Erythropoietin protects against diabetes through direct effects on pancreatic β cells

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A common feature among all forms of diabetes mellitus is a functional β-cell mass insufficient to maintain euglycemia; therefore, the promotion of β-cell growth and survival is a fundamental goal for diabetes prevention and treatment. Evidence has suggested that erythropoietin (EPO) exerts cytoprotective effects on nonerythroid cells. However, the influence of EPO on pancreatic β cells and diabetes has not been evaluated to date. In this study, we report that recombinant human EPO treatment can protect against diabetes development in streptozotocin-induced and db/db mouse models of type 1 and type 2 diabetes, respectively. EPO exerts antiapoptotic, proliferative, antiinflammatory, and angiogenic effects within the islets. Using β-cell–specific EPO receptor and JAK2 knockout mice, we show that these effects of EPO result from direct biological effects on β cells and that JAK2 is an essential intracellular mediator. Thus, promotion of EPO signaling in β cells may be a novel therapeutic strategy for diabetes prevention and treatment.

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Abbreviations used: EPO, erythropoietin; MLDS, multiple low doses of STZ; mRNA, messenger RNA; PI3K, phosphatidylinositol-3 kinase; rHuEPO, recombinant human EPO; STZ, streptozotocin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VEGF, vascular endothelial growth factor.

Type 1 and type 2 diabetes mellitus are chronic disorders of insulin insufficiency resulting in the dysregulation of glucose homeostasis, hyperglycemia, and vascular complications. Although these two types of diabetes have distinct pathogenic mechanisms, a common element among both forms is the insufficient functional pancreatic β-cell mass that is required to maintain euglycemia (Bach, 1994; Mathis et al., 2001; Kahn, 2003; Rhodes, 2005). Thus, one of the overarching goals in the treatment of all types of diabetes is the preservation and growth of β cells.

Erythropoietin (EPO) is best known for its role in promoting red blood cell formation and survival (Krantz, 1991; Lacombe and Mayeux, 1998). Interestingly, recent studies have shown the EPO-R to be present in nonerythroid tissues, including the brain (Digicaylioglu et al., 1995), heart (Depping et al., 2005), small bowel (Juul et al., 1999), uterus (Yasuda et al., 1998), kidney (Westenfelder et al., 1999), and pancreatic islets (Fenjves et al., 2003). Accordingly, the biological effects of EPO in nonerythroid tissues are currently being investigated. In particular, several studies have shown the efficacy of EPO in providing cytoprotection in experimental models of tissue injury (for review see Brines and Cerami, 2006).

Several lines of evidence have prompted us to further investigate the potential cytoprotective role of EPO in mediating protection from diabetes. (a) EPO-R is present in human and rodent pancreatic islets (Fenjves et al., 2003). Furthermore, EPO overexpression in human

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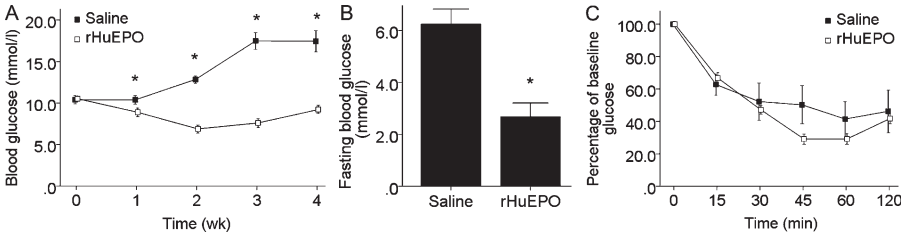

Figure 1. Treatment with rHuEPO protects against hyperglycemia in the MLDS model of diabetes. (A) 6–8-wk-old C57BL/6 mice were injected with rHuEPO or saline three times per week for 4 wk starting on the first day of STZ treatment. Blood glucose levels were measured at the indicated time points (n = 15 per treatment group). (B) Fasting blood glucose levels were measured after 4 wk of rHuEPO or saline treatment (n = 10 per treatment group). (C) Insulin tolerance (insulin sensitivity) was measured after 4 wk of treatment (n = 6 per treatment group). *, $P < 0.05$. Results represent means ± SE.

islets has been shown to prevent cytokine-induced cell death (Fenjves et al., 2004). (b) EPO deficiency and a higher incidence of anemia have been shown in individuals with diabetes, suggesting potential beneficial effects of EPO in the setting of diabetes (Craig et al., 2005; McGill and Bell, 2006; Thomas, 2006). (c) In a recent major clinical trial involving individuals without diabetes with chronic renal failure, EPO treatment was associated with a significant increase in the incidence of hypoglycemia as an adverse effect, which raises the intriguing possibility of a direct effect of EPO on pancreatic β cells (Drüeke et al., 2006). (d) EPO-R belongs to the cytokine class I receptor superfamily and utilizes a similar signal transduction pathway as the receptors for growth hormone and prolactin, knockouts of which show defects in β-cell mass and function (Freemark et al., 2002; Liu et al., 2004). Collectively, these data raise the possibility that EPO signaling may have significant biological effects on β cells and thus may be relevant to diabetes.

Mechanistically, EPO binding to the EPO-R leads to the activation of downstream signaling pathways, including the canonical JAK2–STAT5 pathway, in addition to phosphatidylinositol-3 kinase (PI3K) and Ras–mitogen-activated protein kinase pathways (Damen et al., 1993; He et al., 1993; Quelle et al., 1996). The JAK2–STAT5 pathway leads to the transcription of STAT5-dependent genes that are involved in proliferation, survival, and angiogenesis (Bittorf et al., 2000; for review see Brines and Cerami, 2006). These EPO-mediated signaling pathways are well characterized for the erythroid cell types but are less well defined for the other nonerythroid tissues.

In this study, we investigated the in vivo protective role of EPO against the multiple low doses of streptozotocin (STZ [MLDS]) model of type 1 diabetes and the db/db mouse model of type 2 diabetes. Administration of recombinant human EPO (rHuEPO) resulted in diabetes prevention and reversal in both models of diabetes. Diabetes protection was caused by the direct effects of EPO on the pancreatic β cells in promoting antiapoptosis, proliferation, and angiogenesis within the pancreatic islets, signaling through its cognate receptor and its downstream effector, JAK2.

RESULTS

rHuEPO protects mice against STZ-induced type 1 diabetes

EPO-R messenger RNA (mRNA) and protein are present in isolated islets, pancreas, brain, liver, and kidney of mice as shown by RT-PCR and Western blot, respectively (Fig. S1, A and B). We also confirmed the expression of EPO-R by quantitative real-time PCR in FACS-sorted pancreatic β cells (Fig. S1 C).

To assess the effects of rHuEPO on diabetes protection, we first administered rHuEPO in an MLDS model of type 1 diabetes (Like and Rossini, 1976). rHuEPO or saline was administered to 6–8-wk-old C57BL/6 mice three times weekly for 4 wk starting on the first day of STZ treatment. In contrast to the saline-treated controls that developed hyperglycemia within 1 wk after the start of STZ injections, the rHuEPO-treated group maintained euglycemia throughout the treatment period (Fig. 1 A). Fasting blood glucose levels were also significantly reduced in the rHuEPO-treated group compared with the saline-injected control group (Fig. 1 B). Theoretically, the lower blood glucose levels in the rHuEPO treatment group could be attributed to either increased peripheral insulin sensitivity or beneficial effects of rHuEPO directly on the β cells. The peripheral insulin sensitivity was not increased, as assessed by insulin tolerance tests in the rHuEPO-treated mice (Fig. 1 C). Therefore, these results suggest that rHuEPO may have a direct role on the β cells to provide protection against STZ-induced diabetes.

Indeed, we observed that rHuEPO treatment in the MLDS model of diabetes led to an increase in β-cell area (Fig. 2 A). To investigate the cellular mechanism responsible for the protective role of rHuEPO against STZ-induced diabetes, we examined for β-cell proliferation and apoptosis by Ki67 immunostaining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), respectively. There was an increase in Ki67-positive β cells (Fig. 2 B and Fig. S2 A) and a reduction in TUNEL-positive cells in the islets of the rHuEPO-treated group compared with saline-treated mice (Fig. 2 C and Fig. S2 B). EPO has also been shown to provide cytoprotection through angiogenesis. To assess the vasculature, we examined for the presence of factor VIII (Fig. 2 D) and CD31 (Fig. 2 E and Fig. S2 C), both endothelial cell markers which were present in higher concentrations within the islets of mice in the rHuEPO-treated group compared with saline-injected controls. Quantitative real-time PCR analyses showed enhanced *bcl-xL* and *vascular endothelial growth factor* (*vegf*) mRNA levels in isolated islets of rHuEPO-treated mice compared with saline-treated controls in the STZ-treated cohort (Fig. 2 F). These results are in keeping with the inhibition of apoptosis and enhanced angiogenesis observed from histological analyses of the islets of rHuEPO-treated mice. We also observed a significant attenuation in the induction of IL-1β, TNF, IL-6, and MCP-1 (monocyte chemotactic protein-1) transcript levels in

