complete homozygous knockout of either *Ins1* or *Ins2* in conjunction with heterozygous deletion of the other *INS* gene does not cause diabetes in mice (Leroux et al., 2001), just as several human *INS* gene mutations silencing one *INS* allele are recessive (Garin et al., 2010; Raile et al., 2011). Thus, neither loss of beta cell mass nor loss of one normal *INS* allele is likely to be a sufficient reason for onset of diabetes in MIDY. As discussed above, the beta cells of *Akita* mice do experience ER stress due to expression of misfolded mutant proinsulin. However, neither overall protein synthesis nor specific proinsulin synthesis *in Akita* beta cells is decreased compared with that of wild-type islets, indicating that decreased insulin production is not caused by attenuation of proinsulin synthesis (Izumi et al., 2003; Liu et al., 2007). However, pulse-chase experiments have shown that, two hours after synthesis, at a time when the majority of proinsulin from wild-type islets is processed to mature insulin – a significant amount of newly synthesized wild-type proinsulin in *Akita* islets has been degraded without being processed, leading to dramatically decreased insulin production (Liu et al., 2007). Altogether, the data in in *Akita* mice (and probably in humans with MIDY) indicate that insulin deficiency is initiated by a defect in the biosynthesis pathway from proinsulin to insulin.

Based on the foregoing reasoning, cellular defects in normal proinsulin processing, caused by MIDY mutant proinsulin at a time before there is significant loss of beta cell mass, is likely to be responsible for the initial failure of insulin production in MIDY. In *Akita* mice, a significant amount of wild-type proinsulin itself becomes misfolded and becomes recognized by the ER chaperone, BiP (Liu et al., 2010a), suggesting that misfolded mutant proinsulin can affect the normal folding of co-expressed wild-type proinsulin in the ER. There may be at least two reasons for this. First, ER stress caused by the expression of misfolded mutant proinsulin may change the ER environment, altering the availability of free chaperones, the redox environment, and perhaps even the kinetics with which preproinsulin is translocated and its signal peptide cleaved at the ER membrane – all of which may directly or indirectly affect the normal folding pathway of wild-type proinsulin in the ER. However, in cells pretreated with a low dose of tunicamycin for 16 hours to induce ER stress, wild-type proinsulin continues to exit the ER and proceeds on to secretion (Liu et al., 2010a). Thus, at least mild acute ER stress does not seem to be sufficient to block proinsulin trafficking (although the effect of severe prolonged ER stress on proinsulin folding and trafficking remains to be determined).

Second, misfolded mutant proinsulin directly acts on co-expressed wild-type proinsulin, affecting its folding pathway in the ER. Indeed, both co-immunoprecipitation and an-isotropy FRET microscopy have shown that misfolded mutant proinsulin can intimately interact with co-expressed wild-type proinsulin in the ER (Haataja et al., 2013; Hodish et al., 2010; Liu et al., 2010a, 2012). Importantly, these abnormal interactions appear to include, among others, an intermolecular thiol attack in which cysteine residues from the mutant proinsulin form intermolecular disulfide bonds with wild-type proinsulin. As proinsulin normally uses all of its 6 Cys residues for intramolecular disulfide pairing, any intermolecular disulfide bonds are by definition non-native, and will leave the wild-type partner with at least one unpaired Cys residue that itself may produce further inappropriate interactions, thereby affecting wild-type proinsulin folding and resulting in formation of

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disulfide-linked complexes that contain both mutant and wild-type proinsulin molecules (Liu et al., 2010a, 2012). The consequence of the formation of aberrant protein complexes between mutant and wild-type proinsulin is that the wild-type partner becomes retained in the ER. Thus, even though *Akita* beta cells continuously synthesize normal amounts of proinsulin (Izumi et al., 2003; Liu et al., 2007), the failure of ER export of wild-type proinsulin is a direct cause of diminished insulin production, which is an initiating factor in the onset of insulin-deficient diabetes (Liu et al., 2007, 2012).

