Throughout evolution, proinsulin has exhibited significant sequence variation in both C-peptide and insulin moieties. As the proinsulin coding sequence evolves, the gene product continues to be under selection pressure both for ultimate insulin bioactivity and for the ability of proinsulin to be folded for export through the secretory pathway of pancreatic β-cells. The substitution proinsulin-R(B22)E is known to yield a bioactive insulin, although R(B22)Q has been reported as a mutation that falls within the spectrum of mutant INS-gene–induced diabetes of youth. Here, we have studied mice expressing heterozygous (or homozygous) proinsulin-R(B22)E knocked into the Ins2 locus. Neither females nor males bearing the heterozygous mutation developed diabetes at any age examined, but subtle evidence of increased proinsulin misfolding in the endoplasmic reticulum is demonstrable in isolated islets from the heterozygotes. Moreover, males have indications of glucose intolerance, and within a few weeks of exposure to a high-fat diet, they developed frank diabetes. Diabetes was more severe in homozygotes, and the development of disease paralleled a progressive heterogeneity of β-cells with increasing fractions of proinsulin-rich/insulin-poor cells as well as glucagon-positive cells. Evidently, subthreshold predisposition to proinsulin misfolding can go undetected but provides genetic susceptibility to diet-induced β-cell failure.

Several groups have been pursuing the molecular mechanisms underlying β-cell dysfunction and compensatory cellular responses in the rare genetic syndrome mutant INS–gene–induced diabetes of youth (MIDY) (1–3). The fundamental defect in MIDY is observed in humans (4), large animal models (5), and small animal models (6) and has been replicated in cell culture (7) in vitro (8) and modeled in silico (9). The clinical problem originates from the fact that misfolded proinsulin within the endoplasmic reticulum (ER) can propagate its misfolding and ER retention onto wild-type (WT) bystander proinsulin molecules, thereby impairing insulin production (10,11). Yet, MIDY is a rare disease (12,13).

Proinsulin exhibits significant sequence variation throughout evolution, including within both the C-peptide and the insulin moieties (14,15). The Ins gene product continues to be under selection pressure both for the ultimate bioactivity (of insulin) and for the ability of proinsulin to be folded for export through the secretory pathway of pancreatic β-cells (16). Evidence suggests that with only natural variation provided by evolution, “WT” proinsulin itself is capable of forming nonnative disulfide-linked proinsulin complexes not unlike those triggered by MIDY proinsulin mutations (17). However, massive formation of disulfide-linked complexes of WT proinsulin has thus far been observed only in the islets of db/db or other leptin receptor–deficient mice (or in the islets of normal animals that have been treated with one or more toxins that drastically perturb ER homeostasis [17]). We have also wondered whether it is possible that subtle predisposition to proinsulin misfolding can go unnoticed and yet be a genetic risk to diet-induced diabetes.

With these considerations in mind, we have interest in proinsulin residue 46, i.e., Arg at position 22 of the
insulin B chain. Recent studies indicated that insulin-R(B22)Q is a naturally occurring variant in bats (15), has greater than or equal to half of the normal affinity for insulin receptor binding, and can be found released into the bloodstream of patients who express the INS c.137G>A (R46Q) variant (18). Yet in all three family members bearing this heterozygous mutation, diabetes was ultimately diagnosed at ages 17–20 years (19), suggesting that this substitution, albeit less severe than MIDY mutants triggering neonatal diabetes (12), still trips over the diabetogenic threshold.

It has been reported that modification of R(B22) with a bulky group of the opposite charge does not alter the specific bioactivity of insulin (20) as in the case of R(B22)D substitution (21), which is naturally occurring in mole rats and guinea pigs (15). Similarly, the R(B22)E substitution has also been reported to retain insulin bioactivity (22). Here, we pursued the possible impact of proinsulin-R(B22)E expression in the β-cells of mice in which this variant is knocked into the Ins2 locus. As expected, the substitution creates a proinsulin that is secratable from pancreatic β-cells, and under normal laboratory conditions, all heterozygous animals remain diabetes free. Nevertheless, heterozygous males consistently developed diabetes upon exposure to a high-fat diet (HFD), highlighting the impact of a subthreshold genetic predisposition to proinsulin misfolding on the development of diet-induced diabetes.

RESEARCH DESIGN AND METHODS
Proinsulin Mutagenesis
Plasmids encoding untagged human proinsulin-R(B22)E, untagged human proinsulin-WT, or Myc-tagged proinsulins were generated as previously described (23,24). All proinsulin-expressing constructs were confirmed by direct DNA sequencing.

Cell Transfection, Metabolic Labeling, Immunoprecipitation, Coimmunoprecipitation, Western Blotting
Min6 mouse β-cells (25) (obtained from Dr. D. Stoffers, University of Pennsylvania, Philadelphia, PA) were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin, and 0.05 mmol/L β-mercaptoethanol. Cells at 70–80% confluence were transfected using Lipofectamine 2000 (Thermo Fisher Scientific), with fresh media changed at 6 h posttransfection. Media were removed at 48 h; the cells were washed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 2 mmol/L EDTA) plus protease inhibitor/phosphatase inhibitor cocktail (Sigma-Aldrich). Total protein was measured by bicinchoninic acid assay. Proteins in sample buffer were boiled and resolved by 4–12% NuPAGE Bis-Tris Gel (Invitrogen) and electrotransferred to nitrocellulose (Bio-Rad). Membranes were incubated with primary antibodies (4°C overnight) and then secondary peroxidase-conjugated goat anti-rabbit IgG (111-035-144; Jackson Immunoresearch) or peroxidase-conjugated goat anti-mouse IgG (115-035-174; Jackson Immunoresearch) followed by enhanced chemiluminescence (SuperSignal West Pico PLUS; Thermo Fisher Scientific) with digital image capture.

