Increased Engraftment of Hepatic Progenitors After Activation of the Hepatocyte Growth Factor Signaling Pathway by Protein Transduction

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Cell transplantation has become a major focus in biomedical research. However, efficient engraftment in solid tissues remains a challenge. Hepatocyte growth factor (HGF) signaling increases survival, proliferation, migration, and invasion of many cell types through Met, its cell surface receptor. Therefore, activation of this signaling pathway may improve the ability of many cells to be transplanted. We constructed a constitutively activated form of Met (Tpr-Met) fused to the protein transduction domain of HIV-TAT to activate the HGF/Met pathway for a few hours following cell injection. Matrix-assisted refolding was used to renature TAT-Tpr-Met protein, which was efficiently delivered into cells and recapitulated several biological functions of Met *in vitro*. Furthermore, treatment of hepatic progenitors with this molecule for one hour before transplantation significantly improved engraftment efficiency (31% untreated cells, 58% treated cells). These findings suggest that the transient transfer of Tpr-Met may provide a new approach to increase the proportion of successfully engrafted cells. Exp Biol Med 234:1102–1108, 2009

Key words: HGF; TAT; protein transduction; hepatocyte transplantation

Introduction

Cell transplantation in solid organs like the heart, liver, pancreas, or brain is limited by poor engraftment (1). Expression of the hepatocyte growth factor (HGF) by gene delivery can improve the engraftment efficiency of pancreatic islets (2, 3). HGF elicits multiple cellular responses and specifically mediates the regulation of an invasive growth program through Met, its cell surface receptor (4). The signaling pathways involved in Met-dependent biological responses induce survival, proliferation, migration, and invasion in many cell types (5). Although HGF/Met signaling is an effective way to improve the ability of cells to survive transplantation, HGF-expressing cells may be at high risk of neoplastic transformation (6). Therefore, alternative methods to transiently induce HGF signaling in transplanted cells need to be developed.

Protein transduction domains (PTD) are short, basic peptides, for example, polyarginine or the HIV-TAT PTD sequence. They can deliver cytosolic proteins into almost any cell type (7, 8). Positive charges within these cationic domains facilitate their binding to negatively charged cell-surface proteoglycans. PTD-containing peptides are subsequently internalised by lipid raft macropinocytosis (9, 10).

Neither HGF, a secreted protein, nor Met, which is present throughout the plasma membrane, is a suitable candidate for protein transduction. Tpr-Met is a cytosolic form of Met (11, 12). The Tpr domain promotes dimerisation and autophosphorylation of tyrosine residues and leads to the constitutive activation of the kinase (13). Moreover, the docking site for the E3 ubiquitin-protein ligase CBL, mediating ubiquitylation and degradation of the activated receptor, is absent from Tpr-Met (14–16). Tpr-Met seems to have a longer half-life than activated Met (17). Altogether, these properties make Tpr-Met an attractive candidate to induce HGF/Met signaling by protein transduction.

Protein transduction has many potential uses in organ and cell transplantation (18). In this study, we examined

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whether transduction of TAT-Tpr-Met into hepatic progenitors affects liver cell engraftment.

Materials and Methods

TAT-Tpr-Met Synthesis and Purification. Tpr-Met cDNA was used as a template to PCR amplify the coding sequence of the Tpr-Met-encoding gene with the primers 5′ ATATATCCATGGCGGCGGTGTTGCAGC 3′ and 5′ TTTAGGAATTCCTATGATGTCTCCCAGAAGG 3′ (PCR conditions: 94°C for 5 min followed by 40 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min). We inserted the purified PCR fragment into the *Nco*I/*Eco*RI site of the pTAT-HA vector.

TAT-β-galactosidase and TAT-Tpr-Met were produced in the *E. coli* strain BL21 (DE3) pLysS (Invitrogen) and purified as described (19). Briefly, expression was induced at OD 600 of about 0.8–1 for two hours. Inclusion bodies were then recovered, solubilised in urea buffer (8 *M* urea, Hepes 10 m*M* pH 8, 1.5 M NaCl, 15 m*M* imidasol) and agitated for one hour in a Ni^{2+}-agarose column (QIAGEN, Hilden, Germany). The matrix was washed and incubated for 12–16 hours at 4°C in PBS. Protein was eluted with 500 m*M* imidasol, desalted via PD-10 columns (Amersham Biosciences, Orsay, France), and sterilised by filtration through 0.22-μm pores. The protein was used immediately or stored at −80°C in (DMEM + 10% glycerol). Protein concentration was determined by Coomassie blue staining after SDS-PAGE, followed by comparison with BSA standard.