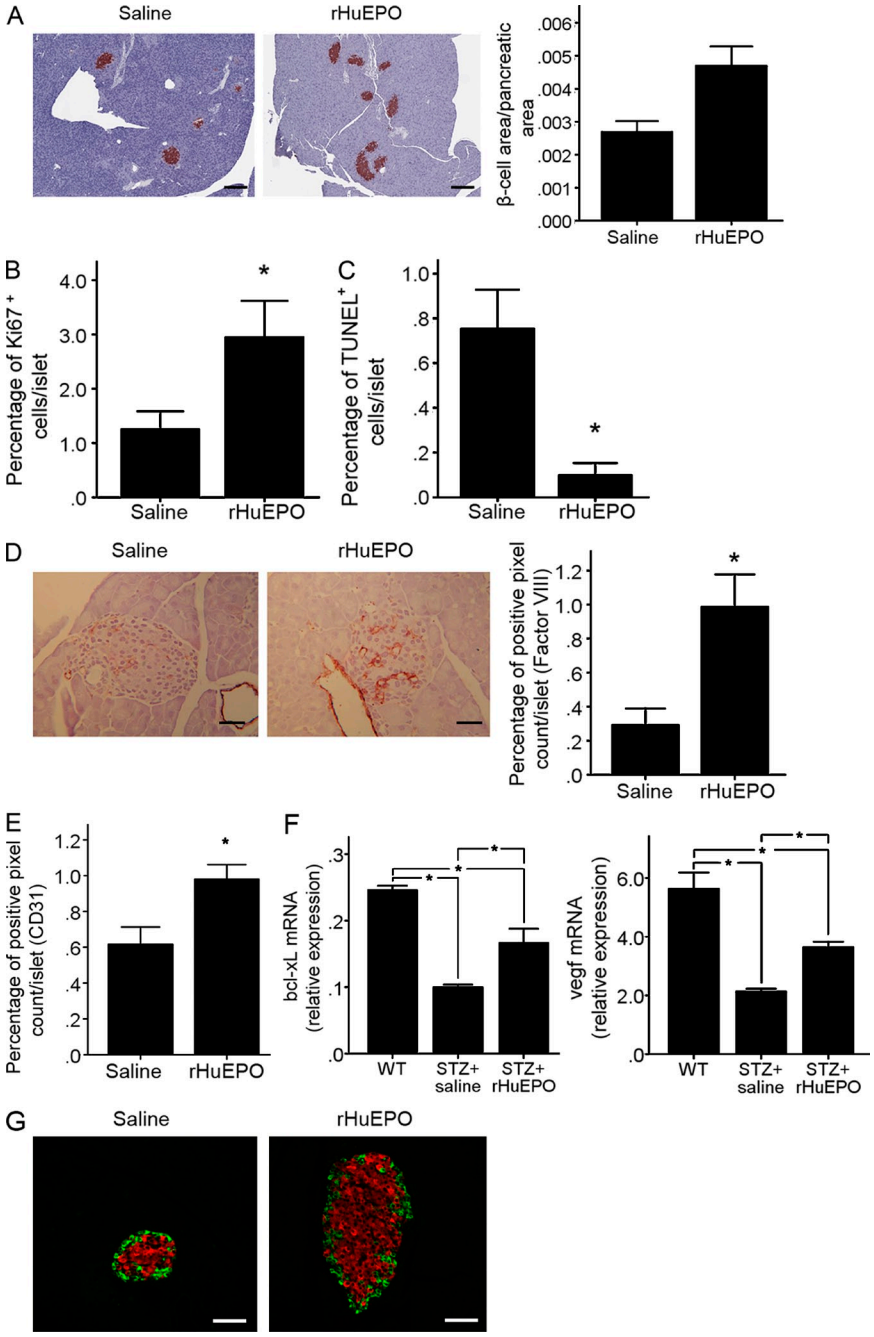

Figure 2. rHuEPO-treated mice have enhanced β-cell mass. (A) β-cell area per pancreatic area was determined on insulin-immunostained sections (n = 11 per treatment group). (B) β-cell proliferation was assessed on Ki67/insulin-coimmunostained sections (n = 11 per treatment group). (C) TUNEL-positive apoptotic β cells were determined on the day after the last STZ injection (n = 3 per treatment group). (D and E) Islet vasculature was assessed on factor VIII (D)– and CD31 (E)-immunostained pancreatic sections (n = 5 per treatment group). Mice were sacrificed after 4 wk of treatment. (F) *bcl-xL* and *vegf* mRNA levels in isolated islets were assessed by quantitative real-time PCR after 1 wk of STZ and either rHuEPO or saline treatments (n = 3 per treatment group). The expression level of each unknown was normalized by the internal control 18S. Each sample was run in triplicate, and each experiment included three nontemplate control wells. Similar results were also found from the normalization by GAPDH and actin (not depicted). (G) Intact islet architecture in both rHuEPO- and saline-treated mice as assessed by insulin (red) and glucagon (green) coimmunostaining (n = 3 per treatment group). Mice were sacrificed after 4 wk of their respective treatments. *, $P < 0.05$. Results represent means ± SE. Bars: (A) 200 µm; (D) 20 µm; (G) 40 µm.

islets of mice in response to STZ in the rHuEPO-treated compared with the saline-treated group (Figs. S2, D–G). These results suggest that rHuEPO also has the role of dampening the inflammatory response at the level of the islet in the context of STZ-induced diabetes. Finally, analysis of insulin and glucagon double immunostaining on pancreatic sections showed intact islet architecture in both the rHuEPO- and saline-treated mice (Fig. 2 G).

We assessed whether rHuEPO treatment can reverse established diabetes by starting rHuEPO treatment 2 wk after the start of STZ injections. We observed that rHuEPO was able to reverse STZ-induced diabetes (Fig. S2 H). 2 wk after the start of STZ injections, we observed a significant decrease in β-cell area in the STZ-treated mice compared with control mice treated with citrate buffer, as assessed by insulin-immunostained pancreatic sections (Fig. S2 I). Mice treated with rHuEPO 2 wk after STZ, after development of diabetes, had enhanced β-cell area compared with saline-treated mice after 4 wk of treatment (Fig. S2 J), which indicates that rHuEPO treatment is able to reverse STZ-mediated β-cell destruction.

rHuEPO treatment reverses hyperglycemia in the db/db mouse model of type 2 diabetes

We also examined the protective effects of rHuEPO against diabetes in the db/db (leptin receptor mutant) mouse model of type 2 diabetes. 5-wk-old male db/db mice were treated with rHuEPO, at which time severe diabetes was already present (>25 mmol/l). Blood glucose levels in the rHuEPO treatment group decreased promptly and remained lower compared with those in the saline-treated control group throughout the treatment period (Fig. 3 A). Fasting blood glucose levels were also significantly reduced in the rHuEPO-treated

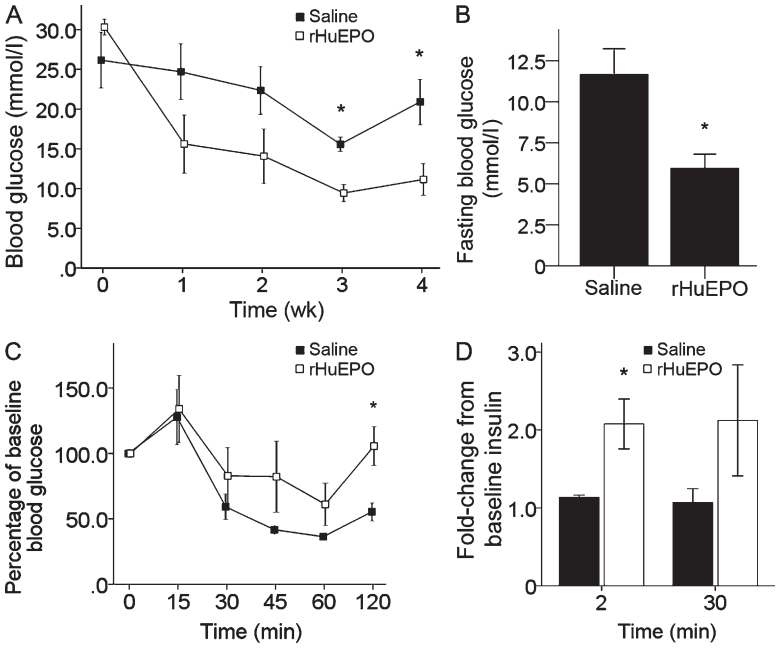

Figure 3. Treatment with rHuEPO reverses type 2 diabetes in the db/db mouse. (A) Random blood glucose levels were measured weekly (n = 10 per treatment group). (B) Fasting blood glucose levels were measured after 4 wk of treatment (n = 10 per treatment group). (C) Insulin sensitivity as assessed by insulin tolerance tests performed after 4 wk of treatment (n = 10 per treatment group). (D) In vivo glucose-stimulated insulin secretion was performed after 4 wk of rHuEPO or saline treatment (n = 9 per treatment group). *, $P < 0.05$. Results represent means ± SE.

