

Hepatocyte Growth Factor Enhances Engraftment and Function of Nonhuman Primate Islets

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OBJECTIVE—Adenoviral delivery of hepatocyte growth factor (HGF) to rodent islets improves islet graft survival and function, markedly reducing the number of islets required to achieve glucose control. Here, we asked whether these prior observations in rodent models extend to nonhuman primate (NHP) islets.

RESEARCH DESIGN AND METHODS—NHP islets were transduced with murine (Ad.mHGF) or human (Ad.hHGF) adenoviral HGF (Ad.HGF) at low multiplicity of infection and studied in vitro. To study the function of Ad.HGF-transduced NHP islets in vivo, a renal subcapsular marginal mass islet transplant model was developed in streptozotocin-induced diabetic NOD-SCID mice.

RESULTS—Baseline glucose values were 454.7 ± 11.3 mg/dl ($n = 7$). Transplant of 500 NHP islet equivalents (IE) had only a marginal effect on blood glucose (369.1 ± 9.7 mg/dl, $n = 5$). In striking contrast, 500 NHP IE transduced with Ad.mHGF promptly and continuously corrected blood glucose (142.0 ± 6.2 mg/dl, $n = 7$) for the 6-week duration of the experiment. Unilateral nephrectomy resulted in an immediate return of glucose to baseline diabetic levels. Interestingly, adenoviral DNA, as well as mouse HGF (mHGF) mRNA derived from the adenovirus, were present for 42 days posttransplantation. Surprisingly, transplant of 500 IE with Ad.hHGF, as compared with Ad.mHGF, resulted in only marginal correction of blood glucose, suggesting that human HGF is less efficient than mHGF in this system.

CONCLUSIONS—These studies demonstrate that mHGF markedly improves islet transplant outcomes in the highest preclinical species examined to date. HGF has promise as an agent that can improve islet mass and function in transplant models and likely in other models of types 1 and 2 diabetes. *Diabetes* 57: 2745–2754, 2008

Hepatocyte growth factor (HGF) is a mesenchyme-derived protein originally identified as a circulating factor involved in liver regeneration (1–7). It is now recognized as a widely expressed mitogen, morphogen, and motogen with actions on many organs, including the liver, kidney, brain, and islets (1–7). Mature HGF is a heterodimeric protein composed of a 69-kDa α -chain and a 34-kDa β -chain. This

mature HGF is generated by cleavage by extracellular proteases of the inactive pro-HGF precursor to the active HGF heterodimer.

Hayek and colleagues (1,2) have shown that HGF is a potent mitogen for fetal and adult human β -cells in vitro. We and others have shown that HGF is a β -cell mitogen in vivo and is also a β -cell survival factor (3–7). Transgenic or viral overexpression of HGF in murine or rat β -cells results in an increased quantity of engrafted β -cells and β -cell proliferation. Moreover, HGF upregulates GLUT-2, glucokinase, and insulin gene expression in β -cells in vivo (4). As a result of these events, HGF-overexpressing islets sense glucose and secrete insulin in a fashion superior to normal islets (3,4), and β -cell-targeted HGF transgenic mice develop mild hypoglycemia (3,4). Moreover, using two different preclinical models, the SCID mouse and the “Edmonton rat,” we have shown that adenoviral delivery of HGF to the mouse and rat islets markedly enhances transplant performance and reduces the number of islets required for successful islet transplantation (5,6). Finally, HGF decreases streptozotocin (STZ)-induced β -cell death in RIP-HGF mice (4), and adenoviral-mediated HGF transfer into normal mouse islets improves graft survival (5).

In light of the promise of HGF as a therapeutic agent, we have now examined the efficacy and safety of HGF in a higher-level preclinical model, using nonhuman primate (NHP) islets transplanted into NOD-SCID mice. The results clearly indicate that NHP islets overexpressing mouse HGF (mHGF) function more effectively than normal islets. These anticipated outcomes were accompanied by three unexpected observations: 1) HGF overexpression and enhanced β -cell engraftment were not associated with increases in β -cell proliferation, 2) abundant adenovirus expression persisted the full 6 weeks of the experiment, and 3) murine HGF was superior to human HGF (hHGF) in this system. These results extend the efficacy of HGF from rodent islets to the primate level and clearly demonstrate that HGF should be explored as a therapeutic agent in human diabetes.

RESEARCH DESIGN AND METHODS

Generation of recombinant adenovirus. Adenoviruses encoding green fluorescent protein (GFP), β -galactosidase (LacZ), and mHGF and hHGF were prepared as described previously (5,6,8,9). Multiplicity of infection (MOI) was determined using optical density (OD_{260}) and plaque assay. MOI calculations assumed 1,000 cells per islet equivalent (IE; 1 IE = 125- μ m-diameter islet). NHP islets were maintained in culture for 24–48 h before they were transduced for 1 h with 250–500 MOI of the adenovirus, as previously described in detail (5,6,8,9) and in Fig. 2. Uninfected and infected islets were used 24–48 h after infection, as indicated in the Figures. To determine the efficiency of adenoviral transduction, relative semiquantitative PCR analysis and immunoblots were performed using standard methods as described previously (5,6,8,9).

NHP islet isolation. NHP islets from cynomolgus donors (*Macaca fascicularis*, aged 3–10 years) were isolated at the University of Miami using modifications (10) of the automated method for human islet isolation (11). All

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procedures were approved by the institutional animal care and use committee of the University of Miami. Isolated islets were shipped in CMRL media overnight to the University of Pittsburgh.

Insulin content and glucose-stimulated insulin secretion were measured as described previously (3–6,9). Results are expressed as a percentage of insulin concentration per IE obtained with uninfected islets at 5 mmol/l glucose.

Islet transplantation. Transduced and nontransduced NHP islets were transplanted under the kidney capsule of STZ-induced diabetic NOD-SCID mice as described previously (4,5). Briefly, NOD-SCID mice were rendered diabetic by injecting twice within 48 h with 150 mg/kg body wt i.p. STZ. Diabetes was confirmed by the presence of hyperglycemia (>300 mg/dl), polyuria, and weight loss. Random nonfasted blood glucose was measured from the snipped tail using a Precision Q.I.D. portable glucometer. After 3 consecutive days of hyperglycemia, diabetic NOD-SCID mice were transplanted under the left kidney capsule with 500, 1,000, or 2,000 IE 24 h after transduction as described in the figures. Blood glucose levels were measured at days 3, 5, and 7 and then weekly until unilateral nephrectomy at day 42. All studies were performed with the approval of, and in accordance with, guidelines established by the University of Pittsburgh institutional animal care and use committee.

Insulin immunohistochemistry. For insulin immunohistochemistry, kidneys were fixed at day 42 after transplantation in Bouin's solution, paraffin embedded, sectioned, and stained with anti-insulin antibody (Zymed, San Francisco, CA) at 1:75 dilution. Visualization was achieved using the avidin-biotin immunoperoxidase complex system (3–6,9). Sections were counterstained using hematoxylin.