Western Blot Analysis. Protein and cell extract were resolved by SDS/PAGE and transferred to PVDF membrane. Primary antibodies were mouse monoclonal anti-HA (Covance, 1:1000), rabbit polyclonal anti-ERK (K-23, Santa Cruz Biotechnology, 1:1000), and mouse monoclonal anti-pERK (E-4, Santa Cruz Biotechnology, 1:1000). For secondary antibodies, we used anti-mouse and anti-rabbit peroxidase–conjugated antibodies (Amersham Biosciences NA931V and NA935V, 1:5000). Chemiluminescent signal was detected using the ECL reagent (Amersham Biosciences, UK Ltd).

Cell Culture and Protein Transduction. The NIH 3T3 cell line was cultured in DMEM/10% newborn calf serum (Eurobio, France). MLP29 cells were cultured in DMEM/10% fetal calf serum. BMEL (bipotential mouse embryonic liver stem cells, cell line 9A1) were cultured on collagen-coated dishes in DMEM/10% fetal calf serum supplemented with 20 ng/ml HGF (kind gift of Genentech). For protein transduction, TAT-Tpr-Met was applied directly to cells for one hour in serum-free DMEM at the concentration of 1 μ*M*.

Proliferation Assay. NIH 3T3 cells were cultured in complete medium containing 5% newborn calf serum. For protein transduction, culture medium was removed and cells were incubated for one hour, with TAT-Tpr-Met (1 μ*M*) in DMEM, or DMEM alone as a control. After one hour, the cultures were replenished with complete medium. The procedure was repeated daily. After four days, triplicates of control and treated cultures were briefly trypsined and quantified by Trypan Blue exclusion, using a Malassez cell.

Apoptosis Induction and TUNEL Assay. Apoptosis was induced by serum withdrawal as described (20). Low density (1/10 confluence) NIH 3T3 cultures were washed twice with serum-free DMEM and incubated for 18 hours in serum-free DMEM supplemented with glutamine. TUNEL assays were performed with the ApopTag Plus Peroxidase kit (S7101, Chemicon).

Cell Transplantation and Degree of Chimerism. Experiments were performed in line with the principles of laboratory animal care following the guidelines approved by INSERM. All surgery was performed using aseptic procedures. Xylazine/ketamine induced anesthesia in mice. Confluent BMEL was trypsinised and washed in DMEM/10% fetal calf serum. Cells were resuspended at 10^7 per ml in DMEM and incubated at 37°C for 30 minutes with Hoechst fluorescent dye (10 ng/ml, Sigma). Cells were washed with DMEM/10% fetal calf serum and incubated for one hour with TAT-Tpr-Met (1 μ*M*) or DMEM as control. Cells were washed three times in PBS. Two million cells in a total volume of 200 μL of PBS were transplanted through the splenic capsule of each six-week-old female FVB mouse (Charles Rivers, France). Mice were killed 24 hours after the operation and livers were frozen in Tissue-Tek. Cryostat sections (7 μm) were mounted in glycerol and Hoechst was visualised under fluorescence microscopy.

Given that 1 g of liver is estimated to contain $\approx 10^8$ hepatocytes, we infused 2×10^6 hepatocytes, representing $\approx 2\%$ of the mouse hepatocytic mass. Hoechst-labeled cells were counted in all transplanted mouse livers in a total of 100 microscopic fields. Assuming that about 225 hepatocytes can be visualised per microscopic field using these same conditions (7 μm liver section; ×400 magnification), the degree of cell chimerism with donor cells was calculated as follows: observed percentage of cell chimerism = [(number of transplanted cells counted/100)/225] × 100.

Statistical Analysis. Values are presented as means ± SE. Differences in cell proliferation and survival *in vitro* were analysed using one-way ANOVA. Statistical significance between transplanted groups was analysed using the Mann-Whitney *U* test. *P* values less than 0.05 were considered significant.

