門脇 糖尿病の治療において，インスリン注射や膵臓・膵島移植には制限や限界があるため，期待されているのが膵臓を細胞から作製する再生医療です。再生医療の実現には，膵β細胞の分化や再生の過程を明らかにすることが重要です。本日は「膵β細胞の再生医療と糖尿病治療への応用」をテーマに，それぞれの先生方の研究過程ならびに最先端の研究についてご紹介いただきたいと思います。

まずは川口先生から，iPS細胞を用いた新規糖尿病治療法の開発について，これまでの研究成果の概説と今後の展望をお話しいただけますか。

iPS細胞を用いた新規糖尿病治療法開発の展望

川口 我々は，再生医療に用いる機能的細胞作製の根本は，「発生現象を培養皿上で再現すること」だと考えています。膵臓の発生はすでに3次元構造を持った原腸から上皮細胞が発芽する膵原基形成に始まり，立体構築の中で，内分泌細胞と外分泌細胞の分化がほぼ同時に起こります。我々はこの原腸から膵臓への運命決定のメカニズムに関して研究を行ってきました。

まず1990年代に，遺伝子ノックアウトマウスの情報から，PDX-1(pancreatic and duodenal homeobox-1)，ngn3 (neurogenin 3) [参照 p.16 キーワード解説]，Ptf1a (pancreas transcription factor 1a)がそれぞれ膵臓，膵内分泌組織，膵外分泌組織の形成に必要であるということが分かっていました。遺伝子ノックアウト技術は有用な手法ですが，ノックアウトされて本来の細胞になれなかった細胞は，細胞死によって除去されたのか，未分化の状態で生存しているのか，あるいは運命を変更して他の細胞として生存しているのかが明らかではないという限界があります。そこで，遺伝子ノックアウト技術と細胞系譜解析を組み合わせるという実験を行いました。転写因子Ptf1aのlocusにCreリコンビナーゼ遺伝子をノックインしたマウスとROSAレポーターマウスを交配すると，Ptf1aを発現した細胞は膵臓になります。一方，両方のアリルをCreアリルに直したマウスは，自動的にPtf1aノックアウトとなります。Ptf1aのノックアウト細胞の大部分が膵臓になれなかったことから，Ptf1aは単に外分泌組織の形成に必要なだけではなく，膵臓決定遺伝子であることが示されました。

次に，ヒトの胃の前庭部大彎側に好発する異所性膵組織形成におけるPtf1aの役割について検証しました。Ptf1aの発現を制御する上位シグナルについて，2004年に筑波大学のグループが「Notch Signalingのeffectorである HES1 (hairy and enhancer of split-1)をノックアウトすると，下部胆管組織が内分泌組織・外分泌組織を含む膵組織に置換された」ことを報告しました[1]。我々は，これはPtf1aの作用によるものだという仮説を立て，ヒト，マウス，ラット，ゼブラフィッシュでPtf1aのプロモーター領域のシークエンスを調べたところ，TATAAボックスを挟んで2ヵ所にHES1結合モチーフが存在し，種を超えて保存されていました。続けて，HES1ワイルドタイプとHES1ノックアウトの条件で，それぞれPtf1a発現細胞の系譜解析を行ったところ，HES1ノックアウトで形成される下部胆管領域の異所性膵組織は全て系譜標識され，Ptf1a発現細胞に由来することがわかりました。さらに，胃の前庭部大彎や十二指腸にも異所性のPtf1a発現を介した異所性膵組織が形成されました。十二指腸の異所性膵組織形成過程では，CDX2 (caudal-related homeobox 2)陽性の十二指腸上皮から異所性のPtf1a発現を介して最終的にはCDX2陰性の膵組織に変わっていることから，発生早期で運命転換が起こることが分かります。胎生早期ですでに異所性Ptf1a発現が認められていることから，これは教科書でいわれる「迷入膵」ではなく，de novoの運命転換であると考えます。

以上の実験から，HES1はPtf1aの発現を制御することで，膵臓の形成の位置決定に関与するというシグナルであることが分かります。つまり，胆管前駆細胞，膵臓の前駆細胞，胃・腸管前駆細胞間には可塑性が存在し，その後の細胞運命がNotchシグナリングによって制御されている可能性を考えました。さらに，HES1は内胚葉組織の広い領域に発現するにも関わらず，ある一定の限られた部位に異所性膵組織が形成さ

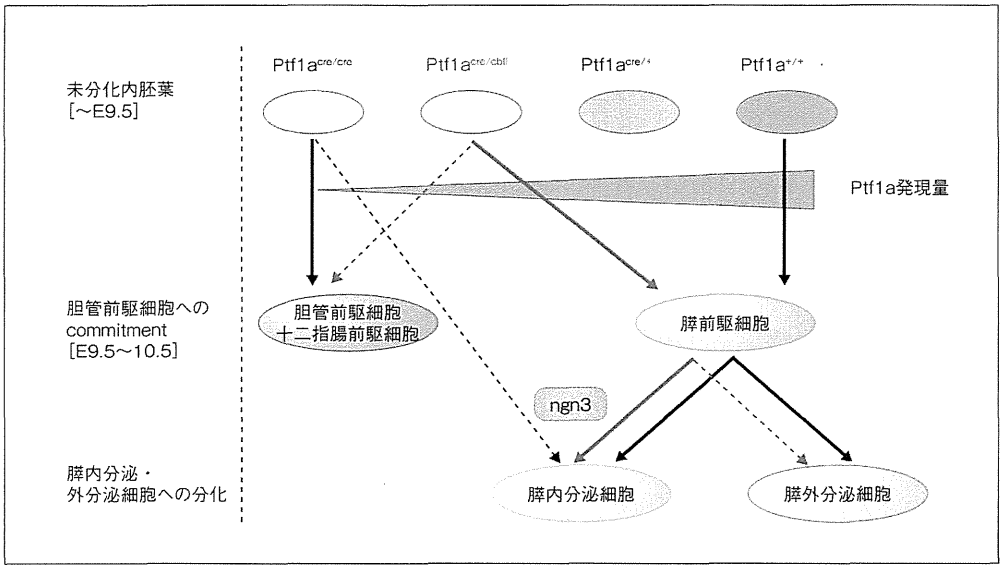


図1　Ptf1a dosage-dependent に細胞運命を規定する

れた点に注目すると，HES1がPtf1aを負に制御しているのに対して，何らかのPtf1aのpositive regulator（正の制御因子）が存在し，様々な上位制御システムによって最終的に決定されたPtf1a発現量が「ある閾値を超えた時のみ」膵細胞へと分化するという仮説が成り立ちます。これを検証するために，Ptf1a低発現マウスを用いた交配により様々なPtf1a発現量を持つマウスラインを作製したところ，Ptf1a低発現マウスはPtf1a低発現細胞の一部が膵臓になれずに，胆管あるいは十二指腸への運命をたどっていることが分かりました。Ptf1a低発現マウスの膵発生過程を見てみると，極端な外分泌組織分化の遅延と膵臓全体の低形成が認められました。内分泌細胞分化開始のタイミングは正常であったものの，圧倒的に細胞の数が少なく，結果的に糖尿病になりました。このことから，ヒトのSNPなどでPtf1aの発現が少ないと，新生児糖尿病になる可能性が示唆されます。以上の結果から，胎生期にはPtf1a発現量依存的な運命制御機構が存在すると考えられます。この制御は2段階で行われており，第一は膵臓になれるかどうかで，Ptf1aが少ないものは膵臓になれずに，胆管あるいは十二指腸になります。第二は膵臓になった後もPtf1a発現量によって，内分泌細胞，外分泌細胞への運命が決定されると思われます（図1）。

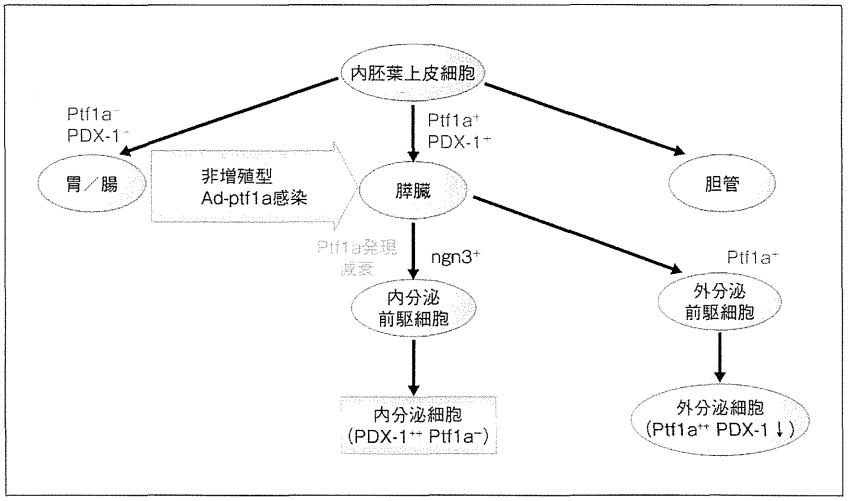


図2　Ptf1aを使って異所性膵を“作る”ことは可能か？

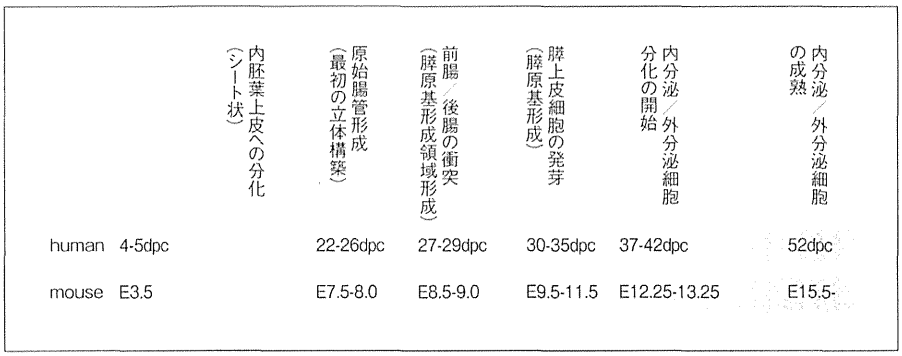


図３　機能的細胞の作製に向けて，発生現象を愚直に再現する

さて，こうした実験結果を踏まえて，いよいよ「Ptf1aを用いて異所性膵を作製することは可能か？」という命題を検証しました（図２）。マウス胎生期11.5日のPDX-1陽性かつPtf1a陰性の胃前庭部から十二指腸にかけての部分を組織培養し，アデノウイルスによりPtf1a発現をひき起こしたところ，ある一定のウイルス力価の時のみ内在性のPtf1a発現が誘導されました。そして，その条件の時のみ３日間の追加培養でインスリンのmRNA発現が確認されました。この組織を解析してみると，腸管細胞とは明らかに性質の異なる細胞が間質方向に発芽しており，免疫染色の結果，アミラーゼ陽性細胞，インスリン陽性細胞に加え，グルカゴン陽性細胞やソマトスタチン陽性細胞が認められました。インスリン陽性細胞はGLUT2（glucose transporter 2）［参照 p.16 キーワード解説］やPDX-1を発現し，細胞塊を形成していました。さて，アデノウイルスによるPtf1a導入で作成された異所性膵の機能をみたところ，*in vitro*で周囲のブドウ糖濃度に反応したインスリン分泌を行うだけではなく，ストレプトゾトシン（STZ）誘発糖尿病ヌードマウスへの移植によって血糖の改善効果が確認され，生理活性を有することが確認されました。

これらの結果を踏まえ，iPS細胞を用いてどのようにして機能的な膵細胞を作るか？を考えた場合，私はまず立体構築を持った原腸オルガノイドを作製し，Ptf1a発現誘導をきっかけとして，内分泌細胞も外分泌細胞も含めた膵臓全体を作るべきだと考えています。つまり，最初にお話ししたとおり，発生現象を愚直に再現することが最も確実な方法だと考えています（図３）。

