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RESEARCH ARTICLE

HSV vector-mediated transduction and GDNF secretion from adipose cells

J Fradette, D Wolfe, WF Goins, S Huang, RM Flanigan and JC Glorioso
Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

The accessibility of adipose tissue and its ability to secrete various bioactive molecules suggest that adipose cells may be attractive targets for gene therapy applications. Here, we report the use of highly defective herpes simplex virus (HSV) vectors as suitable gene transfer agents for adipose cells in culture and fat tissue in animals. Using an in vitro model of human adipose differentiation, we first demonstrated that mature adipocytes and their precursor cells express the two principal HSV viral entry receptors HveA and HveC (nectin-1) and are efficiently transduced at a low multiplicity of infection by HSV-lacZ reporter gene and glial cell line-derived neurotrophic factor (GDNF) gene vectors. Extended expression of β-galactosidase and secretion of GDNF occurred in transduced fat tissue explants from rabbits. In vivo gene transfer to rabbit subcutaneous adipose tissue resulted in local GDNF expression for at least 2 months. These experiments establish the efficient transduction of adipose cells by HSV vectors and suggest that fat tissue may represent a useful site for HSV-mediated gene delivery with potential for therapeutic applications.
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Keywords: *adipose tissue; herpes simplex virus vectors; gene transfer; GDNF; adipocyte*

Introduction

Adipose tissue is the largest energy reservoir of the human body ensuring homeostasis either by storing free fatty acids in the form of triglycerides or by hydrolysis of these stored products on demand. In addition, numerous adipokines are secreted from fat cells into the bloodstream and these bioactive molecules influence a wide range of metabolic functions such as energy balance, glucose oxidation, insulin sensitivity, host defense and reproduction.[1] Adipose tissue is mainly composed of terminally differentiated adipocytes filled with lipid droplets. These mature cells arise from preadipocytes, a fibroblast-like precursor cell present in the stromal-vascular fraction of the tissue. Recently, multipotent stromal cells with lineage potentials similar to bone marrow-derived mesenchymal stromal cells have been identified in adipose tissue, suggesting that this accessible and prevalent tissue could represent a promising alternative to bone marrow as a source of adult multipotent stem cells.[2,3] These stem cells, as well as the more differentiated cell populations constituting adipose tissue, may represent appealing targets for therapeutic gene transfer.

Replication-defective herpes simplex virus (HSV)-based gene vectors are attractive vehicles for both *ex vivo* and *in vivo* gene transfer strategies. HSV vectors can be propagated to high titers on complementing cell lines, have large payload capacity and efficiently transduce cells at low multiplicity of infection (MOI). In addition to their natural tropism for neurons, HSV vectors can infect a wide variety of cell types including ligament, muscle, intestinal and bladder smooth muscle cells, suggesting that HSV may have broad utility for gene transfer applications.[4,5] In the present study, we explored the feasibility of using genomic HSV-based recombinant vectors for gene transfer to adipose cells. Using an *in vitro* model that recreates adipose conversion, we demonstrated the presence of HveA and HveC HSV receptors and report efficient transduction of both human preadipocytes and mature adipocytes. Extended β-galactosidase (β-gal) expression and secretion of the exogenous gene product glial cell line-derived neurotrophic factor (GDNF) was demonstrated using explants of rabbit fat transduced *ex vivo*. Furthermore, direct inoculation of rabbit subcutaneous adipose tissue *in vivo* with an HSV vector expressing GDNF under the control of the LAP2-HCMV chimeric promoter resulted in local transgene expression persisting for at least 2 months.

Correspondence: Dr JC Glorioso, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, E1240 Biomedical Science Tower, 200 Lothrop St, Pittsburgh, PA 15261, USA
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Results

Presence of HSV entry receptors on adipose cells

An *in vitro* model of adipocyte differentiation from human precursor cells (Zen-Bio, Inc., NC, USA)[6] was initially used to evaluate the ability of replication-deficient HSV vectors to infect adipose cells. These experiments employed primary human preadipocytes rather than the extensively studied 3T3-L1 murine-transformed cell line in order to gather insights that could prove relevant to human studies in the future. HSV recognizes two distinct receptors on susceptible cells termed herpesvirus entry mediators A and C (HveA

and HveC).[7] HveA is a member of the TNF receptor family[8] and HveC has been identified as nectin-1α.[7,9] Flow cytometry and indirect immunocytochemistry demonstrated that these receptors were present on the surface of both human preadipocytes and mature adipocytes following differentiation *in vitro* (Figure 1). By flow cytometry, 27±7% of cultured preadipocytes (Figure 1a) expressed HveA, while 90±5% expressed HveC (Figure 1f). Double-labeling experiments using both anti-receptor antibodies indicated that the HveA-positive cells also expressed HveC on their surface (not shown). Nearly all mature adipocytes differentiated *in vitro* expressed both HveA (Figure 1b) and HveC (Figure 1g) as shown by indirect immunofluorescence staining. Thus, both HSV-1 entry receptors are present on adipose cells, making them excellent targets for HSV-based vectors.

Human adipocytes and precursor cells are efficiently transduced by HSV vectors in vitro

Transduction efficiency was evaluated on cultures of preadipocytes infected with a replication-defective HSV double reporter gene vector (QOZHG) in which U_L41 encoding the virus host shut-off function is replaced by a *lac*Z gene under transcriptional control of the HSV ICP0 immediate early (IE) gene promoter, and the essential IE gene ICP27 is replaced by an enhanced green fluorescence protein (EGFP) gene driven by the HCMV IE promoter.[10] The EGFP reporter gene was used to monitor infected cells in culture and quantify cell transduction by flow cytometry, while β-gal detection readily identified infected cells in intact fat tissue.

Figure 2a shows high-level QOZHG-mediated EGFP expression in human preadipocytes. Flow cytometry of these cells infected at low multiplicity (MOI of 3) revealed that 95±1% were transduced 2 days after infection (data not shown). Similarly, primary stromal-vascular cells extracted from rabbit adipose tissue, expanded in culture and infected with virus (Figure 2c) revealed that 93±1% of these cells expressed β-gal after X-gal staining, demonstrating that adipocyte precursors of different origin were similarly susceptible to HSV infection as determined by two independent methods of assessing transduction efficiency. At an MOI of one, 62% of the rabbit preadipocyte population expressed detectable transgene product, confirming that one infectious particle was sufficient to transduce a single cell in accordance with Poisson statistics.

Human preadipocyte cultures were induced to differentiate using a cocktail of adipogenic hormones, and subsequent infection showed the presence of numerous EGFP-fluorescent lipid-filled cells (Figure 2e), representing nearly the entire adipocyte population. A subpopulation of adipocytes (~10–20%) remained EGFP positive 12 days after infection, the latest time point evaluated (data not shown).