group compared with controls (Fig. 3 B). Similar to the experiment in the MLDS model, the improved glucose homeostasis in the rHuEPO-treated db/db mice was not caused by an increase in insulin sensitivity, as indicated by the results of insulin tolerance tests (Fig. 3 C). We then examined for β-cell area in the two treatment groups in the db/db cohort. There was a statistically significant increase in β-cell area per pancreatic area in the rHuEPO-treated mice compared with saline-treated controls (Fig. 4 A). Assessment of Ki67-positive cells showed no differences between the rHuEPO- and saline-treated db/db mice (Fig. 4 B). This may be because of the fact that islet proliferation in db/db mice was already enhanced in response to severe peripheral insulin resistance (DeFronzo, 1997), such that EPO may not have provided a significant additional proliferative stimulus. However, we did observe a decrease in TUNEL-positive β cells on pancreatic sections of the rHuEPO-treated db/db mice (Fig. 4 C), which is suggestive of decreased apoptosis to account for the increased β-cell mass in the rHuEPO-treated db/db mice. In addition, rHuEPO treatment in the db/db mice enhanced blood vessel density in the islets as assessed by factor VIII and CD31 staining on pancreatic sections, which is suggestive of enhanced islet oxygenation (Fig. 4, D and E). Furthermore, there was a significant increase in *bcl-xL* and *vegf* mRNA levels in the islets of rHuEPO-treated db/db mice compared with saline-treated controls, as assessed by quantitative real-time PCR (Fig. 4 F). Interestingly, glucose-stimulated insulin secretion was increased in the rHuEPO-treated db/db mice compared with controls (Fig. 3 D), which is suggestive of a role for EPO in enhancing β-cell function, perhaps through promoting β-cell viability in db/db mice. Finally, islet morphology was normal in both rHuEPO- and saline-treated db/db mice staining (Fig. 4 G).

rHuEPO administration improves glucose homeostasis under basal conditions

Having demonstrated the protective effects of EPO in both type 1 and type 2 diabetes models, we next assessed whether rHuEPO had direct effects on pancreatic islets under basal physiological conditions. Interestingly, rHuEPO had glucose-lowering effects even in nondiabetic conditions, as assessed by random glucose levels (Fig. S3 A). Importantly, the rHuEPO-treated mice did not develop hypoglycemia, which suggests that rHuEPO is able to enhance β-cell secretion while maintaining glucose responsiveness. Their fasting blood glucose levels were also lower after 4 wk of rHuEPO treatment (Fig. S3 B). To assess for changes in peripheral insulin sensitivity, we performed insulin tolerance tests (Fig. S3 C). These tests revealed that the glucose-lowering effects of rHuEPO were not caused by an increase in whole-body insulin sensitivity but likely caused by biological effects of rHuEPO on the pancreatic β cells. These observations further suggest that the improved glucose homeostasis in the rHuEPO-treated mice is primarily caused by the direct biological effects of rHuEPO on the pancreatic β cells.

To further examine whether rHuEPO plays a role in the dynamic process of β-cell homeostasis under basal conditions, we examined the pancreata of C57BL/6 mice treated with rHuEPO for 4 wk. β-cell area per pancreatic area was increased in the rHuEPO-treated mice compared with the saline-treated group (Fig. S3 D), likely because of the increase in the percentage of Ki67-positive β cells observed in rHuEPO-treated mice (Fig. S3 E). Analyses of factor VIII and CD31 immunostaining showed enhanced vasculature in the islets of the rHuEPO-treated mice compared with control mice (Fig. S3, F and G). rHuEPO treatment did not appear to have an effect on islet morphology (Fig. S3 H). In addition, we observed a significant increase in the hematocrit in the rHuEPO-treated mice compared with controls, which reflects the expected biological effect of EPO on erythropoiesis (Fig. S4).

Exogenous EPO signals through the JAK2–STAT5 and PI3K–Akt pathway in β cells

To assess for potential molecular mechanisms responsible for the effect of EPO in pancreatic β cells, we isolated islets from rHuEPO-treated mice and measured their transcript levels of *bcl-xL*, a major target gene in response to STAT5 activation in

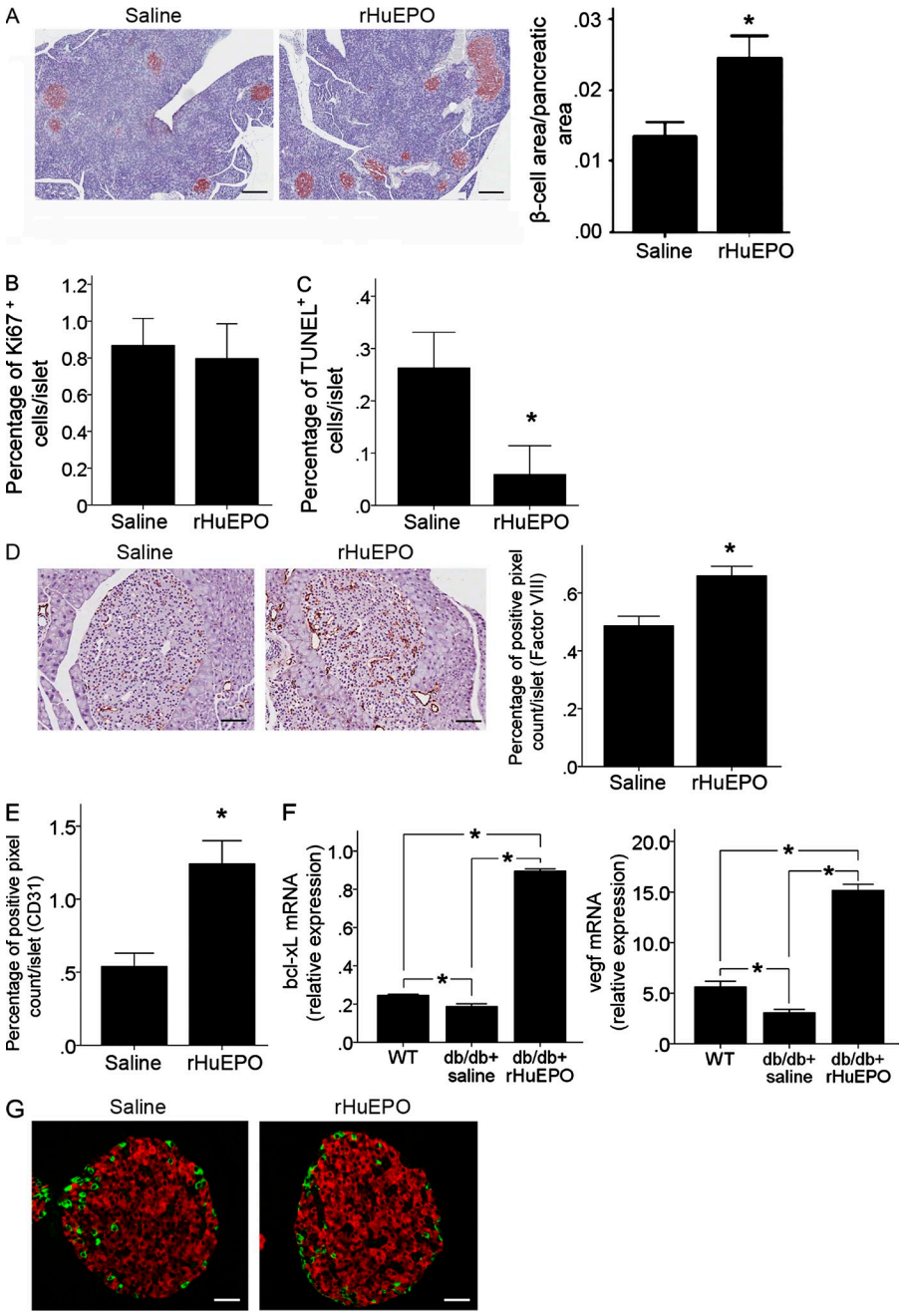

Figure 4. Effect of rHuEPO treatment on the islets of db/db mice. (A) β-cell area per pancreatic area was calculated on insulin-immunostained pancreatic sections (n = 10 per treatment group). (B and C) The percentage of Ki67-positive proliferating β cells (B) and TUNEL-positive apoptotic β cells (C) per islet was assessed by immunohistochemistry (n = 10 per treatment group). (D and E) Islet vasculature was assessed by factor VIII (D) and CD31 (E) immunostaining on pancreatic sections (n = 5 per treatment group). Mice were sacrificed after 4 wk of treatment. (F) *bcl-xL* and *vegf* mRNA levels in isolated islets after 1 wk of rHuEPO or saline treatment were assessed by quantitative real-time PCR (n = 3 per treatment group). The expression level of each unknown was normalized by the internal control 18S. Each sample was run in triplicate, and each experiment included three non-template control wells. Similar results were also found from the normalization by GAPDH and actin (not depicted). (G) Insulin (red) and glucagon (green) coimmunostaining was performed to determine islet architecture (n = 3 per treatment group). Mice were sacrificed after 4 wk of treatment. *, $P < 0.05$. Results represent means ± SE. Bars: (A) 200 µm; (D and G) 40 µm.

providing antiapoptotic effects of EPO (Bittorf et al., 2000). *bcl-xL* mRNA levels were significantly increased in the islets of rHuEPO-treated mice (Fig. 5 A). Expression of *c-myc*, a STAT target gene which has potent proliferative effects, was increased in isolated islets in response to systemic rHuEPO treatment (Fig. 5 A). We also found an increase in mRNA transcript levels of *c-kit* in the isolated islets of rHuEPO-treated mice (Fig. 5 A). *c-kit* is a well-known stem cell marker that is present in regenerating pancreatic β cells after islet destruction (Tiemann et al., 2007). These findings suggest that rHuEPO may promote antiapoptotic and proliferative effects on β cells through these target genes. To analyze the role of EPO in angiogenesis, we measured mRNA levels of *vegf*, which was increased in response to rHuEPO.