The dominant-negative blockade by misfolded mutant proinsulin imposed upon co-expressed wild-type proinsulin appears to be dose-dependent. While the male *Akita Ins2* heterozygous mice begin to have hyperglycemia shortly after weaning, the male *Akita Ins2* homozygous mice develop more severe diabetes shortly after birth (Yoshioka et al., 1997). In contrast, when *Akita* mutant proinsulin is expressed at a lower level from a transgene (not more than 4% of endogenous *Ins2*), diabetes prevalence is low (less than 10% of males) but the animals display pre-diabetes by glucose tolerance testing (Hodish et al., 2011). Similarly, a diabetic phenotype is not observed in transgenic pig lines that express *Akita* mutant proinsulin at levels < 15 % of the endogenous *INS* gene. In contrast, when the expression of *Akita* proinsulin reaches a relative level equivalent to 75% of the expression of the endogenous *INS* gene (i.e., 43% of total), then the transgenic pigs exhibit hyperglycemia within 24 hours after birth (Renner et al., 2013). This dose-dependence is also evident in cell culture studies in which dominant-negative blockade of wild-type proinsulin gradually decreases as the expression ratio of mutant : wild-type proinsulin drops (Liu et al., 2012). Collectively, these studies indicate that diabetogenic MIDY mutants act in a dose-dependent manner.

4.3. Bidirectional effects of mutant and wild-type proinsulin partners in the ER

A recent study not only confirmed the dose-dependent dominant blockade of wild-type proinsulin by MIDY mutants, but also revealed a surprising bidirectional effect of wild-type proinsulin on mutant proinsulin (Wright et al., 2013b). Indeed, by changing the stoichiometry of wild-type and mutant proinsulins to further favor the wild-type, secretion of some of the MIDY mutant gene product was found to be partially rescued by co-expression with wild-type proinsulin. This may explain different results that have been observed in transfected beta cells compared to results in non-beta cells. In transfected beta cells, due to a large amount of endogenous proinsulin, some MIDY mutants (e.g. R89C) have been found to egress from the ER and be transported to insulin secretory granules (Rajan et al., 2010). In contrast, in cells lacking wild-type proinsulin, the same MIDY mutants are entrapped in the ER without detectable secretion (Liu et al., 2010a, 2012).

Intriguingly, this rescue effect appears to be protein specific. Whereas wild-type proinsulin rescued some MIDY mutants, wild-type proinsulin could not rescue secretion of co-expressed mutant thyroglobulin (Tg). Reciprocally, wild-type thyroglobulin could not rescue secretion of co-expressed MIDY mutants, but wild-type thyroglobulin could promote the secretion of two recessive thyroglobulin mutants known as cog-Tg and rdw-Tg, which are misfolded and retained in the ER when expressed alone (Wang et al., 2010; Wright et al., 2013b). Evidently, specific interaction between dimerization partner proteins is needed to

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attain this rescue effect. The physiological and pathological significance of the bidirectional effect in the development and progression of MIDY remains to be further evaluated *in vivo*. However, studies seem to suggest that this bidirectional effect may play a role in the pathogenesis of some genetic diseases. In the heterozygotes of autosomal dominant diseases such as MIDY, the predominant direction is that the mutants block the co-expressed wild-type proteins. Indeed, there are some MIDY mutants that cannot be rescued at all by wild-type proinsulin (Wright et al., 2013b), so in these heterozygous patients, there will be no bidirectionality. In contrast, in autosomal recessive diseases, the predominant direction is that wild-type proteins are not only efficiently exported from ER; they may also promote ER export of their mutant dimerization partners, alleviating potential ER stress caused by these misfolded mutant proteins.

4.4. Cellular responses to untranslocated/mislocated preproinsulin

As discussed in section 3.2, based on the general function and properties of the signal peptide, three preproinsulin signal peptide mutations, R6C, R6H, and L13R are expected to affect SRP recognition, ER targeting, and/or translocation of preproinsulin across the ER membrane. Specifically, our recent study shows that after targeting to the ER, preproinsulin-R6C/H fails to be efficiently translocated across the ER membrane. Untranslocated secretory proteins usually are unstable and rapidly degraded in cells (Ulmer and Palade, 1989a, 1989b). Indeed, most of the newly synthesized untranslocated preproinsulin-R6C/H molecules are degraded (Guo et al., 2014). However, a certain fraction of untranslocated preproinsulin is relocated intracellularly and accumulated within an undefined juxtanuclear compartment with the proinsulin moiety exposed to the cytosol (Fig. 4).