Construction of Knockin Mouse
Initially, the Mouse Genetics Core (National Jewish Health) prepared eggs from superovulated NOD females fertilized with male NOD sperm in vitro. After overnight culture, fertilized embryos were injected with a mixture of 1) Cas9 protein, 2) a CRISPR guide RNA (designed using CRISPOR software; http://crispor.tefor.net) matching a region just upstream of the proinsulin R(B22) codon, and 3) an Ins2-specific 150-base pair repair oligonucleotide covering this region, replacing the R codon (CGT) with E (GAG), plus a silent mutation, creating an Alu I restriction site. Injected embryos were introduced into pseudopregnant mice; DNA from the pups were analyzed by PCR, restriction digest, and sequencing to distinguish specific Ins2 (or offsite Ins1) locus repair with the homologous sequence versus nonhomologous end-joining events. To isolate the proper genetic event, breeding resulted in NOD mice bearing a single heterozygous Ins2 R(B22)E mutation (WT at three other Ins alleles). Breeding was then initiated to move the mutation into the C57BL6/J background. Animals were phenotyped in each of the first five generations of C57BL6/J backcrosses. All data in this article come after five generations of backcrossing (and further backcrosses are ongoing), but because the same phenotype was observed in each backcross generation, the data are reported here. HFD (5.5–11.5 weeks of age) was irradiated rodent chow (60 kcal% fat, #D12492; Research Diets, New Brunswick, NJ).

Circulating Insulin and Proinsulin, In Vivo Glucose Tolerance, and Glucose-Stimulated Insulin Secretion
ELISA was used to measure mouse insulin (80-INSMS-E10; ALPCO) and proinsulin (80-PINMS-E01; ALPCO). For in vivo glucose tolerance, mice were fasted for 6 h; glucose (1 g/kg body weight) was administered intraperitoneally; and tail vein glucose was monitored (One-Touch Ultra blood glucose meter and test strips). For in vivo glucose-stimulated insulin secretion (GSIS), serum was collected under basal conditions and under glucose-stimulated conditions at t = 15 min.

Islet Isolation and GSIS
Islets were isolated by collagenase digestion through the common bile duct followed by pancreatic digestion ex vivo. The digest was washed and spun on a Histopaque 1077 (Sigma-Aldrich) gradient (900g × 20 min without brake). Islets were collected, washed, handpicked, and incubated overnight in RPMI medium plus 10% FBS at 37°C. Recovered islets were preincubated at 2.8 mmol/L glucose for 1 h at 37°C in modified Krebs-Ringer bicarbonate buffer plus...
20 mmol/L HEPES (KR BH) and 0.05% BSA. Fifteen to 17 islets were transferred to microfuge tubes containing 500 μL of KR BH-BSA solution and incubated at 37°C for 30 min at 2.8 mmol/L glucose (basal) followed by 30 min at 16.7 mmol/L glucose (stimulated), with media measured for insulin content.

**Metabolic Labeling of Mouse Pancreatic Islets**

Twenty-five islets isolated from WT and proinsulin-R(B22)E heterozygous and homozygous littermates were washed in prewarmed Met/Cys-deficient RPMI medium and then pulse labeled with 35S-amino acids (Tran35S label) for 30 min at 37°C. Labeled islets were either lysed immediately or chased in complete growth media for 2 h. Islets were sonicated in radioimmunoprecipitation assay buffer (25 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 1% Triton X-100, 0.2% deoxycholic acid, 0.1% SDS, 10 mmol/L EDTA) containing 2 mmol/L N-ethylmaleimide and a protease inhibitor cocktail. Lysates were normalized to trichloroacetic acid–precipitable counts and immunoprecipitated with guinea pig polyclonal anti-insulin and protein A agarose overnight at 4°C. Immunoprecipitates were washed and analyzed by nonreducing/reducing Tris-tricine-urea-SDS-PAGE, followed by phosphorimaging, and bands were quantified with ImageJ software.

**Immunofluorescence**

Paraffin sections of formaldehyde-fixed pancreas were deparaffinized with CitriSolv (Thermo Fisher Scientific) and rehydrated in a decreasing graded series of ethanol followed by heating for antigen retrieval. Slides were washed with PBS and incubated in blocking buffer (Tris-buffered saline [TBS] plus 0.2% Triton X-100 and 3% BSA) for 2 h and incubated in primary antibody (in TBS plus 3% BSA and 0.2% Tween 20) overnight at 4°C. After washes, secondary antibody was incubated for 1 h at room temperature. Slides were washed three times with TBS/0.1% Tween 20, mounted with ProLong Gold plus DAPI, and imaged by epifluorescence on a Nikon A1 confocal microscope with a 60× oil objective.

**Electron Microscopy**

Isolated islets were fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (CB) (pH 7.2), embedded in 2.5% low-melting agarose, trimmed to ~1-mm cubes, washed three times in CB, and postfixed in 2% osmium tetroxide plus 1.5% potassium ferrocyanide in 0.1 mol/L CB for 1 h on ice. After three further washes in CB, cubes were washed three times in 0.1 mol/L sodium acetate buffer (pH 5.2) and then stained in this buffer containing 2% uranyl acetate for 1 h. With agitation, the cubes were washed extensively and then dehydrated in a graded series of ethanol up to 100% and then acetone for 15 min before infiltration with graded concentrations of Spurr’s resin in acetone over 3 days. Islets were finally embedded in fresh Spurr’s resin and polymerized for 45 h at 70–75°C. Ultrathin (70-nm) sections were captured on carbon-coated 200-mesh copper grids, and imaged on a JEM-1400plus transmission electron microscope (JEOL USA Inc., Peabody, MA) at 80 kV, with images captured on an AMT XR401 camera (Advanced Microscopy Techniques, Woburn, MA).

**Statistical Analyses**

Statistical analyses were carried out by two-tailed Student t test or one-way ANOVA followed by multiple comparison testing (GraphPad Prism 8 software). P < 0.05 was taken as statistically significant.