Results

Production and Refolding of Recombinant TAT-Tpr-Met Protein. TAT-Tpr-Met protein was produced at high levels as inclusion bodies in bacteria. It was solubilised with 8 *M* urea and purified by nickel affinity following standard methods (19) (Fig. 1A and B). Eluted TAT-Tpr-Met was very unstable and precipitated within one hour at 37°C. Refolding by removing urea slowly using multi-step dialysis was unsuccessful under several conditions. We used

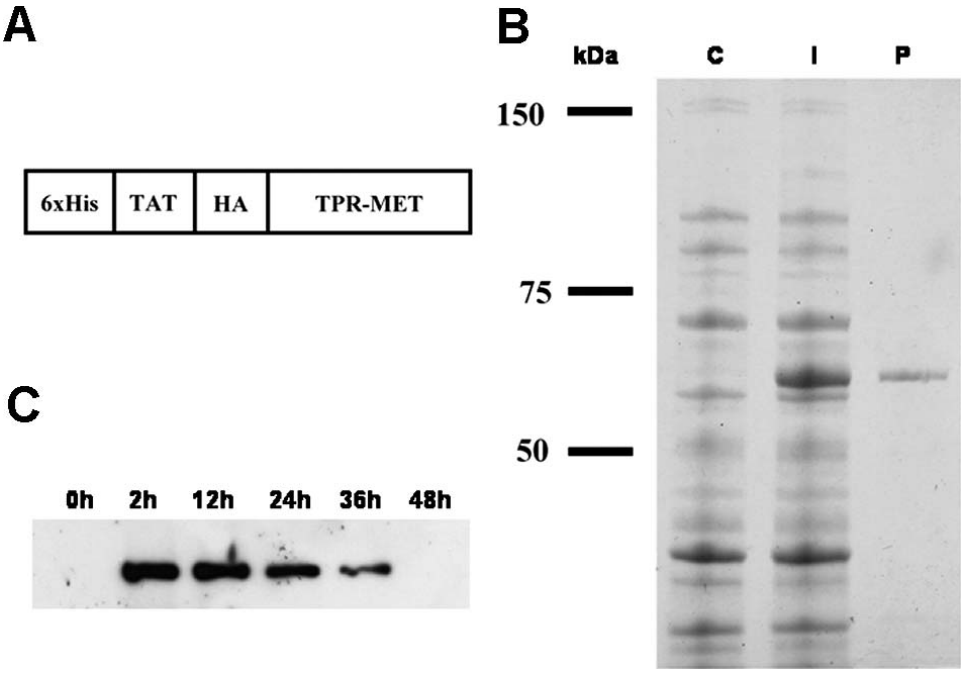

Figure 1. Biochemical properties of TAT-Tpr-Met. (A) Schematic representation of the TAT-Tpr-Met protein. HA, hemagglutinin tag. (B) Production and purification of recombinant TAT-Tpr-Met (67kDa), as detected by SDS-PAGE followed by staining with Coomassie blue. C, control bacterial lysate; I, induced bacterial lysate; P, purified TAT-Tpr-Met. (C) Detection of TAT-Tpr-Met in NIH 3T3 cells over time, by Western blot with anti-HA antibody.

matrix-assisted refolding to help protein folding *in vitro*. In this method, the protein is shifted from denaturing to aqueous media while still bound to the affinity matrix (21, 22). Proteins are physically separated from each other and can no longer interact with misfolded complexes after removal of the urea. Aggregation is thus prevented and each protein refolds on itself, or at least adopts a conformation that is more stable in aqueous solution. TAT-Tpr-Met eluted after 12 hours incubation at 4°C in phosphate-buffered saline was much more stable, giving rise to no or very little aggregation.

Protein Transduction. We tested whether matrix-assisted refolded TAT-Tpr-Met protein can enter cells *in vitro*. NIH 3T3 cells were treated with refolded TAT-Tpr-Met for one hour. Cells were washed and cellular proteins were extracted at different times. TAT-Tpr-Met fusion protein was detected in treated cells by its hemagglutinin (HA) tag. TAT-Tpr-Met seemed to be fairly stable, as it was detected up to 36 hours after treatment (Fig. 1C).