門脇　iPS細胞を用いた機能的な膵細胞を作るためには，まず立体構築を持った原腸オルガノイドを作るというステップを経ることが必要だということでしょうか。

川口　臓器培養で成功した系を単純にiPSの系に持っていくことを考えると，原腸オルガノイドからPtf1a発現の誘導をきっかけとして，膵臓を丸ごと作る戦略は論理的飛躍がないと思います。また，先にも述べたとおり，膵発生はすでに立体構造を持つ原腸から発生します。私の言う“愚直なまでの発生現象の再現”とは，「どの段階から三次元か？」という点にもこだわっています。

門脇　内分泌細胞だけではなく外分泌細胞も同時に作ったほうがいいのはなぜでしょうか。

川口　ヒト臨床膵島移植の２年後の長期臨床成績を見ると，混入した非内分泌細胞数が多いほど良好で，移植された膵β細胞の数とは相関しないことが理由の１つとして挙げられます。さらに，胎生期に外分泌組織特異的にPDX-1をノックアウトする外分泌低形成マウスを作ったところ，出生時の膵β細胞の成熟が遅延し，生後１ヵ月間の膵β細胞増殖が極端に悪く，結果

として糖尿病になったことも理由の1つです。このことは，おそらく幼弱膵島の成熟/増殖や，機能維持に必要なシグナルが外分泌組織から出ているということを示唆しています。

門脇　ありがとうございました。愚直に再現するという意味が，かなり深い洞察といろいろな実験データから導き出されたということがよく分かりました。

マウスES細胞から膵β細胞の作製の研究と今後の展開

門脇　粂先生は2013年にマウスの胚性幹細胞（ES細胞）から，成体膵島と同等の能力を持つ膵臓細胞を作ることに成功されました。その研究課程について詳しくお聞きし，その過程でケミカルバイオロジーにより発見された分化誘導効率を向上させる化合物についてもご説明いただければと思います。

粂　私も川口先生と同様に，発生と同じ環境を試験管内で作り出すことで，発生時の細胞系譜に沿って膵β細胞を分化誘導できると考えています。分化に必要なシグナルや発生の環境についての過去の論文では，内胚葉だけが自律に分化するわけではなく，常に隣接している組織からのシグナルにより分化が誘導されると報告されています。

我々は，支持細胞との共培養によりES細胞を分化させる実験で，特に分化誘導活性が良好であったM15細胞［参照p.16 キーワード解説］を発見しました。このM15細胞を用いて初期の分化ステップに必要な因子について解析したところ，M15細胞との接着が必要であること，さらに成長増殖因子を加えると分化が促進されることから，M15細胞は液性因子だけでなく細胞基底膜を提供する役割も担っていることを明らかにしてきました。このようにして，膵前駆細胞まではPDX-1陽性細胞を高い効率で作製できるようになりました。しかしながら，十分な効率でPDX-1からngn3陽性細胞，さらに膵β細胞を分化誘導することが困難でした。また，誘導された膵β細胞はグルコース濃度に応じてインスリン分泌能が弱いことが問題点でした。そのため，ケミカルバイオロジーの手法を用いて低分子化合物のライブラリーのスクリーニングを開始しました。実際に臨床で使われている1,120個程度の化合物を前駆細胞作製後に加えて，膵β細胞への分化誘導効率を向上する因子を探索しました。その結果，2つの化合物が見つかりました。これらはそれぞれ単独投与でインスリン陽性細胞を増やす効果があり，さらに，同時投与により相加・相乗作用を示します。

化合物の1つは，VMAT2（Vesicle Monoamine Transporter 2）阻害剤［参照p.16 キーワード解説］です。VMAT2は，分泌小胞を多く有する膵β細胞などの組織に発現し，細胞質内のモノアミンを分泌小胞に取り込むトランスポーターです。VMAT2によって取り込まれないモノアミンは，最終的にモノアミン酸化酵素（MAO）によって分解されます。我々は，ES細胞においてVMAT2阻害剤によってモノアミン（ドパミンなど）含量が低下することを見出しました。さらに，ES細胞培養系にモノアミンを加えて膵β細胞分化に対する作用を確認したところ，予想通り膵β細胞の分化抑制結果を示しました。

もう1つの化合物は，グルコース応答性β細胞への分化を促進する細胞膜透過性のcAMPアナログ，dBu-cAMPです。この化合物を加えないと，分化・培養してもグルコース応答性インスリンを分泌する細胞にはなりません。これらのことから，モノアミンが分化に対して抑制的に働き，その抑制を解除することで膵前駆細胞から内分泌前駆細胞に分化することが示されました。さらに，グルコース応答性のインスリン分泌能を獲得するためにdBu-cAMPが必要だということが結論づけられました。

ES細胞由来の膵前駆細胞を純化してインスリン含量を調べたところ，VMAT2阻害剤を加えた分化細胞には成体膵島の60%ほどのインスリン含量が認められましたが，dBu-cAMPにはこの効果は見られませんでした。一方，dBu-cAMPを加えた分化細胞には成体膵島の40%ほどのグルコース応答性のインス

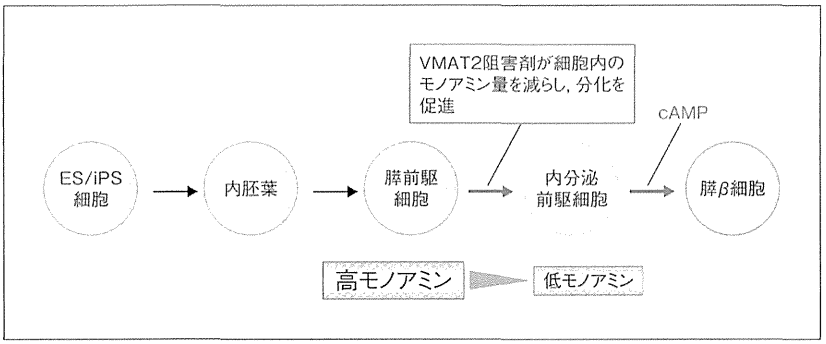


図4　ES細胞から機能的な膵β細胞を創る
VMAT2阻害剤とcAMPの作用による分化効率と成熟度の向上

リン分泌能があることが示されましたが，VMAT2阻害剤にはこの効果は見られませんでした。以上の所見をもとに，これらの化合物で分化誘導した細胞をAKITAマウス（若年性糖尿病マウス）の腎被膜下に移植しました。その結果，約2週間で血糖値が正常値まで降下し，効果が数ヵ月に及ぶことが分かりました。

まとめますと，VMAT2阻害剤は細胞内のモノアミン量を抑制して分化を促進し，さらにcAMPを活性化させると，グルコース応答性のインスリン分泌能を持った細胞に分化することができます。これらから分化した膵β細胞は成体膵島と同等のインスリン含量およびインスリン分泌能を持ち，糖尿病マウスに移植すると低血糖化が達成できました（図4）。今後は，VMAT2阻害剤によるモノアミンの抑制による分化促進作用の機序や，ヒトiPS細胞における作用を解明していくことが課題と考えています。

門脇　ありがとうございました。最近のケミカルバイオロジーによる非常にクリアな結果を見せていただきました。モノアミン抑制シグナルやcAMP上昇シグナルの経路として，外分泌細胞，内分泌細胞，他のβ細胞などいろいろな可能性が考えられますが，化合物を使いながらも生理学的な仕組みを再現するということですね。

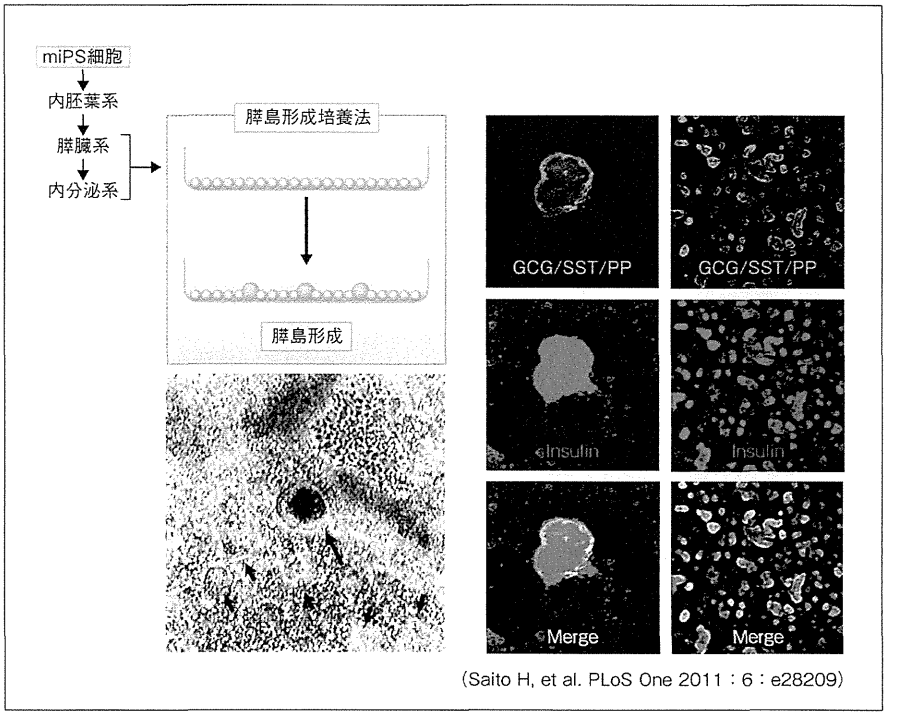
マウスiPS細胞から膵島への分化誘導の研究と今後の展開

門脇　続いて宮島先生に，マウスiPS細胞からの膵島作成についてお話しいただきます。

宮島　ES細胞やiPS細胞からの膵島作製に関するほとんどの論文は，マウスの膵島発生のプロセスを模倣して何段階もの培養をした後，最終的にヒトのインスリン産生細胞が若干できたというものです。しかし，これらの報告ではインスリン産生細胞数が少なく，膵島の構造もできていません。このままでは移植してもすぐには血糖降下作用を発現せず，成熟させるためには生体内の環境に数週間おくことが必要であると考えられます。

そもそも「*in vitro*での膵島構造が作製できるのか」という疑問については，マウス胎児膵臓組織の培養の研究があります。マウス胎児の膵臓組織を分離して培養すると，まず細胞のシートができ，16～18日ほどで膵島様の細胞塊ができてきます。そこから盛り上がった構造を染色すると，インスリン産生細胞の周りにグルカゴン，ソマトスタチン，膵臓ポリペプチドを発現する細胞が存在し，この細胞塊はマウスの膵島によく似ています（図5）。また，STZ誘発高血糖マウスの腎臓の被膜下に移植する場合，マウス胎児から分離した細胞をそのまま移植すると血糖値は上昇し続けますが，培養した細胞を移植すると血糖値が徐々に降

下しました。さらに3ヵ月後，腎臓に膵島様の構造が残っていることが分かりました。すなわち，*in vitro* でも膵島を形成することが可能であることが示されました。そこで，既報のES細胞の膵臓細胞への分化誘導系でマウスiPS細胞を内分泌系まで分化誘導し，細胞を一度分離してマウスの胎児膵臓細胞の培養系に移して培養したところ，こうして得られた細胞では構造的に膵島に似た細胞塊が形成されました。高濃度のグルコースに応答したインスリン分泌が認められました。さらに，これを高血糖誘発マウスの腎臓の被膜下に移植すると血糖値が降下しました。このようにマウスiPS細胞からでも機能的な膵島が作製できる可能性が示されました。次に，マウスiPS細胞と同様の培養系を用いてヒトiPS細胞から膵島様構造物を作製できるかどうかを検証したところ，極めて効率が悪く，グルコース応答性も測定できませんでした[2]。そこで低分子化合物などを加えたり，培養の条件を変更したりすることで，最終的に複数の内分泌細胞で構成される膵島様構造物ができるようになりました。この膵島では，マウス膵島と異なりインスリン産生細胞とグルカゴン産生細胞が混合していました。また，大きさが一定ではなく，膵島の定量は困難でしたが，グルコース濃度に応答してCペプチドの分泌が見られました。これをSTZで高血糖を誘発した免疫不全マウスの腎臓の被膜下に移植すると，迅速に血糖値が降下し，正常化された血糖値が継続しました。