**Data and Resource Availability**

Key resources are shown in Table 1. Data generated and analyzed during the current study are contained within the figures. Additional data are available from the corresponding author upon request.

**RESULTS**

**Expression of Recombinant Proinsulin-R(B22)E**

Proinsulin-R(B22)E (Fig. 1A) should lead to bioactive insulin (20–22), yet it may not necessarily support efficient proinsulin protein folding (16,26–29). We first examined the behavior of recombinant human proinsulin-R(B22)E. Min6 pancreatic β-cells express their own endogenous mouse proinsulin, but we probed transfected cells, and media, with an antibody that recognizes only human proinsulin protein. Thus, from Min6 cells transfected with empty vector, no proinsulin band was detected (Fig. 1B). Both human WT and R(B22)E proinsulins were secreted from Min6 cells (Fig. 1B), and under these conditions, proinsulin-R(B22)E did not block WT proinsulin secretion (Fig. 1C). Nevertheless, from Western blotting of reducing SDS-PAGE, it was clear that the secretion efficiency (extracellular-to-intracellular ratio) for WT proinsulin was greater than that for proinsulin-R(B22)E, whereas Akita mutant proinsulin was not secreted at all (Fig. 1B and C). Thus, in Min6 β-cells, proinsulin-R(B22)E passes ER quality control, and the dominant-negative phenotype of MIDY (1) is inapparent.

**Endogenous Islet Expression of Ins2-Proinsulin-R(B22)E**

A CRISPR/Cas9-mediated knockin of Ins2-proinsulin-R(B22)E was back-bred to C57BL6/J mice. From 5.5 to 11.5 weeks of age, WT and heterozygous Ins2-proinsulin-R(B22)E mice gained weight on a normal chow diet and as expected, gained more weight on an HFD, without statistical differences seen between the genotypes (Fig. 2A). Glucose tolerance in heterozygous Ins2-proinsulin-R(B22)E mice at 5.5 weeks of age was normal (Fig. 2B) with a normal area under the curve (Fig. 2C). However, within 2 weeks of HFD, male heterozygotes
had an average random blood glucose $\geq 350$ mg/dL, suggesting onset of diabetes (Fig. 2D). At 11.5 weeks of age, male heterozygotes on HFD for 6 weeks had clearly diagnosed diabetes (either in terms of fasting hyperglycemia, 2-h glucose tolerance, or area under the curve), whereas those on a normal chow diet did not meet any diabetes criteria (Fig. 2E and F). Despite that random glucose in HFD-fed male heterozygotes was higher, serum insulin was not elevated (Fig. 2G); actually, the random serum insulin-to-glucose ratio was significantly lower in HFD-fed male heterozygotes (Fig. 2H). Indeed, after 6 weeks of HFD, isolated islets from male Ins2-proinsulin-R(B22)E heterozygotes exhibited abnormally low insulin secretion (unstimulated and glucose-stimulated) despite a normal fold change (Fig. 2I). Islets from male heterozygotes also developed diminished insulin content, and in HFD-fed animals, insulin content fell farther (Fig. 2J). Interestingly, proinsulin in these mice (Fig. 2J) was preserved, although proinsulin formed enhanced nonnative disulfide-linked complexes (Supplementary Fig. 1A and B). In contrast, female Ins2-proinsulin-R(B22)E heterozygotes gained weight normally and did not develop random hyperglycemia on HFD (Fig. 2K and L).

Figure 1—Insulin B-chain substitution R(B22)E. A: Schematic representation of insulin chains showing the respective disulfide bonds and the R(B22)E substitution. B: Transfection of Min6 cells with empty vector (EV), untagged WT human proinsulin, hPro-R(B22)E-CpepMyc, or hPro-C(A7)Y-CpepMyc (Akita-Myc). The media-bathing transfected cells were collected overnight, and both cell lysates and media were resolved by SDS-PAGE under reducing conditions and immunoblotting with anti-human-proinsulin or cyclophilin B (CypB) (loading control) (bottom panels). Cell lysates were similarly analyzed under nonreducing conditions to reveal disulfide-linked proinsulin complexes (top panel). C: Transfection of Min6 cells as in B (first four lanes) or cotransfection with both untagged WT human proinsulin and hPro-R(B22)E-CpepMyc (last lane). Proinsulin-R(B22)E is secreted from Min6 cells, and under these conditions, proinsulin-R(B22)E does not block the secretion of coexpressed untagged WT proinsulin.
Figure 2—Heterozygous (Het) Ins2-proinsulin-R(B22)E mice fed normal chow (NC) or HFD. A: Weekly body weight measurements in male mice (n = 4–8 per group). B: Intraperitoneal glucose tolerance test in 5.5-week-old males on NC (n = 4–5 per group). C: Area under the curve (AUC) from the data in B. D: Weekly random blood glucose in males on NC or HFD (n = 4–8 per group), with Ins2-proinsulin-R(B22)E-Het on NC shown in blue and on HFD in red. E: Intraperitoneal glucose tolerance test in 11-week-old males on NC or HFD for 6
When Ins2-proinsulin-R(B22)E was bred to homozygosity (still leaving both WT Ins1 alleles), both males and females at 5 weeks of age exhibited random blood glucose that averaged in the diabetic range, even on a normal chow diet (Fig. 3A), and fasting blood glucose values were similarly elevated. (Random blood glucose in homozygotes rose even higher by 8 weeks of age, whereas heterozygotes remained normoglycemic [Fig. 3B].) Despite the higher blood glucose, neither homozygous males nor females had raised endogenous serum insulin; with both sexes combined, it was apparent that homozygotes had decreased insulinemia despite ongoing hyperglycemic stimulation (Fig. 3C), with a profound lowering of serum insulin-to-glucose ratio (Fig. 3D). These three genotypes were clearly separable when comparing acute GSIS in vivo (Fig. 3E). Moreover, isolated islets from these subgroups tested for GSIS in vitro were distinct (Fig. 3F), despite that the fold change of GSIS was not statistically different. The data indicate a decline of releasable insulin beginning with Ins2-proinsulin-R(B22)E heterozygotes and worsening in homozygotes.