Residual vector cytotoxicity on adipose cells in vitro

MTT assay was used to measure the metabolic activity of infected cultures as an indicator of vector toxicity. Cultured human preadipocytes and Vero cells were infected at an MOI of 3 with the QOZHG vector, and the cellular metabolic activity was measured at days 2 and 4 post-infection (Figure 3a). At 2 days after transduction, the QOZHG vector was less cytotoxic for

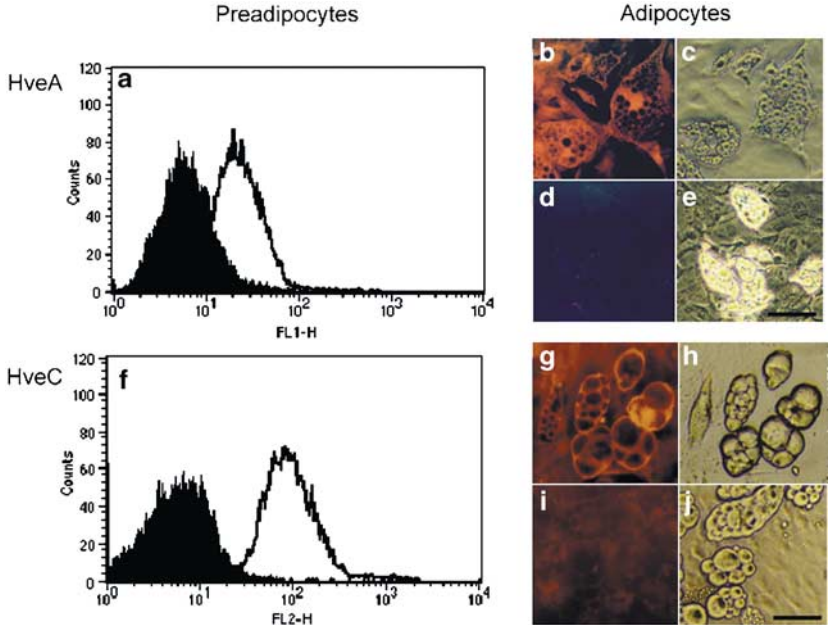

Figure 1 *Expression of HSV receptors HveA and HveC on the cell surface of cultured human preadipocytes and in vitro differentiated adipocytes. Representative flow cytometry experiment from cultured human preadipocytes labeled with (a) R140, an antiserum against HveA (open profile, 27±7% (mean±s.d.), n=6) or (f) monoclonal antibody CK6 directed against the HveC receptor (open profile, 90±5% (mean±s.d.), n=4), demonstrating the presence of both HSV-1 entry receptors on these cells. Solid profiles represent labelings with the appropriate secondary antibodies only. In vitro differentiated human adipocytes expressing HveA (b) and HveC (g) by indirect immunofluorescence staining with the same antibodies used for flow cytometry experiments. Micrographs of negative control (d,i) omitting the primary antibody, and phase contrast micrographs (c, e, h, j) corresponding to immunofluorescent staining (b, d, g and i) respectively. Bar, 20 μm.*

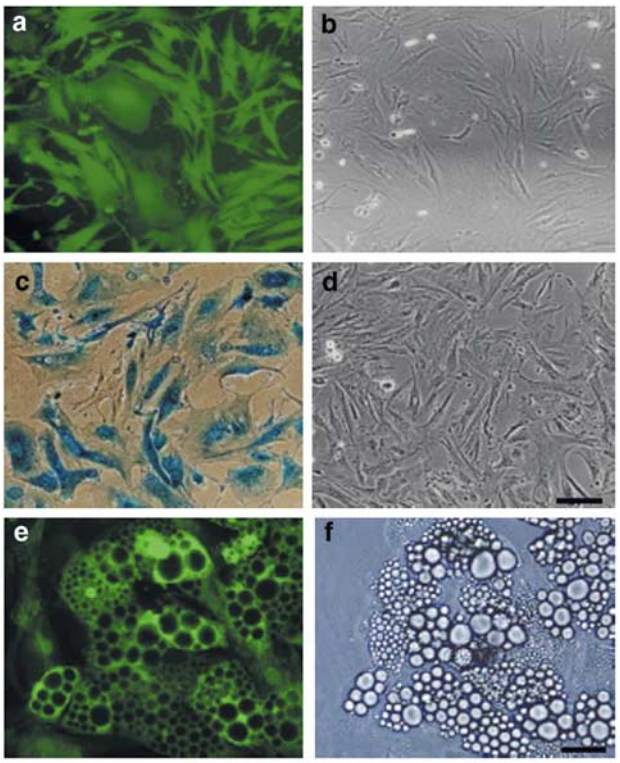

Figure 2 *Cultured preadipocytes and in vitro differentiated adipocytes are readily transduced by HSV vectors. Human preadipocytes (a, fluorescence; b, phase-contrast) were infected at an MOI of 3 with QOZHG. At 2 days after infection, cells were expressing EGFP (a), while mock-infected cultures completely lacked fluorescence. Flow cytometry analysis revealed that 95±1% (mean±s.d.) of the preadipocytes were transduced. Rabbit adipocytes precursor cells (c, d) were also readily transduced (93±1%) by QOZHG 2 days after infection as revealed by X-gal staining of infected cells (c) compared to mock-infected cells (d). Lipid-filled in vitro differentiated human adipocytes (e, fluorescence; f, phase-contrast) were infected at an MOI of 5 and more than 90% expressed EGFP 24 h post-infection while noninfected adipocyte cultures displayed a complete lack of fluorescence. Bars: a–d, 33 μm; e and f, 17 μm.*

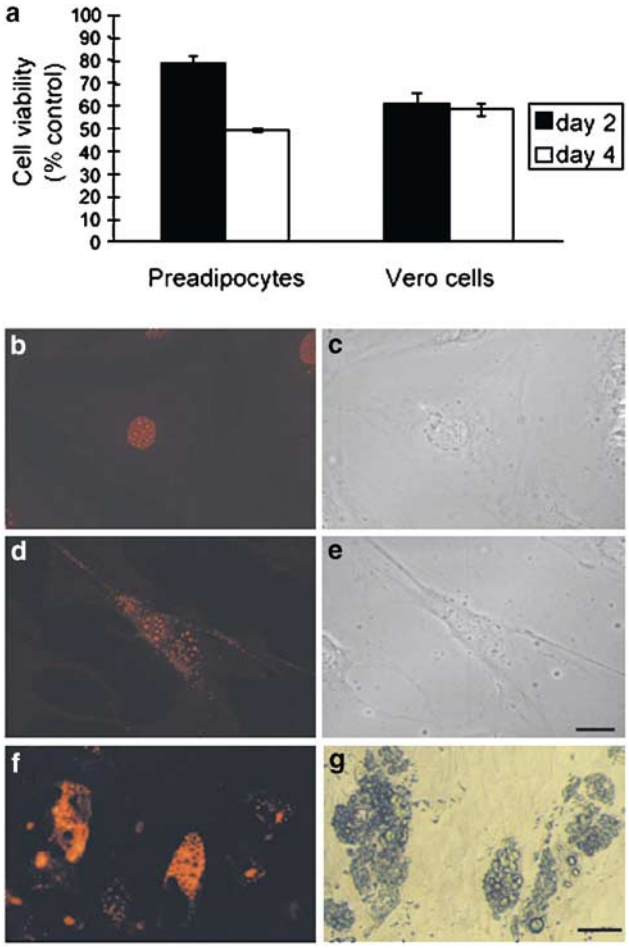

Figure 3 *Residual cytotoxicity of the QOZHG vector after in vitro transduction of adipocyte precursor cells. Cultured human preadipocytes and Vero cells were infected at an MOI of 3 with the replication-deficient vector QOZHG. The viability of the infected cells (a) was evaluated by MTT assay at days 2 and 4 after infection. Values are represented in comparison to mock-infected cells (100% viability) at the same time point. Immunohistochemistry on QOZHG-infected cells for ICP0 detection (b–g). The immediate early HSV protein ICP0 is localized in the nuclei of QOZHG-infected human preadipocytes 2 days after infection (b). The characteristic intranuclear punctate appearance of ICP0 staining extends to the cytoplasm of the cells over time (d). ICP0 is also expressed in QOZHG-transduced in vitro differentiated adipocytes 24 h after infection (f). Bars: b–e, 78 μm; f and g, 17 μm.*