Accumulation of juxtanuclear puncta has been observed upon expression of cytosolic misfolded and aggregation-prone proteins. Such a disturbance in cytoplasmic proteostasis with juxtanuclear accumulation is thought to induce cytoplasmic stress (Kaganovich et al., 2008; Kocik et al., 2012). In contradistinction to the ER stress response, preproinsulin-R6C leads to induced expression of HSP70 (Guo et al., 2014), a major cytosolic stress-inducible chaperone, which can bind to misfolded proteins to prevent their aggregation and promote their degradation (Kakkar et al., 2012; Saibil, 2013). Importantly, cytosolic/juxtanuclear accumulation of misfolded proteins has been linked to cell death that contributes to the development and progression of various neurodegenerative diseases including Huntington’s, Parkinson’s, Alzheimer’s and prion diseases (Debure et al., 2003; Kaganovich et al., 2008; Miesbauer et al., 2010; Rane et al., 2010; Reiner et al., 2011; Rubinsztein, 2006; Schaffar et al., 2004; Strom et al., 2006; Viswanathan et al., 2011). Consistent with this, the expression of preproinsulin-R6C leads to increased beta cell death, which contributes to late-onset diabetes caused by inefficient preproinsulin translocation (Guo et al., 2014). Thus, unlike defects in the secretory pathway caused by other autosomal dominant insulin gene mutations, the preproinsulin cytosolic accumulation caused by R6C/H appears to lead to beta cell failure and diabetes through a completely novel mechanism (Guo et al., 2014).

The cellular defect caused by preproinsulin-L13R has not been experimentally examined. Unlike preproinsulin-R6C/H associated with late-onset diabetes, preproinsulin-L13R causes *in utero* growth retardation and severe insulin-deficient diabetes on the second day after

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birth (Hussain et al., 2013). Although the L13R mutation is predicted to affect preproinsulin translocation, the much more severe clinical presentation than that seen with two other signal peptide mutations suggests that additional mechanisms may be involved in beta cell failure and diabetes. Similar to the findings noted above for preproinsulin-R6C, studies of other proteins have shown that blocking SRP-dependent protein translocation induces the expression of heat shock responses, attenuates cell growth, and promotes cell death (Hall et al., 2014; Mutka and Walter, 2001). Since the L13R mutation may affect SRP-dependent translocation, it will be important to determine whether preproinsulin-L13R results in beta cell growth arrest. Similar mutations that disrupt the hydrophobic core of the signal peptide have been reported to cause several human diseases (Jarjanazi et al., 2008). Examples of this include a heterozygous L25R mutation of type V collagen proα1-chain, which results in Ehlers-Danlos syndrome (Chan et al., 2001); a homozygous L15R mutation of bilirubin UDP-glucoronyltransferase (B-UGT), which causes type II Crigler-Najjar disease (Seppen et al., 1996); and a heterozygous C18R mutation of preproparathyroid hormone (preProPTH), which leads to hypoparathyroidism (Arnold et al., 1990). Among these mutants, preProPTH-C18R has been experimentally examined. The preProPTH-C18R exhibits a translocation defect due to an impaired recognition of the mutant signal peptide by SRP (Karaplis et al., 1995). Interestingly, however, the intracellular accumulation of untranslocated preProPTH-C18R is largely colocalized with the ER, inducing ER stress, and promoting cell death (Datta et al., 2007). Reasoning by analogy, it remains possible that preproinsulin-L13R might cause beta cell failure and diabetes through ER stress, with beta cell death – this possibility has yet to be investigated.

5. Perspectives and strategies for preventing diabetes caused by defects in the early steps of insulin biosynthesis

More than 50% of all insulin gene mutations (30 out of 51) cause proinsulin misfolding in the ER and MIDY. Over the past decade, considerable advance has been made to better understand the molecular mechanisms of beta cell failure caused by misfolded proinsulin. This establishes a foundation for developing strategies to delay/prevent the onset of diabetes. As discussed above, although ER stress and beta cell death are a hallmark of MIDY, an earlier event (i.e., before a significant loss of beta cell mass) that contributes to insulin deficiency includes the failure of insulin production from wild-type proinsulin due to its blockade by misfolded mutant proinsulin (Gupta et al., 2010; Hodish et al., 2010; Liu et al., 2007; Renner et al., 2013). Given the fact that one normal *INS* gene allele is sufficient to produce enough insulin to maintain normoglycemia (Garin et al., 2010; Leroux et al., 2001), strategies that rescue wild-type proinsulin from blockade may therefore potentially restore sufficient insulin production to delay/prevent the onset of insulin-deficient diabetes.

Two distinct approaches might be employed to rescue wild-type proinsulin from blockade by mutant proinsulin. First, because the dominant-negative effect of mutant proinsulin is dose-dependent (Liu et al., 2012; Renner et al., 2013; Wright et al., 2013b), one potential therapeutic strategy for the MIDY syndrome is to target the mutant proinsulin for degradation, so that the stoichiometry of mutant and wild-type proinsulin becomes more favorable to the wild-type – thereby allowing wild-type proinsulin exit from the ER.