(Saito H, et al. PLoS One 2011：6：e28209)

図5　miPS細胞から膵島3次元構造の形成

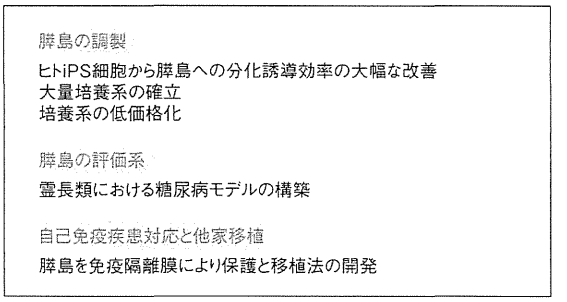


図6　iPS細胞由来の膵島の臨床応用に向けての課題

さて，このヒトiPS細胞から作製した膵島が臨床応用できるのかということが非常に重要です。課題として，①臨床応用に必要な数の膵島をreasonableなコストで調製できるか，②iPS-膵島は安全か，③対象とする患者（1型糖尿病）の免疫学的排除をどう回避するか，ということが挙げられます。こうした課題を解決して臨床応用に向けての研究を進めるために，東京大学は「iPS細胞を基盤とする次世代型膵島移植療法の開発拠点」の拠点機関となりました。免疫制御の問題に関しては，生産技術研究所で免疫隔離ファイバーを研究されている竹内先生と興津先生のグループにも

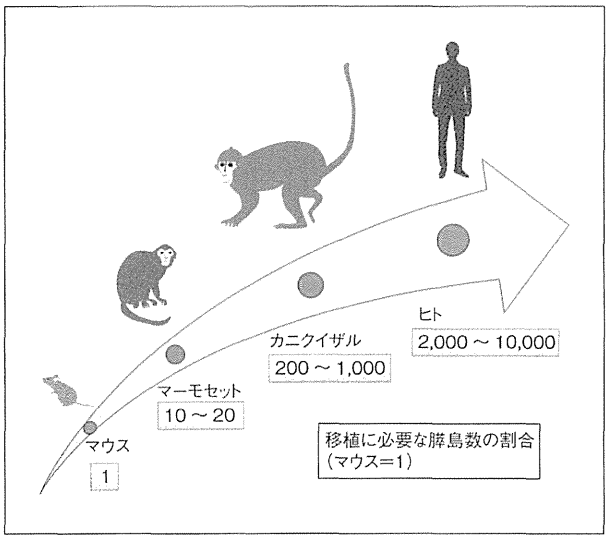


図7　有効性および安全性の検証とスケールアップ

参加していただき，膵島を免疫隔離膜で被って移植する方法を検討中です。この方法が確立されれば，免疫抑制剤を使用せずに，移植膵島の生着性を向上し，かつ安全性を向上させることが可能となります。また，臨床応用に向けて，マーモセット糖尿病モデルでの有効性および安全性の評価を目標に，国際医療研究センターの霜田先生らが実中研の佐々木先生らと検討を始めているところです（図6）。一番問題となる移植に必要な膵島数の確保について，分化誘導系の改良とともに生産技術研究所の酒井先生ら培養工学の力を借りて研究を進めていますが，必要な膵島数はマウスを1とした場合にマーモセットでは10～20倍，カニクイザルでは200～1,000倍，ヒトでは2,000～10,000倍の膵島数が必要になるため，必要数を充足させることはそう簡単ではありません（図7）。さらに，動物成分を含まない培養系の開発や化合物や外分泌細胞などについてもまだまだ課題が多いのが現状です。これらの課題に対する研究を並行して進めることで臨床応用の早期実現を目指しています。

門脇　培養の方法で工夫されたのはどのような点ですか。

宮島　マウスの膵臓組織を分離して細胞を分散させてから培養したことで膵島様の構造ができましたので，おそらく細胞を一度バラバラにして培養し直したことで内分泌細胞が凝集しやすくなったと考えています。このメカニズムはまだはっきりとは解明されていませんが，この培養系のほうが従来の培養系よりも多くの膵島様構造物が得られ，インスリンの分泌能が向上しました。

川口　膵島形成前に細胞シートが自然に盛り上がってくるということでしたが，フラットな細胞はどのような細胞ですか。特に外分泌細胞はありませんでしたか。

宮島　アミラーゼの分泌能しか測定していないので明言はできませんが，おそらく他の細胞だと考えています。外分泌細胞については，下に貼りついた多くの細胞の中に少し見られるという状況だったと思います。

異なるアプローチを融合して同じ目標へ

門脇　それぞれの研究について詳細にお話しいただき，ありがとうございました。

万能細胞を出発点にする可能性という点では先生方のお考えは共通していますが，その後のアプローチが異なるようです。しかし，「糖尿病の治療」という同じ目標を目指しているため参考になることも多いと思いますし，それぞれの先生の構想の中に他の先生の考え方を取り入れることもできるでしょう。これまでお話しいただいた内容を踏まえながら，それぞれのアプローチについて議論したいと思います。

宮島　粂先生はVMAT2阻害剤とdBu-cAMPという2つの非常に素晴らしい化合物を発見されましたが，ヒトへの応用についてはどのようにお考えですか。

粂　ヒトに対してはちょうどアッセイ系を立ち上げたところです。既報のヒトiPS細胞から膵β細胞への分化誘導条件についてもいろいろ試してみましたが，我々が使っているヒトiPS細胞株では適用が困難だったため，新たに条件を検討しました。今後，この点につい

て着手するつもりです。

川口　cAMPに関する研究ですが，今後は成熟β細胞でのグルコース応答性インスリン分泌経路へのcAMPの関与の詳細や，cAMPによる機能獲得が発生期のどの段階で達成されるのかなどについても研究される予定でしょうか。

粂　cAMPだけではなく，いくつかのシグナルがレセプターを介して相互作用することもあり得ると考えており，現在探索しているところです。

川口　今後につながる重要な研究ですね。これは国を挙げて協力していくべきです。

粂　Ptf1aの上位制御システムとして，Ptf1aのpositive regulator（正の制御）として何か心当たりはありますか。その辺についての研究は進められていますか。

川口　これまでに，正の制御の1つは血管からのシグナルだということが分かっています。膵原基形成部位が必ず血管と接していることは古い論文でも示されています。我々のみたHES1ノックアウトでの異所性膵形成部位も血管と近接しています。Ptf1aを正に制御できる低分子化合物を見つけることができれば，遺伝子導入に頼らない膵誘導因子として，再生研究に使えると思います。

宮島　胆管が分化して胆管の一部から膵臓が形成できる点が興味深いです。最近，peribiliary gland（肝内胆管腺）が注目されているようですが，肝内胆管腺でもPtf1aが発現していることを確認されましたか。

川口　肝内胆管腺でPtf1aの発現を詳しく確認したわけではありませんが，肝内胆管腺とPDX-1との関連はあると思っています。その根拠として，PDX-1をノックアウトすると膵芽の発育が途絶し膵臓無形成になるだけではなくて，肝内胆管腺や十二指腸のPAS陽性細胞が完全に欠損することが挙げられます。Ptf1aとPDX-1がお互い転写調節しているというデータも考えると，肝内胆管腺形成にPtf1aが関与する可能性は否定しきれません。

粂　膵島の移植時期として，なぜ14～16日辺りが適しているのでしょうか。

宮島　マウスの発生過程で膵臓組織を採取して培養した時に，膵島形成の直前の細胞がよかったのでそれと関係があるのかもしれません。

今後の課題と展望

門脇　先生方のこれまでの先端的で画期的な研究／アプローチに加えて，臨床応用に向けてあと何段階かの飛躍が必要だと思われます。最後に，今後どのようなブレークスルーや飛躍が求められているのか，また，それぞれの先生が抱いておられる今後の課題や展望についてお聞きしてまとめにしたいと思います。川口先生，いかがでしょうか。

川口　このような研究開発においては，まず最初にゴールをどこに設定するかが重要であり，我々が研究を行っている再生医療の当面の第一目標として，膵島移植に準じた治療効果を目指すことが実際的だと考えています。現状の膵島移植では，移植後の長期成績は臓器移植に比べて格段に劣るにも関わらず，極めて低侵襲であるなどのメリットがあります。つまり，患者のニーズがそこにあるため臨床医療として定着しているのだと思います。膵島移植の治療目標は，低血糖発作の消失です。低血糖で何度も意識を失う患者が，膵島移植をすることによってインスリン離脱が困難でも，基礎分泌があれば，注射ごとのインスリン量が少なくて済むため，低血糖が発症しなくなります。この点こそが，臨床現場で切実に望まれているニーズだと考えます。そのためには，宮島先生がおっしゃったように，移植できる膵島数を確保するということが非常に重要で，大量培養の技術革新というのは確実に必要

だと思います。そしてそれが我々に求められていることだと考えています。

粂 発生に沿って非常に厳密に分化が制御されている部分が多いと考えています。近年はその一端が明らかにされてきていますが，まだまだ分からない部分が多く，今後いかに解明していくかが課題だと思います。発生に沿った制御が完全に明らかになれば，量の問題についても自然に解決できるのではないかと思います。

門脇 発生の生理学的な核心を解明して分化効率を上げることが，量の問題をクリアする近道ではないかということですね。

宮島 私も同様に，量の確保が優先課題だと思います。分化効率を上げるためには原理をきちんと理解することがもちろん非常に重要だと思います。同時に，いかに低コストで大量培養できるかを検討していくべきです。今の培養系を単純に数千倍に上げることは，高額な費用が必要となりあまり現実的ではありません。この問題については，我々だけではなく，安価なサイトカインの供給など国全体としても積極的に関与してほしいと思っています。再生医療に関する研究は様々な研究を集約して国レベルで目標に向けて邁進していく必要があるのではないでしょうか。

門脇 本日は糖尿病における再生医療の最先端におられる先生方のお話をお聞きして，再生医療が差し迫ったニーズを持っていること，そのニーズに応えることを目指して研究をされているという原点がよく分かりました。3人の先生方はそれぞれ違ったアプローチをされているため，それぞれについて議論いただくのが有益だと思っていましたが，まさに先生方の持っている技術や考え方をうまく融合させて組み合わせることによって，この分野での研究や実用化が急速に進むのではないかと非常に強く印象づけられました。3人の先生方には我が国のリーダーとして，協同しながら刺激を与え合ってさらに研究を進めていただきたいと思います。どうもありがとうございました。

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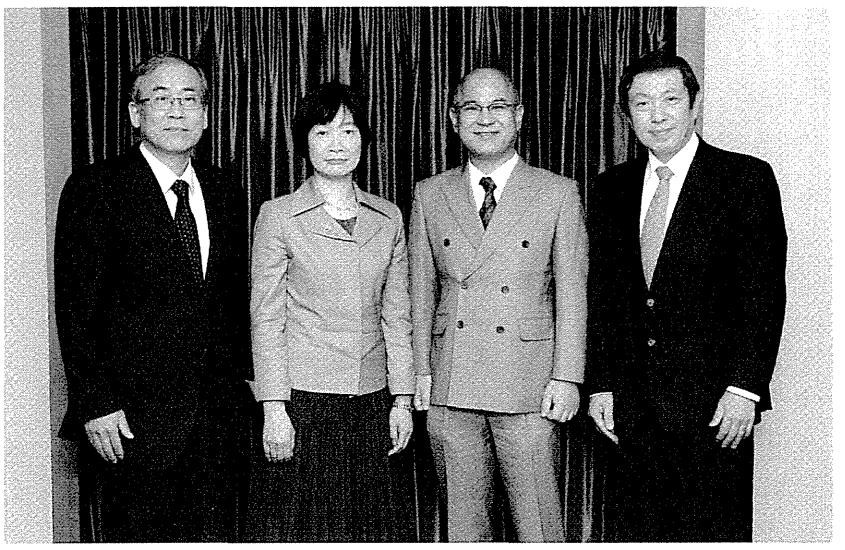

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Deregulation of Pancreas-Specific Oxidoreductin ERO1β in the Pathogenesis of Diabetes Mellitus

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A growing body of evidence has underlined the significance of endoplasmic reticulum (ER) stress in the pathogenesis of diabetes mellitus. ER oxidoreductin 1β (ERO1β) is a pancreas-specific disulfide oxidase that is known to be upregulated in response to ER stress and to promote protein folding in pancreatic β cells. It has recently been demonstrated that ERO1β promotes insulin biogenesis in β cells and thus contributes to physiological glucose homeostasis, though it is unknown if ERO1β is involved in the pathogenesis of diabetes mellitus. Here we show that in diabetic model mice, ERO1β expression is paradoxically decreased in β cells despite the indications of increased ER stress. However, overexpression of ERO1β in β cells led to the upregulation of unfolded protein response genes and markedly enlarged ER lumens, indicating that ERO1β overexpression caused ER stress in the β cells. Insulin contents were decreased in the β cells that overexpressed ERO1β, leading to impaired insulin secretion in response to glucose stimulation. These data indicate the importance of the fine-tuning of the ER redox state, the disturbance of which would compromise the function of β cells in insulin synthesis and thus contribute to the pathogenesis of diabetes mellitus.