Porte and Kahn (30) as well as others (31) reported that patients with type 2 diabetes (T2D) demonstrate disproportionately elevated levels of circulating proinsulin that tends to be worse with the degree of hyperglycemia and correlates with a diminished maximal insulin secretion capacity. Yet, recent reports have suggested that serum proinsulin-to-insulin ratio measurements may be of limited value across populations (32,33), although they may still be of value within selected subgroups (33). Because model organisms have reduced genetic heterogeneity, we observed that in normal chow-fed animals at 5.5 weeks of age (Fig. 3G), male and female mice solely expressing WT proinsulin (black symbols), Ins2-proinsulin-R(B22)E heterozygotes (blue symbols), and homozygotes (red symbols) tended to fall into three distinct groups when plotting circulating proinsulin levels versus simultaneous random blood glucose. Ins2-proinsulin-R(B22)E heterozygotes showed the greatest variability of circulating proinsulin among mice, which was not well-correlated with random glucose, and overall, these values were nearly normal (Fig. 3G). However, homozygotes formed a discrete group, suggesting a positively sloped relationship of circulating proinsulin with simultaneous random blood glucose. Given the developing insulin deficiency (Fig. 3C), these differences were amplified when considering the circulating proinsulin-to-insulin ratio in homozygotes (Fig. 3H), although only small differences remained between WT and heterozygous animals. (A separate set of heterozygous HFD-fed males at 11 weeks of age [distinct from the animals shown in Fig. 3G and H] progressed into diabetestes, yet the mean value for circulating proinsulin or proinsulin-to-insulin ratio was not increased [Supplementary Fig. 2].) Altogether, the data support that circulating proinsulin-to-insulin ratio relative to prevailing glucose does serve to distinguish animal subgroups.

We next examined islet insulin and proinsulin content and localization in fixed tissues. In WT mice, β-cells exhibit robust insulin immunofluorescence, and proinsulin is concentrated in a juxtanuclear subregion (Fig. 4A). In Ins2-proinsulin-R(B22)E heterozygous males, again most islet cells are β-cells exhibiting obvious insulin immunofluorescence, but there were a few cells with diminished insulin signal and more expansive proinsulin immunofluorescence (suggesting depletion of insulin stores yet maintenance of biosynthetic activity [Fig. 4B]). Young Ins2-proinsulin-R(B22)E homozygotes that were not yet diabetic did not initially appear very different from heterozygotes. Animals with random blood glucose <200 mg/dL exhibited a subset of bright insulin-positive cells plus other islet cells with diminished insulin accompanied by increased proinsulin immunofluorescence (Fig. 4C), but this was not a major change (quantified in Fig. 4F). Even after homozygotes developed frank diabetes, there were still a few islet cells brightly immunofluorescent for insulin, but an enlarged fraction of cells showed diminished insulin signal (quantified in Fig. 4F) while exhibiting robust proinsulin immunofluorescence, as seen in both larger and smaller islets (Fig. 4D and E).

By electron microscopy, β-cells from WT and nor-moglycemic heterozygous Ins2-proinsulin-R(B22)E mice had a similar, well-granulated appearance (Supplementary Fig. 3A and B). Even in HFD-fed animals, the secretory pathway of WT β-cells exhibited the normal cisternal ER, ER-Golgi vesicular tubular clusters (pre-Golgi intermediates), well-developed Golgi stacks, and immature secretory granules and mature insulin granules (Supplementary Fig. 3C). These features were also present in Ins2-proinsulin-R(B22)E heterozygotes on a normal chow diet (Supplementary Fig. 3A and B). However, on HFD, a β-cell subpopulation in
Ins2-proinsulin-R(B22)E heterozygotes developed an expanded ER and underfilled, low-electron-density granule contents (Supplementary Fig. 3D, “cell B”). In homozygotes, it was easy to identify a subset of β-cells bearing some insulin granules, while other β-cells exhibited few granules but increased ER (Fig. 5 and Supplementary Fig. 3E) as well as cells bearing underfilled low-electron-density “microgranules” and related secretory pathway organelles (Supplementary Fig. 3P and G). The changes in granule number and appearance are quantified in Fig. 5C.

Insulin/proinsulin double-immunofluorescence images of heterozygous and homozygous Ins2-proinsulin-R(B22)E animals also revealed some islet cells unlabeled for either marker (Fig. 4B–E). Three-color immunofluorescence in Ins2-proinsulin-R(B22)E-positive islets labeled glucagon-positive cells initially at the perimeter of normoglycemic WT islets (Fig. 6A), which appeared increasingly within the islet interior, and corresponded to most of the remaining cells (Fig. 6B–E), especially in homozygous mice (quantified in Supplementary Fig. 4). Together, the data indicate that...
while progressing from WT to heterozygous to homozygous diabetic animals, a decrease of insulin-storing β-cells (Figs. 4 and 5) is seen with increasing proinsulin-enriched cells (Figs. 4 and 6) and glucagon-enriched cells (Supplementary Fig. 4). The loss of insulin in homozygotes was also observed by immunoblotting of islet lysates analyzed by reducing SDS-PAGE. Insulin deficiency was less noticeable when the homozygotes were still at the euglycemic stage but was exacerbated in parallel with the progression of hyperglycemia (Fig. 7A and B).

Remarkably, islets of Ins2-proinsulin-R(B22)E homozygotes did not exhibit proinsulin deficiency, even as hyperglycemia progressed into the 400–500 mg/dL range (Fig. 7A). However, in homozygous Ins2-proinsulin-R(B22)E mice, a greater fraction of proinsulin was contained in aberrant disulfide-linked complexes (Fig. 7B), which have been reported both in stressed human islets and murine diabetes models (17). Indeed, even before development of frank diabetes, we noted a tendency toward increased islet BiP protein (as well as the BiP cochaperone p58ipk [Fig. 7C and D]), although the magnitude of the effect on these downstream targets of ER stress response was only approximately twofold. ER resident proteins (reactive with anti-KDEL) (Supplementary Fig. 5A) became more apparent in proinsulin-enriched cells of heterozygotes (mean ~30% of β-cells) and homozygotes (mean ~50% of β-cells) (Supplementary Fig. 5B), consistent with cellular heterogeneity that includes an increased subfraction of β-cells maintaining an expanded or activated ER compartment.