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Compared to the extensive studies of glycoprotein degradation in the secretory pathway (Goldberg, 2003; Hebert and Molinari, 2007; Oda et al., 2003; Smith et al., 2011; Ushioda et al., 2008; Vembar and Brodsky, 2008), the degradation pathway of unglycosylated secretory proteins including proinsulin remains largely unexplored. Although both the ubiquitin-proteasome system and autophagy have been suggested to play roles in degradation of proinsulin in *Akita* mice (Allen et al., 2004; Bachar-Wikstrom et al., 2013a, 2013b), the key intracellular molecules that regulate degradation of misfolded proinsulin are unknown. Identifying those molecules may provide potential targets to modulate the degradation of misfolded mutant proinsulin and thereby rescue wild-type proinsulin.

The second approach is to target wild-type proinsulin for improved oxidative folding in the ER. Because abnormal inter-molecular disulfide bonds between mutant and wild-type proinsulins contribute to the dominant negative effect of mutant proinsulin (Liu et al., 2010a, 2012), approaches that accelerate the oxidative folding of wild-type proinsulin may promote its folding, limiting its interactions with co-expressed mutant proinsulin, and allowing it to escape from the ER prior to its retention induced by aberrant interaction with the mutant gene products. Protein disulfide isomerase (PDI) and ER oxidoreducin-1 (ERO1) play central roles in maintaining the oxidizing environment to promote disulfide pairing (Bulleid, 2012; Sevier, 2010). Recently, studies show that overexpressing (ERO1) can improve oxidative folding of wild-type proinsulin even when it is co-expressed with MIDY mutants (Liu et al., 2012; Wright et al., 2013a). This results in a significant increase of wild-type proinsulin export from the ER, accompanied by increased insulin production. Mutation of the cysteines in ERO1 that negatively regulate this enzyme (to create a constitutively active oxidase) enhances this effect, while mutation of the catalytic cysteines of ERO1 abolishes most of the rescue, suggesting that enhanced oxidation of proinsulin downstream of ERO1 contributes to proinsulin escape from the ER.

One concern about strategies to enhance ERO1 activity in order to rescue proinsulin export from the ER is that ER hyperoxidation by ERO1 may potentially cause ER stress. However, in cells expressing mutant proinsulin, although overexpressing wild-type or activated ERO1 increases oxidation of the ER lumen, it does not augment ER stress induced by mutant proinsulin – indeed, this maneuver appears to lead to a significant diminution of mutant proinsulin-induced ER stress (Wright et al., 2013a). Decreased ER stress may result from improved oxidative folding of proinsulin with its increased export from the ER. Despite these promising initial studies, the long-term effect of increasing oxidation of the ER lumen on beta cell function still needs to be evaluated *in vivo*.

Surprisingly, overexpression of PDI does not improve oxidative folding of proinsulin or improve insulin production. As PDI does not mediate rescue of proinsulin, PDI per se might not play a role in native proinsulin disulfide bond formation *in vivo* (Liu et al., 2012; Rajpal et al., 2012). Of course, there are many other PDI-like ER oxidoreductase family members that might serve this function (Bulleid, 2012; Ellgaard and Ruddock, 2005).

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6. Conclusion

The discovery of new insulin gene mutations has stimulated renewed interest in insulin biosynthesis in pancreatic beta cells. These mutants provide unique tools with which to explore the molecular and cellular processes that go awry during the onset and progression of diabetes. Although many questions remain unanswered, we are beginning to get a deeper understanding of the molecular mechanisms of beta cell failure and diabetes caused by defects in the early events of insulin biosynthesis, including preproinsulin targeting and translocation across the ER membrane, signal-peptide cleavage to proinsulin, and the folding of newly made proinsulin molecules in the ER (Fig. 5). Studies are still needed to understand the proinsulin folding pathway in the ER of pancreatic beta cells, because accruing evidence suggests that misfolded proinsulin may be able to “propagate” its misfolding onto “bystander” proinsulin molecules. Factors including the stoichiometry of misfolded and bystander proinsulin forms, the redox environment of the ER lumen, and the activity of ERAD machinery in pancreatic beta cells, are all essential components to the development of novel therapeutic approaches for MIDY. At the same time, a new realization that proinsulin misfolding may occur under pathological conditions in the absence of any mutations, opens the door to the possibility that MIDY may be an extreme example of the kind of ER stress generated in more common forms of type 2 diabetes. Future work is needed to explore these links.