Quantification of transplanted NHP β -cell proliferation and cell death. To assess NHP β -cell proliferation *in vivo*, animals were injected with bromodeoxyuridine (BrdU) (3–6,9,12,13), and graft-containing kidneys were harvested on the 5th day after transplantation at 6 h after BrdU injection, fixed in 4% paraformaldehyde overnight at 4°C, paraffin embedded, and sectioned. In the graft-containing kidneys, three serial sections separated by 25 μ m were deparaffinized, rehydrated, treated in prewarmed 1 mmol/l HCl for 1 h at 37°C, blocked in 2% BSA/PBS for 1 h, and then incubated overnight with anti-BrdU (Abcam, Cambridge, MA) and anti-insulin antibodies (Zymed). After serial washing in PBS containing Tween, slides were incubated for 1 h with secondary antibodies, gel mounted, and coverslipped. Two sections per graft were analyzed. Three fields per section were quantified for BrdU- and insulin-positive cells. To assess β -cell survival, identical procedures were followed, and sections from the same paraffin blocks were costained for insulin and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). The percent of TUNEL- and insulin-positive nuclei were quantified as described previously (14) and as described in the legend to Fig. 7.

Adenovirus persistence *in vitro* and *in vivo*. For the *in vitro* studies, uninfected and infected islets were harvested 24 and 48 h after infection and were frozen at –80°C until extraction. For the *in vivo* studies, the left kidneys containing the NHP islet graft and the right (nontransplanted) kidneys were harvested on days 1, 7, 14, 28, and 42 and were frozen at –80°C until extraction. RNA and DNA extraction were performed using Trizol (Invitrogen, Carlsbad, CA). RNA samples were treated with DNase to prevent DNA contamination. PCR for adenoviral mouse HGF was performed with the oligonucleotides described in Fig. 5. As a control to insure that no contaminated DNA was amplified, PCR was performed on RNA samples treated with DNase with no previous reverse transcription.

Mitogen-activated protein kinase assays. Total p42 and p44 extracellular signal-regulated kinase (ERK), and their phosphorylated counterparts, were examined using extracts of primary cultures of mouse arterial smooth muscle treated with mouse HGF (R&D Systems, Minneapolis, MN) or hHGF (RDI, Concord, MA) at doses of 25 μ g/ml as we have described in detail previously (15) and as described in the legend to Fig. 8. Murine primary arterial smooth muscle cultures were prepared and documented to represent arterial smooth muscle cells as described previously (16).

Statistics. Statistical analysis was performed using Student's *t* test. All values are expressed as the means \pm SE. *P* \leq 0.05 was considered significant.

RESULTS

Immunohistochemical characterization of NHP islets.

Rodent islets, as used in prior rat and mouse studies, are predominantly homogeneous collections of β -cells containing a minority of α -, δ -, PP, and ductal cells, as well as endothelial cells and contaminating exocrine cells. To determine the composition of the NHP islets used in the current studies, and to confirm the purity of the preparations we used, we examined the β -cell composition of our

NHP islets, comparing the number of total islet cells, as assessed using DAPI (4',6-diamidino-2-phenylindole) fluorescence, to those that also stained for insulin. As exemplified in Fig. 1, and as described by Cabrera et al. (17), the large majority of islet cells (Fig. 1B and D for Hoechst staining) in the NHP islet preparations were insulin-containing β -cells (Fig. 1A and C). To confirm this subjective impression, 66 islets were counted from five separate NHP islet preparations, comprising 10,590 total cells. Of these 10,590 total islet cells, 7,467 (72.3 \pm 1.9%) were insulin-positive cells, a percentage comparable or superior to typical isolated human islet preparations (Fig. 1E and F) (9,17–20).

Efficiency of transduction with Ad.HGF. We next sought to transduce the NHP islets with adenoviruses encoding mHGF, hHGF, and two control adenoviruses, Ad.GFP and LacZ. As in our prior studies with mouse and rat islets (5,6,9), infection time was 1 h, followed by 24 h of culture, in an effort to mimic conditions that might be used with human islet transplantation. To determine the optimal conditions for the adenoviral transduction of NHP islets that would permit overexpression without adversely affecting the insulin release, we examined glucose-stimulated insulin secretion 24 h after infection. As shown in Fig. 2A, transducing NHP islets for 1 h at 250 MOI of each of the viruses did not adversely affect the insulin secretory response to glucose. However, exposure of NHP islets to 500 MOI of each of the viruses for 1 h caused mild to markedly attenuated glucose-stimulated insulin secretion, suggesting that, at 500 MOI of adenovirus, NHP β -cell function was impaired.

As can be seen in Fig. 2B and C, 250 MOI of Ad.mHGF and Ad.hHGF followed by 24 h in culture resulted in easily measurable HGF expression at both the mRNA level as well as the protein level. Low levels of HGF were apparent in nontransduced and Ad.lacZ- and Ad.GFP-transduced islets, but the level of overexpression in the Ad.mHGF- and Ad.hHGF-transduced islets was far higher, perhaps some 10–30 times higher. In addition, Ad.HGF transduction with 250 MOI had no adverse effects on NHP islet insulin content: normal, Ad.LacZ-, Ad.mHGF- and Ad.hHGF-transduced islets all contained comparable quantities of insulin, ranging from ~10 to 12 ng/IE. Therefore, infection of NHP islets for 1 h at 250 MOI was selected as optimal for transducing NHP islets in subsequent experiments.

Adenoviral-mediated mHGF transfer into NHP islets enhances islet transplantation. Our previous studies had shown that mHGF overexpression improves islet transplant outcomes in two mouse models and a rat model of islet transplantation (4–6). The principal goal of the current study was to determine whether this applies to islets of higher species. We first needed to define a fully therapeutic as well as a marginal mass of NHP islets in NOD-SCID mice. Sham-transplanted mice remained severely hyperglycemic throughout the study (Fig. 3A). In contrast, mice transplanted with 500 IE achieved borderline improvement in blood glucose, and 1,000 IE or 2,000 IE was sufficient to achieve stable euglycemia. Therefore, we selected 500 IE as the marginal mass. To determine whether adenoviral infection influenced NHP islet function or engraftment, we also studied 500 NHP IE transduced with Ad.LacZ. As shown in Fig. 3B, these behaved comparably with 500 nontransduced normal NHP islets. In marked contrast to these controls, 500 IE infected with Ad.mHGF were able to reduce blood glucose concentrations to the near-normal postprandial range (Fig. 3B).