TAT-Tpr-Met *In Vitro* Biological Activity. As NIH 3T3 is not a transformed cell line and so does not express activated oncogenes known to activate mitogenic pathways, we used it as a model to characterize the effects of TAT-Tpr-Met on proliferation, survival, and ERK activation. TAT-Tpr-Met, added daily for four days, enhanced cell proliferation (2.9-fold increase; $P < 0.05$) (Fig. 2A). We also assessed the effect of TAT-Tpr-Met on cell survival. Cells were incubated with TAT-Tpr-Met for one hour. Apoptosis was then induced by shifting cells to serum-free medium. The level of DNA fragmentation was significantly lower in cultures pretreated with TAT-Tpr-Met than in controls (14-fold; $P < 0.005$) (Fig. 2B). NIH 3T3 cells were also incubated in low serum concentration to reduce background activation of extracellular signal-regulated kinase (ERK), a well-known intracellular target of Met

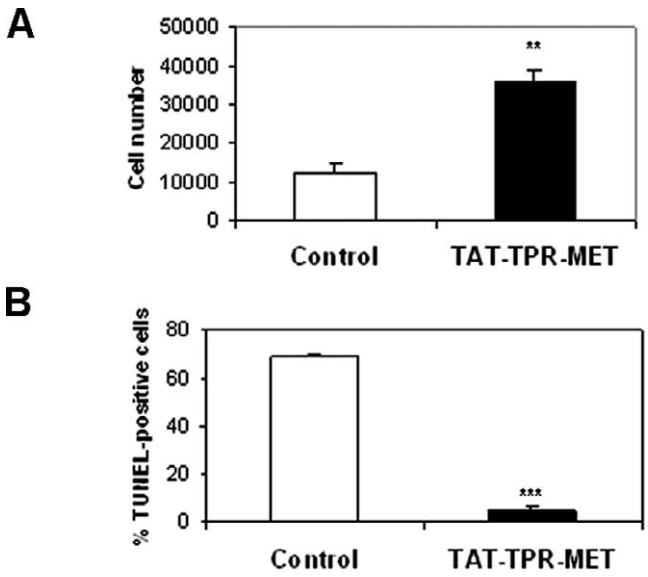

Figure 2. TAT-Tpr-Met biological activity in NIH 3T3 cells. (A) TAT-Tpr-Met caused a 2.9-fold increase in proliferation ($P < 0.05$) and (B) protected cells from apoptosis induced by serum removal ($P < 0.005$). Data shown are representative of at least two independent experiments. (C) TAT-Tpr-Met promotes ERK phosphorylation. Serum-starved NIH 3T3 cells were incubated with TAT-Tpr-Met or stimulated with 10% serum as a positive control.

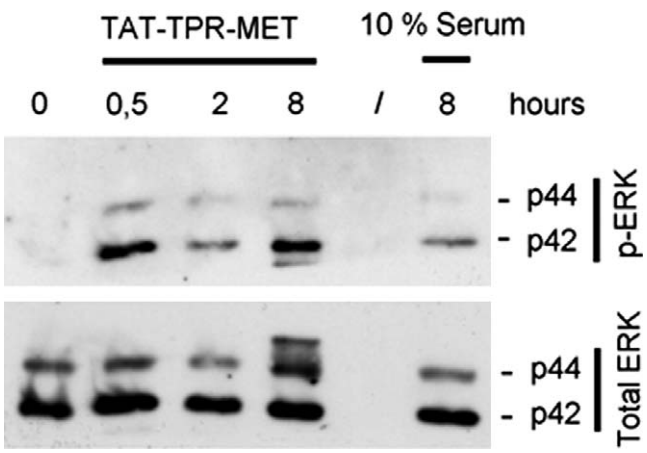

Figure 3. TAT-Tpr-Met promotes ERK phosphorylation. Serum-starved NIH 3T3 cells were incubated with TAT-Tpr-Met, and ERK phosphorylation was detected at different times. Stimulation with 10% serum was used as a positive control.

signaling (15). TAT-Tpr-Met treatment promoted the rapid and prolonged phosphorylation of ERK in these cells (Fig. 3).