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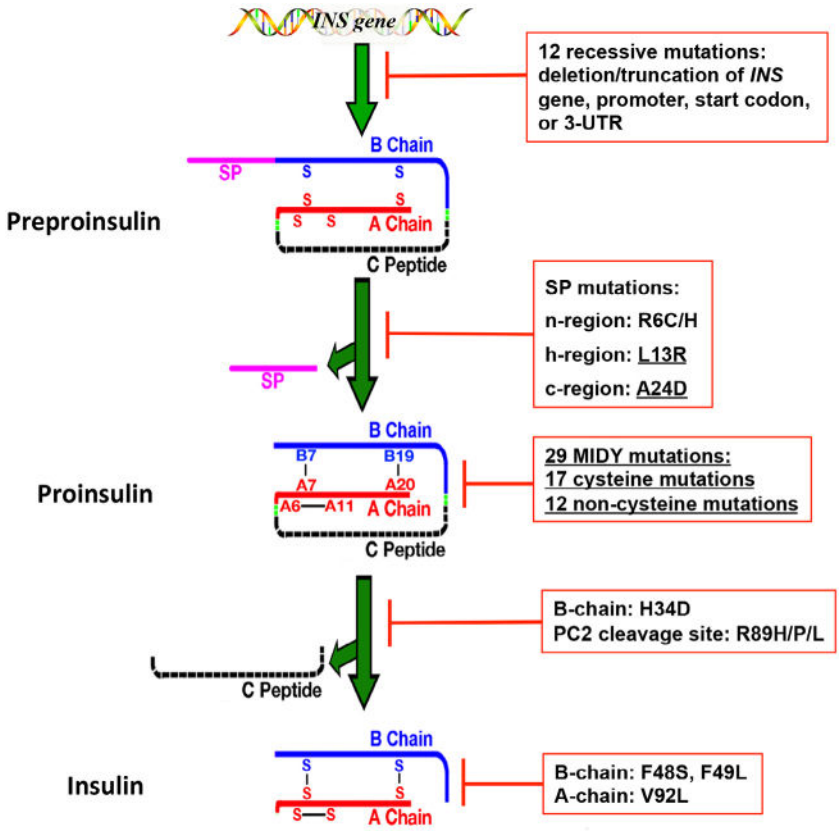

Fig. 1.

The effects of *INS*-gene mutations on the major steps of insulin biosynthesis. *INS*-gene mutations have been identified in the untranslated regions of *INS* gene and the coding sequence encoding all functional domains of preproinsulin molecule, including signal peptide (SP, pink), insulin B-chain (blue), C-peptide (black), insulin A-chain (red), and the proteolytic cleavage sites of signal peptidase (SPase) as well as prohormone convertases (PC1/3 and PC2, green). The mutations affect all major steps of insulin biosynthesis. Twelve recessive mutations in the untranslated regions result in more than 80% decrease of insulin production due to either *INS* gene deletion or truncation, or failure of insulin translation initiation, or instability of insulin mRNA. SP mutations in the n-region (R6C/H) or h-region (L13R) cause defective translocation of preproinsulin into the ER. The mutation at SP cleavage site (A24D) impairs SP cleavage. The largest group of *INS*-gene mutations are the mutations that affect proinsulin folding in the endoplasmic reticulum (ER), impairing formation of three evolutionarily conserved native disulfide bonds, B7-A7, B19-A20, and A6-A11. H34D affects sorting efficiency of proinsulin into regulated secretory pathway. The non-cysteine mutations at the PC2 cleavage site (R89H/P/L) impairs processing of proinsulin. The mutations in the B-chain (F48S and F49L) and A-chain (V92L) affect insulin binding to the insulin receptor. All mutations that cause Mutant *INS*-gene-induced Diabetes of Youth (MIDY) are underlined.

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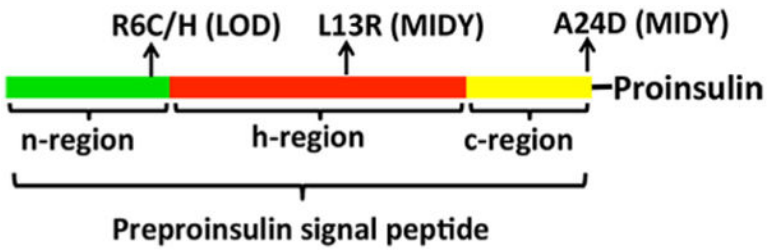

Fig. 2.

Three functional regions of preproinsulin signal peptide and the mutations associated with diabetes. Preproinsulin signal peptide has three functional regions: n-region (green), h-region (red), and c-region (yellow). Diabetogenic *INS*-gene mutations have been found in all three regions. The mutations in the n-region (R6C/H) cause inefficient translcocation of preproinsulin across the ER membrane and lead to late-onset diabetes (LOD). The mutation in the h-region (L13R) causes mutant *INS*-gene-induced diabetes of young (MIDY). The cellular defect caused by L13R remains to be determined, but it is predicted to affect the targeting and translocation of the mutant preproinsulin. The mutation at the signal peptide cleavage site (A24D) impairs normal signal peptide cleavage and causes proinsulin misfolding in the ER, leading to MIDY.