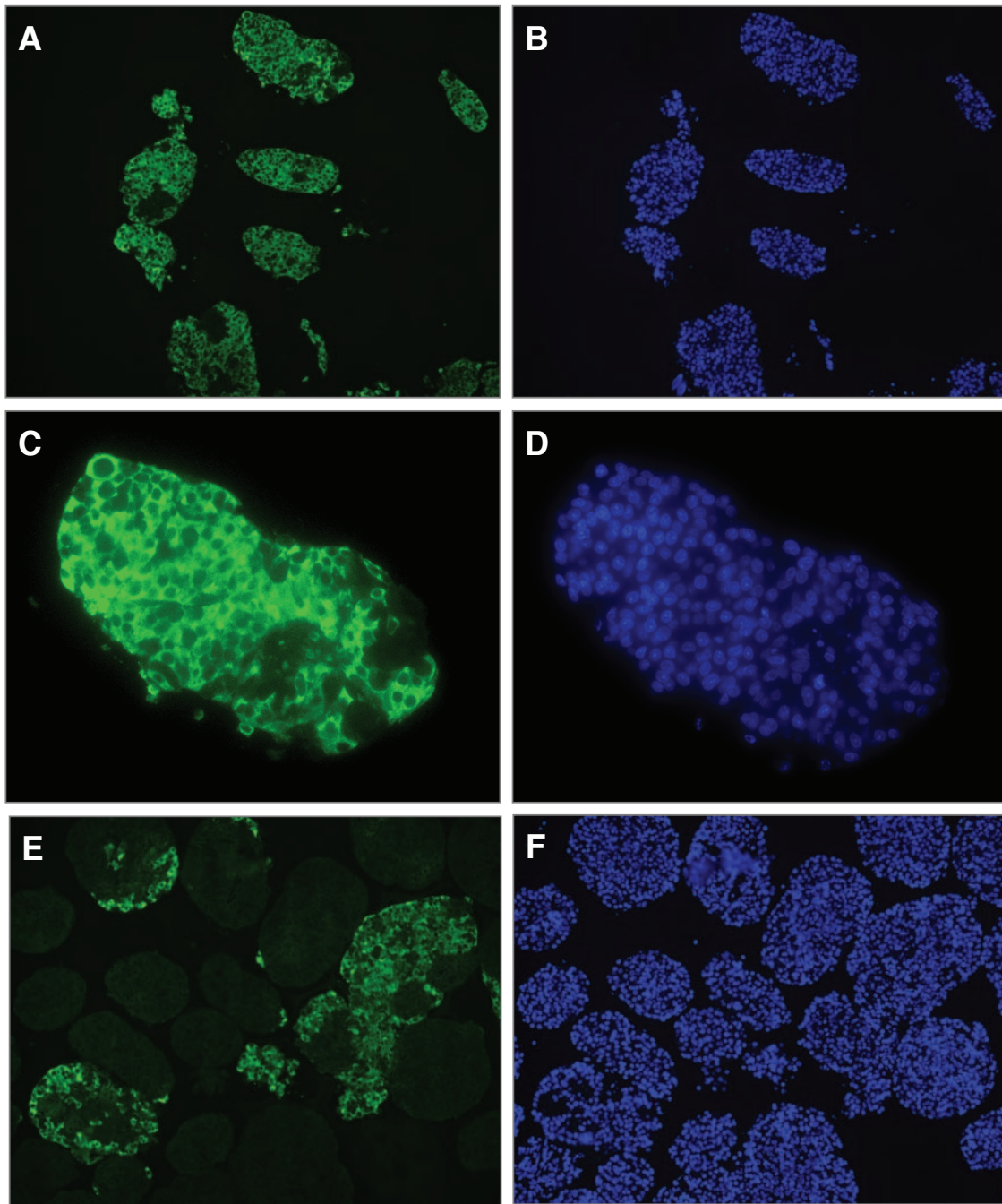


FIG. 1. β -Cell composition in NHP islets. *A–D*: Insulin immunofluorescence (green) and Hoechst (blue) staining in NHP islets. *E* and *F*: Insulin immunofluorescence (green) and Hoechst (blue) staining in human islets. Note that the large majority of NHP islets contain $\sim 70\%$ β -cells, which compares very favorably with isolated human islets (9,17,18,20). (Please see <http://dx.doi.org/10.2337/db08-1085> for a high-quality representation of this figure.)

These findings clearly demonstrate that Ad.mHGF enhances NHP islet engraftment and function.

The left kidney bearing the NHP graft was removed 42 days after transplantation. As shown in Fig. 3*B*, blood glucose levels rapidly returned to the pretransplantation values, indicating that the NHP islets engrafted under the kidney capsule were responsible for the euglycemia observed in these mice.

Immunohistochemical analysis of kidneys containing the grafts obtained at 42 days (Fig. 4) revealed the presence of abundant insulin-positive cells in each kidney examined from six of the seven Ad.mHGF mice. In contrast, insulin-positive cells were not identified in any of the

10 kidneys from mice transplanted with Ad.LacZ-transduced or uninfected islets.

Adenoviral mHGF DNA and mRNA persist up to 42 days posttransplantation. The preceding data suggested that gene delivery of mHGF improves NHP islet transplantation outcome and that this effect is sustained for 42 days. Therefore, we wanted to determine whether this was associated with long-term expression of viral mHGF DNA and mRNA. To address this question, we designed primers that specifically recognize adenoviral mHGF DNA but not murine host mHGF DNA or NHP HGF DNA (Fig. 5*A*). As shown in Fig. 5*B*, adenoviral mHGF DNA was easily detectable 48 h after infection *in vitro*. Similarly, adenovi-