Figure 4—Proinsulin and insulin double immunofluorescence showing random blood glucose (BG) at the time of euthanasia. A: WT control. B: Ins2-proinsulin-R(B22)E heterozygote (Het) (6-week-old female). C: Normoglycemic 4-week-old female Ins2-proinsulin-R(B22)E homozygote (Hom). D and E: Ins2-proinsulin-R(B22)E Hom 7-week-old female. F: Quantitation of insulin-positive cytoplasmic area as a percentage of total β-cell cytoplasmic area in independent islets from the genotypes indicated (mean ± SD; P = 0.0002). ***P < 0.001.
We conducted three independent pulse-chase radiolabeling experiments to look at the efficiency of insulin biosynthesis. First, the amount of newly synthesized pro-insulin (made in a 30-min pulse labeling with $^{35}$S-amino acids) was analyzed by immunoprecipitation with anti-insulin, reducing SDS-PAGE, and autoradiography (Fig. 8A, right). At 2 h of chase, cells and media were combined before immunoprecipitation, and the yield of newly made insulin (Fig. 8A, left) derived from labeled proinsulin in WT control islets was nearly 100%, whereas insulin generation in euglycemic homozygous \textit{Ins2-proinsulin-R(B22)E} islets was less efficient (quantitation in Fig. 8D). In the next experiment, labeled heterozygous \textit{Ins2-proinsulin-R(B22)E} islets were compared against those from a homozygote with random blood glucose of 400 mg/dL (Fig. 8B). From the euglycemic heterozygote, recovery of labeled insulin at 2-h chase from the original newly synthesized proinsulin was excellent (Fig. 8B, left), approaching 100% (Fig. 8D). However, in the homozygote, little labeled insulin was produced (Fig. 8B), with a yield of only 24% (Fig. 8D). In a third experiment, recovery of mature insulin from newly synthesized proinsulin in a euglycemic heterozygote again approached 100% (Fig. 8C), whereas in a homozygote with a random blood glucose of 537 mg/dL, insulin yield was only 4% (Fig. 8D). Remarkably, in each case, newly synthesized proinsulin in the homozygous \textit{Ins2-proinsulin-R(B22)E} islets was detected by nonreducing SDS-PAGE as a ladder of aberrant disulfide-linked proinsulin complexes that exceeded the recovery of monomeric proinsulin (Fig. 8A–C).
DISCUSSION

We report that unlike Akita-proinsulin-C(A7)Y, proinsulin-R(B22)E can pass ER quality control to become secreted from Min6 pancreatic β-cells (Fig. 1B), and its expression does not efficiently block export of coexpressed WT proinsulin in these cells (Fig. 1C). Moreover, in a normal laboratory environment, heterozygous Ins2-proinsulin-R(B22)E males gained weight postweaning and exhibited normal glucose tolerance, and diabetes did not develop (Fig. 2A–D) for as long as we followed the animals (up to 6 months of life). Nevertheless, different cell lines may exhibit different degrees of ER quality control of mutant proinsulin (34); moreover, proinsulin-R(B22)E showed evidence of misfolding in the ER (Figs. 1, 7, and 8, and Supplementary Fig. 1A). Additionally, when transitioned to an HFD, all male heterozygotes developed progressive hyperglycemia/diabetes (Fig. 2D–F) with an inadequate insulin secretory response (Fig. 2G and H) because of diminished islet insulin content (Fig. 2J), accounting for impaired GSIS (Fig. 2I) despite the persistence of proinsulin (Fig. 2J and Supplementary Fig. 1B) in a growing percentage of β-cells (Fig. 4B). The pathological effects of HFD may include contributions from 1) increased insulin demand that exceeds insulin storage, 2) increased proinsulin misfolding secondary to upregulated proinsulin biosynthesis, and/or 3) adverse effects on ER homeostatic function secondary to nutrients contained within HFD.

The sexually dimorphic effects of proinsulin misfolding seen in the heterozygotes is amply documented in mouse models of MIDY as well as several other forms of diabetes. Akita mice, which bear one allele encoding misfolded proinsulin-C(A7)Y, exhibit a selective diabetes phenotype in males (35). A similar sexual dimorphism is observed in
Kuma mice bearing proinsulin-Q(A15)del (36). Male KINGS mice bearing one allele encoding proinsulin-G(B8)S are overtly diabetic at ~5 weeks, whereas females have only slightly elevated nonfasting glycemia (6). Furthermore, in Munich mice bearing proinsulin-C(A6)S, even at 6 months of age, males have diabetes (by fasting glucose criteria) and females do not (37). One explanation may be that estrogen helps to promote the successful degradation of mutant proinsulin (38). Interestingly, even in the absence of any Ins gene mutations, low-dose streptozotocin injections in five different strains of mice (C57BL/6, MRL/Mp, BALB/c, DBA/2, and 129/SvE) results in males developing diabetes to a greater extent than females (35). Our current studies of Ins2-proinsulin-R(B22)E mice are thus consistent with the established sexually dimorphic hyperglycemia phenotype in many murine models.