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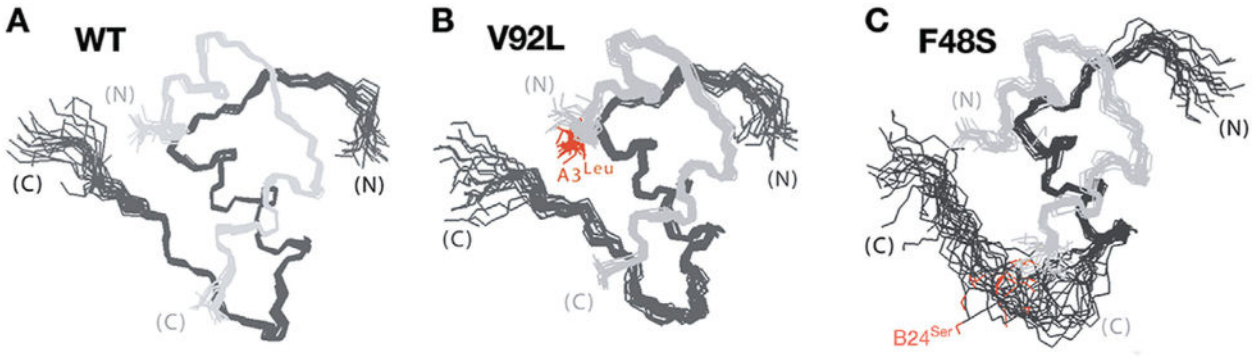

Fig. 3.

Solution structures of insulin analogs. A. Ensemble of NMR-derived structures DKP-insulin wild-type (WT). The A- and B-chains are shown in light and dark gray, respectively. B. Solution structures of V92L-DKP-insulin. The side chain of the mutant residue leucine (Leu) at the 3rd residue of insulin A-chain is shown in red. C. Solution structures of F48S-DKP-insulin. The side chain of the mutant residue serine (Ser) at the 24th residue of insulin B-chain is shown in red. Whereas V92L is compatible with native-like structure in accord with results of X-ray crystallography, F48S destabilizes the C-terminal strand of the B-chain. This figure is modified from Liu et al. (2010a).

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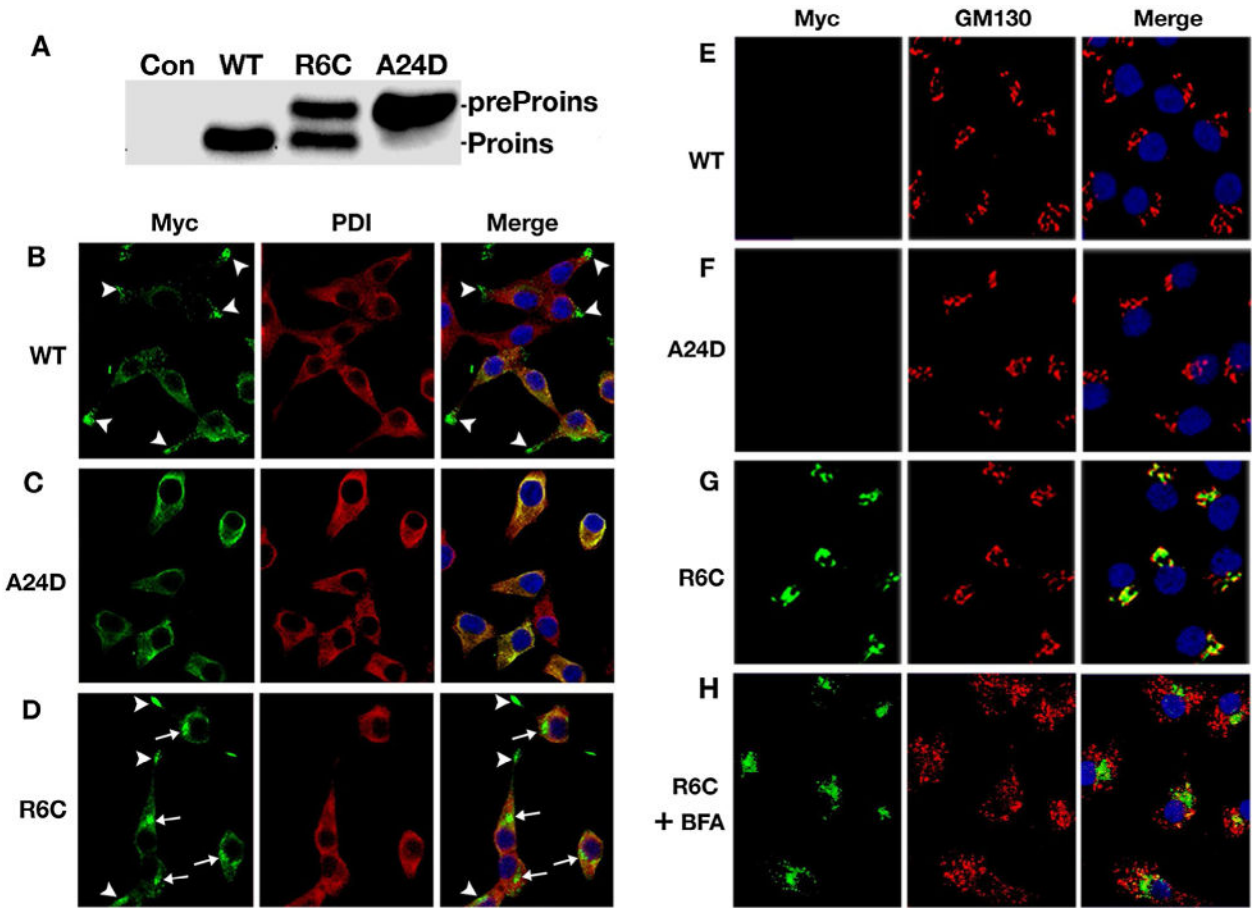