Diabetes mellitus has long been a worldwide threat. One of the essential aspects of diabetic pathogenesis is the progressive dysfunction of pancreatic β cells. It is widely believed that during the course of diabetes progression, insulin secretion from β cells gradually declines, eventually leading to hyperglycemia with an insufficient insulin supply to compensate for the increased insulin demand imposed by peripheral insulin resistance (1, 2). This state is called pancreatic β cell failure, the pathophysiology of which has, however, still not been fully elucidated. Endoplasmic reticulum (ER) stress is one of the strong candidates for the mechanisms underlying β cell failure (3, 4), and thus, the molecules and signaling pathways involved in the ER stress response have been intensively investigated as possible therapeutic targets for diabetes mellitus (5–7).

ER stress is known to be induced in response to multiple stimuli, all of which essentially interfere with proper protein folding in the ER. These mechanisms include impairing protein glycosylation, causing malfunctions of chaperones, or compromising oxidized protein folding, and they eventually lead to an accumulation of unfolded proteins (8, 9). Oxidized protein folding, or disulfide bond formation within a nascent polypeptide, is a facilitated process aided by protein disulfide isomerases (PDIs) (10) that is dependent on the highly oxidizing condition of the ER (11). Recently it has been reported that several ER resident proteins play essential roles in maintaining the ER oxidizing condition (12, 13), among which are a family of conserved genes termed ER oxidoreductin 1 (ERO1). ERO1p, the protein encoded by ERO1, couples the oxidizing power of molecular oxygen to generate disulfide bonds, which are eventually transferred from PDIs to client secretory proteins (11). Thus, ERO1 loss-of-function mutants of *Saccharomyces cerevisiae* accumulate reduced misfolded proteins in the ER (14, 15). Previous reports have shown that in *S. cerevisiae*, ERO transcripts are induced upon ER stress in the course of the unfolded-protein response (UPR), establishing that EROs are members of the UPR gene family (14, 15). In contrast, mammals have two isoforms of ERO, ERO1α and ERO1β, which have distinct functions with different tissue distributions (16). Importantly, only ERO1β transcripts are induced upon ER stress (16), whereas the regulation of ERO1α expression seems to be associated with hypoxia (17, 18). Furthermore, ERO1β transcripts are abundant in the pancreas (16), with preferentially higher expression in the islets than in the exocrine cells (19). Together with the facts that β cells are highly professionalized cells for insulin synthesis, with proinsulin accounting for up to 50% of the total protein (20, 21), and that the folding of proinsulin requires three intrachain disulfide bond formations (4, 22), it has been speculated that ERO1β would play significant roles in the physiological function of pancreatic β cells and not any less in the pathogenesis of diabetes mellitus.

Recently, Zito et al. have reported that whole-body deletion of ERO1β specifically affects pancreatic β cells, compromising the oxidative folding of insulin and thus leading to glucose intolerance in mice (23). Another report has demonstrated that suppressed ERO1β expression in pancreatic β cells leads to an increased susceptibility to ER stress and a reduction of insulin content (24). While these data clearly indicate that ERO1β plays an important role in insulin biogenesis in β cells and contributes to physiological glucose homeostasis, it is as yet unclear how the

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expression or function of ERO1β is changed during pancreatic β cell failure and what its precise roles in the pathogenesis of diabetes are. Here we report that, unlike the expression of other UPR genes, that of ERO1β transcripts in the islets paradoxically declines during the course of diabetes progression despite increased ER stress. However, mice overexpressing human ERO1β specifically in pancreatic β cells showed impaired glucose tolerance due to reduced insulin secretion. In β cells overexpressing ERO1β, the expression of UPR genes was upregulated and the ER lumens were markedly enlarged, indicating that ERO1β overexpression caused ER stress in the β cells.

MATERIALS AND METHODS

Animals. BKS.Cg-$m^{+/+}$ $Lepr^{db}$/J (*db/db*) mice and control *misty/misty* mice were purchased from Japan CLEA. Akita mice were purchased from Japan SLC. For the generation of hERO1βTg mice, a fusion gene was designed that comprised the rat insulin promoter and human ERO1LB cDNA coding sequences with a Flag tag at its C terminus so that its expression was targeted to β cells. The linearized construct was microinjected into the pronuclei of fertilized C57BL/6 mouse (Japan CLEA) eggs. Transgenic founder mice were identified by PCR analysis by using a primer for the Flag sequence, which was also used to determine the tissue distribution of the transgene by PCR after reverse transcription (RT). All experiments were conducted with heterozygote male mice. High-fat diet (HFD) feeding was started at 7 weeks of age where required. The Animal Care Committee of the University of Tokyo approved the animal care conditions and experimental procedures used.

Quantitative real-time PCR. Total RNA was prepared with the RNeasy kit (Qiagen). RT reagents (Applied Biosystems) were used to prepare cDNA. Quantitative real-time PCR was performed with ABI Prism and PCR Master Mix reagent (Applied Biosystems). The sequences of the primers and probe used for the simultaneous detection of human *ERO1B* and mouse *Ero1b* were as follows: forward primer, TGGAGTTCTGGAT GATTGCTT; reverse primer, TCTTCTGCCCAGAAAGGACA; probe, CGTTATTACAAGGTTAATCTGAA. All of the other primers and probes used were purchased from Applied Biosystems. The levels of mRNAs were normalized to that of cyclophilin (25).

Immunoblotting. Immunoblotting was conducted as previously described (25). The antibodies used for immunoblotting were anti-phospho-PERK antibody (Thr980; Cell Signaling Technology); anti-phospho-eukaryotic transcription initiation factor 2 alpha subunit (anti-phospho-eIF2α) antibody (Ser51; Cell Signaling Technology); anti-4E-BP1 antibody (Cell Signaling), and anti-β-actin antibody (Sigma-Aldrich).

Metabolic assays. A glucose tolerance test (GTT) was performed as described previously (26). The mice were fasted for 16 h, and blood samples were obtained at the indicated time points after the intraperitoneal injection of 1 g/kg body weight of D-glucose (WAKO). Blood glucose levels were checked at indicated time points (Glutest Pro; Sanwa Kagaku Kenkyusho).

Immunohistochemical and morphometric analyses of the pancreas. Immunohistochemical and morphometric analyses of pancreas sections were performed as described earlier (27) with a slight modification. Six mice under each condition at 11, 22, and 36 weeks of age were subjected to morphometric analysis. Sections were stained with antibodies as indicated. For morphometric analysis, the images of islets were traced manually and analyzed by ImageJ software (NIH). The mean of four different sections of each pancreas was used for the analysis.

Islet isolation. Islets were isolated by Liberase RI (Roche) with pancreatic perfusion and subsequent digestion for 24 min at 37°C (28). Islets were picked manually in Hanks' balanced salt solution (Sigma) buffer supplemented with 10% fetal calf serum and 25 mM HEPES buffer and then immediately used for further experiments, except for the pulse-chase analysis, where the islets were subjected to the experiments after overnight incubation in RPMI 1640 medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO).

Glucose-stimulated insulin secretion (GSIS) assay. Freshly isolated islets were maintained in Krebs-Ringer bicarbonate (KRB) buffer (129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES [adjusted to pH 7.4]) containing 0.2% bovine serum albumin supplemented with 2.8 mM glucose for 30 min at 37°C. The islets were then incubated for 30 min in the same buffer containing 22.4 mM glucose or 50 mM KCl as indicated. For the MIN6 β cell experiment, cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO). The cells were incubated with KRB buffer with 2.8 mM glucose for 60 min at 37°C, and then the medium was changed to KRB buffer with 22.4 mM glucose for further incubation for 30 min. For measurement of insulin content, insulin was extracted from islets or cultured cells by overnight incubation with acid ethanol at −20°C. Insulin concentrations were measured with an insulin radioimmunoassay kit (Institute of Isotopes) according to the manufacturer's instructions.

Electron microscopy. Two mice of each genotype were anesthetized and subjected to cardiac perfusion with 0.1 M sodium phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 2% paraformaldehyde. The pancreas was excised from each mouse, cut into small pieces, and immersed overnight in the same fixative. The tissue was then exposed to 2% osmium tetroxide, stained with 2% uranyl acetate, dehydrated with ethanol, and embedded in Epon812 (TAAB). Thin sections were stained with uranyl acetate and lead citrate before examination with a Hitachi 7100 electron microscope (Hitachi). The quantification of ER luminal areas was done by a previously described method (29) in which 22 to 34 pictures were taken per animal and then by using a double-lattice test system with a spacing of 1 cm, the points that fell on the ER lumen were counted. The ratio of the points falling on the ER lumen to the points falling in the entire 20-by-20 double lattice was recorded as the ER luminal area percentage.

Detection of superoxide. Superoxide was detected in frozen pancreas sections with dihydroethidium (DHE; 10 μmol/liter) in phosphate-buffered saline (PBS) for 30 min at 37°C in a humidified chamber protected from light. DNA-bound ethidium bromide, which was formed from DHE on reaction with superoxide, was detected as red fluorescence (30).

Generation and infection of adenoviruses. Adenovirus encoding human ERO1β was generated according to the manufacturer's protocol (TaKaRa Biotechnology) by using the same construct as that used to generate hERO1βTg mice. An adenovirus encoding LacZ was purchased from TaKaRa Biotechnology and used as the negative control. Prior to use, all adenoviruses were purified on a cesium chloride gradient and dialyzed into PBS plus 10% glycerol. MIN6 β cells were infected with the adenoviruses at a multiplicity of infection (MOI or number of viral particles per cell) of 3,000 PFU/cell. The cells were subjected to experiments 48 h after adenovirus infection.

Pulse-chase analysis. A total of 65 islets were preincubated in 500 μl of methionine- and cysteine-free RPMI 1640 medium (GIBCO) for 1 h and then labeled in the same medium containing [^{35}S]methionine-cysteine (EXPRE^{35}S^{35}S protein labeling mix; PerkinElmer) at a concentration of 10 μCi/ml for 30 min. When necessary, a subsequent radiolabel-free chase was performed with complete RPMI medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO) after the islets were washed twice with the same medium, and islets were frozen in liquid nitrogen at the indicated times. The lysates were immunoprecipitated with anti-insulin antibody (ab8304 insulin plus proinsulin antibody; Abcam). Immunoprecipitated proteins were resolved by Tricine-SDS-PAGE with 15% polyacrylamide gel and detected by autoradiography with a phosphorimager (Typhoon FLA 7000; GE Healthcare).