A similar phenotype develops in male and female homozygotes even without HFD (Figs. 3A–F and 4C–E). An increase of circulating proinsulin-to-insulin ratio has been described in human T2D (39,40); an intra-islet increase of proinsulin-to-insulin has also been reported during development of spontaneous T2D in animal models (41). We also observed an increasing proinsulin-to-insulin ratio in the circulation (Fig. 3H) and in the islets themselves (Figs. 4C–E and 7A) in diabetic homozygotes. Proinsulin misfolding is causal for diabetes in the homozygotes, with obviously aberrant disulfi de-linked proinsulin complexes forming immediately upon synthesis and accompanying diminished insulin biosynthesis (Figs. 7B and 8). Whereas the ability of isolated islets from WT versus heterozygous versus homozygous mice to respond to a glucose challenge exhibits no obvious defect in fold stimulation, a progressive deficiency of insulin secretion under both unstimulated and stimulated conditions is apparent in vivo and in vitro (Figs. 2I and 3E and F), consistent with a failure to maintain the insulin storage pool (42).

Figure 8—Biosynthesis of proinsulin and insulin in WT control and Ins2-proinsulin-R(B22)E heterozygous (Het) and homozygous (Hom) males with progression of diabetes (age 4–8 weeks). The random blood glucose of each animal at the time of euthanasia is indicated. Isolated islets were pulse labeled with 35S-amino acids for 30 min and then chased for 2 h, as indicated. Cells (C) and chase media (M) were either combined (so that no protein was lost) or analyzed separately. Samples (normalized to trichloroacetic acid–precipitable counts in the cell lysates) were immunoprecipitated with anti-insulin followed by Tris-tricine-urea-SDS-PAGE under nonreducing or reducing conditions followed by fluorography. A line is drawn separating the nonreduced and reduced samples, but these images show the complete gels, and no lanes have been excised. A–C: Three independent experiments with the genotypes shown (ages 4, 5, and 8 weeks, respectively). D: Quantitative recovery of newly synthesized insulin derived from pulse-labeled proinsulin, as derived from the preceding phosphorimages; genotype and random blood glucose are indicated. Proinsulin bands (at chase time 0) were quantitated from reducing gels; newly synthesized insulin derived from the pulse-labeled samples were quantified from nonreducing gels. (Insulin is a two-chain protein that “falls apart” under reducing conditions; thus, nonreducing gels are preferable for this analysis.) ox, oxidized; R, reduced.
| Reagent type or resource | Designation | Source or reference | Identifier | Additional information |
|--------------------------|-------------|---------------------|------------|------------------------|
| Cell line (mouse)        | Min6 (mouse β-cell line) | Soleimanpour et al.⁵⁶ |            |                        |
| Antibody                 | Anti-rat proinsulin monoclonal CCI-17 | ALPCO | CCI-17 | 1:1,000 |
| Antibody                 | Anti-human proinsulin | Abmart (Haataja et al.³⁴) | 1B24, 3L10 | 1:1,000 |
| Antibody                 | Guinea pig anti-insulin | Covance |            | 1:500 |
| Antibody                 | Mouse antiglucagon | Abcam | ab109888 | 1:1,000 |
| Antibody                 | Antiglucagon (rabbit polyclonal) | Millipore | AB932 | 1:500 |
| Antibody                 | Anti-KDEL (rabbit monoclonal) | Novus Biologicals | NBP2–75549 | 1:300 |
| Antibody                 | Anti-Myc (rabbit polyclonal) | Immunology Consultants Laboratory | RMYC-45A | 1:500 |
| Antibody                 | Rabbit anti-cyclophilin B | Thermo Fisher Scientific | PA1-027A | 1:1,000 |
| Antibody                 | Alexa Fluor 488, 555, 647 (secondary) | Invitrogen |            | 1:500 |
| Antibody                 | HRP guinea pig, rabbit, or mouse ² | Jackson ImmunoResearch |            | 1:5,000 |
| Recombinant DNA reagent  | pTARGET (vector) | Promega | A1410 |            |
| Recombinant DNA reagent  | Myc-WT proinsulin (plasmid) | Liu et al.⁵⁷ |            | Human proinsulin |
| Commercial assay or kit  | QuikChange II Site-Directed Mutagenesis Kit | Agilent Technologies | Agilent: 200524 |            |
| Commercial assay or kit  | Mouse insulin ELISA | ALPCO | 80-INSMS-E10 | |
| Commercial assay or kit  | Enhanced chemiluminescence Immobilon or Clarity | Millipore or Bio-Rad, respectively |            | |
| Chemical compound, drug  | N-ethylmaleimide | Sigma-Aldrich | E3876 | |
| Chemical compound, drug  | Trans 35S Label | Perkin-Elmer | PerkinElmer: NEG072007MC | |
| Chemical compound, drug  | ProSieve 50 Gel Solution | Lonza | Lonza: 50618 | |
| Chemical compound, drug  | cOmplete Mini Protease Inhibitor Cocktail | Roche | Roche: 11836153001 | |
| Chemical compound, drug  | Collagenase P | Sigma-Aldrich | 11249002001 | |
| Chemical compound, drug  | Protein A agarose | Invitrogen |            | |
| Chemical compound, drug  | Dithiothreitol, pansorbin, urea | Sigma-Aldrich or Thermo Fisher Scientific |            | |
| Reagent                  | Lipofectamine 2000 | Thermo Fisher Scientific |            | |
| Reagent                  | Tissue culture reagents | Thermo Fisher Scientific |            | |
| Reagent                  | Met/Cys-deficient RPMI medium | Sigma-Aldrich |            | |
| Reagent                  | ProLong Gold Antifade Reagent with DAPI | Thermo Fisher Scientific | Invitrogen P36931 | |
| Reagent                  | Unmasking solution | Thermo Fisher Scientific | Cell Signaling 14747P | |
| Cell culture imaging      | Nunc LabTek-II Chambers | Thermo Fisher Scientific |            | |
| Software, algorithm       | GraphPad Prism 8.0 | GraphPad Software |            | |
The progression of dysglycemia/diabetes is linked to the emergence of intra-islet heterogeneity, with increasing proinsulin-rich cells (43–46) bearing little or no stored insulin—distinct from the subpopulation of β-cells rich in stored insulin (Fig. 4). Heterogeneity within the β-cell population is increasingly recognized at the mRNA level (47), including the description of “extreme” β-cells with lower insulin levels and higher juxtacrine proinsulin immunostaining (48), which is believed to represent the Golgi region from which new secretory granules emerge (23,49). However, the diabetogenic progression in HFD-challenged heterozygous Ins2-proinsulin-R(B22)E mice (or homozygotes) involves islet cells exhibiting a proinsulin localization that fills the cytoplasm (Fig. 4) with expansion of the ER (Fig. 5 and Supplementary Fig. 3) and with increased generation of misfolded proinsulin (Figs. 7 and 8) accompanied by increased KDEL-containing ER resident proteins (Supplementary Fig. 5) and deficient insulin biosynthesis (Fig. 8) with deficient insulin content (Fig. 2J). Curiously, this is what has been observed for the localization and misfolding of proinsulin in islets of T2D-like mice with hyperphagia-induced dysglycemia without any Ins gene variant (17). The total life span of proinsulin in β-cells is limited to ~4 h (23), suggesting that β-cells lacking insulin but bearing proinsulin (which may be entirely overlooked in β-cell mass measurements [50]) remain biosynthetically active right up to the time of our analysis.