Fig. 4.

Two preproinsulin signal peptide mutations cause distinct cellular defects in beta cells. A. Processing of Myc-tagged preproinsulin R6C and A24D in INS1 cells were examined by western blotting using anti-Myc antibody While most of preproinsulin-A24D presents as uncleaved preproinsulin, preproinsulin-R6C produces two populations: proinsulin and unprocessed preproinsulin. C–D. INS1 cells expressing Myc-tagged preproinsulin-WT, R6C or A24D were fully permeabilized and immunoblotted with anti-Myc (green) and anti-PDI (red) antibodies. Nuclei were counterstained with DAPI. In most cells expressing Myc-tagged preproinsulin-WT (A), anti-myc immunoreactable molecules presented as punctate insulin granule-like pattern (arrowheads) that were distinct from PDI. Preproinsulin-A24D lost the granule pattern and largely overlapped with PDI (B). Preproinsulin-R6C produces two major intracellular pools: one did indeed concentrate in distal tips (arrowheads) while another accumulated in a juxtanuclear location (arrows), and neither pool overlapped with PDI (C). E–G. The parallel sets of INS1 cells were selectively permeabilized by 0.01% digitonin and immunoblotted with anti-Myc (green) and anti-GM130 (red) antibodies. Nuclei were counterstained with DAPI. Unlike B–D, anti-myc immunoreactable molecules were detected only in the cells expressing Myc-tagged R6C (G). Such molecules appeared to accumulate in a juxtanuclear region close to Golgi marker GM130. H. A parallel well of the cells of G were pre-treated with 5uM brefeldin A (BFA) before being partially permeabilized and immunoblotted as in G. This figure is modified from Guo et al. (2014).

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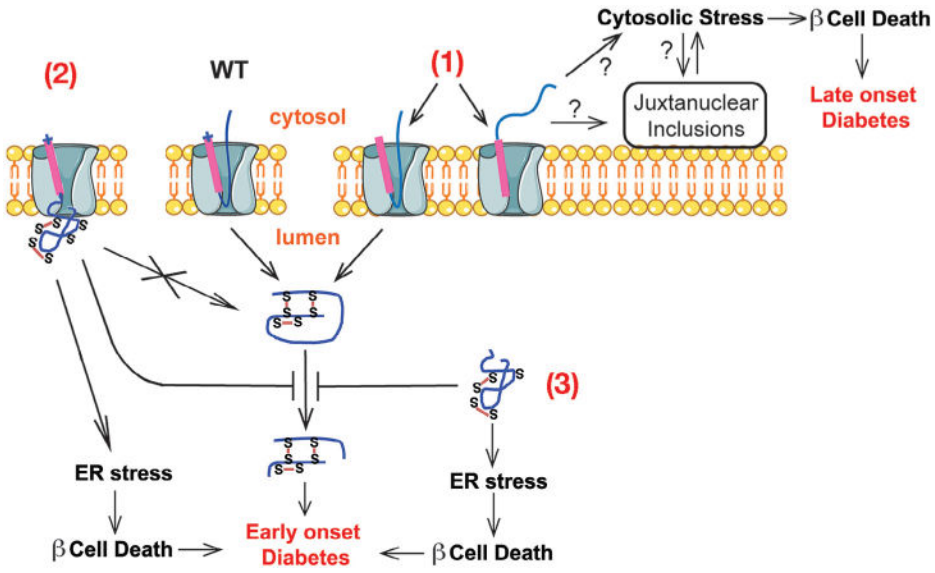

Fig. 5.