preadipocytes (80% viable) than Vero cells (61%), a simian kidney cell line routinely used for HSV propagation. At 4 days after infection, 50% of the human preadipocytes remained metabolically active (Figure 3a). However, vector-related cytotoxicity was sufficient to prevent infected preadipocytes from differentiating into mature fat cells, a process requiring 2 weeks of culture under adipogenic conditions (data not shown). QOZHG infection of *in vitro* differentiated mature adipocytes also led to declining cell viability but this effect was more protracted than observed for subconfluent preadipocyte cultures (data not shown).

Considering that ICP0, the only HSV IE gene expressed in the vector, is known for its ability to alter cell functions,[11,12] we examined the presence and distribution of this protein in the transduced cells by immunocytochemistry (Figure 3b-g). Early after infection (9–24 h), ICP0 was observed in an intranuclear punctate staining pattern within infected preadipocytes (Figure 3b), a distribution characteristic of HSV infection of non-neuronal cells.[10] This punctate ICP0 staining expanded into the cytoplasm over time (Figure 3d). In QOZHG-transduced human adipocytes differentiated *in vitro*, ICP0 was detected in the nucleus as early as 9 h post-infection and staining of the entire cell was seen from day 1 (Figure 3f) until day 4 post-infection. Although ICP0 is known to have toxic effects on dividing cells,[13] it is likely that this viral function mediates cytotoxic effects on both dividing preadipocytes and quiescent adipocytes.

Ex vivo transduction of adipose tissue explants by HSV replication-defective vectors

Since HSV efficiently transduced adipocytes in monolayer culture, experiments were carried out to determine whether fat cells in intact tissue explants were also susceptible to virus-mediated transduction. Dorsal adipose tissue excised from rabbits was divided into fragments (~0.6 g each), injected with the QOZHG vector *in vitro*, and maintained as explants floating in adipocyte culture media (Figure 4a–f). X-gal staining was

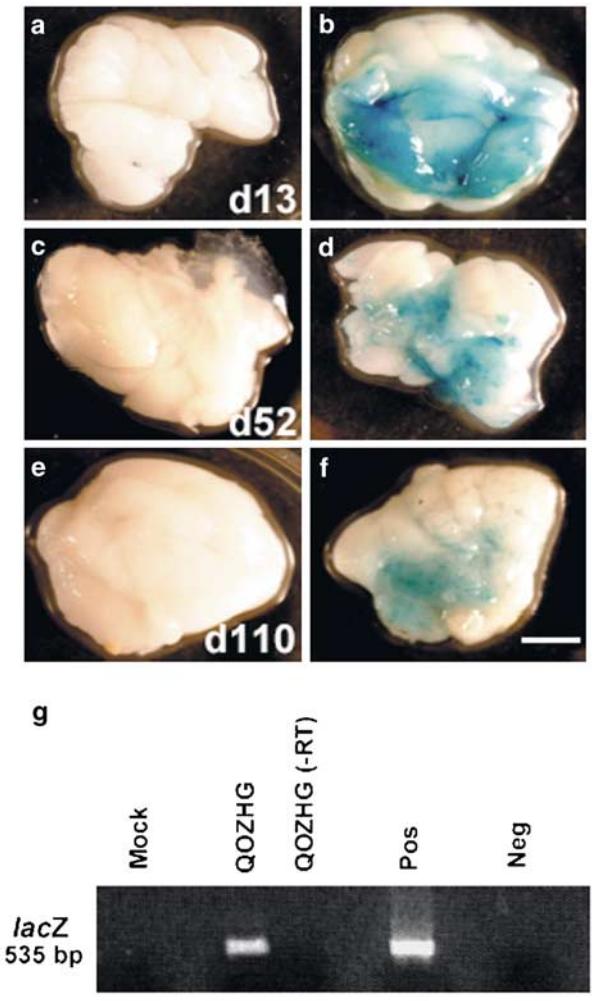

Figure 4 *Long-term expression of β-gal in explants of rabbit adipose tissue injected with QOZHG. Adipose tissue was injected ex vivo with 1×10^7 PFU of viral vector QOZHG and maintained in culture for up to 110 days. X-gal staining of PBS-injected (a, c, e) and QOZHG-injected (b, d, f) explants at day 13 (a, b), 52 (c, d) and 110 (e, f) post-infection. Bar, 4 mm. RT-PCR analysis (g) revealed vector-derived β-gal transcripts 90 days after infection in QOZHG-transduced explants (QOZHG) but not in PBS-injected explants (Mock). No product was detected is the absence of reverse-transcriptase (QOZHG-RT). Pos, control RNA; Neg, water.*

performed at various time points to monitor transgene expression. The results showed that β-gal was expressed in QOZHG-injected explants only and for up to 110 days after infection, the latest time point examined (Figure 4f). RT-PCR analysis revealed vector-derived β-gal transcripts 90 days after infection in the QOZHG-transduced explants (Figure 4g, QOZHG), but not in PBS-injected explants (Figure 4g, Mock).

HSV-mediated in vivo transduction of rabbit subcutaneous adipose tissue

The prolonged expression of β-gal in fat tissue samples *ex vivo* prompted further examination of fat transduction using an *in vivo* route of administration. The QOZHG vector was injected directly into the abundant and easily accessible subcutaneous dorsal fat pad of rabbits. Vector inoculation (2×10^8 total particles in PBS) was performed in 7–8 injections for a total volume of 150 μl. Detection of β-gal expression was carried out by X-gal staining of the total adipose tissue at different time points. At 2 days after infection, the QOZHG-injected regions showed high levels of β-gal activity (Figure 5b), while the adipose tissue from mock-injected animals was negative (Figure 5a). Cryosections of β-gal-expressing regions after X-gal staining (Figure 5d) revealed the presence of QOZHG-transduced adipocytes around the injection site. Analysis of adipose tissue from animals at later time points showed substantially reduced β-gal expression with only faint X-gal staining observed at 57 days post-inoculation (Figure 5c, arrows).

The detection of viral genomes in injected fat tissue was correlated with β-gal expression (Figure 5e, lanes 2 and 6). While regions lacking X-gal staining were generally negative for HSV sequences (Figure 5e, lanes 1, 3 and 5), some β-gal-negative samples were positive for viral genomes at later time points (Figure 5e, lanes 4 and 7). This observation suggested that the decline in β-gal expression was due to gradual silencing of the ICP0 IE promoter that controls *lac*Z expression rather than to loss of viral genomes.