EPO has been shown to use multiple intracellular signaling pathways in promoting tissue protection, including the JAK2–STAT5, PI3K–Akt, and mitogen-activated protein kinase pathways, although the JAK–STAT pathway is considered to be the principal signal downstream of EPO (Bittorf et al., 2000; for review see Brines and Cerami, 2006). To determine the intracellular signaling proteins in response to rHuEPO treatment in vivo, we treated C57BL/6 mice with rHuEPO for 1 wk and isolated islets to examine for proteins in the EPO-R signaling cascade. Phospho-JAK2, phospho-STAT5, and Bcl-xL expression was increased in the islets of the rHuEPO-treated group (Fig. 5 B), showing that EPO elicits its effects on the pancreatic β cells through the JAK2–STAT5–Bcl-xL pathway. The PI3K–Akt pathway has also been implicated in EPO-mediated tissue protection (Leverrier et al., 1999), and this pathway has been reported to play a major role in β-cell growth and survival (Bernal-Mizrachi et al., 2001, 2004; Nguyen et al., 2006; Stiles et al., 2006). We found that rHuEPO treatment resulted in increased Akt phosphorylation in β cells

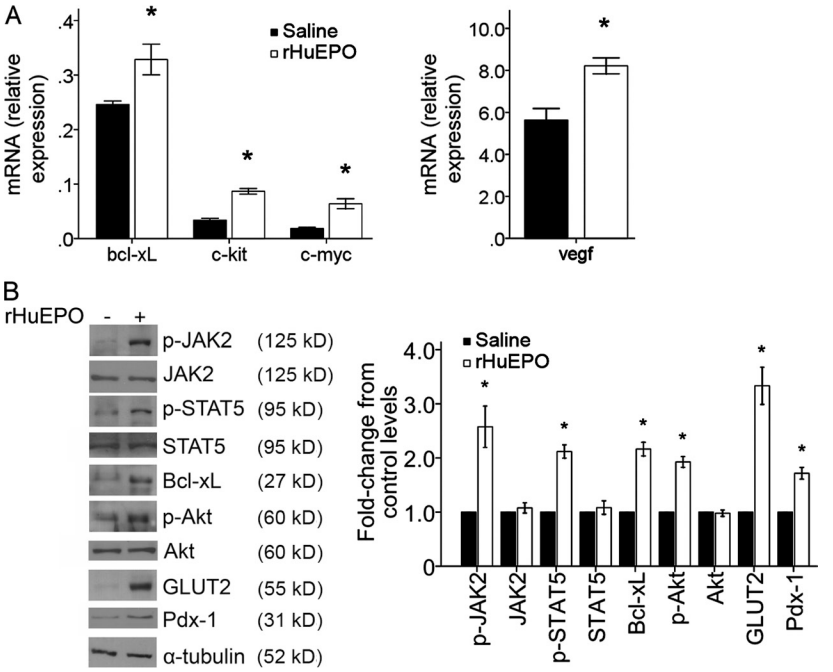

Figure 5. Effect of rHuEPO administration on islet signaling and gene transcription. (A) Quantitative real-time PCR results of *bcl-xL*, *c-myc*, *c-kit*, and *vegf* mRNA levels in islets isolated after 1 wk of rHuEPO treatment (n = 3 per treatment group). The expression level of each unknown was normalized by the internal control 18S. Each sample was run in triplicate, and each experiment included three nontemplate control wells. Similar results were also found from the normalization by other internal controls, including GAPDH and actin (not depicted). (B) Western blots of islet protein lysates were performed after 1 wk of rHuEPO or saline administration to assess phospho (p)-JAK2, phospho-STAT5, and Bcl-xL expression levels as well as phospho-AKT, PDX-1, and GLUT2 levels (n = 3 per treatment group). *, $P < 0.05$. Results represent means ± SE.

(Fig. 5 B). In keeping with the observation of enhanced insulin signaling, the islets of rHuEPO-treated mice showed greater expression of PDX-1 (pancreatic and duodenal homeobox 1), a downstream transcriptional target of insulin signaling which is important for β-cell growth and differentiation (Fig. 5 B; Kulkarni et al., 2004). Furthermore, GLUT2, a β-cell differentiation marker which is important for glucose sensing, was also increased in the islets of rHuEPO-treated mice compared with controls (Fig. 5 B).

Ex vivo effects of rHuEPO on β-cell function

To assess whether EPO may have direct effects on β-cell function, which may account for the improved glucose-stimulated insulin secretion in the rHuEPO-treated db/db mice, we performed islet perifusion experiments and examined for changes in membrane capacitance on individual β cells. Our results showed similar insulin secretion between the islets treated with rHuEPO or saline in response to high glucose (16.7 mM) or KCl during perifusion of individual islets (Fig. S5, A and B). By patch-clamp capacitance measurements on individual β cells, we assessed for changes in membrane capacitance (Fig. S5, C–E). The first two depolarization pulses, which determine the initial readily releasable pool of insulin granules, or first phase of insulin secretion, showed no difference in membrane capacitance of rHuEPO-treated β cells (6.89 ± 1.70 fF/pF) compared with control cells (6.47 ± 0.88 fF/pF). Similarly, the incremental and cumulative increases in cell membrane capacitance between the 3rd and 10th pulses, which measure the rate of insulin granule mobilization to the releasable pool, or the second phase of insulin secretion, also showed similar increments between the rHuEPO-treated and saline-treated β cells (20.13 ± 4.25 fF/pF in rHuEPO-treated β cells vs. 22.35 ± 1.79 fF/pF in saline-treated cells; Fig. S5 E). These results suggest that rHuEPO does not have direct enhancing effects on the insulin secretion machinery but rather provides diabetes protection through the enhancement of islet mass and viability.

EPO-R in the β cells is not required for glucose homeostasis under basal conditions

To further assess the direct effects of EPO on pancreatic β cells, we next took a genetic approach by assessing β-cell–specific EPO-R knockout mice (Fig. S6, A–C). We first assessed whether endogenous EPO action on β cells would have a direct impact in basal glucose homeostasis by measuring random and fasting blood glucose levels in the mice at 1 and 2 mo of age. Our results show that there were no differences in both measurements between the RIP$cre^{+}EpoR^{+/+}$ and RIP$cre^{+}EpoR^{fl/fl}$ mice (Fig. S6, D and E). Furthermore, glucose and insulin tolerance tests revealed no significant differences between the genotypes (Fig. S6, F and G), suggesting that β-cell EPO-R does not play an essential role in the maintenance of glucose homeostasis in vivo. Similarly, there were no differences in β-cell mass and islet morphology between the RIP$cre^{+}EpoR^{+/+}$ and RIP$cre^{+}EpoR^{fl/fl}$ mice at 2 mo of age (Fig. S6, H and I). These findings demonstrate that endogenous EPO signaling in β cells is not essential in regulating β-cell mass under homeostatic conditions.

EPO-R is required for rHuEPO-mediated diabetes protection against the MLDS model of type 1 diabetes

We next assessed whether direct effects of EPO in pancreatic β cells were essential for the diabetes protection provided by exogenous EPO. RIP$cre^{+}EpoR^{fl/fl}$ mice and their control littermates were subjected to MLDS-induced diabetes while on either rHuEPO or saline treatment. In contrast to the RIP$cre^{+}EpoR^{+/+}$ mice, which were protected against STZ-induced diabetes while on rHuEPO treatment (Figs. 1 A and 6 A), diabetes protection was not observed in rHuEPO-treated RIP$cre^{+}EpoR^{fl/fl}$ mice (Fig. 6 A). Similarly, pancreatic morphometry analyses showed enhanced β-cell area per