Additionally, even in “off-scale high-glucose” Ins2-proinsulin-R(B22)E homozygotes, a subset of β-cells with substantial stored insulin content and only modest proinsulin persists, although they represent a shrinking fraction of the total (Figs. 4 and 6). Conceivably, such cells could represent immature β-cells with deficient glucose sensing (51,52) that may not release stored insulin, but this remains to be determined. Additionally, the diseased islets develop increased glucagon-positive cells within the islet interior (Fig. 6), a feature noted in several diabetes models. Altogether, these data are consistent with the existence of islet β-cell subpopulations exhibiting heterogeneity in ER homeostasis (53) and an increase in islet α-to-β-cell ratio (54).

In summary, the main observation in this article is that proinsulin misfolding can be entirely subclinical, yet dramatic pathology emerges upon HFD exposure, triggering rapid insulin deficiency. HFD-induced β-cell failure has been proposed to be alternatively associated with glucolipotoxicity, β-cell senescence, dedifferentiation, transdifferentiation, or apoptosis (55). This article does not resolve those alternatives but highlights intra-islet heterogeneity, with decreasing insulin-high/proinsulin-low cells, increasing proinsulin-high/insulin-low cells, and glucagon-positive cells during disease progression. Unequivocally, our data in these models show that development of hyperglycemia runs antiparallel with pancreatic insulin storage (i.e., biosynthesis of new insulin secretory granules is inadequate to replace the depletion of stored insulin used to meet the body’s metabolic needs. Fascinating work is ongoing worldwide to understand the changes in islet cell heterogeneity during the development of diabetes (53–55). We merely emphasize that all the pathological changes identified herein can be triggered by a genetic predisposition to proinsulin misfolding. Therefore, we conclude that predisposition to proinsulin misfolding serves as an important potential risk factor to diet-induced diabetes.

Acknowledgments. We thank Leroux Devon, Michigan Biomedical Research Electron Microscopy Core Facility, for support and the Michigan Tissue and Molecular Pathology (Histology) Core for help with sample preparation.

Funding. This work was supported by National Institutes of Health grants R01-DK-48280 and P01-Al-118688, the Michigan Diabetes Research Center Morphology Core (P30-DK-020572), the University of Michigan and Protein Folding Diseases Initiative, and Howard Hughes Medical Institute, University of Colorado Anschutz Medical School, and National Jewish Health Institutional funds.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. M.A., A.A., L.H., M.T., and N.J. generated research data, reviewed data, and contributed to discussion. D.L. developed analysis techniques. J.K. and P.A. initiated and designed the research project. N.J. collaborated with Dr. Jennifer Matsuda and the team in the National Jewish Mouse Genetics Core Facility in the design and creation of the original NOD knockin mouse that gave rise to the mice used in these studies. P.A. wrote the manuscript. All authors edited and reviewed the manuscript. P.A. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented as a symposium talk at the 81st Scientific Sessions of the American Diabetes Association, 25–29 June 2021.