HSV-mediated GDNF expression from adipose cells

Given that direct inoculation of fat tissue *in vivo* resulted in the accumulation of transgene product in adipose cells, a potential use of fat transduction might involve the synthesis of a therapeutic product suitable for release into the circulation. Accordingly, we examined the capacity of adipose cells to secrete a transgene product, glial cell line-derived neurotrophic factor (GDNF), which is a potent neurotrophin capable of supporting neuron survival in culture.[14] We recently reported that a component of the viral latency promoter, latency-associated transcript 2 (LAP2), provides long-term transgene expression in neurons.[15] Moreover, the activity of LAP2 can be enhanced when used in combination with a strong promoter such as the HCMV IE gene promoter (SH and WFG, unpublished data). Thus in our current study, we engineered a new double gene vector, QL2HGDNF, in which the GDNF cDNA was placed under transcriptional control of a chimeric LAP2-HCMV promoter in the UL41 locus and the EGFP reporter gene driven by the HCMV IE promoter was retained in the deleted ICP27 locus (Figure 6a). This new vector was examined for its ability to provide sustained production of GDNF in adipose cells.

We first compared human adipocyte cultures differentiated *in vitro* and their undifferentiated precursors for GDNF secretion after infection with QL2HGDNF or the QOZHG control vector (MOI = 5) (Figure 6b). High amounts of GDNF were released into the culture media from either cell type for at least 10 days following QL2HGDNF infection. The secretion levels obtained from mature adipocytes were approximately three-fold higher than from preadipocytes during the first week post-infection (Figure 6b).

The *ex vivo* rabbit fat explant model was then used to determine whether inoculated intact fat tissue was capable of secreting GDNF in a dose-dependent manner. Explants injected with a range of vector doses (2×10^5, 2×10^6 or 2×10^7 total particles) demonstrated high levels of secreted GDNF for each set of explants (Figure 7). Both the persistence and level of GDNF expression were dose-dependent. The high-dose samples (2×10^7 PFU) peaked at 2500 pg of GDNF/mg of protein on day 2. The

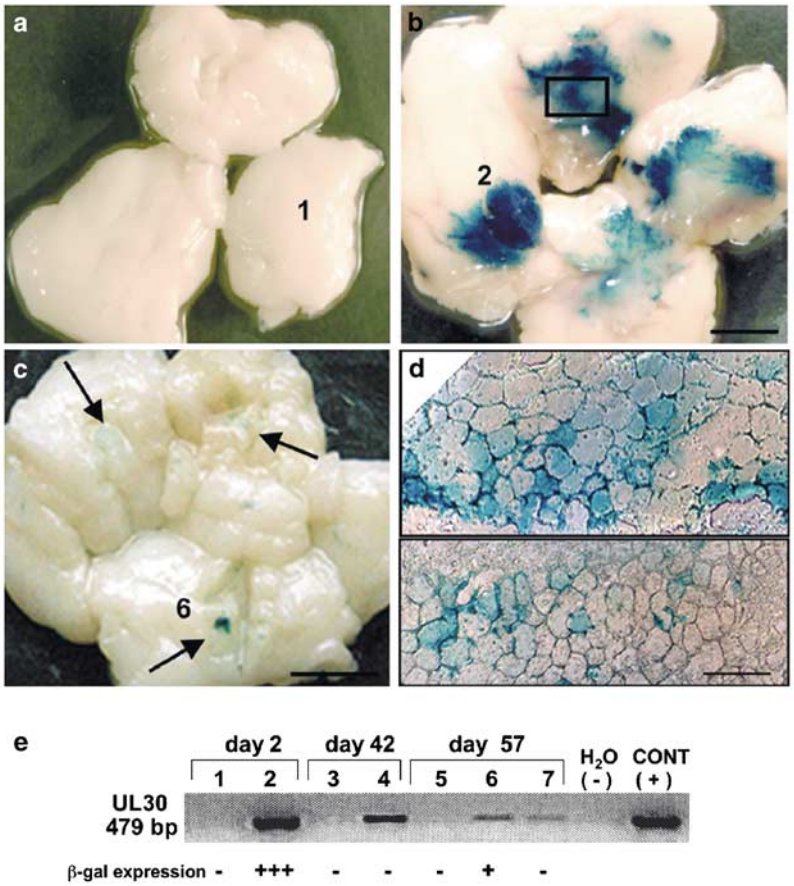

Figure 5 *In vivo transduction of rabbit adipose tissue by QOZHG vector. Two days after injection of the rabbit dorsal fat pad with 2×10^8 total particles of QOZHG, regions strongly expressing β-gal were visualized by X-gal staining (b) compared to the lack of staining observed for mock-injected animals (a). Adipose tissue harvested 57 days after infection (c) revealed only faint residual β-gal expression following X-gal staining (arrows). Adipose tissue cryosections (d) from a QOZHG-injected area (Figure 5b, box) depicts X-gal-stained adipocytes near the injection site. Areas marked 1, 2 and 6 correspond to samples examined under (e). Bar, 5 mm. PCR analysis of virus-injected tissues (e) demonstrated the persistence of HSV genomes at all time points examined. Lane 1, mock-injected fat; lane 2, X-gal staining positive tissue 2 days post-injection; lanes 3, 4, staining-negative tissues 42 days post-injection; lanes 5, 7, staining-negative tissues 57 days post-infection; lane 6, staining-positive tissue 57 days post-infection. H_2O, water, negative control; CONT, purified viral DNA, positive control.*

expression was reduced by 90% on day 20 and was undetectable by day 30. No GDNF was detected in media from either the PBS-injected or control vector-injected explants.

In vivo experiments were conducted in which rabbit dorsal subcutaneous fat tissue was injected with either the QL2HGDNF or control vector, and the presence of GDNF in fat tissue extracts was compared to extracts from untreated animals. Vector preparations (2×10^8 total particles in PBS) were administered at 7–8 injection sites into the fat pad, which was later excised, divided into multiple samples (~1 g each) and analyzed at days 2, 42 and 57 after injection. The amount of GDNF present in these tissue extracts was determined by ELISA. Figure 8 depicts the GDNF content of each sample constituting the dorsal fat pad injected with QL2HGDNF at day 2 (a), 42 (b) or 57 (c). The dotted zone on graphs b and c represents the baseline range of GDNF values measured for adipose tissue from mock-injected animals. No significant differences in GDNF concentrations were seen between tissues from noninjected or control vector QOZHG-injected animals ($P > 0.05$, Mann–Whitney test). At 2 days after transduction, high amounts (10-fold above baseline levels) of vector-derived GDNF were measured (Figure 8a, $P < 0.001$). The highest GDNF-expressing samples (up to 6.8 ng GDNF/mg total proteins) likely corresponded to the injected sites. At 42 days post-injection (Figure 8b), GDNF levels were considerably reduced (80% reduction compared to day 2; $P < 0.01$) but were still higher on average than values obtained from mock-injected animals ($P < 0.05$). Adipose tissue harvested 57 days after injection (Figure 8c) also displayed greatly reduced GDNF levels compared to day 2 ($P < 0.05$) but was still positive for GDNF expression ($P < 0.001$). Expression at day 57 still represented 20% of the initial expression level, was not different from GDNF levels seen at day 42 ($P > 0.05$), and corresponded to a two-fold increase over baseline.