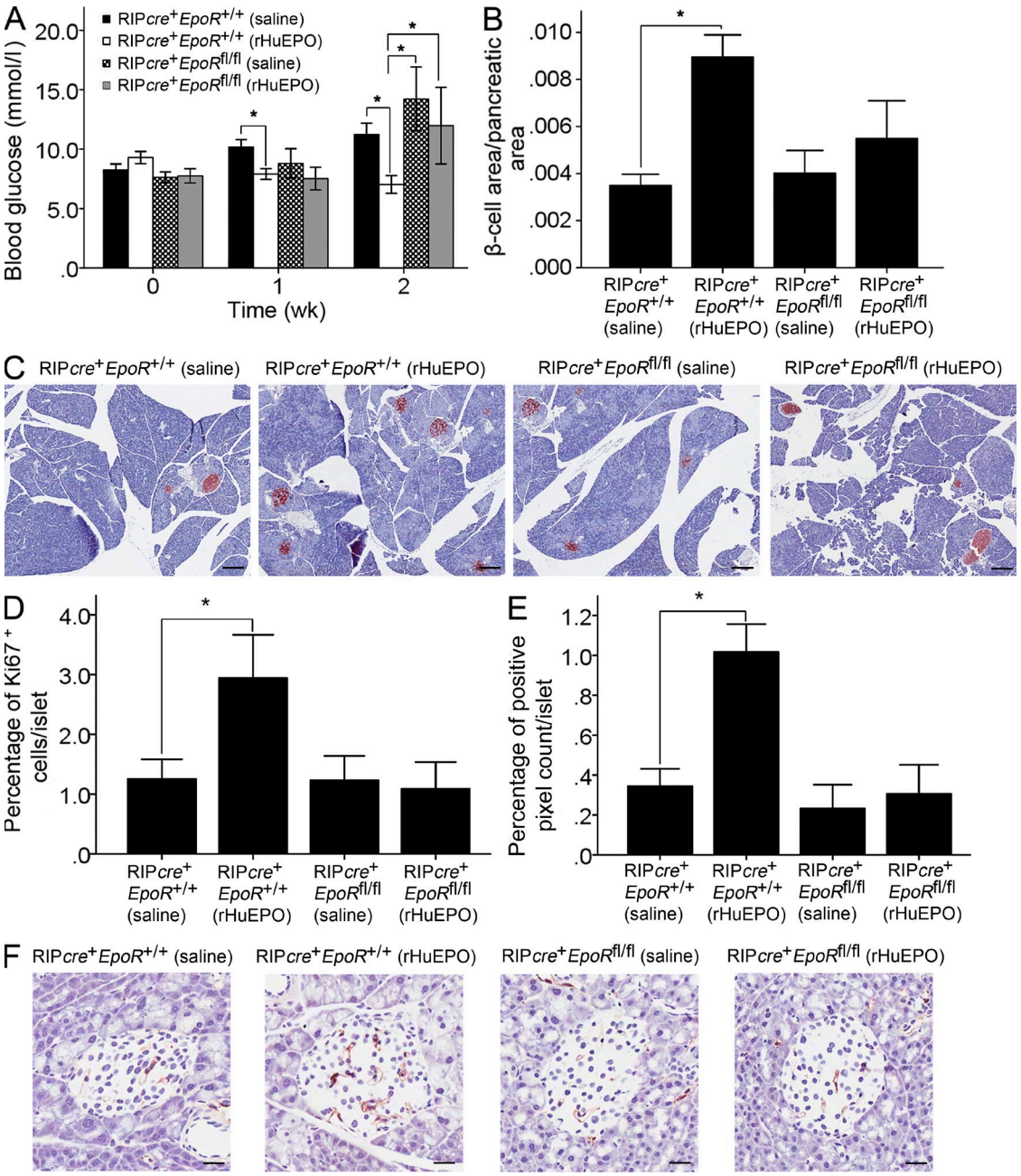

Figure 6. EPO-R is required for rHuEPO-mediated diabetes protection against STZ treatment. (A) Blood glucose levels were measured on a weekly basis throughout the treatment period (n = 8 per treatment group). (B and C) Insulin-immunostained pancreatic sections were performed to assess β-cell area per pancreatic area (n = 8 per treatment group). (D) Ki67 immunostaining on pancreatic sections was performed to assess the percentage of Ki67-positive β cells per islet (n = 8 per treatment group). (E and F) Islet vasculature was assessed by factor VIII immunostaining on pancreatic sections (n = 5 per treatment group). Mice were sacrificed 4 wk after their respective treatments. *, $P < 0.05$. Results represent means ± SE. Bars: (C) 200 µm; (F) 20 µm.

pancreatic area in the rHuEPO-treated RIP*cre*$^{+}$*EpoR*$^{+/+}$ mice, but this effect of rHuEPO on pancreatic β cells was abolished in the RIP*cre*$^{+}$*EpoR*$^{fl/fl}$ mice (Fig. 6, B and C). Furthermore, the increase in Ki67-positive β cells (Fig. 6 D) and increase in islet vasculature observed in the rHuEPO-treated RIP*cre*$^{+}$*EpoR*$^{+/+}$ mice were also abolished in islets of RIP*cre*$^{+}$*EpoR*$^{fl/fl}$ mice (Fig. 6, E and F).

Direct effects of rHuEPO on pancreatic β cells for target gene regulation

rHuEPO treatment in mice was shown to enhance the expression of *bcl-xL*, *c-myc*, *c-kit*, and *vegf* in isolated islets (Fig. 5). To determine whether the enhanced expression of these genes in isolated islets was caused by direct effects of rHuEPO on the pancreatic β cells, we treated RIP*cre*$^{+}$*EpoR*$^{+/+}$ and

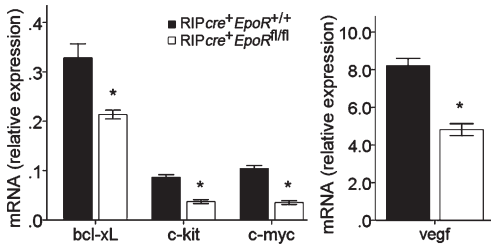

Figure 7. Role for EPO-R in EPO-mediated gene transcription in islets. Quantitative real-time PCR was performed to measure expression levels of *bcl-xl*, *c-kit*, *c-myc*, and *vegf* in isolated islets after 1 wk of rHuEPO or saline treatment ($n = 3$ per genotype). The expression level of each unknown was normalized by the internal control 18S. Each sample was run in triplicate, and each experiment included three nontemplate control wells. Similar results were also found from the normalization by GAPDH and actin (not depicted). *, $P < 0.05$. Results represent means ± SE.

RIP*cre*$^{+}$*EpoR*$^{fl/fl}$ mice with rHuEPO for 1 wk and analyzed the expression of these genes in the isolated islets. Our results by RT-PCR showed that the islets of RIP*cre*$^{+}$*EpoR*$^{fl/fl}$ mice treated with rHuEPO did not show an induction in these genes, and the gene levels were lower compared with rHuEPO-treated RIP*cre*$^{+}$*EpoR*$^{+/+}$ mice (Fig. 7), which is indicative of the requirement of EPO-R in β cells for the transcriptional regulation by rHuEPO.

RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ mice have no defects in glucose homeostasis

JAK2 has been identified as a canonical essential intracellular mediator of EPO action in erythrocyte precursors; however, EPO-mediated signaling events in pancreatic β cells have remained elusive. Therefore, we assessed β-cell–specific JAK2 knockout mice (Fig. S7, A–C) to determine its essential role in EPO signaling in β cells. To assess for changes in glucose homeostasis, we measured monthly fed and fasting blood glucose, which showed no differences between the RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ mice and their littermate controls (Fig. S7, D and E). Glucose and insulin tolerance tests also showed similar results between the mutant mice and their littermate controls (Fig. S7, F and G).

JAK2 is described to function downstream of various growth factors, including growth hormone, prolactin, and placental lactogen, which have all been shown to play an essential role in pancreatic β-cell homeostasis (Sorenson and Stout, 1995; Cousin et al., 1999; Fujinaka et al., 2007). Surprisingly, analyses of insulin-immunostained pancreatic sections revealed no differences in β-cell area per pancreatic area and islet architecture in the RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ mice compared with those of control RIP*cre*$^{+}$*Jak2*$^{+/+}$ littermates (Fig. S7, H and I).

JAK2 in pancreatic β cells is required for diabetes protection by rHuEPO

RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ and control mice were subjected to STZ-induced diabetes with or without rHuEPO treatment to assess for the essential direct role of JAK2 in EPO signaling in pancreatic β cells. rHuEPO provided protection in the RIP*cre*$^{+}$*Jak2*$^{+/+}$ mice; however, diabetes protection was not observed in the RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ mice, as assessed by measurements of postprandial blood glucose levels (Fig. S8 A). In keeping with the lack of diabetes protection, RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ mice did not have a significant increase in β-cell area per pancreatic area in response to rHuEPO treatments, in contrast to RIP*cre*$^{+}$*Jak2*$^{+/+}$ mice treated with rHuEPO, in which the increase was observed (Fig. S8, B and C). Similarly, the increase in Ki67-positive β cells (Fig. S8 D) and increase in islet vasculature present in the rHuEPO-treated RIP*cre*$^{+}$*Jak2*$^{+/+}$ mice compared with saline-treated controls were absent in the islets of RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ mice (Fig. S8, E and F). These data, which parallel those of EPO-R mutant mice, collectively indicate that the rHuEPO-mediated diabetes protection occurs through direct activation of EPO signaling in pancreatic β cells and that JAK2 is its essential mediator. Similarly, the islet gene modulation by rHuEPO appears to also be mediated through JAK2 in β cells. As such, *bcl-xL*, *c-myc*, *c-kit*, and *vegf* in the islets of rHuEPO-treated RIP*cre*$^{+}$*Jak2*$^{fl/fl}$ mice were lower than those of RIP*cre*$^{+}$*Jak2*$^{+/+}$ mice (Fig. S8 G).

DISCUSSION

Central to both type 1 and type 2 diabetes is a pancreatic β-cell defect, as manifested by β-cell death and dysfunction. rHuEPO was previously shown to have cytoprotective effects in nonerythroid tissues (for review see Brines and Cerami, 2006); therefore, we investigated whether the cytoprotective effects of EPO extended to the pancreatic β cells in diabetes models. Intriguingly, we found that systemic rHuEPO administration confers diabetes protection by exerting a direct biological effect on β cells through its cognate receptor, EPO-R, and its immediate downstream kinase, JAK2, as we have demonstrated using β-cell–specific EPO-R and JAK2 knockout mice. Importantly, no other downstream kinase was able to compensate for JAK2 to mediate EPO-R signaling in β cells of the JAK2 knockout mice. Thus, our results show that pancreatic β cells may use similar EPO-mediated signaling machinery as that which is used during embryogenesis, as indicated by the similar phenotypes of embryonic lethality caused by fetal anemia observed in both JAK2- and EPO-R–null mice (Wu et al., 1995; Neubauer et al., 1998).