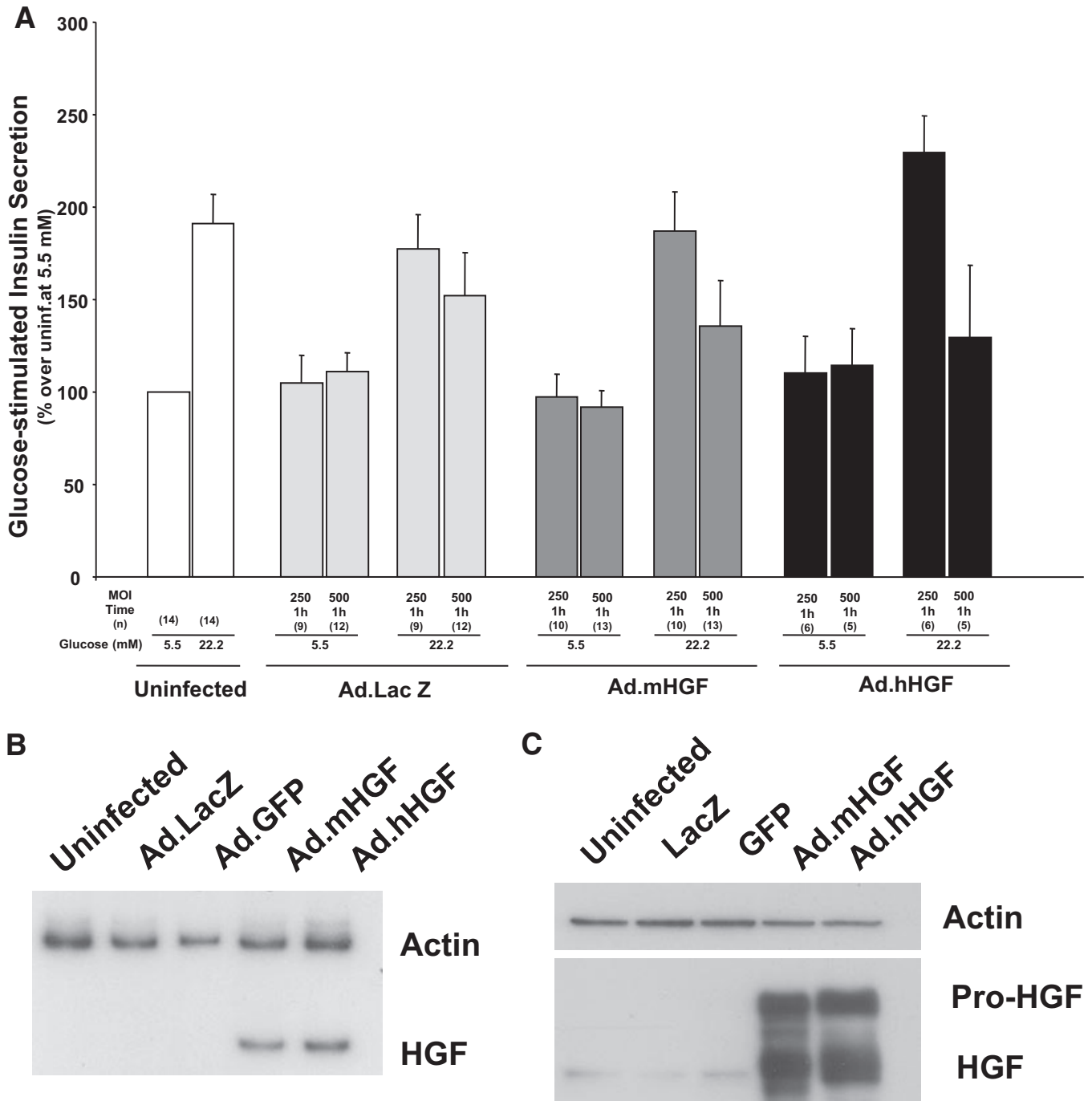


FIG. 2. Glucose-stimulated insulin secretion and HGF expression in nontransduced and transduced NHP islets. **A:** NHP islets were exposed to 250–500 MOI for 1 h. At 24 h after transduction, groups of 15 IE of similar size were incubated with 5 or 22 mmol/l glucose for 30 min, and insulin secreted into the media was measured by radioimmunoassay. Results are the means \pm SE of 5–13 experiments performed in triplicate. The data are presented as the percentage above the insulin secreted by uninfected islets (100%). **B** and **C:** HGF expression as assessed by RT-PCR (**B**) and Western blot (**C**) analysis of RNA extracts and protein extracts obtained from nontransduced NHP islets and islets transduced with 250 MOI of Ad.LacZ, Ad.GFP, Ad.mHGF, or Ad.hHGF. At 24 h after transduction, islets were harvested and mRNA and protein were extracted. For the RT-PCR, primers were specifically designed to amplify either mHGF or hHGF cDNA, as appropriate. HGF was detected using an antibody recognizing both mHGF and hHGF. Actin was used as an internal control for loading. Note that both Ad.mHGF and Ad.hHGF were able to transduce NHP islets equally as assessed by RT-PCR and Western blot.

ral mHGF DNA was clearly abundant for the entire 42 days in the kidneys that received the graft (Fig. 5C). The presence of the NHP islet graft was confirmed using primers specific for NHP actin. As expected, contralateral kidneys that did not receive any NHP islet grafts were negative for the presence of mHGF and NHP actin DNA. Importantly, as shown in Fig. 5D and E, similar results were obtained for the adenoviral mHGF mRNA expres-

sion, indicating not only that the Ad.mHGF virus was present but that it also was functional for the duration of the study.

Adenovirus-mediated mHGF transfer into NHP islets did not induce β -cell proliferation. HGF has been shown to stimulate β -cell proliferation when added in vitro (1,2) and in vivo in the RIP-HGF transgenic mouse (3). We therefore assumed that mHGF would cause increased

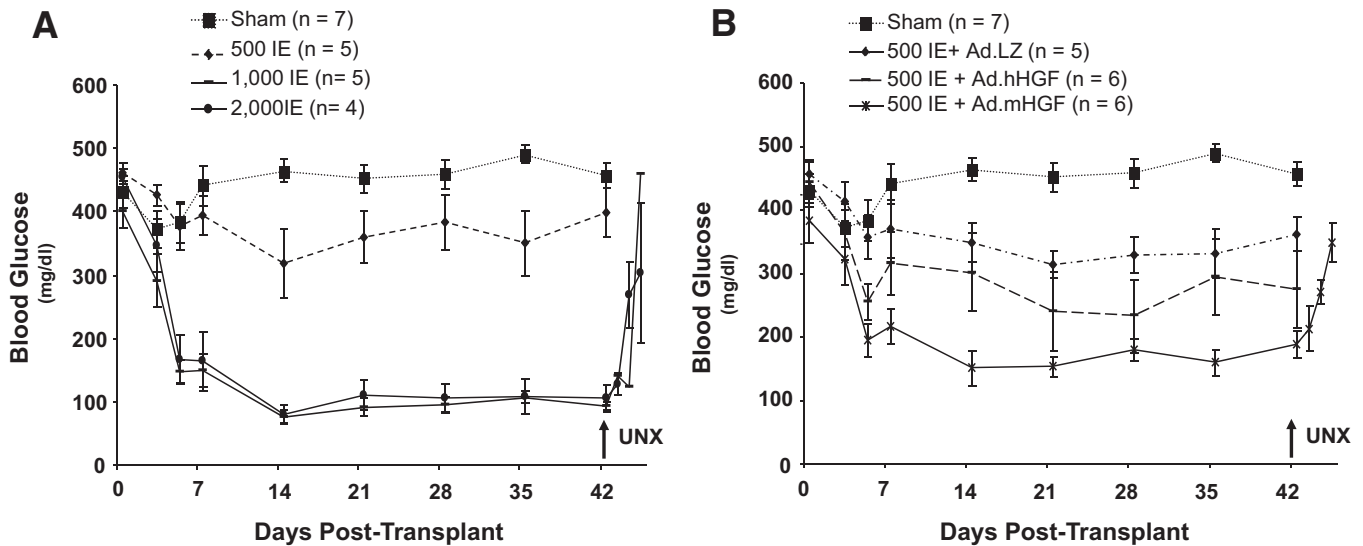


FIG. 3. Blood glucose levels in STZ-induced diabetic NOD-SCID mice transplanted with uninfected or infected NHP islets. **A:** Defining the NHP marginal mass. At 24 h after mock transduction, 500, 1,000, or 2,000 IE of NHP islets were transplanted under the kidney capsule of STZ-induced NOD-SCID mice. Identical surgery was performed on the sham-operated animals, except that they received saline instead of islets. Results are the means \pm SE of four to seven animals per condition. At day 42, unilateral nephrectomy (UNX) was performed in mice transplanted with either 1,000 or 2,000 control IE or with 500 Ad.mHGF IE, and the blood glucose returned immediately to pretransplant diabetic levels. **B:** Effect of Ad.HGF on NHP islet transplant outcome. At 24 h after transduction, 500 Ad.LacZ, Ad.mHGF, or Ad.hHGF IE were transplanted under the kidney capsule of STZ-induced NOD-SCID mice. Results are the means \pm SE of five to seven animals per condition.

proliferation as assessed using BrdU incorporation in NHP islets *in vivo*, in a transplant setting, as it does *in vitro* or when overexpressed under the RIP promoter. Figure 6 shows unexpectedly that Ad.mHGF-transduced NHP β -cells did not proliferate at a higher rate than uninfected NHP β -cells, at least at the 3-, 5-, and 10-day time points after transplantation.