The presence of viral genomes in DNA extracted from these samples was investigated by PCR, as before. Samples from QL2HGDNF-injected animals were positive at each time point analyzed (Figure 8d). Table 1 shows that the percentage of vector genome-positive samples was consistent for all three sampling times and represents approximately 40% of the injected fat pad. Of the genome-positive samples, the percentage of GDNF-containing extracts declined from approximately 80% at day 2 to 50% at day 57, a proportion higher than that

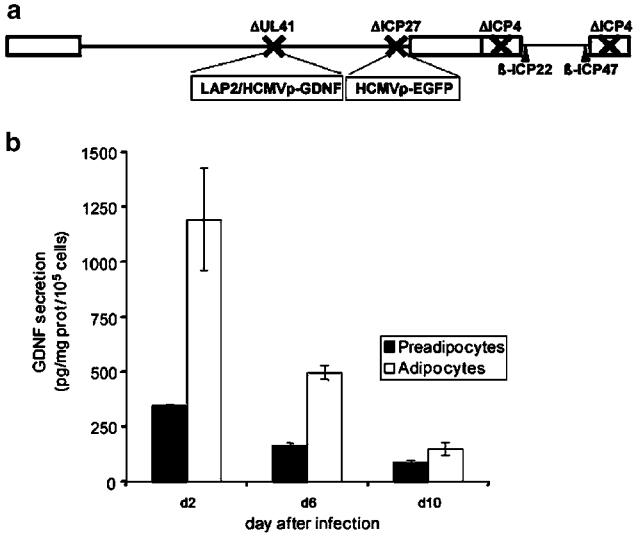

Figure 6 *HSV-mediated secretion of GDNF from adipose cells in vitro. Schematic representation (a) of the replication-deficient QL2HGDNF vector. Secretion of GDNF (b) from in vitro differentiated adipocytes (20 days differentiation; open bars) compared to undifferentiated preadipocytes (passage 2; solid bars) cultured for the same length of time, after infection at an apparent MOI of 5 with the QL2HGDNF vector or QOZHG control vector. No GDNF was measured by ELISA in the media of uninfected or QOZHG-infected cultures (not shown), while high levels of GDNF (up to 1.2 ng/mg protein per 10^5 cells) were released from QL2HGDNF-transduced cultures (bars). Data presented as mean ± s.d. for a representative experiment.*

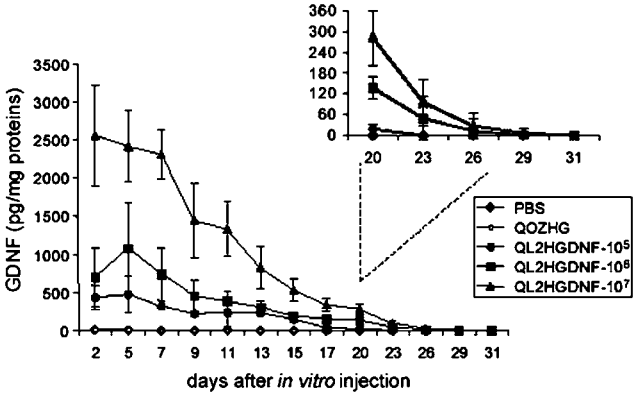

Figure 7 *GDNF dose–response secretion curve from rabbit adipose tissue explants transduced in vitro. Explants (n = 3 per series) were injected with increasing amounts of QL2HGDNF (2×10^5, 2×10^6, 2×10^7 PFU), the control vector QOZHG (1×10^7 PFU) or PBS. Secretion of GDNF in the culture media was measured by ELISA for up to 30 days for the explants injected with the highest vector dose (2×10^7 PFU). GDNF was detected only for the QL2HGDNF-injected explants and not for those injected with PBS or QOZHG.*

observed for β-gal expression from QOZHG. This finding may be related to the sensitivity of the detection method of the two transgene products, the nature of the transgenes or to the behavior of the two different promoters used to drive transgene expression. In either case, the reduction in transgene product over time was unlikely the result of extensive vector genome loss at the injected sites.

To determine if circulating levels of GDNF were achieved after vector injection, plasma samples from

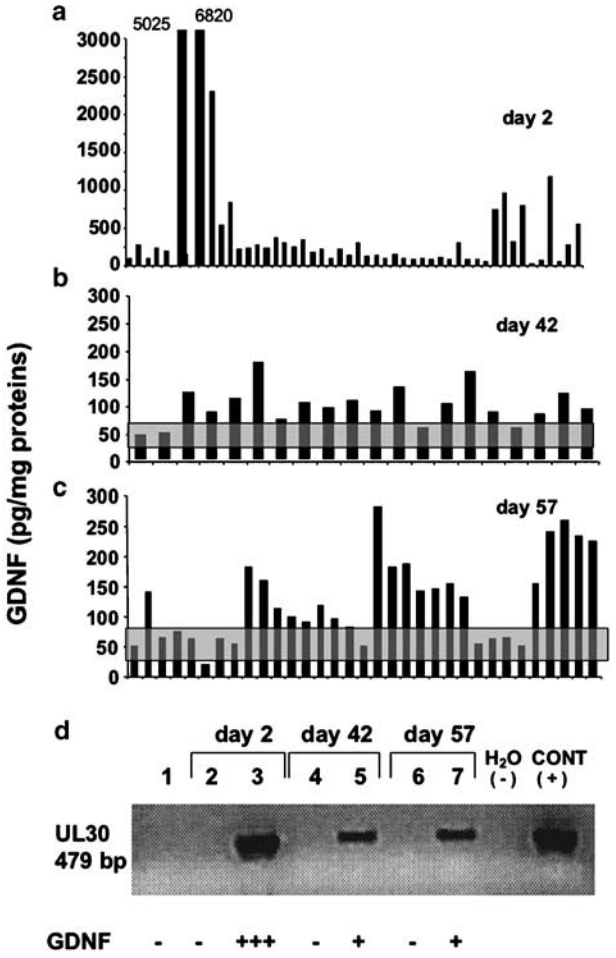

Figure 8 *GDNF detection in protein extracts from adipose tissue of QL2HGDNF-injected rabbits. Total GDNF protein expression (pg/mg total proteins) was measured in adipose tissue samples from each rabbit at day 2 (a) day 42 (b) and day 57 (c) after direct injection of 2×10^8 particles of viral vector. Each graph represents the expression of GDNF protein for each sample (~1 g fragments) constituting an entire dorsal fat pad. The dotted zone represents the amount of GDNF measured for mock-injected animals (n = 4, 35 samples). At later time points after injection (b and c), vector-derived expression of GDNF in adipose tissue was reduced (~80%) compared to day 2 after injection (a) but was significantly higher ($P < 0.05$) than values measured for mock-injected animals. PCR analysis of fat samples from injected animals (d) demonstrated the persistence of HSV genomes at all time points examined. Samples are naive rabbit fat (lane 1), tissues harvested 2 days (lane 2, 3), 42 days (lanes 4, 5) or 57 days (Lanes 6, 7) after injection. H_2O, water, negative control; CONT, purified viral DNA, positive control.*