It appears that the protective effect of rHuEPO on the β cells under diabetes conditions occurs largely through the promotion of β-cell growth and survival rather than through direct effects on β-cell function. Indeed, the islet perifusion and patch-clamp capacitance measurements show that rHuEPO does not have a significant enhancing effect on β-cell function per se. Instead, our data show that rHuEPO treatment has antiapoptotic, proliferative, and angiogenic effects on the pancreatic islets.

The increase in *bcl-xL* expression induced by EPO may be the critical molecular mechanism through which EPO inhibits apoptosis in β cells during diabetes progression. A previous study has shown that increased *bcl-xL* expression is a key mediator of EPO action in maintaining erythrocyte mass in an organism, as *bcl-xL*–null mice die embryonically as the result of anemia and defects in erythropoiesis (Motoyama et al., 1995). Furthermore, EPO has been shown to up-regulate

bcl-xL expression in various erythroleukemic cells lines (Silva et al., 1999).

We have also shown that rHuEPO treatment increases β-cell proliferation as well as enhances the transcription of *c-myc* and *c-kit*. EPO has been previously shown to induce the proliferation and expression of *c-myc* in normal human erythroid progenitors (Umemura et al., 1988). In addition, *c-kit* has been reported to be present on progenitor cells of the pancreas and play an important role during pancreatic development and during regeneration after STZ-induced diabetes (Tiemann et al., 2007). Furthermore, a recent study has also shown a physiological role of *c-kit* in the growth and maintenance of β-cell mass in mice (Krishnamurthy et al., 2007). Therefore, *c-kit* may be an important mediator through which effects of rHuEPO on pancreatic β cells are achieved.

Furthermore, rHuEPO administration increases *vegf* expression in the pancreatic islets, and EPO-mediated angiogenesis in the islets is dependent on β-cell EPO-R and JAK2. Islets are highly vascular, and previous work has shown that the dense vasculature is dependent on VEGF that is produced by the pancreatic β cells (Lammert et al., 2003). VEGF is known to play an essential role in maintaining the integrity of the microvasculature and in stimulating angiogenesis to aid in tissue healing (Ribatti et al., 2003; Galeano et al., 2004). VEGF has been shown to increase not only oxygenation and nutrient delivery to the islets but also to have a direct impact on pancreatic β-cell function by mechanisms that are not yet entirely clear (Heeschen et al., 2003). Given the potent effect of rHuEPO on the up-regulation of VEGF within islets, this important angiogenic factor may be another key mediator through which EPO promotes the survival and maintenance of function of pancreatic β cells.

Collectively, our in vivo data demonstrate potent protective effects of EPO against diabetes development by direct actions of EPO on β cells, thereby increasing β-cell mass through antiapoptotic, proliferative, and angiogenic mechanisms. In the context of the central role of β-cell insufficiency in the pathogenesis of diabetes, these biological effects of EPO reflect ideal properties for a potential new therapeutic agent for diabetes. Indeed, EPO is a safe, widely used hormone, and strategies to generate nonerythropoietic EPO-related agents are currently being actively investigated. Thus, further understanding of the biology of EPO may hold promise for the development of a potential novel strategy for diabetes prevention and treatment.

MATERIALS AND METHODS

Mice. Experiments were performed on 6–8-wk-old C57BL/6 mice and 5-wk-old db/db mice in the C57BLKS/J background obtained from The Jackson Laboratory. RIP*cre*$^{+}$ (Postic et al., 1999) and *EpoR*$^{fl/fl}$ (Tsai et al., 2006) or *Jak2*$^{fl/fl}$ (Wagner et al., 2004) mice were bred to generate RIP*cre*$^{+}$*EpoR*$^{+/fl}$ (or *Jak2*$^{+/fl}$) mice, which were then intercrossed to generate RIP*cre*$^{-}$ and RIP*cre*$^{+}$*EpoR*$^{+/+}$(or *Jak2*$^{+/+}$), RIP*cre*$^{+}$*EpoR*$^{+/fl}$ (or *Jak2*$^{+/fl}$), and RIP*cre*$^{+}$*EpoR*$^{fl/fl}$ (or *Jak2*$^{fl/fl}$) mice. These mice were examined at 8 wk of age and maintained on a 129J-C57BL/6-FVB/N mixed background, and RIP*cre*$^{+}$*EpoR*$^{+/+}$ (or *Jak2*$^{+/+}$) littermates were used as controls. Mice were maintained on a 12-h light–dark cycle with free access to water and standard irradiated rodent chow (5% fat; Harlan Teklad) and housed in pathogen-free barrier facilities at the central animal facility in the Ontario Cancer Institute (Toronto, Ontario, Canada). All animal experiments were approved by the Ontario Cancer Institute Animal Care Facility.

rHuEPO treatment. rHuEPO (Epoetin-α; Eprex; Ortho-Biotech) was used. Mice were injected i.p. three times per week at a dose of 50 μg/kg body weight (Savino et al., 2006) between 9 a.m. and 10 a.m.

Hematocrit measurement. 30 μl of tail vein blood was collected using EDTA-coated capillary tubes. Samples were kept at room temperature before analysis at the Toronto Centre for Phenogenomics (Toronto, Ontario, Canada).

STZ protocol. MLDS were i.p. injected into mice (40 mg/kg body weight) for a consecutive 5 d (Liadis et al., 2005). Blood glucose was measured weekly after i.p. MLDS injections from tail vein blood with an automated glucose monitor (Precision Xtra; Abbott Laboratories).

Metabolic experiments and hormone measurements. Overnight fasts were 14–16 h in duration. Glucose tolerance tests were performed on overnight-fasted animals using a glucose dose of 1 g/kg body weight injected i.p., and measurements of blood glucose were taken at 0, 15, 30, 45, 60, and 120 min after the injection. Insulin tolerance tests were performed using human regular insulin (Novo Nordisk) at a dose of 1 U/kg body weight, and blood glucose levels were measured at 0, 15, 30, 45, 60, and 120 min after the injection of insulin. Glucose-stimulated insulin secretion in vivo was performed on overnight-fasted animals after an i.p. injection of glucose at a dose of 3 g/kg body weight, with tail vein blood collected at 0, 2, 10, and 30 min after the injection. Insulin levels were measured by an ELISA kit using a rat insulin standard (Crystal Chem Inc.).

Islet morphometry, immunohistochemistry, and immunofluorescent staining. Pancreatic tissue was fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. 7-μm-thick paraffin sections were obtained at 150-μm intervals on three levels and stained for insulin, glucagon, Ki67, factor VIII (Dako), and CD31 (Santa Cruz Biotechnology, Inc.). TUNEL (ID Labs) was performed by immunohistochemistry. Immunofluorescence was visualized using an inverted fluorescent microscope (Axio Observer; Carl Zeiss, Inc.). Insulin- and factor VIII–immunostained sections were scanned using a ScanScope ImageScope system and analyzed with ImageScope version 9.0.19.1516 software (Aperio Technologies). Factor VIII immunostaining was calculated per β-cell area by an automated positive pixel count algorithm. Total β-cell area per total pancreatic area was determined on insulin-stained sections.

Islet isolation and β-cell sorting. Islets were isolated from mice as previously described (Liadis et al., 2005). In brief, 3 ml of 3 mg/ml collagenase (Sigma-Aldrich) was injected into the common bile duct for pancreatic digestion. Isolated islets were maintained in suspension culture in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For β-cell sorting, freshly isolated islets were dispersed and subjected to flow cytometry based on high autofluorescence, as previously described (Allison et al., 2005; Liadis et al., 2007).

Islet perifusion secretory assay. Islets were isolated from C57BL/6 mice as previously described (Liadis et al., 2005) and incubated overnight in either 100 U/ml rHuEPO or saline. The next day, batches of 50 islets, on average, per group were placed in 37°C perifusion chambers (with rHuEPO for the rHuEPO-treated islets) and perifused at a flow rate of 1 ml/min with a Krebs-Ringer bicarbonate buffer. Islets were initially equilibrated for 30 min in Krebs-Ringer bicarbonate Hepes buffer supplemented with 2.8 mmol/l glucose. They were then stimulated with 2.8 mmol/l glucose for 10 min, which was followed by stimulation with 16.7 mmol/l glucose for 40 min. After 40 min with 16.7 mmol/l glucose alone, the islets were subjected to an additional 25-min stimulation period with 30 mmol/l KCl. Fractions were

collected for insulin determination using an RIA kit (Linco Research, Inc.). At the end of each perifusion, islets were collected to assess insulin content.

Capacitance measurements. Isolated islets from C57BL/6 mice were dispersed into single β cells using 0.05% trypsin-EDTA (Invitrogen) for 10 min in a 37°C incubator and treated with rHuEPO or saline overnight. Cells were patch-clamped in conventional whole-cell configuration at 33–34°C. rHuEPO-treated cells were treated again with rHuEPO during the capacitance measurements. β cells were identified by cell size (>4 pF) and by their Na^+ current inactivation properties. Capacitance measurements were performed using EPC-9 amplifier and PULSE software from HEKA. Patch pipettes had typical resistances of 3–6 MΩ when fire polished and filled with an intracellular solution containing 120 mM CsCl, 20 mM tetraethylammonium chloride, 1 mM $MgCl_2$, 0.05 mM EGTA, 10 mM Hepes, 0.1 mM cAMP, and 5 mM MgATP, pH 7.3, with CsOH. After whole-cell configuration was established, intracellular solution was dialyzed into the cells via patch pipette for 1 min, and then a train of 10 500-ms depolarization pulses from −80 to 0 mV was applied to determine the cell membrane capacitance change (ΔCm). Extracellular solutions contained 118 mM NaCl, 20 mM tetraethylammonium chloride, 2.6 mM $CaCl_2$, 5 mM Hepes, 5.6 mM KCl, 1.2 mM $MgCl_2$, and 3 mM glucose, pH 7.4, with NaOH. Patch-clamp data were analyzed with IGOR Pro 3.12 software (WaveMetrics).