HGF has many effects on the cytoskeleton, cell shape, and motility (4, 23, 24). TAT-Tpr-Met treatment (>300 nM) of NIH 3T3 cells shifted to 0.1% serum immediately after protein transduction prevented cell flattening due to serum starvation and promoted sprouting of cellular extensions during an eight- to 48-hour period after protein transduction (Fig. 4A). Cells had reverted to normal morphology after three days, suggesting that the effect is transient. Transduction using TAT-β-galactosidase as a control had no effect (data not shown).

On MLP29 cells, a hepatic mouse cell line known to show a strong scattering effect in response to HGF (25), TAT-Tpr-Met treatment induced morphological changes characteristic of epithelial-mesenchymal transition (Fig. 4B). A one-hour incubation period with TAT-Tpr-Met was sufficient to bring about these changes. This confirmed Western blot data and previous reports demonstrating that TAT-mediated protein transduction is a rapid process (10).

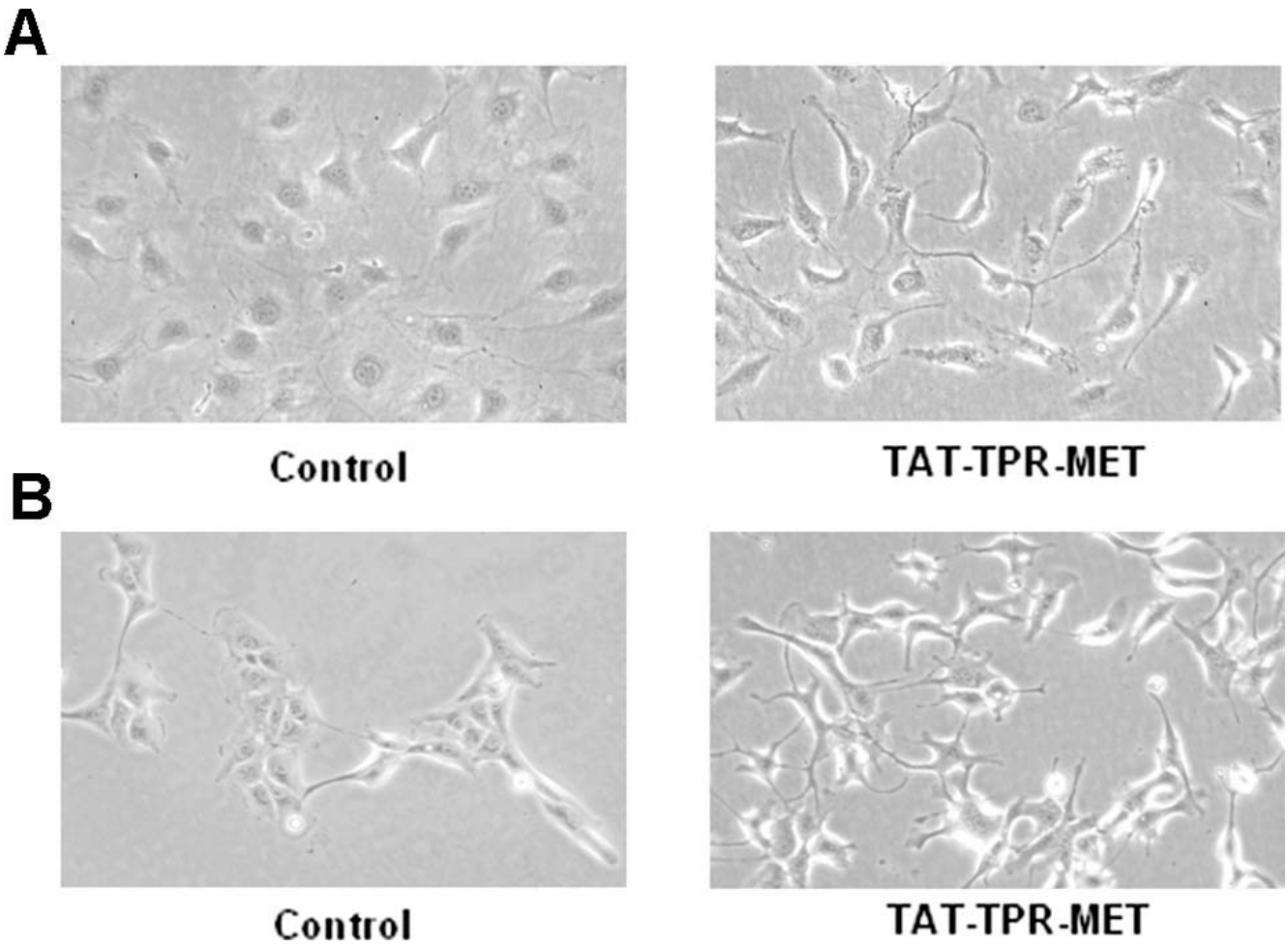

Figure 4. Morphological changes induced by TAT-Tpr-Met. Photographs 24 hours after delivery of TAT-Tpr-Met into (A) NIH 3T3 or (B) MLP29 cell lines.