HGF enhances β -cell survival *in vivo*. To determine the effects of HGF on β -cell survival, TUNEL and insulin costaining were performed both on isolated islets transduced with Ad.mHGF as well as on islet grafts from transplanted mice 3 days after transplantation. As can be seen in Fig. 7A and B, β -cell death rates *in vitro* were lower in Ad.mHGF-transduced islets than in controls, and this was also true of islet grafts *in vivo* (Fig. 7C and D).

Ad.hHGF is less effective than Ad.mHGF in NHP islets. From immunological and therapeutic points of view, it seemed preferable a priori to use a human version of HGF in NHP human islet transplantation. On the other hand, human and mouse HGF share a 93% homology at the amino acid level and might be presumed to interact with the c-Met receptor interchangeably. To determine whether hHGF might be superior in efficacy to mHGF in enhancing

NHP islet transplant outcomes, we prepared an adenovirus encoding hHGF. As shown in Fig. 2B and C, both Ad.mHGF and Ad.hHGF transduced NHP islets with comparable efficiency, as assessed by RT-PCR and Western blotting. Surprisingly, however, as shown in Fig. 3B, 500 NHP IE transduced with Ad.hHGF failed to reduce blood glucose nearly as effectively as 500 NHP IE transduced with Ad.mHGF. These observations indicate that Ad.hHGF is unexpectedly less effective than Ad.mHGF for improving NHP islet transplant outcomes in this murine recipient model.

In a preliminary effort to explore the mechanisms responsible for the superior performance of mHGF compared with hHGF in this system, we examined the ability of recombinant mHGF and hHGF proteins to stimulate p42 and p44 ERK phosphorylation in NHP islets and in primary murine vascular smooth muscle cells. Whereas both peptides failed to elicit significantly different p42/44 ERK phosphorylation in NHP Islets (not shown), mHGF was substantially more effective in stimulating ERK phosphorylation in murine arterial smooth muscle cells (Fig. 8). These observations suggest that, at least in part, some of the beneficial effects of HGF may be mediated via host murine arterial or endothelial cells or their precursors, rather than on transplanted islet cells, and that mHGF may be more effective than hHGF in this regard.

DISCUSSION

Whereas transgenic and viral overexpression of HGF in rodent β -cells markedly improves β -cell proliferation, survival, and function in three different *in vivo* rodent models (3–6), a necessary intermediate step toward bringing HGF to human clinical use is demonstrating efficacy in a species higher in the phylogenetic tree than rodents. Here, we demonstrate for the first time that HGF improves the quantity of engrafted β -cells as well as their function in the highest preclinical species, the NHP. These studies are significant, for they demonstrate that the efficacy of HGF clearly merits further study as a therapeutic agent for

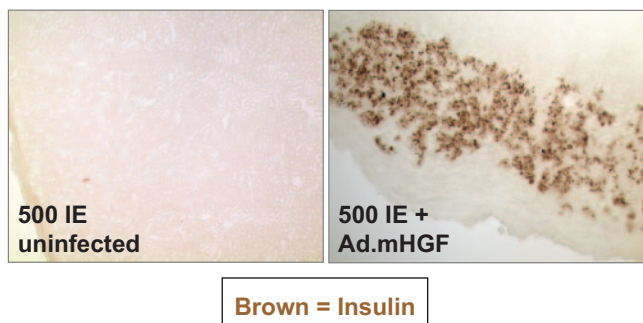


FIG. 4. Islet graft histology 42 days after transplantation. Representative photomicrographs of kidney sections containing the grafts at day 42 after transplant and staining for insulin. (Please see <http://dx.doi.org/10.2337/db08-1085> for a high-quality representation of this figure.)