Western blotting. Islets, pancreas, brain, liver, muscle, hypothalamus, and kidney tissue were isolated, and protein lysates were obtained as previously described (Wijesekara et al., 2005). Lysates were separated by Na dodecyl sulfate–10% PAGE and immunoblotted with antibodies against JAK2 (Millipore), Akt, phospho-Akt (Ser473), phospho-JAK2 (Cell Signaling Technology), STAT5 (Invitrogen), phospho-STAT5 (BD), GLUT2, PDX-1 (Millipore), Bcl-xL, and EPO-R (Santa Cruz Biotechnology, Inc.). Western blot signal densities were analyzed using ImageJ software (National Institutes of Health). Protein levels were normalized to α-tubulin levels and expressed in arbitrary units relative to littermate control levels.

Quantitative real-time PCR. RNA from FACS-sorted β cells or isolated whole islets were extracted using the RNeasy mini plus RNA extraction kit (QIAGEN), and cDNA was synthesized by reaction with MMLV RT (Invitrogen) and random primers, according to standard protocols. *bcl-xL*, *c-kit*, *c-myc*, *vegf*, *EpoR*, *IL-1β*, *TNF*, *IL-6*, *MCP-1*, and *18S* levels were quantified (ABI Prism 7900HT real-time PCR system; Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reactions were performed in a final volume of 10 µl containing 0.3 µM gene-specific primers and SYBR green master mix (Applied Biosystems). PCR cycles consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. To confirm amplification specificity, PCR products from each primer pair were subjected to a melting curve analysis. A standard curve was generated from cDNA (from 100 ng to 10^{-2} ng RNA) serial dilutions for each PCR reaction and was used to determine the relative gene expression level in each sample. The expression level of each unknown was normalized by the internal control 18S, GAPDH, or actin. Each sample was run in triplicate, and each experiment included three nontemplate control wells.

RT-PCR. mRNA was extracted from isolated islets, pancreas, brain, liver, and kidney tissues by TRIZOL (Invitrogen) according to the manufacturer's protocol and treated with RNase-free DNase (Invitrogen). Semiquantitative RT-PCR was performed with a one-step RT-PCR kit (Invitrogen). *EpoR*, *bcl-xL*, *vegf*, *c-myc*, *c-kit*, and *β-actin* cDNA were amplified by PCR using specific primers. mRNA levels were normalized to β-actin levels and expressed in arbitrary units relative to littermate control levels using ImageJ software.

Statistical analysis. Data are presented as means ± SEMs and were analyzed by one-sample *t* test, independent-samples *t* test, or one-way analysis of variance, as appropriate, using the statistical software SPSS (version 17.0 for Windows). P-values of ≤0.05 were accepted as statistically significant.

Online supplemental material. Fig. S1 shows the expression of EPO-R in pancreatic β cells and islets. Fig. S2 shows the effects of rHuEPO on STZ-induced diabetes. Fig. S3 demonstrates the improvement of glucose homeostasis as a result of rHuEPO treatment. Fig. S4 shows increased hematocrit in mice treated with rHuEPO compared with saline-treated mice. Fig. S5 demonstrates that rHuEPO does not have an effect on β-cell function. Fig. S6 shows that EPO-R is not required for glucose homeostasis. Fig. S7 shows that JAK2 is not essential for glucose homeostasis under basal conditions. Fig. S8 demonstrates that β-cell JAK2 is required for rHuEPO-mediated protection against STZ-induced diabetes. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100665/DC1.

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REFERENCES

Allison, J., H.E. Thomas, T. Catterall, T.W. Kay, and A. Strasser. 2005. Transgenic expression of dominant-negative Fas-associated death domain protein in beta cells protects against Fas ligand-induced apoptosis and reduces spontaneous diabetes in nonobese diabetic mice. *J. Immunol.* 175:293–301.

Bach, J.F. 1994. Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr. Rev.* 15:516–542.

Bernal-Mizrachi, E., W. Wen, S. Stahlhut, C.M. Welling, and M.A. Permutt. 2001. Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J. Clin. Invest.* 108:1631–1638.

Bernal-Mizrachi, E., S. Fatrai, J.D. Johnson, M. Ohsugi, K. Otani, Z. Han, K.S. Polonsky, and M.A. Permutt. 2004. Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J. Clin. Invest.* 114:928–936.

Bittorf, T., J. Seiler, B. Lüdtke, T. Büchse, R. Jaster, and J. Brock. 2000. Activation of STAT5 during EPO-directed suppression of apoptosis. *Cell. Signal.* 12:23–30. doi:10.1016/S0898-6568(99)00063-7

Brines, M., and A. Cerami. 2006. Discovering erythropoietin's extra-hematopoietic functions: biology and clinical promise. *Kidney Int.* 70:246–250. doi:10.1038/sj.ki.5001546

Cousin, S.P., S.R. Hügl, M.G. Myers Jr., M.F. White, A. Reifel-Miller, and C.J. Rhodes. 1999. Stimulation of pancreatic beta-cell proliferation by growth hormone is glucose-dependent: signal transduction via janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) with no crosstalk to insulin receptor substrate-mediated mitogenic signalling. *Biochem. J.* 344:649–658. doi:10.1042/0264-6021:3440649

Craig, K.J., J.D. Williams, S.G. Riley, H. Smith, D.R. Owens, D. Worthing, I. Cavill, and A.O. Phillips. 2005. Anemia and diabetes in the absence of nephropathy. *Diabetes Care.* 28:1118–1123. doi:10.2337/diacare.28.5.1118

Damen, J.E., L. Liu, R.L. Cutler, and G. Krystal. 1993. Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-Kd tyrosine phosphorylated protein. *Blood.* 82:2296–2303.

DeFronzo, R.A. 1997. Pathogenesis of type 2 diabetes mellitus: metabolic and molecular implications for identifying diabetes genes. *Diabetes Rev.* 5:177–269.

Depping, R., K. Kawakami, H. Ocker, J.M. Wagner, M. Heringlake, A. Noetzold, H.H. Sievers, and K.F. Wagner. 2005. Expression of the erythropoietin receptor in human heart. *J. Thorac. Cardiovasc. Surg.* 130:877–878. doi:10.1016/j.jtcvs.2004.12.041

Digicaylioglu, M., S. Bichet, H.H. Marti, R.H. Wenger, L.A. Rivas, C. Bauer, and M. Gassmann. 1995. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proc. Natl. Acad. Sci. USA*. 92:3717–3720. doi:10.1073/pnas.92.9.3717

Drüeke, T.B., F. Locatelli, N. Clyne, K.U. Eckardt, I.C. Macdougall, D. Tsakiris, H.U. Burger, and A. Scherhag. 2006. Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N. Engl. J. Med*. 355:2071–2084. doi:10.1056/NEJMoa062276

Fenjves, E.S., M.S. Ochoa, O. Cabrera, A.J. Mendez, N.S. Kenyon, L. Inverardi, and C. Ricordi. 2003. Human, nonhuman primate, and rat pancreatic islets express erythropoietin receptors. *Transplantation*. 75:1356–1360. doi:10.1097/01.TP.0000062862.88375.BD

Fenjves, E.S., M.S. Ochoa, C. Gay-Rabinstein, R.D. Molano, A. Pileggi, A.J. Mendez, L. Inverardi, and C. Ricordi. 2004. Adenoviral gene transfer of erythropoietin confers cytoprotection to isolated pancreatic islets. *Transplantation*. 77:13–18. doi:10.1097/01.TP.0000110422.27977.26

Freemark, M., I. Avril, D. Fleenor, P. Driscoll, A. Petro, E. Opara, W. Kendall, J. Oden, S. Bridges, N. Binart, et al. 2002. Targeted deletion of the PRL receptor: effects on islet development, insulin production, and glucose tolerance. *Endocrinology*. 143:1378–1385. doi:10.1210/en.143.4.1378

Fujinaka, Y., K. Takane, H. Yamashita, and R.C. Vasavada. 2007. Lactogens promote beta cell survival through JAK2/STAT5 activation and Bcl-XL upregulation. *J. Biol. Chem*. 282:30707–30717. doi:10.1074/jbc.M702607200

Galeano, M., D. Altavilla, D. Cucinotta, G.T. Russo, M. Calò, A. Bitto, H. Marini, R. Marini, E.B. Adamo, P. Seminara, et al. 2004. Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse. *Diabetes*. 53:2509–2517. doi:10.2337/diabetes.53.9.2509

He, T.-C., H. Zhuang, N. Jiang, M.D. Waterfield, and D.M. Wojchowski. 1993. Association of the p85 regulatory subunit of phosphatidylinositol 3-kinase with an essential erythropoietin receptor subdomain. *Blood*. 82:3530–3538.