Statistical analysis. Statistical analysis was performed by using the paired two-sample *t* test for means. Analysis of variance (ANOVA) and Tukey's *post hoc* analyses were used when more than two groups were compared. Repeated-measures ANOVA was used for analyzing the results of metabolic assays. Statistical significance was accepted at P values <0.05.

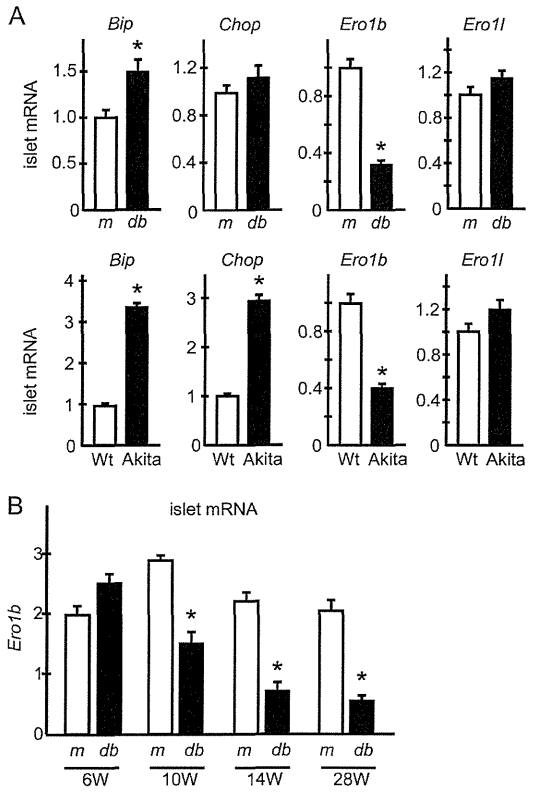

FIG 1 *Ero1b* expression in the islets of diabetic model mice. (A) *Ero1b* expression in the islets of *db/db* and Akita mice. Pancreatic islets were isolated from *db/db* (*db*) and *misty/misty* (*m*) mice at 14 weeks of age or from Akita and control C57BL/6 mice at 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of the genes indicated. $n = 4$ to 6; *, $P < 0.05$. (B) Pancreatic islets were isolated from *db/db* (*db*) or *misty/misty* (*m*) mice at the indicted weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of *Ero1b* expression. $n = 4$ to 6; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

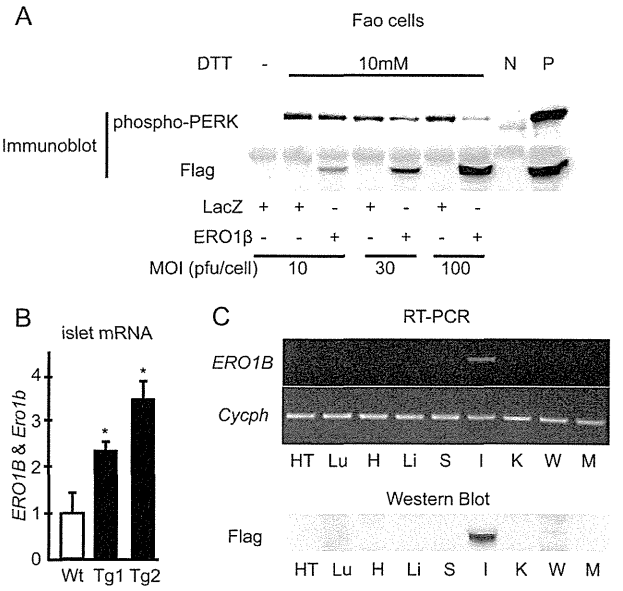

FIG 2 Human *ERO1B* overexpression in Fao cells and hERO1βTg mouse islets. (A) Adenoviral overexpression of human *ERO1B* in Fao cells. Fao cells were infected with adenovirus encoding human ERO1β or the control LacZ at the indicated MOIs. The cells were incubated with 10 mM DTT for 0.5 h. Total cell lysates were prepared and subjected to immunoblotting with anti-phospho-PERK or anti-Flag antibody. N, negative control; P, positive input. (B) *Ero1b* and *ERO1B* expression in the islets of hERO1βTg mice. Islets were isolated from hERO1βTg (Tg1 and Tg2) or control Wt mice at 14 weeks of age. Total mRNA was extracted from the islets and subjected to RT-PCR analysis, which detects mouse *Ero1b* and human *ERO1B*, as described in Materials and Methods. $n = 6$ to 9; *, $P < 0.05$. The data shown are means ± the standard errors of the means. (C) Distribution of the transgene *ERO1B* in the tissues of hERO1βTg mice. Each tissue type was removed from hERO1βTg mice at 9 weeks of age. Total mRNA was extracted from the tissues, 0.2 μg of which was subjected to RT and subsequent PCR analysis amplifying Flag-tagged human *ERO1B* cDNA or cyclophilin (upper panel). Lysate of protein from each tissue type was prepared and subjected to immunoblotting with Flag antibody at 10 μg/lane (lower panel). Tissue types: HT, hypothalamus; Lu, lung; H, heart; Li, liver; S, spleen; I, islet; K, kidney; W, epididymal white adipose tissue; M, skeletal muscle.

RESULTS

ERO1β expression was decreased in the islets of diabetic model mice despite evidence of ER stress. To investigate the roles of ERO1β in the pathogenesis of diabetes, we first examined ERO1β mRNA expression in the islets of diabetic *db/db* mice. As widely accepted, the expression of UPR genes such as *Bip* and *Chop* tended to be upregulated in *db/db* islets, most likely reflecting the increased ER stress in the β cells. In contrast, the expression of *Ero1b* was paradoxically lower than that in control *misty/misty* mouse islets (Fig. 1A, upper panels). The expression levels of *Ero1l*, which encodes the other isoform of ERO1 protein, ERO1α, were relatively similar in *db/db* and control *misty/misty* mouse islets (Fig. 1A, upper panels). We next investigated the islets of Akita mice as another diabetic model mouse that harbors a C96Y mutation in the insulin-2 gene resulting in misfolded proinsulin accumulation and progressive β cell loss due to ER stress-induced apoptosis (7, 31). Again, the expression of *Ero1b* was paradoxically decreased despite the robust upregulation of other typical UPR genes (Fig. 1A, lower panels). Moreover, the reduction of *Ero1b* in *db/db* islets occurred in an age-dependent manner, which was consistent with the time course of diabetes progression, with its expression being maintained, or tending to be higher, at early ages (Fig. 1B). These results highlight the special nature of ERO1β among UPR genes, namely, its lack of any upregulation under ER-stressed conditions. These data prompted us to hypothesize that ERO1β overexpression in β cells would benefit the β cells and rescue the glucose intolerance seen under pathological conditions such as those experienced during HFD feeding.

First we overexpressed Flag-tagged human ERO1β with adenovirus in Fao rat hepatoma cells. The Flag tag was added at the C terminus of the construct so that the tag would not interfere with the signal sequence at the N terminus of ERO1β (32). Overexpression of ERO1β in Fao cells ameliorated the dithiothreitol (DTT)-induced UPR response in a dose-dependent manner, as revealed by reduced pancreatic ER kinase (PERK) phosphorylation during DTT treatment (Fig. 2A), suggesting not only that human ERO1β was functionally valid as a redox regulator in rodent cells but also that ERO1β overexpression could counteract the reducing effects of DTT in Fao cells. Thus, we created a mouse line overexpressing Flag-tagged human ERO1β specifically in β cells under the control of a rat insulin promoter (hERO1βTg mice). We obtained two

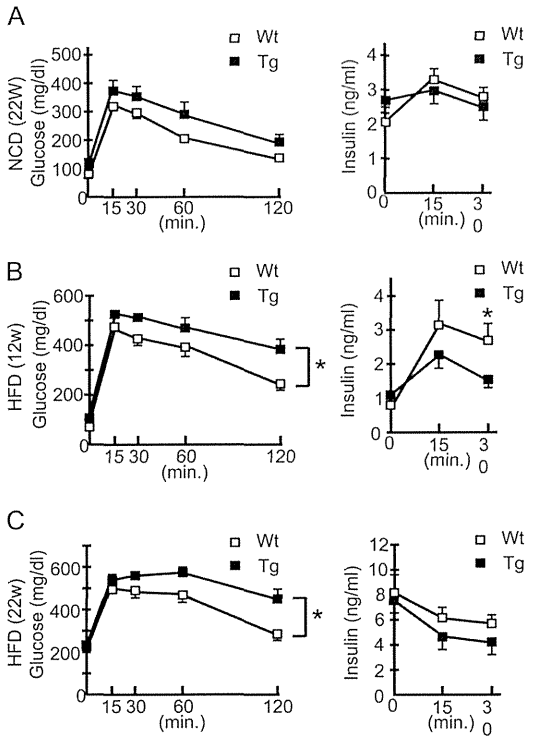

FIG 3 Metabolic phenotypes of hERO1βTg mice. Shown are blood glucose levels (right panels) and serum insulin concentrations (left panels) after the intraperitoneal injection of glucose. hERO1βTg (Tg) or Wt control mice were intraperitoneally injected with glucose at 1 g/kg body weight during NCD feeding at 22 weeks of age (A), during HFD feeding at 12 weeks of age (B), or during HFD feeding at 22 weeks of age (C). Blood samples were collected at the indicated time points and subjected to glucose and insulin measurements. $n = 10$; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

lines (Tg1 and Tg2) with different but similar levels of overexpression of the *ERO1B* gene, the mRNA expression levels of which were quantified by an RT-PCR analysis designed to amplify the mRNA region common to human *ERO1B* and mouse *Ero1b* (Fig. 2B). In the following experiments we essentially used the Tg1 line (referred to as Tg in this report) to characterize our overexpression model, while key experiments were also repeated with the Tg2 line. The expression of human ERO1β in these Tg mice, which was determined by measuring Flag expression, was detected specifically in pancreatic islets (Fig. 2C). These mice were born normally and showed no obvious abnormalities in their appearance.

hERO1βTg mice showed impaired glucose tolerance with reduced insulin secretion. To explore the effects of ERO1β overexpression in β cells under physiological as well as pathological conditions, we fed hERO1βTg mice with a normal chow diet (NCD) or an HFD and examined their metabolic phenotypes. hERO1βTg mice showed a body weight gain similar to that of control wild-type (Wt) mice during both NCD and HFD feeding (data not shown). Unexpectedly, hERO1βTg mice showed impaired glucose tolerance in the GTT compared to findings for the control Wt mice, with a statistically significant difference only under the HFD feeding condition (Fig. 3A to C, left panels). The exacerbated glucose intolerance seen in the HFD-fed hERO1βTg mice was due to their lower insulin secretion than that of the Wt control mice (Fig. 3B and C, right panels). No difference in insulin sensitivity between hERO1βTg and Wt control mice was detectable in an insulin tolerance test (data not shown). To confirm that ERO1β overexpression does not benefit β cells, we also created hERO1βTg mice with a *db/db* background (Wt/*Lepr*^{db} or Tg/*Lepr*^{db} mice) by crossing Tg2 line mice with C57BLKS-*Lepr*^{db} heterozygotes. We then tested their glucose tolerance with a GTT, in which we observed no improvement or worsening of the glucose levels in Tg/*Lepr*^{db} mice compared with those of Wt/*Lepr*^{db} mice, where the blood glucose level had already reached >500 mg/dl after a half-dose glucose challenge (data not shown).

hERO1βTg islets showed an impaired GSIS response with reduced insulin content. To explore the mechanisms whereby ERO1β overexpression led to reduced insulin secretion in glucose challenge tests during HFD feeding, we first examined the morphology of hERO1βTg islets by light microscopy. Microscopic analyses of hERO1βTg mouse islets showed no morphological changes detectable by insulin and glucagon staining (Fig. 4A, upper left panels). Insulin staining showed that the insulin-positive areas of hERO1βTg and control mouse islets were similar under both of the feeding conditions at 12 and 22 weeks, the time points when the glucose intolerance phenotype was already observed in hERO1βTg mice, whereas there was a nonsignificant reduction of the insulin-positive areas of HFD-fed hERO1βTg mouse islets only at 36 weeks of age (Fig. 4A, upper right and lower panels). Single-stranded DNA (ssDNA) staining and proliferating cell nuclear antigen (PCNA) staining showed no evidence of accelerated apoptosis or cell proliferation in the islets under any of the conditions (Fig. 4B). These data indicated that the exacerbated glucose intolerance in hERO1βTg mice was not associated with β cell mass reduction.