Table 1 GDNF expression in relation to the distribution and persistence of HSV viral genomes detected by PCR for the UL30 component of HSV in adipose tissue samples from rabbits injected with the QL2HGDNF vector

Days after transduction	*Percentage of adipose tissue samples containing HSV genome (%)*	*Percentage of PCR-positive samples displaying GDNF values above baseline levels (%)*
2	43	80
42	36	75
57	36	50

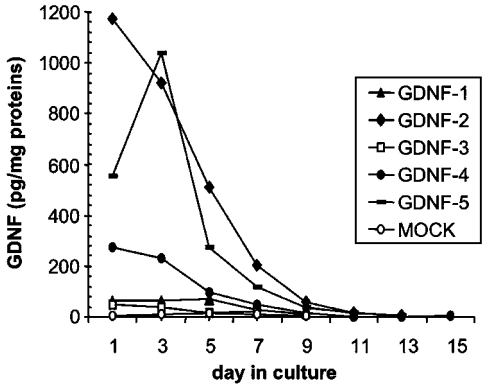

Figure 9 *GDNF secretion from adipose tissue after in vivo transduction. Rabbit adipose tissue inoculated in vivo with QL2HGDNF or QOZHG was excised 2 days after injection, cut in ~1 g fragments and maintained in culture as explants. GDNF secretion was monitored and quantified by ELISA assay and related to total protein concentration. QOZHG-injected explants served as controls (MOCK) to determine baseline values of GDNF ($n=5$). Different secretion profiles were observed among the five QL2HGDNF-derived explants. High amounts of GDNF (~1 ng/mg) were measured for explants GDNF-2 and -5 one day after establishment in culture (3 days post-inoculation), while moderate (GDNF-4) or low levels of GDNF were secreted by explants GDNF-1 and -3. GDNF production persisted for up to 12 days of culture after which GDNF levels reached baseline values.*

the QL2HGDNF-injected rabbits were analyzed and compared to the QOZHG-injected and naive animals. The ELISA assay detected no increase in plasma GDNF levels above baseline values for any animal from 1 to 8 weeks after injection. To verify that an impairment in secretion *in vivo* was not responsible for the lack of GDNF detection in the serum, pieces of rabbit fat injected *in vivo* were excised 2 days post-injection, maintained in culture as explants and the levels of secreted GDNF measured (Figure 9). Among the tissue fragments ($n=5$) obtained from the QL2HGDNF-injected animal, different expression profiles were observed, with high levels of expression (up to 1.1 ng/mg protein) for two fragments (GDNF-2 and -5). Others exhibited moderate (GDNF-4) to low (GDNF-1 and -3) GDNF secretion for up to 12 days in culture, after which the values returned to baseline levels measured for adipose tissue fragments ($n=5$) obtained from QOZHG-injected rabbit. These observations indicated that the absence of detectable GDNF in the circulation was unlikely the result of impaired GDNF secretion from adipose tissue transduced *in vivo*.

Discussion

Previously, we reported that an HSV-derived nerve growth factor (NGF) gene vector administered *in vivo* to inguinal adipose tissue resulted in sustained circulating levels of NGF that prevented peripheral nerve degeneration in a mouse model of diabetic neuropathy.[16] While these experiments pointed out that inoculation of murine fat tissue with recombinant HSV vectors could provide sustained expression of a secreted transgene product, the vector distribution or nature of the transduced cells remained to be characterized. The goals of the current study were to examine in detail the ability of HSV vectors possessing more extensive IE gene deletions to infect and transduce adipose cells derived from rabbits and humans, and to evaluate potential vector-related toxicity, genome persistence and durability of transgene expression. We demonstrate here for the first time that both precursors and mature adipocytes of rabbit and human origin are readily transduced by recombinant genomic HSV-based vectors *in vitro*, requiring only a single infectious particle per cell to express and/or secrete detectable transgene products. Virus infectivity correlated with the presence of well-characterized HSV receptors (HveA and HveC) not previously shown to be present on adipose cells. Using β-gal and GDNF as transgene products, we observed GDNF secretion from adipose cells *in vitro* and local transgene expression in rabbit fat tissue following vector delivery *in vivo*. For example, at 2 days post-inoculation, the GDNF content of the dorsal fat pad injected with the GDNF gene vector was 10-fold higher than tissue inoculated with the control vector. However, the total amount of GDNF measured in the tissue represents only 30% of the amount predicted from our *in vitro* data. Approximately 40% of the tissue samples contained vector genomes (Table 1), a finding consistent with transgene expression at the site of injection with limited virus diffusion to neighboring cells. Optimization of vector dosage and delivery will be needed to improve vector distribution and the outcome of transgene expression, particularly for applications involving larger animals.

Transduction of adipose cells with HSV vectors compares favorably to the data available from other vector systems. Although recombinant SIV vectors have recently been shown to infect human adipocytes, the efficiency was substantially lower, requiring an MOI of 5000 to infect approximately 60% of cells in culture.[17] Adenoviral-mediated gene transfer has also been demonstrated in differentiated adipocytes *in vitro*[18] and following adipose tissue injection *in vivo*, with transgene expression persisting for approximately 2 weeks.[19–22] However, preadipocytes of either human or murine origin were not transduced unless either very high multiplicities were applied or the cells were modified to express the coxsackie-adenovirus receptor (CAR) normally lacking on the surface of preadipocytes.[22,23] In contrast, retroviral-mediated transduction is limited to replicating preadipocytes, while nondividing adipocytes are not infected.[24] After cell transduction, the HSV genome persists as an intranuclear episomal element,[25] eliminating risks related to insertional mutagenesis. The efficiency of HSV infection allows for repeat vector dosing, a possibility that is difficult to achieve with other vector systems.[16,26]

Our previous results demonstrated that a replication-defective HSV:NGF vector introduced into inguinal fat tissue of mice resulted in expression of NGF detectable in serum for 11 months.[16] In contrast, circulating vector-derived GDNF was not detected in rabbit plasma samples. While the precise reason for this outcome is unknown, the most likely explanation is that the rabbit study employed a vector dose that was 10 times lower on a per kilogram basis, resulting in plasma GDNF levels below the detection limit of the ELISA. Taking into account our previous data where only ~10% of the transgene product expressed in rabbit tissue reached the bloodstream,[27] that a total of 26 ng/mg of GDNF was

measured in the dorsal fat (representing 30% of the amount predicted from *in vitro* data), and that the volume of plasma for the animals used in these studies was 180 ml for rabbits and 800 μl for mice, the resulting plasma GDNF concentration fell below the detection limit of the ELISA (32 pg/ml). As mentioned, strategies to improve diffusion of the vector and overall transduction of voluminous fat pads may result in detectable serum levels. Other mitigating variables may include the short half-life of GDNF, the strength of the chimeric LAP2-HCMV promoter and the anatomical location of the targeted fat pad.