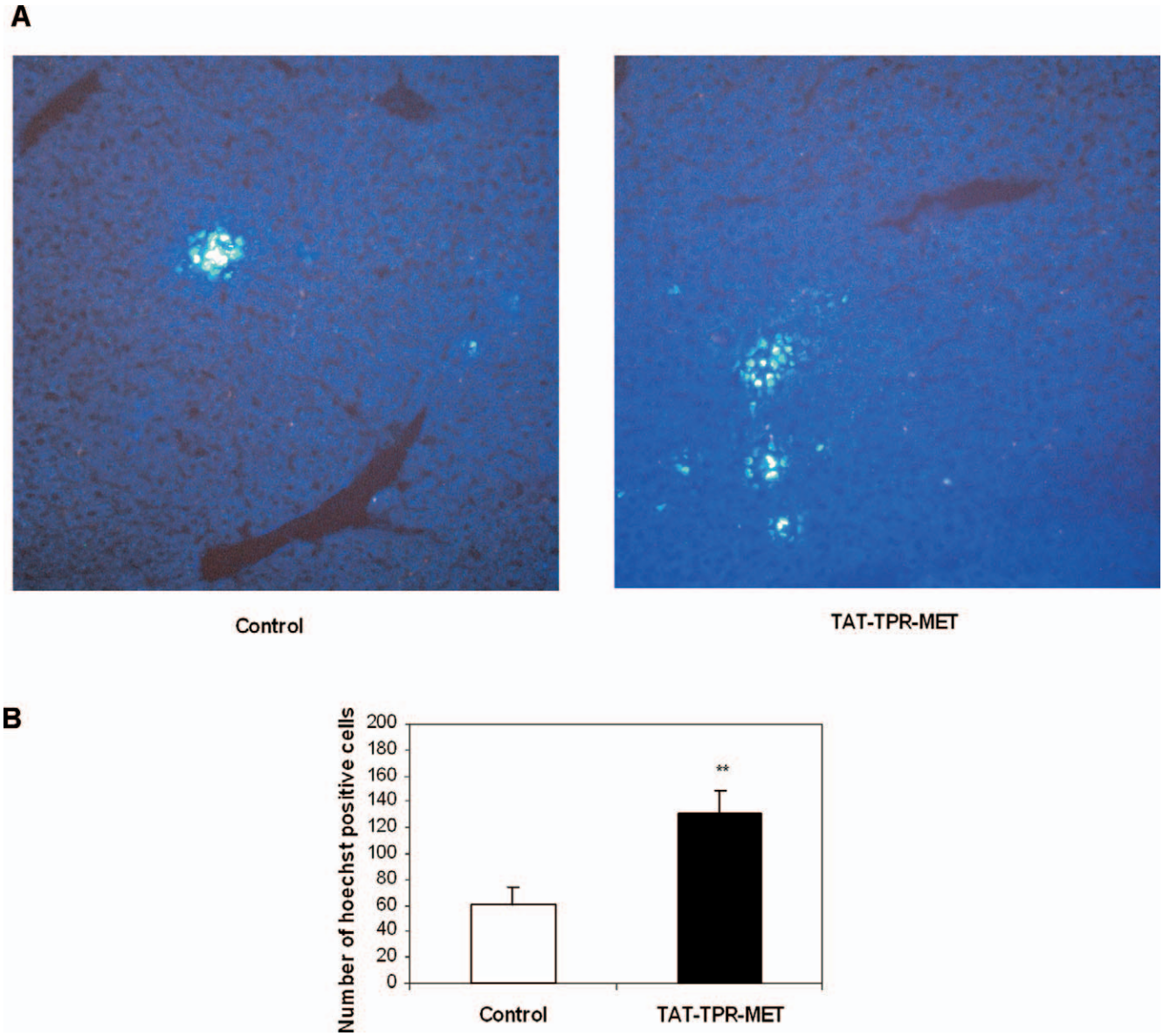

Figure 5. Hepatic progenitor transplantation. (A) Representative microscopic field showing Hoechst-labeled BMEL detected in liver, 24 hours after transplantation. (B) TAT-Tpr-Met improves hepatocyte engraftment. Bar represents mean number of Hoechst-positive cells in 50 fields ± SE. Control group ($n = 11$ mice); TAT-Tpr-Met group ($n = 9$ mice) ($P < 0.005$). A color verson of this figure is available in the online journal.

Transplantation of Bipotential Mouse Embryonic Liver Stem Cells. Bipotential mouse embryonic liver stem cells (BMEL) engraft and differentiate into bile ducts and hepatocytes after transplantation (26). We therefore used these cells to assess the effect of TAT-Tpr-Met on transplantation efficiency. BMEL cells were labeled with Hoechst and treated for one hour with TAT-Tpr-Met or DMEM as a control. Cells were transplanted via intrasplenic injection in mice. Twenty-four hours after transplantation, engraftment efficiency was measured by counting the number of labeled cells on liver slides (Fig. 5A). Statistical analysis revealed that the number of engrafted hepatocytes in the livers of mice receiving cells pretreated with TAT-Tpr-Met was 1.9 times higher than that of controls ($P <$ 0.005) (Fig. 5B). The degree of organ chimerism was 0.62% in controls and 1.17% in the treated group. Assuming that about 2% of the liver mass was transplanted, 31% of control cells and 58% of TAT-Tpr-Met treated cells were found in the liver parenchyma. Thus, transient activation of Met signaling improved the engraftment efficiency of hepatic progenitors.

Discussion

The precipitation of unfolded proteins presents a major challenge in successful protein transduction (19). Matrix-assisted refolding has not been considered for correct refolding of TAT-fused proteins in previous studies. Our findings demonstrate the potential benefit of this technique

and suggest that it may be useful as an alternative strategy to renature large proteins fused to a PTD (21, 22).