We next investigated the GSIS response of islets isolated from HFD-fed hERO1βTg mice. Islets isolated from HFD-fed hERO1βTg mice showed a weaker GSIS response than those from HFD-fed control mice, and the difference was more pronounced and reached statistical significance after longer HFD feeding (Fig. 5A and B, left panels). The weaker GSIS responses in Tg islets were due to reduced insulin contents in the islets (Fig. 5A and B, right panels), as insulin secretion did not differ between hERO1βTg and control Wt islets when normalized to their insulin contents (Fig. 5A and B, middle panels). The analyses of mRNA expression in Tg islets revealed a marginal reduction in *Ins1* and *Ins2* expression by about 15%, the degree of which was, however, relatively small compared to the reduction in the insulin contents of Tg islets (Fig. 5C). Collectively, these data suggested that HFD-fed hERO1βTg mice showed exacerbated glucose intolerance, which was attributed to the reduced islet insulin contents with the possible involvement of posttranscriptional mechanisms.

ERO1β overexpression caused ER stress in pancreatic β cells. To further characterize the phenotypes of ERO1β-overexpressing β cells, we investigated the morphology of hERO1βTg β cells in detail with an electron microscope. Electron microscopic analyses revealed severely enlarged ER lumens in the β cells of hERO1βTg mice (Fig. 6A and B), showing a sharp contrast to the scarce changes observed in the light microscopic analyses. The ER dilation of hERO1βTg β cells was already observed under NCD-fed conditions, the magnitude of which did not change further under HFD-fed conditions (data not shown). No apparent changes were detected in the organelles other than the ER, such as the Golgi apparatus or insulin-containing granules, with regard to

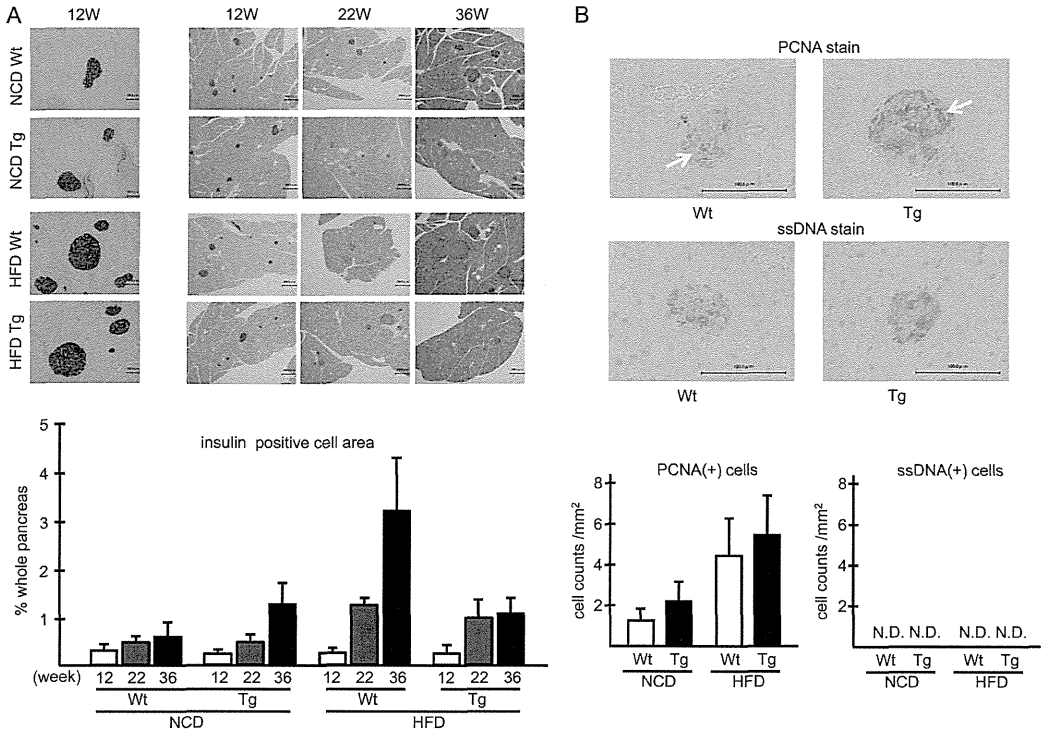

FIG 4 Histological analysis of islets of hERO1βTg mice. (A) Representative images of islets of hERO1βTg mice. Pancreas sections of hERO1βTg (Tg) or Wt control mice under NCD or HFD feeding conditions at 12 weeks of age were stained with insulin (red) or glucagon (dark red) antibody (left panels). Pancreas sections of the mice at the indicated weeks (W) of age were stained with insulin antibody (brown) (right side), and β cell areas determined as insulin-positive areas by staining were quantified (bottom). The occupancy of pancreatic β cells in the whole pancreas was determined as described in Materials and Methods. $n = 6$. Bars, 100.0 μm (left column) and 300.0 μm (right three columns). (B) Cell proliferation and apoptosis markers in hERO1β islets. Pancreas sections of hERO1βTg (Tg) or Wt control mice under NCD or HFD feeding conditions at 12 weeks of age were stained with insulin (brown) and PCNA or ssDNA (dark purple) antibody (representative images are shown at the top). The arrows indicate PCNA-positive cells. The PCNA- or ssDNA-positive cells were counted. Bars, 100.0 μm. Cell counts normalized to the insulin-positive area are shown at the bottom. $n = 6$. The data shown are means ± the standard errors of the means. N.D., not detected.

their numbers or their morphology. As previous reports have indicated, cells under ER stress or with compromised ER homeostasis often show ER enlargement (31, 33, 34), suggesting that the β cells of hERO1βTg mice were also subjected to ER stress. In fact, the analyses of mRNA expression showed upregulation of the expression of multiple UPR genes in Tg islets, including *Bip*, *Chop*, *Derl3*, and *Trb3* (Fig. 7A). Upregulation of UPR genes was again observed in the islets of NCD-fed hERO1βTg mice, the degree of which tended to be higher in the islets of HFD-fed Tg mice. These data collectively indicated that ERO1β overexpression caused ER stress in β cells. Interestingly, however, phosphorylation of PERK and the α subunit of eukaryotic transcription initiation factor 2 (eIF2) or upregulation of 4E binding protein 1 (4E-BP1) was not evident in hERO1βTg islets (Fig. 7B).

Next we investigated whether reactive oxygen species (ROS) could contribute to the β cell dysfunction of hERO1βTg mice. As previously described, in the relay of oxidative equivalents among EROs, PDIs, and client proteins during oxidative protein folding, the final acceptor of the electron is molecular oxygen; thus, ERO-mediated oxidative protein folding could lead to ROS production (35). However, we did not observe any evidence of ROS accumulation in hERO1βTg islets, as revealed by DHE staining of hERO1βTg islets (Fig. 7C). In addition, the mRNA expression of genes involved in the antioxidant pathway, such as *Sod1*, *Sod2*, and *Cat*, was unaltered in hERO1βTg islets compared to that in control Wt islets (Fig. 7D). These results suggested the absence of ROS overproduction in hERO1βTg islets.

ERO1β overexpression caused impaired insulin secretion with ER stress in MIN6 cells. To investigate whether ERO1β overexpression in cultured cells could lead to phenotypes similar to those in islets, we next overexpressed human ERO1β with adenovirus in MIN6 β cells. The amount of insulin secreted under the high-glucose condition was significantly lower in ERO1β-overexpressing MIN6 cells, while the insulin secretion ratio, normalized to the insulin content, did not decrease with ERO1β overexpression (Fig. 8A). These results indicated that the reduced insulin secretion under ERO1β overexpression was due to reduced insulin contents in MIN6 β cells, essentially mimicking the phenotypes of hERO1βTg islets. The mRNA analyses showed that ERO1β overexpression caused UPR gene upregulation (Fig. 8B), while mRNA expression of antioxidant pathway genes such as *Sod1*, *Sod2*, and *Cat* was unaltered (data not shown), suggesting that ERO1β overexpression led to ER stress in MIN6 cells without collateral ROS overproduction, again showing characteristics similar to those in hERO1βTg islets. Importantly, mild DTT treatment paradoxically led to attenuation of UPR gene upregulation (Fig. 8B, gray bars), which was associated with restored insulin contents under ERO1β overexpression (Fig. 8C). These data sug-

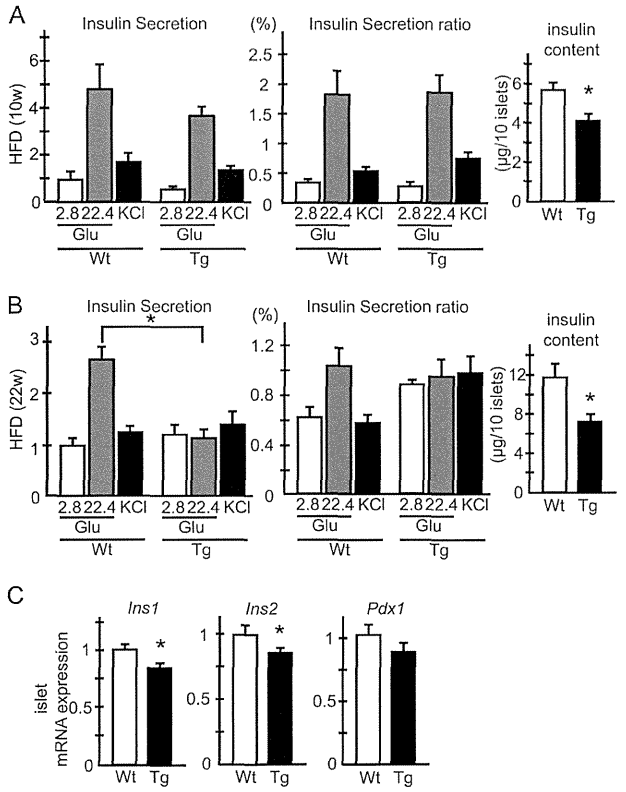

FIG 5 Static-incubation study of islets from hERO1βTg mice. (A and B) GSIS of hERO1βTg islets. Islets were freshly isolated from 10-week-old (A) or 22-week-old (B) hERO1βTg (Tg) or control Wt mice fed an HFD from 7 weeks of age. The islets were incubated for 30 min in KRB buffer containing 2.8 mM glucose (Glu), 22.4 mM glucose, or 50 mM KCl, respectively, and the media were collected. The insulin concentrations in the incubation media were measured with an insulin radioimmunoassay kit. Insulin secretion is displayed as a ratio normalized to the basal secretion of Wt mice (left panels). Insulin secretion was determined as the ratio of secreted insulin to the insulin content of the islets (middle panels). $n = 6$ to 18; 6 islets for each condition. For measurements of the insulin contents of islets, insulin was extracted from the islets by overnight incubation with acid ethanol and measured with an insulin radioimmunoassay kit (right panels). $n = 18$ to 54; 6 islets for each condition; *, $P < 0.05$. (C) mRNA expression of insulin-related genes in hERO1βTg islets. Pancreatic islets were isolated from 10-week-old Tg or control Wt mice fed an HFD from 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of *Ins1*, *Ins2*, and *Pdx1* mRNA expression. $n = 6$ to 8; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