We previously reported that HSV-mediated delivery of GDNF to the peripheral and central nervous system under control of the HCMV promoter was therapeutic in short-term models of spinal cord injury, neuropathic pain and Parkinson's disease,[26,28] but long-term therapeutic gene expression was only achieved using the LAP2 promoter.[15] In our experience, the activity of this promoter in adipocytes is very low (JF, unpublished observation). However, LAP2 used in combination with a strong promoter such as HCMV has shown enhanced activity (SH and WG, unpublished data). Accordingly, we tested the LAP2-HCMV chimeric promoter to examine the possibility of prolonged transgene expression in adipose cells. We observed continued local GDNF production for at least 2 months in rabbit adipose tissue *in vivo*. However, initial high levels of transgene expression gradually declined during this time period, suggesting that LAP2 could not sustain maximum levels of transgene expression. Nevertheless, for highly active neurotrophins such as GDNF, this lower level of expression may be sufficient to achieve biological effects *in vivo*, as recently observed in rodent CNS models of Parkinson's disease.[15]

Considerable progress has been made in reducing HSV vector-associated cellular toxicity.[11,29] Among the immediate early genes that affect cell viability, the ICP0 gene product has been shown to arrest cell cycle progression and disrupt nuclear domain 10 structures that contain a number of cellular products including DNA repair functions, histone deacetylases and promyelocytic leukemia antigen (PML).[30–32] Additionally, ICP0 possesses ubiquitin ligase activity and has been shown to target particular cellular functions for proteosome degradation as a prelude to viral replication.[13] Mutant viruses that fail to express the IE genes including ICP0 are noncytotoxic, but their genomes are strongly repressed in standard fibroblast cultures and transgenes are rapidly silenced.[11] In contrast to the situation in non-neuronal cells, ICP0 is rapidly degraded in neurons, which may contribute to the ability of HSV to establish latency in these cells.[10] Based on the above considerations, we selected for the current study a mutant virus background that would provide the most efficient transduction and transgene expression. The highly defective vectors we used express a single viral function, ICP0, with previously unknown potential toxicity for nondividing, mature adipocytes. As observed with other cell types,[10] the ICP0 gene product accumulated in the nucleus of both preadipocytes and mature adipocytes with attendant cytotoxicity. Compared to adipocyte cultures, preadipocytes plated at low density were highly sensitive to the cytotoxic effects of ICP0, which included interference with both cell division and differentiation. In contrast, vector injections into adipose tissue *in vivo* suggested that intact fat tissue was more resistant to the toxic effects of this viral product since vector genome stability and persistent transgene expression were observed. While these *in vivo* findings may be more promising, it is clear that a better understanding of the role of ICP0 in preventing promoter silencing will be required to engineer vectors that are broadly applicable to non-neuronal cells.

The central role of obesity in the development of adult onset diabetes and cardiovascular diseases suggests that genetic modification of fat tissue will likely emerge as an important therapeutic strategy in the future. The feasibility of targeting adipose tissue is supported by several practical considerations. First, fat is found in most body locations under the skin surface and is readily accessible for nonsurgical vector administration. Second, fat is largely dispensable, allowing safe removal of the injected tissue in the event of a possible gene transfer-related adverse reaction. Third, fat is often well vascularized and secretes a wide range of molecules into the blood, providing opportunities to either supply therapeutic products to the circulation or alter adipose tissue metabolism locally. And fourth, multipotent precursor stem cells can be readily isolated from fat tissue with the potential for application to selected forms of tissue repair. Here, we provide evidence that HSV vectors may be developed for gene transfer to adipocytes and their precursors, which could ultimately translate into the development of new strategies for the treatment of obesity-related diseases.

Materials and methods

Cell culture

Human subcutaneous preadipocytes and culture media were purchased from Zen-Bio Inc. (Research Triangle Park, NC, USA) and cells were differentiated according to the provided instructions. Rabbit stromal-vascular cells were extracted from dorsal adipose tissue according to a modification of standard protocols[33,34] and expanded in preadipocyte media. Briefly, rabbit fat was digested for 30 min at 37°C in a 0.05% collagenase A (Roche, Indianapolis, IN, USA) solution containing 0.5 mM glucose (Sigma, St-Louis, MO, USA) and 4% bovine serum albumine (BSA, Sigma) in phosphate saline buffer (PBS). The resulting cellular suspension was filtered through a 250 μm tissue sieve, pelleted at 700 *g* for 10 min and contaminating red blood cells were eliminated by hypo-osmotic shock. Vero cells (African Green monkey kidney cells, ATCC (CCL81), Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS, Sigma). CHO-HveA and CHO-HveC cells (kindly provided by Dr Patricia Spear, Northwestern University) were cultured in F-12K medium supplemented with 10% FBS, 100 U/ml Penicillin and Streptomycin and 250 μg/ml G418 (Invitrogen).

Flow cytometry analysis of HSV entry receptors

Human preadipocytes (passages 3–6) were analyzed for surface expression of the two HSV-1 receptors by flow cytometry (FACSCalibur, Becton-Dickinson, San Diego,

CA, USA). Cultured cells were trypsinized and stained with the following primary antibodies (Abs) diluted in PBS containing 1% FBS: R140, a rabbit antiserum directed against the HveA receptor ($n=6$) and two mouse monoclonal Ab directed against the human HveC molecule: CK6 ($n=2$) and CK41 ($n=3$) (kindly provided by Drs Gary Cohen and Roselyn Eisenberg, University of Pennsylvania). Secondary Abs included an FITC-conjugated goat anti-rabbit immunoglobulins (IgG) (Sigma) and a goat RPE-conjugated anti-mouse IgG (Dako, Denmark). For double-labeling experiments ($n=2$), biotinylated CK41 antibody was used in combination with a streptavidin-APC conjugated secondary antibody (Sigma). Negative controls were performed using secondary Abs only. Signal for nonspecific background was tested on Chinese Hamster Ovary (CHO) cells, while CHO cell lines expressing human HveC (M3A) and HveA (M1A) were used as positive controls.

Histochemistry

Cultured cells were fixed with 1% formol (Sigma) in PBS at room temperature for 5 min, then rinsed with PBS and permeabilized with methanol for 2 min at -20°C. Indirect immunofluorescence staining was performed by first blocking with 10% horse serum (HS) (Invitrogen) in PBS for 30 min followed by incubation for 3 h at room temperature or overnight at 4°C with primary antibody in PBS/1% FBS. Primary antibodies used were CK41 (40 μg/ml), CK6 (175 μg/ml), R140 (1:200) or the mouse anti-ICP0 mAb (1:5000, Virusys, East Coast Biologics Inc., North Berwick, ME, USA). Cells were then incubated with a Cy3-conjugated anti-mouse or anti-rabbit secondary antibody (1:500, Sigma) for 45 min at room temperature. Cells were observed with a Nikon Diaphot 300 inverted fluorescent microscope (Nikon Inc., Melville, NY, USA).