Several models of cell transplantation have shown that the engraftment efficiency of cells is low. Approximately 0.5% of the total recipient liver cells engraft successfully under normal circumstances (27). Our findings are consistent with these previous reports. Liver cell graft loss occurs during the early stages of transplantation (28, 29). Less than 30% of these cells is found within the liver parenchyma in rodent models, 24 hours after injection (29). It is caused primarily because the cells do not enter the sinusoids and do not efficiently cross the endothelial cell sinusoidal barrier (29). Therefore, we hypothesised that transient stimulation of the HGF/Met pathway would improve cell survival and engraftment. Met is a pleiotropic factor, acting on multiple pathways (4); thus, the increased engraftment efficiency observed here may be the result of a combination of several effects. In particular, increased adhesion and survival may have been important factors involved in this improvement. Contrary to hepatic progenitors, adult hepatocytes are well differentiated and are poorly responsive to mitogenic/motogenic signals. Thus, we believe that our strategy will work better with hepatic progenitors like the ones recently isolated from human adult liver or obtained after differentiation of ES or iPS cells (30).

The addition of a cytokine cocktail to cell cultures has also been used in some studies to shift cells to a state that is more favorable for engraftment (1). However, the biological effect of cytokine stimulation does not last more than several hours, whereas cell engraftment takes approximately 20 hours in liver (29); thus, cytokine effects are likely to be minimal during cell integration within the parenchyma. Protein transduction overcomes these limitations. This is demonstrated in our study: TAT-Tpr-Met entered cells rapidly and significant amounts of this fusion protein remained stable for at least 24 hours.

Interestingly, Met signaling is present in many cell types and can be activated by ectopic Met in some cell types that do not express it (15). Therefore, TAT-Tpr-Met transduction may be a useful tool for applications in many tissues. Moreover, the toxicity of protein transduction does not appear to be a limitation, and so it may be possible to incorporate the transduction of several proteins simultaneously to obtain synergic effects in future strategies. Further work will be needed to investigate the effects of our engineered TAT-Tpr-Met fusion protein in preclinical animal models and to assess its potential use in cell therapy.

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