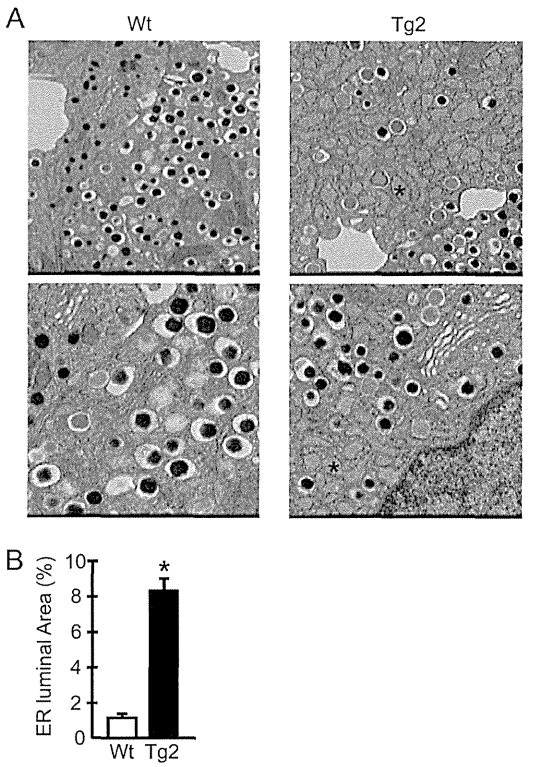

FIG 6 Electron microscopic analysis of pancreatic β cells of hERO1βTg mice. Representative electron micrographs (A) and quantifications of ER luminal areas (B) of pancreatic β cells of hERO1βTg (Tg2) or Wt control mice during NCD feeding at 12 weeks of age are shown. The asterisks indicate the markedly dilated ER lumens. ER lumen areas were quantified as described in Materials and Methods.

gested the possibility that ERO1β overexpression caused ER stress by shifting ER redox states toward overly oxidizing conditions, which was countersuppressed by the reducing effect of the mild DTT treatment.

Insulin maturation was not compromised in hERO1βTg β cells. How did the overexpression of ERO1β cause ER stress in β cells? Generally, ER stress can result from an accumulation of misfolded proteins, which is due to either accelerated misfolding of client proteins or impaired removal of irreparably misfolded proteins from the ER lumens by a mechanism called ER-associated degradation (ERAD). Recent studies have pointed out that the reduction of protein disulfides is required for the dislocation and degradation of misfolded proteins targeted for ERAD (36, 37). To directly address these issues, we conducted a pulse-chase analysis with hERO1βTg islets and investigated proinsulin processing and insulin maturation with an antibody detecting proinsulin and insulin with equal efficiency. The pulse-chase analyses showed that there was no delay in the appearance of processed insulin, which was reflected in the band shift downward (Fig. 9A, upper panel), suggesting that the proinsulin maturation with the C-peptide cleavage occurred in hERO1βTg β cells as smoothly as that in the control Wt β cells. Additionally, no delay in the disappearance of proinsulin was observed, as reflected in the similarly remaining upper bands in Tg and Wt islets until the end of the chase period. The decrease in insulin content in the islets of hERO1βTg mice was confirmed in the proinsulin immunoblot assay, as detected by anti-C-peptide immunoblotting of the same membrane (Fig. 9A, lower panel). In fact, the amount of newly synthesized proinsulin, which was investigated by collecting islets just after 30 min of metabolic "pulse" labeling, was lower in the islets of hERO1βTg mice than in control Wt mouse islets (Fig. 9B). These data collectively suggested the possibility that the decrease in insulin contents in the islets of hERO1βTg mice could be accounted for by reduced protein synthesis, while the conversion of proinsulin to insulin occurred normally in the hERO1βTg β cells, and that the misfolded proinsulin, if it existed, could be removed from the ER with similar efficiency in Tg β cells compared to its clearance from control Wt β cells.

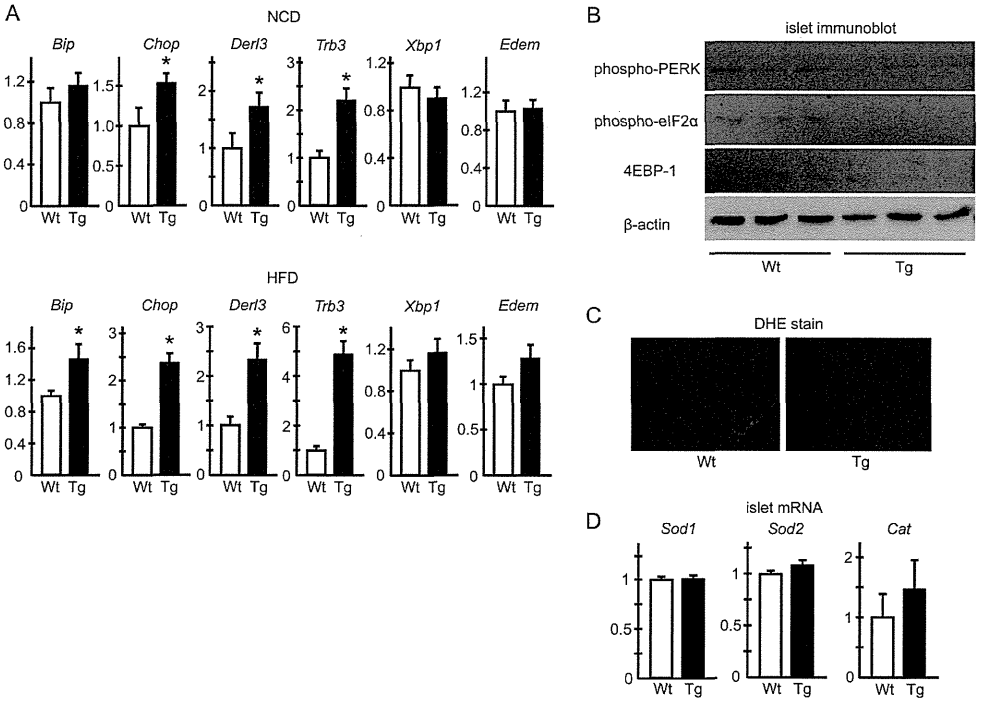

FIG 7 ER stress and oxidative stress markers in hERO1βTg islets. (A) UPR gene mRNA expression in hERO1βTg islets. Pancreatic islets were isolated from 12-week-old hERO1βTg (Tg) or control Wt mice fed either an NCD or an HFD from 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of the genes as indicated. $n = 7$ or 8; *, $P < 0.05$. (B) UPR signaling pathways in hERO1β islets. Pancreatic islets were freshly isolated from 12-week-old hERO1βTg (Tg) or control Wt mice fed an NCD. Total cell lysates were prepared from the islets, and the same amounts of protein were loaded and subjected to immunoblotting with anti-phospho-PERK, anti-phospho-eIF2α, or anti-4E-BP1 antibody. The same membrane was reblotted with anti-β-actin antibody. Representative images of immunoblotting of hERO1β islets are shown. (C and D) Oxidative stress in hERO1β islets. (C) Representative images of DHE staining of islets of hERO1βTg mice. Pancreas sections of hERO1βTg (Tg) or Wt control mice during NCD feeding at 12 weeks of age were stained with DHE. (D) Antioxidant pathway gene mRNA expression in hERO1βTg islets. Pancreatic islets were isolated from 12-week-old hERO1βTg (Tg) or control Wt mice fed an HFD from 7 weeks of age. Total mRNA was extracted and subjected to RT-PCR analysis of *Sod1*, *Sod2*, and *Cat* mRNA expression ($n = 6$ to 8). The data shown are means ± the standard errors of the means.

DISCUSSION

Here we report for the first time the phenotypes of mice with ERO1β overexpression specifically in pancreatic β cells. While it has been well documented that EROs play critical roles in ER protein folding or in ER homeostasis (12, 14, 15), their roles in the pathogenesis of diseases such as diabetes mellitus have remained obscure, with only one report showing that the disruption of ERO1β expression compromised oxidative folding of insulin and thus led to glucose intolerance in mice (23).

In the first place, we observed a special feature of ERO1β among other UPR genes, which showed a paradoxical decrease in its expression in the islets of *db/db* and Akita mice despite the evidence of increased ER stress. Considering that the β cell mass itself is decreased in these model mice and that ERO1β is specifically expressed in β cells, it would be reasonable to assume that the observed reductions in ERO1β expression could be partly accounted for by the reduction in β cell mass itself. Nevertheless, the reduction of ERO1β showed a striking contrast to findings for other UPR genes like *Bip*, the mRNA upregulation of which in response to ER stress is due to its induction exclusively within β cells (31), indicating that there occurred either a reduction or, more precisely, an inadequate upregulation of ERO1β expression in the stressed β cells in these models.

EROs are essentially double-bladed molecules; they are necessary proteins for the cells to facilitate disulfide protein folding but at the same time could be toxic to the cells by imposing oxidative stress, as EROs produce ROS as by-products when they couple the oxidizing power to molecular oxygen during disulfide bond formation (11). In *Saccharomyces cerevisiae*, cell death under ER stress is attributed partly to ROS production resulting from ERO1 upregulation (35), while $Perk^{-/-}$ cells, in which protein synthesis is not properly attenuated under ER stress, accumulate ROS, leading to apoptosis (33), which is ameliorated by ERO1 abrogation (38).

Interestingly, ERO1β overexpression did not lead to ROS accumulation in the β cells in our model, nor was upregulation of antioxidant pathways observed. In contrast, we observed evidence of severe ER stress induced by ERO1β overexpression. However, despite the upregulation of proapoptotic genes such as *Chop* or *Trb3*, as well as the severe dilation of the ER lumen of hERO1βTg β cells, which is generally regarded as indicative of unfolded protein accumulation and ER stress (31, 33, 34), hERO1βTg islets did not show evidence of ER stress-induced β cell death; thus, the glucose intolerance of hERO1βTg mice was mild and became obvious only after an HFD load. This lack of apoptosis could simply be explained as a consequence of successful compensations achieved through the strongly invoked UPRs, possibly via the downregulation of insulin synthesis, leading to a sort of balanced and maintained status within the ER.