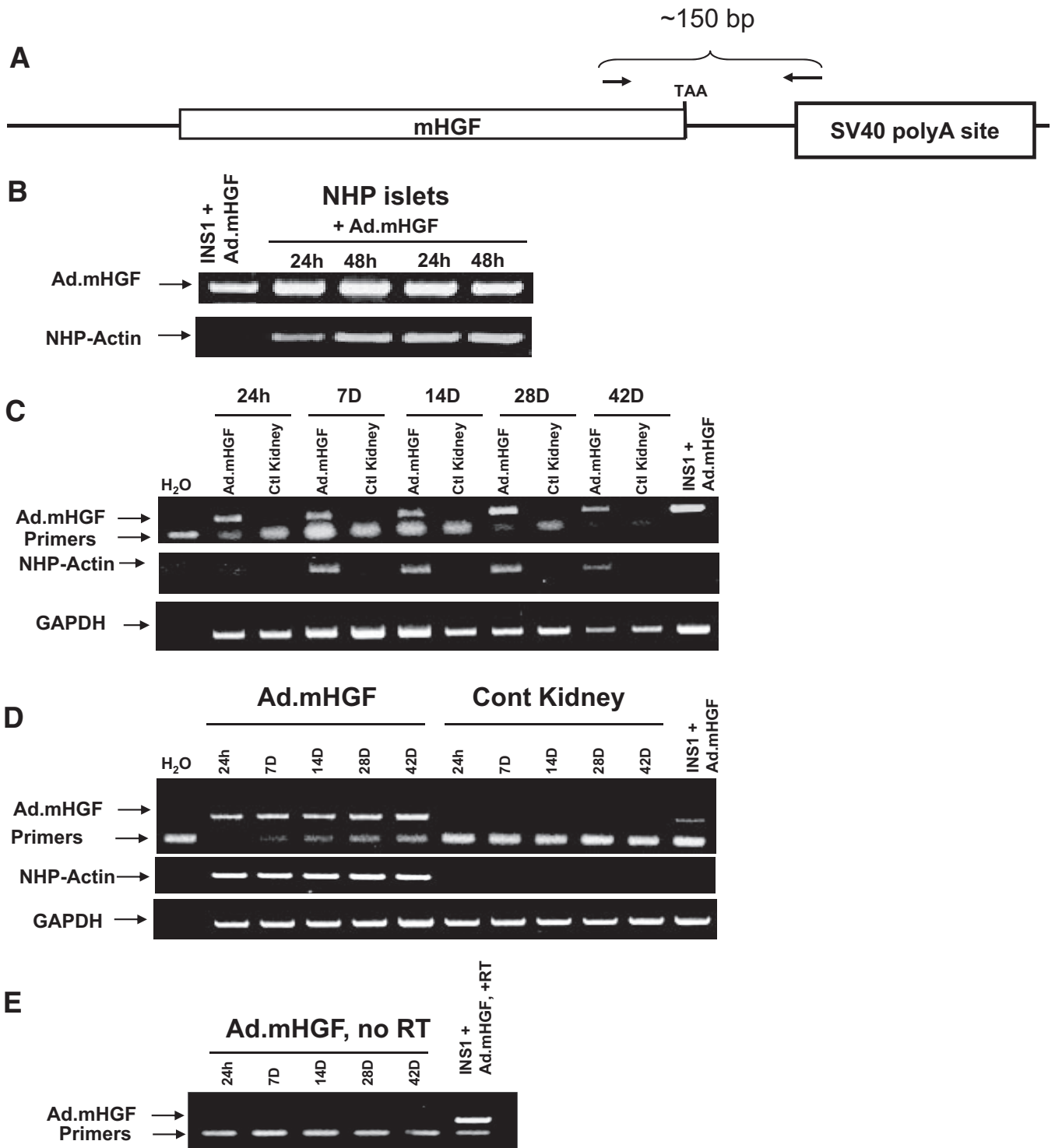


FIG. 5. Adenoviral persistence in islet grafts after transplantation. **A:** Viral constructs and primers. The primers were designed to specifically amplify only mHGF DNA derived from the adenovirus. Primers used were 5'-GCC ATG CCA AAT CGT CCT GG-3' and reverse 3'-GTA GTT TGT CCA ATT ATG TCA CAC CAC-5' for Ad.mHGF and 5'-CAT TCC AAA TAT GAG ATG CAT TG-3' and reverse 5'-TAA AAA AGT ATT AAG GCG AAG ATT A-3' for NHP actin. **B:** Persistence of viral DNA in vitro. NHP islets transduced with Ad.mHGF at 250 MOI were harvested 24 h after transduction. INS1 cells were transduced with Ad.mHGF at 1,000 MOI for 1 h and were used as a positive control. Note that NHP actin is only present in NHP islets, but not in INS1 cells. **C:** Assessment of viral DNA in vivo. Kidneys bearing the NHP islet graft transduced with Ad.mHGF, and contralateral kidneys containing no islet graft were harvested 24 h and 7, 14, 28, and 42 days after transplant. DNA and RNA were extracted. INS1 cells infected with Ad.mHGF were used as positive controls. Note that the adenoviral DNA was still abundant 42 days after transplant in kidneys bearing the graft. The presence of the graft was confirmed by the presence of NHP actin. Kidneys with no islet grafts were negative for mouse HGF and NHP actin but were positive for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **D:** Assessment of viral mRNA in vivo. The presence of viral mouse HGF and NHP actin mRNAs was confirmed in kidneys bearing the NHP grafts, whereas contralateral kidneys were negative. INS1 cells transduced with Ad.mHGF were used as positive controls. **E:** Control PCR from mRNA samples used in **D** with no reverse transcriptase (RT). To confirm that no genomic DNA contaminated the samples used in **D**, PCR was performed on the same samples used in **D** but with no reverse transcriptase. INS1 cells transduced with Ad.mHGF were used as a positive control. RT was performed before the PCR. Note that only the INS1 cells were positive, confirming that samples used in **D** were free of contaminating DNA.

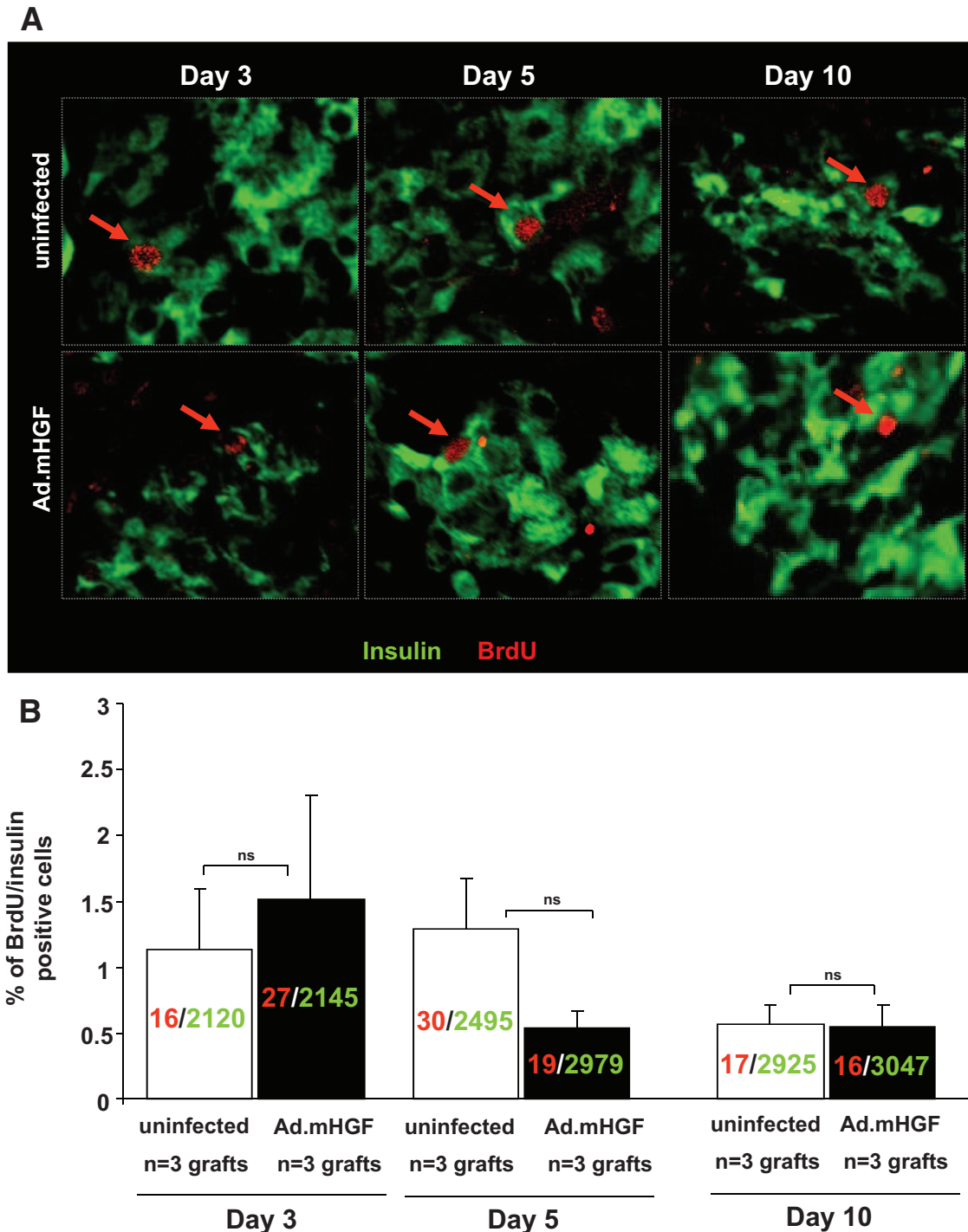


FIG. 6. β -Cell proliferation and insulin content in islet grafts 5 days after transplant. **A:** Representative photomicrographs of renal subcapsular grafts obtained at days 3, 5, and 10 after transplant in control islet grafts or islet grafts transduced with Ad.mHGF showing BrdU (red) and insulin (green) staining. Arrowheads indicate BrdU-positive cells. **B:** Quantification of BrdU proliferation in the islet grafts. The numbers in green indicate the numbers of β -cells counted, and those in red, the number of BrdU-positive cells. There were no statistically significant differences between Ad.mHGF and control islet grafts. Results are the means \pm SE. Three different grafts per condition were examined. (Please see <http://dx.doi.org/10.2337/db08-1085> for a high-quality representation of this figure.)

expanding β -cell mass and function in humans with diabetes.