Heeschen, C., A. Aicher, R. Lehmann, S. Fichtlscherer, M. Vasa, C. Urbich, C. Mildner-Rihm, H. Martin, A.M. Zeiher, and S. Dimmeler. 2003. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood*. 102:1340–1346. doi:10.1182/blood-2003-01-0223

Juul, S.E., A.E. Joyce, Y. Zhao, and D.J. Ledbetter. 1999. Why is erythropoietin present in human milk? Studies of erythropoietin receptors on enterocytes of human and rat neonates. *Pediatr. Res*. 46:263–268. doi:10.1203/00006450-199909000-00003

Kahn, S.E. 2003. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia*. 46:3–19. doi:10.1007/s00125-002-1009-0

Krantz, S.B. 1991. Erythropoietin. *Blood*. 77:419–434.

Krishnamurthy, M., F. Ayazi, J. Li, A.W. Lyttle, M. Woods, Y. Wu, S.P. Yee, and R. Wang. 2007. c-Kit in early onset of diabetes: a morphological and functional analysis of pancreatic beta-cells in c-KitW-v mutant mice. *Endocrinology*. 148:5520–5530. doi:10.1210/en.2007-0387

Kulkarni, R.N., U.S. Jhala, J.N. Winnay, S. Krajewski, M. Montminy, and C.R. Kahn. 2004. PDX-1 haploinsufficiency limits the compensatory islet hyperplasia that occurs in response to insulin resistance. *J. Clin. Invest*. 114:828–836.

Lacombe, C., and P. Mayeux. 1998. Biology of erythropoietin. *Haematologica*. 83:724–732.

Lammert, E., G. Gu, M. McLaughlin, D. Brown, R. Brekken, L.C. Murtaugh, H.P. Gerber, N. Ferrara, and D.A. Melton. 2003. Role of VEGF-A in vascularization of pancreatic islets. *Curr. Biol*. 13:1070–1074. doi:10.1016/S0960-9822(03)00378-6

Leverrier, Y., J. Thomas, A.L. Mathieu, W. Low, B. Blanquier, and J. Marvel. 1999. Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells. *Cell Death Differ*. 6:290–296. doi:10.1038/sj.cdd.4400492

Liadis, N., K. Murakami, M. Eweida, A.R. Elford, L. Sheu, H.Y. Gaisano, R. Hakem, P.S. Ohashi, and M. Woo. 2005. Caspase-3-dependent beta-cell apoptosis in the initiation of autoimmune diabetes mellitus. *Mol. Cell. Biol*. 25:3620–3629. doi:10.1128/MCB.25.9.3620-3629.2005

Liadis, N., L. Salmena, E. Kwan, P. Tajmir, S.A. Schroer, A. Radziszewska, X. Li, L. Sheu, M. Eweida, S. Xu, et al. 2007. Distinct in vivo roles of caspase-8 in beta-cells in physiological and diabetes models. *Diabetes*. 56:2302–2311. doi:10.2337/db06-1771

Like, A.A., and A.A. Rossini. 1976. Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science*. 193:415–417. doi:10.1126/science.180605

Liu, J.L., K.T. Coschigano, K. Robertson, M. Lipsett, Y. Guo, J.J. Kopchick, U. Kumar, and Y.L. Liu. 2004. Disruption of growth hormone receptor gene causes diminished pancreatic islet size and increased insulin sensitivity in mice. *Am. J. Physiol. Endocrinol. Metab*. 287:E405–E413. doi:10.1152/ajpendo.00423.2003

Mathis, D., L. Vence, and C. Benoist. 2001. β-Cell death during progression to diabetes. *Nature*. 414:792–798. doi:10.1038/414792a

McGill, J.B., and D.S. Bell. 2006. Anemia and the role of erythropoietin in diabetes. *J. Diabetes Complications*. 20:262–272. doi:10.1016/j.jdiacomp.2005.08.001

Motoyama, N., F. Wang, K.A. Roth, H. Sawa, K. Nakayama, K. Nakayama, I. Negishi, S. Senju, Q. Zhang, S. Fujii, et al. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*. 267:1506–1510. doi:10.1126/science.7878471

Neubauer, H., A. Cumano, M. Müller, H. Wu, U. Huffstadt, and K. Pfeffer. 1998. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell*. 93:397–409. doi:10.1016/S0092-8674(00)81168-X

Nguyen, K.T., P. Tajmir, C.H. Lin, N. Liadis, X.D. Zhu, M. Eweida, G. Tolasa-Karaman, F. Cai, R. Wang, T. Kitamura, et al. 2006. Essential role of Pten in body size determination and pancreatic β-cell homeostasis in vivo. *Mol. Cell. Biol*. 26:4511–4518. doi:10.1128/MCB.00238-06

Postic, C., M. Shiota, K.D. Niswender, T.L. Jetton, Y. Chen, J.M. Moates, K.D. Shelton, J. Lindner, A.D. Cherrington, and M.A. Magnuson. 1999. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J. Biol. Chem*. 274:305–315. doi:10.1074/jbc.274.1.305

Quelle, F.W., D. Wang, T. Nosaka, W.E. Thierfelder, D. Stravopodis, Y. Weinstein, and J.N. Ihle. 1996. Erythropoietin induces activation of Stat5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. *Mol. Cell. Biol*. 16:1622–1631.

Rhodes, C.J. 2005. Type 2 diabetes-a matter of β-cell life and death? *Science*. 307:380–384. doi:10.1126/science.1104345

Ribatti, D., A. Vacca, A.M. Roccaro, E. Crivellato, and M. Presta. 2003. Erythropoietin as an angiogenic factor. *Eur. J. Clin. Invest*. 33:891–896. doi:10.1046/j.1365-2362.2003.01245.x

Savino, C., R. Pedotti, F. Baggi, F. Ubiali, B. Gallo, S. Nava, P. Bigini, S. Barbera, E. Fumagalli, T. Mennini, et al. 2006. Delayed administration of erythropoietin and its non-erythropoietic derivatives ameliorates chronic murine autoimmune encephalomyelitis. *J. Neuroimmunol*. 172:27–37. doi:10.1016/j.jneuroim.2005.10.016

Silva, M., A. Benito, C. Sanz, F. Prosper, D. Ekhterae, G. Nuñez, and J.L. Fernandez-Luna. 1999. Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. *J. Biol. Chem*. 274:22165–22169. doi:10.1074/jbc.274.32.22165

Sorenson, R.L., and L.E. Stout. 1995. Prolactin receptors and JAK2 in islets of Langerhans: an immunohistochemical analysis. *Endocrinology*. 136:4092–4098. doi:10.1210/en.136.9.4092

Stiles, B.L., C. Kuralwalla-Martinez, W. Guo, C. Gregorian, Y. Wang, J. Tian, M.A. Magnuson, and H. Wu. 2006. Selective deletion of Pten in pancreatic beta cells leads to increased islet mass and resistance to STZ-induced diabetes. *Mol. Cell. Biol*. 26:2772–2781. doi:10.1128/MCB.26.7.2772-2781.2006

Thomas, M.C. 2006. The high prevalence of anemia in diabetes is linked to functional erythropoietin deficiency. *Semin. Nephrol*. 26:275–282. doi:10.1016/j.semnephrol.2006.05.003

Tiemann, K., R. Panienka, and G. Klöppel. 2007. Expression of transcription factors and precursor cell markers during regeneration of β cells in pancreata of rats treated with streptozotocin. *Virchows Arch*. 450:261–266. doi:10.1007/s00428-006-0349-4

Tsai, P.T., J.J. Ohab, N. Kertesz, M. Groszer, C. Matter, J. Gao, X. Liu, H. Wu, and S.T. Carmichael. 2006. A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. *J. Neurosci.* 26:1269–1274. doi:10.1523/JNEUROSCI.4480-05.2006

Umemura, T., K. Umene, H. Takahira, N. Takeichi, M. Katsuno, Y. Fukumaki, J. Nishimura, Y. Sakaki, and H. Ibayashi. 1988. Hematopoietic growth factors (BPA and Epo) induce the expressions of c-myc and c-fos proto-oncogenes in normal human erythroid progenitors. *Leuk. Res.* 12:187–194. doi:10.1016/0145-2126(88)90135-X

Wagner, K.U., A. Krempler, A.A. Triplett, Y. Qi, N.M. George, J. Zhu, and H. Rui. 2004. Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice. *Mol. Cell. Biol.* 24:5510–5520. doi:10.1128/MCB.24.12.5510-5520.2004

Westenfelder, C., D.L. Biddle, and R.L. Baranowski. 1999. Human, rat, and mouse kidney cells express functional erythropoietin receptors. *Kidney Int.* 55:808–820. doi:10.1046/j.1523-1755.1999.055003808.x

Wijesekara, N., D. Konrad, M. Eweida, C. Jefferies, N. Liadis, A. Giacca, M. Crackower, A. Suzuki, T.W. Mak, C.R. Kahn, et al. 2005. Muscle-specific Pten deletion protects against insulin resistance and diabetes. *Mol. Cell. Biol.* 25:1135–1145. doi:10.1128/MCB.25.3.1135-1145.2005

Wu, H., X. Liu, R. Jaenisch, and H.F. Lodish. 1995. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell.* 83:59–67. doi:10.1016/0092-8674(95)90234-1

Yasuda, Y., S. Masuda, M. Chikuma, K. Inoue, M. Nagao, and R. Sasaki. 1998. Estrogen-dependent production of erythropoietin in uterus and its implication in uterine angiogenesis. *J. Biol. Chem.* 273:25381–25387. doi:10.1074/jbc.273.39.25381