References

1. Liu M, Hodish I, Haataja L, et al. Proinsulin misfolding and diabetes: mutant INS gene-induced diabetes of youth. Trends Endocrinol Metab 2020;21:652–659
2. Sun J, Cui J, He Q, Chen Z, Arvan P, Liu M. Proinsulin misfolding and endoplasmic reticulum stress during the development and progression of diabetes. Mol Aspects Med 2015;42:105–118
3. Arunagiri A, Haataja L, Cunningham CN, et al. Misfolded proinsulin in the endoplasmic reticulum during development of beta cell failure in diabetes. Ann N Y Acad Sci 2018;1418:5–19
4. Ameth B. Insulin gene mutations and posttranslational and translocation defects: associations with diabetes. Endocrine 2020;70:488–497
5. Blutke A, Renner S, Flenkenthaler F, et al. The Munich MIDY Pig Biobank—a unique resource for studying organ crosstalk in diabetes. Mol Metab 2017;6:931–940
6. Austin ALF, Daniels Gatward LF, Cnop M, et al. The KINGS Ins2+/G32S mouse; a novel model of β-cell endoplasmic reticulum stress and human diabetes. Diabetes 2020;69:2667–2677
7. Sprunger ML, Jackrel ME. Quality control in the ER: misfolded prohormones get a checkup. Mol Cell 2019;75:415–416
8. Kim YH, Kastner K, Abdul-Wahid B, Izaguirre JA. Evaluation of conformational changes in diabetes-associated mutation in insulin a chain: a molecular dynamics study. Proteins 2015;83:662–669
9. Ionescu-Tirgoviste C, Despa F. Biophysical alteration of the secretory track in β-cells due to molecular overcrowding: the relevance for diabetes. Integr Biol 2011;3:173–179
10. Hodish I, Liu M, Rajpal G, et al. Misfolded proinsulin affects bystander proinsulin in neonatal diabetes. J Biol Chem 2010;285:685–694
11. Sun J, Xiong Y, Li X, et al. Role of proinsulin self-association in mutant INS gene-induced diabetes of youth. Diabetes 2020;69:954–964
12. Barbetti F, Mammi C, Liu M, et al. Neonatal diabetes: permanent neonatal diabetes and transient neonatal diabetes. In Diabetes associated with single gene defects and chromosomal abnormalities. Barbetti F, Ghizzoni L, Guaraldi F, Eds. Basel, Switzerland, Karger AG, 2017, pp. 1–25
13. Liu M, Sun J, Cui J, et al. INS-gene mutations: from genetics and beta cell biology to clinical disease. Mol Aspects Med 2015;42:3–18
14. Steiner DF. The proinsulin C-peptide—a multirole model. Exp Diabesity Res 2004;5:7–14
15. Irwin DM. Evolution of the mammalian insulin (Ins) gene; changes in proteolytic processing. Peptides 2021;135:170435
16. Rege NK, Liu M, Yang Y, et al. Evolution of insulin at the edge of foldability and its medical implications. Proc Natl Acad Sci U S A 2020;117:29618–29628
17. Arunagiri A, Haataja L, Pottekat A, et al. Proinsulin misfolding is an early event in the progression to type 2 diabetes. eLife 2019;8:e44532
18. Stay J, Olsen J, Park SY, Gregersen S, Hjørringgaard CU, Bell GI. In vivo measurement and biological characterisation of the diabetes-associated mutant insulin p.R46Q (GlnB22-insulin). Diabetologia 2017;60:1423–1431
19. Molven A, Ringdal M, Nordbø AM, et al.; Norwegian Childhood Diabetes Study Group. Mutations in the insulin gene can cause MODY and autoantibody-negative type 1 diabetes. Diabetes 2008;57:1131–1135
20. Liu M, Wan ZL, Chu YC, et al. Crystal structure of a monomeric insulin. Acta Biochim Biophys Sin (Shanghai) 2006;38:537–542
21. Haataja L, Snapp E, Wright J, et al. Proinsulin intermolecular interactions during secretory trafficking in pancreatic β cells. J Biol Chem 2013;288:1896–1906
22. Liu M, Lara-Lemus R, Shan SO, et al. Impaired cleavage of proinsulin signal peptide linked to autosomal-dominant diabetes. Diabetes 2012;61:828–837
23. Miyazaki J, Araki K, Yamato E, et al. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to immature β-cells lacking Glut2 precedes the expansion of single human β-cells reveals states of insulin production and unfolded protein response. Diabetes 2018;67:1783–1794
24. Farack L, Golan M, Egozi A, et al. Transcriptional heterogeneity of β-cells in the laboratory Nile rat model of type 2 diabetes. J Endocrinol 2016;229:343–356
25. Liu M, Huang Y, Xu X, et al. Normal and defective pathways in biogenesis and maintenance of the insulin storage pool. J Clin Invest 2021;131:e142240
26. Schuit FC, In’t Veld PA, Pipeleers DG. Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. Proc Natl Acad Sci U S A 1988;85:3865–3869
27. Kiekens R, In’t Veld P, Mahler T, Schuit F, Van De Winkel M, Pipeleers D. Differences in glucose recognition by individual rat pancreatic β-cells are associated with intercellular differences in glucose-induced biosynthetic activity. J Clin Invest 1992;89:117–127
28. Pipeleers D, Kiekens R, Ling Z, Wilikens A, Schuit F. Physiologic relevance of heterogeneity in the pancreatic beta-cell population. Diabetologia 1994;37(Suppl. 2):S57–564
29. Bosco D, Meda P. Actively synthesizing beta-cells secrete preferentially after glucose stimulation. Endocrinology 1991;129:3157–3166
30. Xín Y, Domínguez Gutiérrez O, Okamoto H, et al. Pseudotime ordering of single human β-cells reveals states of insulin production and unfolded protein response. Diabetes 2018;67:1783–1794
31. Farack L, Golan M, Egozi A, et al. Transcriptional heterogeneity of beta cells in the intact pancreas. Dev Cell 2019;48:115–125.e4
32. Orri L, Ravazzola M, Amherdt M, Madsen O, Vassalli JD, Perrelet A. Direct identification of prohormone conversion site in insulin-secreting cells. Cell 1995;62:671–681
33. Oram RA, Sims KE, Evans-Molina C. Beta cells in type 1 diabetes: mass and function; sleeping or dead? Diabetologia 2019;62:567–577
34. Beamish CA, Zhang L, Szlipinski SK, Strutt BJ, Hill DJ. An increase in immature β-cells lacking Glut2 precedes the expansion of β-cell mass in the pregnant mouse. PLoS One 2017;12:e0182256
35. van der Meulen T, Mawla AM, DiGruccio MR, et al. Virgin beta cells reveal states of insulin production and unfolded protein response. Diabetes 2018;67:1783–1794
36. Wang YJ, Kaestner KH. Single-cell RNA-seq of the pancreatic islets—a promise not yet fulfilled? Cell Metab 2019;29:539–544
37. Gromada J, Chabosseau P, Rutter GA. The α-cell in diabetes mellitus. Nat Rev Endocrinol 2018;14:694–704
55. Hudish LI, Reusch JE, Sussel L. β Cell dysfunction during progression of metabolic syndrome to type 2 diabetes. J Clin Invest 2019;129:4001–4008
56. Soleimanpour SA, Gupta A, Bakay M, et al. The diabetes susceptibility gene Clec16a regulates mitophagy. Cell 2014;157:1577–1590
57. Liu M, Haataja L, Wright J, et al. Mutant INS-gene induced diabetes of youth: proinsulin cysteine residues impose dominant-negative inhibition on wild-type proinsulin transport. PLoS One 2010;5: e13333