In some senses, the results observed might have been anticipated, based on the efficacy of HGF in rat and mouse islets. On the other hand, there were three major unanticipated surprises in the current studies. First, HGF has been shown to drive replication in mouse, rat, as well as fetal, neonatal, and adult human β -cells (1–3). Given the strikingly higher quantity of engrafted β -cells in the HGF-

treated grafts (Fig. 4), we had anticipated that β -cell replication would have been increased. On the other hand, HGF has well-documented salutary effects on β -cell survival as well as β -cell function, increasing glucokinase, GLUT-2, and insulin gene expression (4–6), and each of these might have contributed to the increase in the quantity of engrafted β -cells and/or β -cell function. Indeed, HGF delivery also resulted in a significant decrease in NHP β -cell death, both in vitro as well as in vivo (Fig. 7). It is

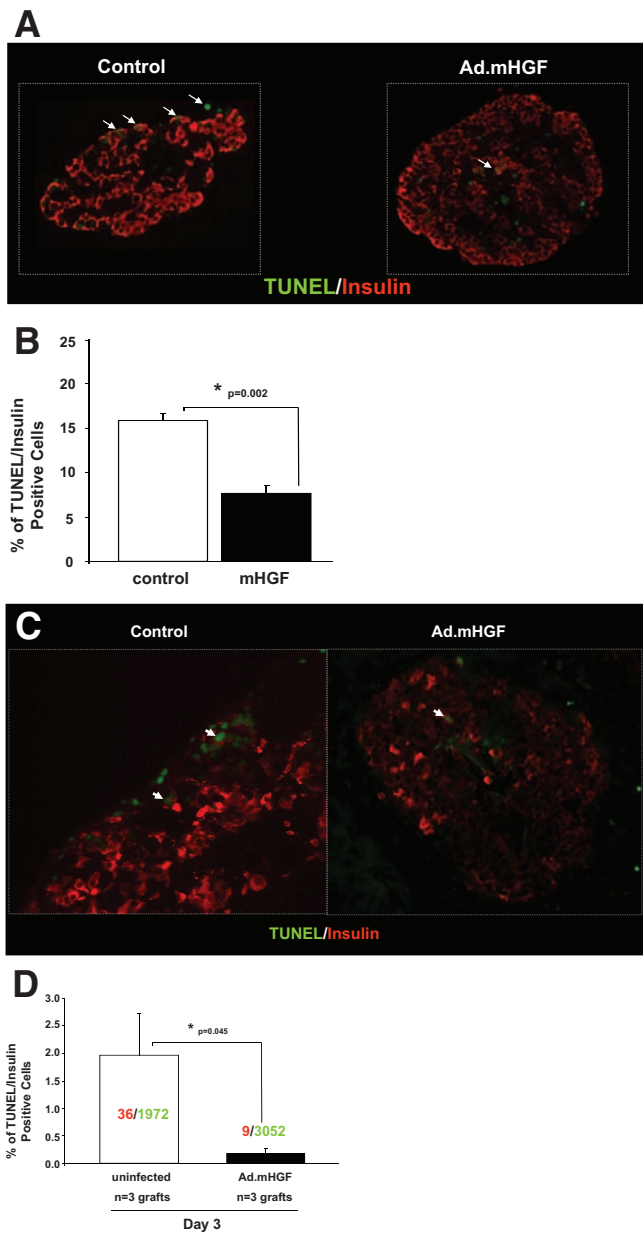


FIG. 7. Cell death rates in Ad.mHGF-treated islets in vitro and in vivo. **A:** Representative photomicrographs of TUNEL staining in vitro in isolated control islets or those transduced with Ad.mHGF 48 h previously. Insulin is shown in red and TUNEL staining in green. Arrows indicate examples of TUNEL-positive β -cells. **B:** Quantification of TUNEL-positive, insulin-positive cells. The numbers indicate the numbers of cells counted as in Fig. 6. Bars indicate SE. **C:** Examples of TUNEL-positive β -cells in islet grafts 3 days after transduction and transplantation. Arrows indicate examples of TUNEL-positive β -cells. **D:** Quantitation of TUNEL-positive/insulin-positive cells in islet grafts in vivo. These observations demonstrate that Ad.mHGF treatment reduces cell death in vitro and in vivo. hHGF, human HGF; mHGF, mouse HGF. (Please see <http://dx.doi.org/10.2337/db08-1085> for a high-quality representation of this figure.)

also plausible that β -cell replication did contribute to the increased quantity of engrafted β -cells but that this occurred earlier or later than the 3- to 10-day time points examined here. Although these are critically important mechanistic questions, we believe that they are better addressed using human islets in NOD-SCID mice, for two reasons: 1) additional studies on NHP islets would require the unnecessary death of additional NHP donors; and 2) in the end, answering these questions using human islets is

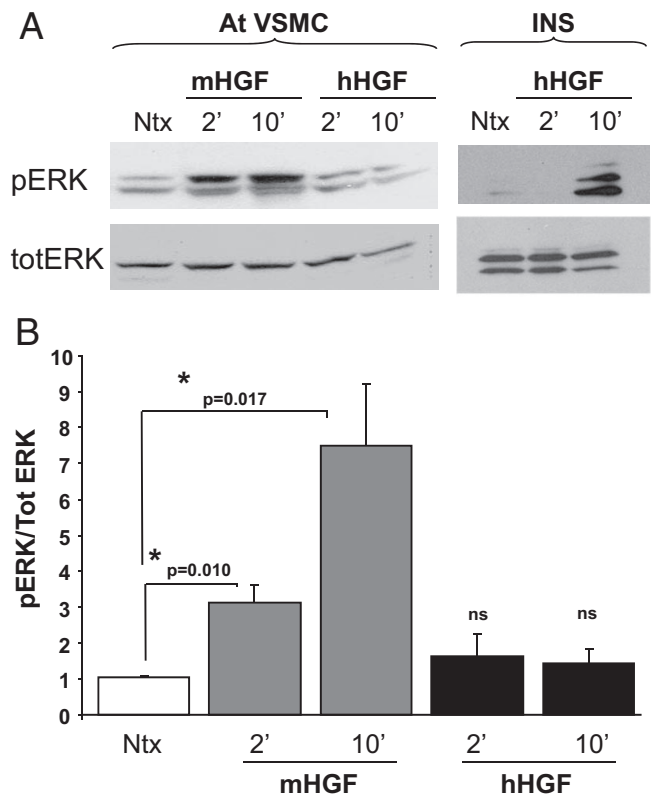


FIG. 8. Differential effects mouse HGF and human HGF on p42/44 mitogen-activated protein kinase activity in primary murine arterial smooth muscle cells. **A:** Effects of human HGF (hHGF) and mouse HGF (mHGF) peptides at 25 ng/ml on total ERK and phospho-ERK in primary cultures of murine aortic smooth muscle cells. Note that human HGF fails to activate mitogen-activated protein kinase in mouse arterial smooth muscle cells, whereas mouse HGF readily activates mitogen-activated protein kinase. To insure that the human HGF was active, it was also assayed in rat INS-1 cells: the panel on the far right demonstrates that the identical human HGF preparation that failed to activate mitogen-activated protein kinase in the vascular smooth muscle cells (VSMC) was robustly active in INS-1 cells. **B:** Densitometric quantification of three experiments as outlined in **A**. These studies demonstrate that whereas the human HGF peptide is active, it is unable to activate mitogen-activated protein kinase in murine arterial smooth muscle cells, in contrast to mouse HGF, which is active in these same cells. NTx, no treatment.

more relevant to human type 1 and type 2 diabetes than additional studies using NHP islets.