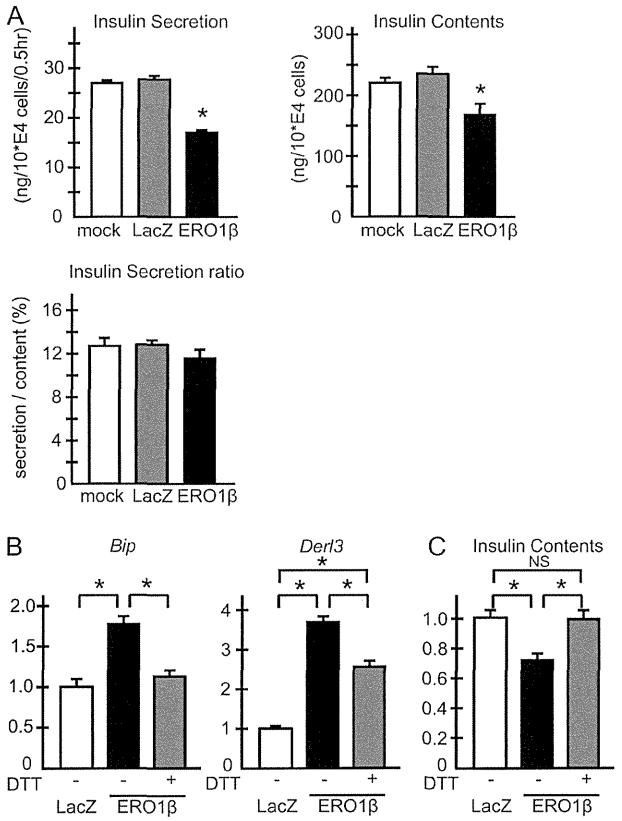

FIG 8 Adenoviral overexpression of *ERO1B* in MIN6 cells. (A) Insulin secretion rate of MIN6 cells with human *ERO1B* overexpression under high-glucose conditions. MIN6 cells infected with adenovirus as indicated were incubated with KRB buffer with 2.8 mM glucose for 60 min at 37°C, and then the medium was changed to KRB buffer with 22.4 mM glucose for a further 30 min of incubation. The medium was then subjected to insulin concentration measurement (left upper panel). Insulin was extracted from the cells by overnight incubation with acid ethanol at −20°C for the measurement of insulin content (right upper panel). Insulin concentrations were measured with an insulin radioimmunoassay kit. Insulin secretion was determined as the ratio of secreted insulin to the insulin content of the cells (lower panel). Representative results of two independent experiments are shown. $n = 4$; *, $P < 0.05$. (B) UPR gene expression of MIN6 cells with human *ERO1B* overexpression. MIN6 cells infected with the adenovirus indicated were incubated for 4 h with or without 0.5 mM DTT. Total mRNA was extracted from the cells and subjected to RT-PCR analysis of *Bip* and *Derl3*. $n = 4$; *, $P < 0.05$. (C) Restored insulin contents via mild DTT treatment under *ERO1B* overexpression. MIN6 cells infected with adenovirus as indicated were incubated for 12 h with or without 0.1 mM DTT. Insulin was extracted from the cells by overnight incubation with acid ethanol at −20°C and subjected to insulin measurement. $n = 9$; *, $P < 0.05$. The data shown are means ± the standard errors of the means.

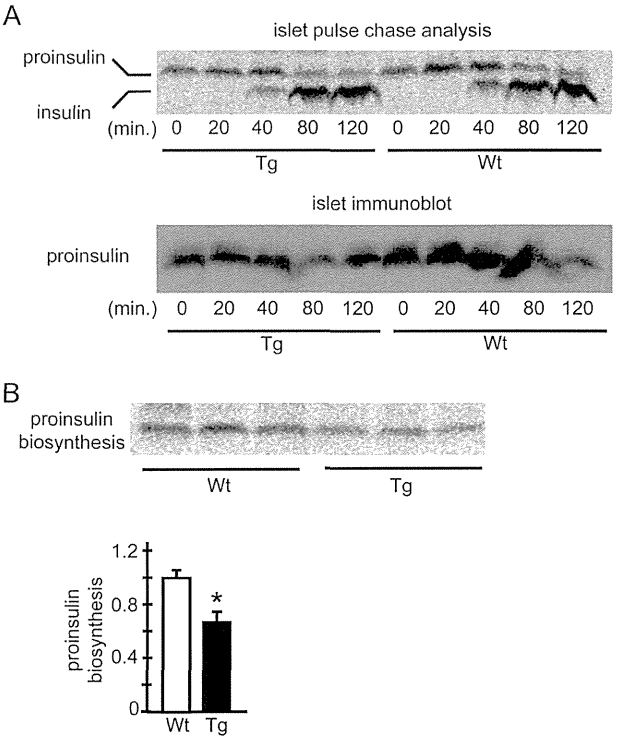

FIG 9 Analysis of insulin synthesis in hERO1βTg islets by pulse-chase experiments. Pancreatic islets were isolated from 12-week-old hERO1βTg (Tg) or control Wt mice. On the day after isolation, the islets were subjected to pulse-labeling with [^{35}S]methionine-cysteine for 30 min. (A) Subsequently, the islets were incubated in radiolabel-free medium for the indicated periods. The lysates were immunoprecipitated with anti-insulin/proinsulin antibody. Immunoprecipitated proteins were resolved by Tricine-SDS-PAGE and detected by autoradiography (top). The same membrane was subjected to immunoblotting for proinsulin with a C-peptide antibody (bottom). (B) The islets were directly collected. The lysates were immunoprecipitated with anti-insulin/proinsulin antibody. (Top) Immunoprecipitated proteins were resolved by Tricine-SDS PAGE and detected by autoradiography. (Bottom) Quantification ($n = 3$; *, $P < 0.05$). The data are means ± the standard errors of the means.

The exact mechanisms whereby ERO1β overexpression caused ER stress in β cells were uncertain. The ER stress caused by ERO1β overexpression seems to be due to the oxidizing actions of ERO1β, instead of being a nonspecific artifact, as DTT treatments reversed UPR gene upregulation, as well as reduced insulin contents by ERO1β overexpression in MIN6 β cells. One possible mechanism is that inappropriately high oxidizing conditions in the ER created by ERO1β overexpression resulted in aberrant disulfide formation within client proteins and thus led to the accumulation of misfolded proteins. Another possibility is that ERO1β overexpression caused ER stress by impairing the ERAD system. Given that the reduction of protein disulfides is required within misfolded proteins before they are dislocated and successfully degraded (36, 37), ERO1β overexpression could have hampered the reduction of misfolded proteins and thereby interfered with the ERAD system. Although we could not demonstrate delayed insulin maturation or compromised ERAD in the pulse-chase analysis, these two possibilities are still not excluded as causes of the increased ER stress in our model. Importantly, one of the most upregulated UPR genes under ERO1β overexpression was *Derl3*, which is essential to the machinery of the ERAD system (39). Therefore, it is tempting to suspect that ERAD functions were enhanced in our ERO1β overexpression model as a compensatory response by which healthy insulin handling was, if impaired by EROβ overexpression, successfully restored.

ERO1β overexpression led to impaired glucose tolerance due to impaired insulin secretion. Insulin staining in mice showed that there was a tendency toward reduction of the insulin-positive areas of hERO1βTg islets only at 36 weeks under HFD feeding, which did not reach statistical significance because of their large variations. At earlier time points of HFD feeding, ERO1β overex-

pression did not lead to changes in the insulin-positive areas, when impairment of insulin secretion was already observed upon a glucose challenge, indicating that the mechanism of impaired insulin secretion could be explained not by the changes in β cell mass but only by the altered functions of β cells. A GSIS study with isolated islets suggested that the functional impairment of ERO1β Tg islets was associated with a reduction of islet insulin contents. We observed no consistent changes in basal insulin secretion under low-glucose status in our experimental settings, including GTT of mice or GSIS of islets or MIN6 cells. Although the reason for this is unclear, there might be a specific mechanism whereby ERO1β overexpression preferentially affected the insulin granules responsible for phase 1 and 2 insulin release or, more plausibly, with the relatively small decrease in the insulin contents in any of our models, it might be due to a mere lack of enough sensitivity to detect the difference in the basal states. In fact, previous models with a more pronounced decrease in islet insulin contents do not consistently show a decrease in basal insulin secretion at low glucose concentrations (40, 41).

There could be more than one mechanism whereby ERO1β overexpression caused the reduction of islet insulin contents. *Ins1* and *Ins2*, as well as *Pdx1*, gene expression was significantly downregulated, while the magnitude of the reduction was relatively smaller than the magnitude of the insulin content reduction. Considering that ERO1β overexpression led to ER stress and that one of the fundamental ER stress responses is to downregulate protein synthesis (42), it is tempting to speculate that global repression of protein synthesis is taking place as well. In fact, the pulse-chase analysis showed a significant decrease in insulin biosynthesis in ERO1βTg islets, the magnitude of which was greater than the decrease in *Ins1* and *Ins2* mRNA levels and comparable to the decrease in insulin contents. However, the exact mechanism of insulin synthesis suppression, as well as the mechanism of decreased *Ins1* and *Ins2* mRNA levels, during ERO1β overexpression is unclear and remains to be further investigated and clarified.

So, how is ERO1β involved in the pathogenesis of diabetes mellitus? Here we have shown that ERO1β expression gradually decreases with age in the islets of *db/db* mice in parallel with the progression of glucose intolerance and that ERO1β expression was also decreased in the islets of Akita mice. The reductions in ERO1β expression are in a sharp contrast to the expression of other UPR genes, which were all upregulated in the islets of these model mice possibly because of the increased ER stress. These results indicate that ERO1β has a special place among the UPR components in the islets of diabetic model mice and that ERO1β regulation during diabetes progression is subject to mechanisms distinct from those of UPR, which are currently unknown and need to be clarified by further research. Given that ERO1β suppression leads to decreased insulin content and increased susceptibility to ER stress in β cells (24), we speculate that the reduced expression of ERO1β, or its paradoxical response to ER stress during diabetes progression, could be associated with β cell dysfunction and the inability to synthesize adequate insulin to compensate for peripheral insulin resistance. However, as we have reported here, simply upregulating ERO1β in β cells would not benefit β cell homeostasis and, on the contrary, could worsen ER stress and lead to the suppression of insulin synthesis. Although there remains the possibility that the overexpressed levels of ERO1β in our Tg models are beyond the physiological range and pathologically damaged β cell homeostasis, these results nevertheless clearly illustrate the importance of the fine-tuning of ERO1β regulation required in the maintenance of ER homeostasis, the disturbance of which compromises β cell function for insulin synthesis and could contribute to the pathogenesis of diabetes mellitus.

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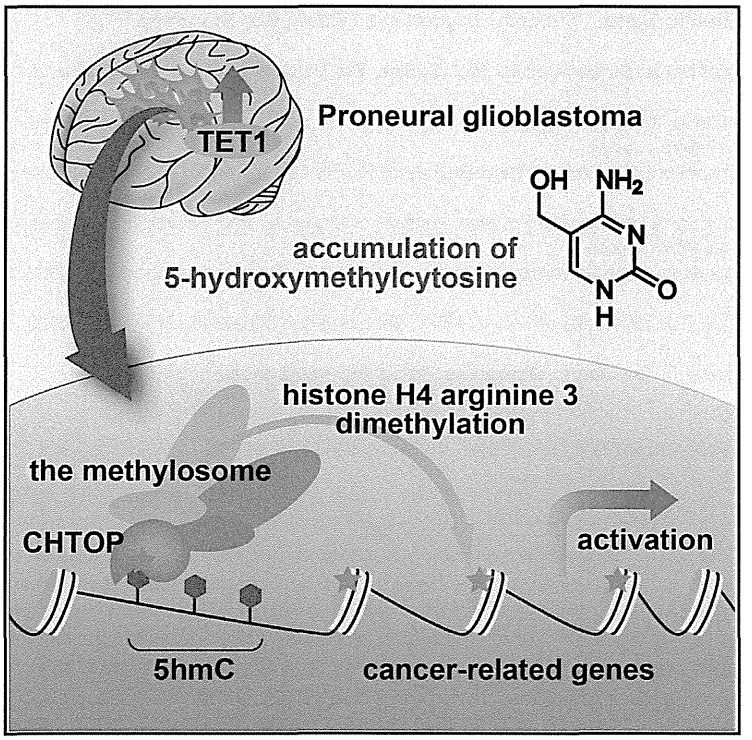
Article

Cell Reports

5-Hydroxymethylcytosine Plays a Critical Role in Glioblastomagenesis by Recruiting the CHTOP-Methylosome Complex

Graphical Abstract



Highlights

Glioblastoma cells contain elevated levels of 5hmC and TET1

TET1-mediated production of 5hmC is required for glioblastomagenesis

5hmC recruits the CHTOP-methylosome complex

The CHTOP-methylosome complex methylates H4R3 and transactivates cancer-related genes

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In Brief

The development of cancer is driven not only by genetic mutations but also by chromatin and DNA modification changes. Takai et al. now show that proneural glioblastomas contain high levels of 5hmC and TET1. Production of 5hmC is required for the tumorigenicity of glioblastoma cells. Furthermore, 5hmC recruits the CHTOP-methylosome complex to selective sites on the chromosome, where it methylates H4R3 and activates the transcription of cancer-related genes.

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