A proposed model of beta cell failure and diabetes caused by the defects in the early events of insulin biosynthesis. The preproinsulin signal peptide mutations in the n-region, i.e. R6C/H, result in a translocation defect of newly synthesized preproinsulin across the ER membrane (1). A certain fraction of untranslocatd preproinsulin relocates and accumulates in the juxtanuclear compartment, activating cytosolic response, promoting beta cell death, and leading to beta cell failure. The preproinsulin mutation at the signal peptide cleavage site, i.e. A24D, impairs normal cleavage of the signal peptide (2). This results in misfolding of downstream proinsulin domain. Twenty-eight reported gene mutations, e.g. C(A7)Y, lead to proinsulin misfolding in the ER (3). Misfolded mutant proinsulins not only induce ER stress and beta cell death, but also block the ER export of co-expressed proinsulin-WT, resulting in decreased insulin production from proinsulin-WT, leading to MIDY. This figure is modified from Guo et al. (2014).

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Table 1

Insulin gene mutations and their affects on insulin biosynthesis.

Affects on insulin biosynthesis		Mutations	Re/Dn	Age of onset	Ref
Transcription and/or Translation[a]	Deletion/truncation	c.−366_−343del, c.−370-_186 + del, c.−65_581del	Re	<6 mo[c]	1–2
	Promoter	c.−331 C > A/G, c.−332C>G + c.−331 C > G, c.−218 A > C	Re	<6 mo[b]	1
	3′UTR	c.*59 A > G	Re	<6 mo[b]	1
	Translational initiation	c. 3G>T, c.3G>A	Re	<6 mo[b]	1
	Premature stop	c. 184C>T (Q62X)	Re	<6 mo[b]	1
Translocation and/or SP cleavage	Targeting/translocation	L13R	Dn	<6 mo	3
	Translocation	R6C/H	Dn	15–65 yr	4–6
	SP cleavage	A24D	Dn	<6 mo[c]	6–8
Proinsulin folding in the ER	Non-cysteine mutations	H29D, L30M/P/V/Q, G32R/S, L35P, R46Q, L39Y40delinsH, G47V	Dn	<6 mo[c]	6–12
	Cysteine mutations	C31Y, C43G, F48C, R55C, G84R, R89C, G90C, C95Y, S101C, C96Y/S/R, Y103C, Y108C/X, C109F/Y/R	Dn	<6 mo[c]	6–14
Proinsulin trafficking/processing	Sorting/trafficking	H34D	Dn	Hyperpro-insulinimia	15–16
	Processing	R89H/P/L	Dn	Variable[d]	17–20
Insulin bioactivity	Binding to IR	V92L, F48S, F49L	Dn	Adult-onset	21–23

Re: recessive; Dn: Dominant; 3-UTR: 3′-untranslated region; IR: insulin receptor; ER: endoplasmic reticulum; SP: signal peptide; Ref: References.

[a]The nomenclature of the nucleotide changes is based on the *INS* gene coding sequence where nucleotide 1 represents translational start site.

[b]The patients that are homozygote or compound heterozygote for those recessive INS gene mutations develop diabetes in 6 mo after birth.

[c]Few patients carrying those mutations develop diabetes after 6 mo.

[d]Hyperproinsulinemia, borderline glucose intolerance, or late-onset mild diabetes.

References: 1 (Garin et al., 2010); 2 (Raile et al., 2011); 3 (Hussain et al., 2013); 4 (Boesgaard et al., 2010); 5 (Meur et al., 2010); 6 (Edghill et al., 2008); 7 (Stoy et al., 2007); 8 (Polak et al., 2008); 9 (Colombo et al., 2008); 10 (Molven et al., 2008); 11 (Rubio-Cabezas et al., 2009);12 (Bonfanti et al., 2009); 13 (Moritani et al., 2013); 14 (Ozturk Mehmet et al., 2014); 15 (Chan et al., 1987); 16 (Gruppuso et al., 1984); 17 (Oohashi et al., 1993); 18 (Yano et al., 1992); 19 (Ozturk Mehmet et al., 2014); 20 (Warren-Perry et al., 1997) 21 (Sakura et al., 1986); 22 (Shoelson et al., 1983a); 23 (Tager et al., 1979).