The second surprise was that hHGF seems to function less effectively than mHGF in this system, which at first glance more closely approximates a human rather than a murine β -cell transplant model. In theory, this unexpected species preference might have been attributable to differences in efficiency of transduction or expression of the two peptides. This seems an unlikely explanation because both RNA and protein analyses (Fig. 2B and C) suggested that expression of the two peptides was comparable, and, since each peptide is a secreted product, that it would diffuse through an intact islet regardless of the actual percentage of cells transduced. It is also possible that mHGF is a superior agonist compared with hHGF for the NHP HGF receptor, c-Met. Most interestingly, and in our view perhaps most likely a third explanation, it is also possible, as also suggested in Fig. 8, that the target of the HGF in these experiments is not limited to the NHP β -cell c-Met receptor, but also includes murine c-Met receptors on vascular cells, renal cortical cells, etc., in the murine recipient. In support of this possibility, both Dong and colleagues (19) as well as Powers and colleagues (20) have

shown that successful islet engraftment depends on stimulation of angiogenesis and vascular invasion by host vasculature into the grafted islets. In this scenario, graft-derived mHGF could more efficiently drive vascular invasion from the host murine kidney than hHGF, which might be a poor agonist for the murine c-Met receptor in murine kidney and vasculature. Indeed, as shown in Fig. 8, mHGF is superior to hHGF as an agonist for at least one signaling pathway in murine primary arterial smooth muscle cells. Clearly, the relative effects of mHGF and hHGF on vascularization of islet grafts in this diabetic NOD-SCID mouse model deserves additional study. The most relevant answers would come from future studies using either a human-mouse system or a human-human system.

The third surprise is the prolonged duration and apparently high degree of adenovirus expression. We used adenovirus here as a “poor man’s” protein expression system to inexpensively produce and locally deliver hHGF and mHGF to transplanted β -cells *in vitro* and *in vivo*. Now-classic studies in humans with cystic fibrosis and familial hypercholesterolemia have suggested that adenovirus is a poor vector for human gene therapy because of its poor duration of expression and its eliciting of a host immune response (21,22). On the other hand, most studies using adenovirus have used immunocompetent hosts. Here, as in patients with type 1 diabetes who receive islet transplants (18), the hosts lacked a competent immune system. This allowed assessment as to how long adenovirus would persist and remain functional in the absence of immune surveillance. To our surprise, there was little difference in the presence of adenovirus or the expression of adenovirally delivered HGF mRNA between the day after transduction and up to 6 weeks thereafter.

This has both positive and negative implications. On the positive side, it means that adenovirus may be a very reasonable gene therapy vector for pancreatic islets in a transplant setting, where immunosuppression is the norm. In this setting, adenovirus might provide weeks or months of effective gene delivery without the risk of permanent alteration of the host genome, and without the risk of integration into tumor suppressor genes, as has occurred with humans receiving lentiviral gene therapy for severe combined immunodeficiency syndrome (23).

On the negative side, to the extent that oncogenic peptides might be delivered using adenovirus in an immunodeficient setting, one might now need to worry about prolonged expression and the risk of tumor development. In the case of HGF and the β -cell, this seems like a small concern, for lifelong (2 years) expression of HGF in RIP-HGF mice is not associated with tumor development (3,4). Again, to address these questions, studies currently ongoing in human islets are exploring the ultimate duration of expression of adenovirus in human islets in a comparable human islet–NOD-SCID mouse model.

The studies described here used an islet transplant model. However, whereas the results may apply conceptually most easily to islet transplantation, the model is really a laboratory for β -cell proliferation, regeneration, and recovery of β -cell function that applies to all forms of diabetes. It extends far beyond the specific or narrow goal of improving islet transplant outcomes, to type 1 and type 2 diabetes.

The complete absence of β -cells in control islet grafts contrasts with the apparent abundance of β -cells in the Ad.mHGF grafts (Fig. 4). This was a consistent finding; no grafts were identified in any of the 10 control kidneys

examined, but they were easily identified in 6 of 7 Ad.mHGF kidneys examined. We have observed this “all or none” phenomenon previously in transplant experiments with RIP-HGF or Ad.mHGF-treated transplants into STZ-induced diabetic mice and rats (3–6). Coupled with the glycemic data (Fig. 3B), we interpret this to mean that mHGF enhances engraftment, and if one looked at early time points (perhaps 7–14 days), grafts would be present in experimental and control groups, but larger in the HGF groups. When one waits until day 42, as was the case in this study, it is reasonable to hypothesize that chronic hyperglycemia has taken its glucotoxic toll on the control islets and either abolishes them or makes these grafts so small as to be nonidentifiable. These are questions that can be addressed by sampling at multiple time points after transplantation, and we are addressing them in current studies in human islets transplanted/transduced with Ad.HGF.

Recombinant production of therapeutic quantities of complex, posttranslationally processed heterodimeric peptides such as the HGF family is so expensive as to be impractical, particularly when, as unexpectedly illustrated here, the optimal peptide—hHGF versus mHGF—is unknown. Thus, the production of adenoviral constructs encoding several HGF family members is relatively inexpensive, and, as also shown here, permits direct and sustained delivery of the HGF peptide to the β -cells. Whether HGF peptides will ultimately best be deployed by gene delivery or by parenteral injection remains an unsettled question. It may be, for the reasons outlined above, that adenovirus will prove to be the optimal means of HGF delivery in the setting of diabetes and immunosuppression. It is equally possible that injection of HGF peptides, as has been used in rodent models (7), is preferable. Again, these are questions that are most relevantly addressed in studies using human islets, which are underway.

Finally, as has been illustrated so nicely with the epidermal growth factor–gastrin combination (24), it is in theory possible to use HGF peptides in combination with other peptides or small molecules that may enhance β -cell mass and function. For example, combining a peptide that stimulates β -cell replication with one that stimulates β -cell neogenesis may yield synergistic or complimentary effects.

In summary, these studies demonstrate the efficacy of HGF peptides in enhancing the quantity of engrafted β -cells and their function in the highest preclinical species, and they provide strong support for pursuing comparable studies using human islets cells to address the questions summarized above.

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