Construction and purification of replication-defective genomic HSV-1 vectors

The backbone vector QOZHG was created by a genetic cross between TOZ.1 (ICP4$^-$, ICP27$^-$, ICP22$^-$, UL24$^-$::ICP4p-tk, UL41$^-$::ICPOp-lacZ) and d106 (ICP4$^-$, ICP27$^-$::HCMV IEp-EGFP, β-ICP22, β-ICP47).[10,11] The construction of the GDNF expression vector QL2HGDNF was similar to QL2HNT-3, a vector with a chimeric LAP2/HCMV promoter driving NT-3 in the QOZHG backbone.[35] The rat GDNF PCR product[28] was ligated with *Xba*I linkers, digested with *Xba*I, and inserted into the *Xba*I site of the plasmid p41L2H*lac*Z to create p41L2HGDNF. Plasmid p41L2H*lac*Z contains a LAP2HCMV*lac*Z polyA cassette in which the *lac*Z sequences are flanked by *Xba*I sites. The entire expression cassette is flanked by HSV sequences nt. 90145–91605 and nt. 92193–93857. Plasmid p41L2HGDNF was co-transfected with QOZHG viral DNA and clear plaques were purified by three rounds of single plaque isolation[36] and the genetic structure confirmed by Southern blot analysis using GDNF and UL41-specific probes. Viral stocks were purified in a continuous nycodenze gradient (Optiprep, Life Technologies Inc., Gaithersburg, MD, USA), resuspended in PBS, aliquoted, stored at -80°C and titered on 7b cells.

In vitro transduction of cultured cells and rabbit adipose tissue explants with replication-deficient HSV vectors

Subconfluent cell monolayers were infected with HSV vectors and analyzed at different times after infection. Transduction efficiency was quantified either by direct determination of the percentage of β-gal positive cells after X-gal staining of 0.25% glutaraldehyde-fixed cell monolayers or by flow cytometry of EGFP-expressing infected cells (FACSCalibur, Becton-Dickinson). The viability of infected cultures was evaluated by MTT ($n=4$) assays (Sigma). Adipose tissue derived from naive rabbits was cultured as explants (~0.6 g per sample) in 12-well plates containing 2–3 ml of adipocyte media (Zen-Bio). *In vitro* injection of the explants with HSV vectors was carried out with a 3/10 cc syringe in one 20 μl-injection containing the desired amount of viral particles.

Quantification of GDNF secretion after transduction

Quantification of GDNF production by transduced cultured cells and adipose tissue explants was measured in culture media using the GDNF Emax ImmunoAssay System (Promega, Madison, WI, USA). Supernatants were regularly harvested for quantification of transgene secretion by ELISA as well as protein content determination by MicroBCA assay (Pierce, Rockford, IL, USA). Samples from later time points of culture were concentrated using YM-10 centrifugal filter devices (Centricon, Millipore, Bedford, MA, USA).

In vivo transduction of rabbit adipose tissue with HSV vectors

New Zealand White rabbits (Myrtles Rabbitry, Thompson Station, TN, USA) were anesthetized with ketamine/acepromazine and injected through the skin into the dorsal fat pad with 7–8 injections of 20 μl of vector diluted or not in PBS, for a total of 2×10^8 particles (~160 μl) of Optiprep-purified vector. The animals were divided in two groups and compared to uninjected animals. One group received the QL2HGDNF vector encoding the secreted GDNF protein, while the control group received the QOZHG vector encoding the β-gal reporter gene. Blood samples were harvested each week and one animal per group was killed at day 2, day 42 and day 57 after infection. The entire dorsal fat pad was then excised, cut in ~1 g fragments and processed for various analysis: some samples were (a) frozen in liquid nitrogen for protein, DNA and RNA extraction, (b) X-gal stained, (c) fixed in 10% formalin and embedded in OCT compound (Tissue-Tek, Sakura Finetek USA, Torrance, CA, USA) for cryosection (20 μm) analysis using the CryoJane® Tape-Transfer System or (d) cultured as explants in adipocyte media.

Assessment of transgene expression in adipose tissue

X-gal staining was performed on unfixed adipose tissue samples by overnight incubation at 37°C in a solution of 1.1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Roche), 14.7 mM $K_4Fe(CN)_6$, 14.7 mM $K_3Fe(CN)_6$ in 25 mM Tris buffer at pH 8. Total proteins were extracted from frozen adipose tissue of each rabbit by incubation (1:3 w:v ratio) for 2 h at 4°C in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA,

1% Nonidet P-40, 1 mM PMSF and 5 μg/ml leupeptin) followed by sonication and centrifugation at 4°C for 15 min at 2100 *g* (adapted from Nisoli *et al*[37]). The infranatants were kept at −80°C for further analysis and GDNF ELISA quantification.

Nucleic acid isolation and analysis

Genomic DNA was isolated from rabbit adipose tissue fragments (~1 g) using a Qiagen DNeasy tissue Kit (Qiagen, Valencia, CA, USA). Frozen or protein-extracted adipose tissue samples were processed according to supplier's instructions. PCR analysis was performed on a Robocycler (Stratagene, La Jolla, CA, USA) with *Taq* platinum (Invitrogen) using 150 ng of genomic DNA from each sample in a reaction volume of 50 μl. The following primer pairs were used: (Primer1/Primer2 (product size, product identity, annealing temperatures)): U_L30U/U_L30L (479 bp, HSV U_L30, 63/58°C), CAGTAC GGCCCCGAGTTCGTGAC/GTCGTAGATGGTGCGGG TGATGTT; *LacZU*/*LacZL* (535 bp, *lacZ*, 63/58°C), CACC AGCAGCAGTTTTTCCAGTTCC/GGGTCGCTTCACTT ACGCCAATGT; RabGAPDHU/RabGAPDHL (262 bp, rabbit GAPDH 65/58°C) TCTGGGAA-GCTGTGGCGTG AC/CCTCGGTGTAGCCCAGGATGC; GDNFU/GDNFL (289 bp, GDNF, 61/55°C), ATGGCAGTGCTTCCTAGA AGAGAGC/AACATGCCTGCCCTACTTTGTCA; PCR conditions were as described with the appropriate annealing temperatures for each primer set.[27] The PCR reaction (12 μl) was subjected to electrophoresis on 1% agarose TBE/EtBr gels and images were acquired with a Kodak DC120 Scientific Imaging System (Kodak, Rochester, NY, USA).

RNA extraction and RT-PCR

Total RNA was extracted from rabbit adipose tissue, explants or cultured cells using an RNA/DNA tissue kit (Qiagen) following homogenization with a tissue homogenizer (Tissue tearer, Biospec Products Inc.). DNase I (Promega) treatment was performed on RNA samples to avoid genomic DNA contamination. A first-strand cDNA synthesis kit was used with Oligo dT primers (Invitrogen) to generate cDNAs. In all, 2 μl of the reaction mixture was amplified in a 50 μl PCR reaction with specific primers (20 pmol) and *Taq* Platinum Polymerase as described above in the presence or absence of reverse transcriptase.

Statistical analysis

Data is presented as mean ± s.d. A two-tailed nonparametric Mann–Whitney test (GraphPadPrism3.0 software) was used for statistical comparisons between rabbits. Multiple groups were compared using the Kruskal-Wallis test followed by Dunn's post-tests. In all statistical tests, the confidence interval was set at 95% ($P < 0.05$).

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