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ORIGINAL ARTICLE

Local gene transfer to calcified tissue cells using prolonged infusion of a lentiviral vector

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Gene transfer using viral vectors offers the potential for the sustained expression of proteins in specific target tissues. However, in the case of calcified tissues, in vivo *delivery remains problematic because of limited accessibility. The aim of this study was to test the efficiency of lentiviral vectors (LVs) on osteogenic cells* in vitro, *and determine the feasibility of directly transducing resident bone cells* in vivo. *LVs encoding for green fluorescent protein (GFP) and ameloblastin (AMBN), a protein associated with mineralization not reported in bone, were generated. The transduction efficiency of the LVs was evaluated using the MC3T3 cell line and primary calvaria-derived osteogenic cells. For* in vivo *delivery, the LVs were infused using osmotic minipumps through holes created in the bone of the rat hemimandible and tibia. The production of GFP and AMBN* in vitro *and* in vivo *was monitored using fluorescence microscopy. Both transgenes were expressed in MC3T3 and primary osteogenic cells.* In vivo, *GFP was detected at the infusion site and fibroblast-like cells, osteoblasts, osteocytes and osteoclasts expressed AMBN. Our data demonstrate, for the first time, that primary osteogenic cells are efficiently transduced with LVs and that their infusion is advantageous for locally delivering DNA to bone cells.*
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Keywords: *ameloblastin; bone; osteogenic cell culture; gene transfer; green fluorescent protein; lentiviral vector*

Introduction

Gene transfer is particularly advantageous for sustained delivery of proteins and offers an exciting avenue for the treatment of a variety of musculoskeletal problems. Expected clinical applications include treatment of orthopedic and periodontal diseases, healing of cartilage and bone defects, and ligament and tendon repair.[1–3] So far, the most efficient and commonly used vectors for gene delivery have been viruses.[4] Viral vectors have been administered to skeletal sites in animals, mainly by local injections in vasculature trunks,[5] muscle,[6–9] natural cavities (e.g. joints and synovial spaces)[10–14] and in post-surgical, traumatic or congenital areas of bone defects.[7,15–18] Systemic delivery via the portal vein has also been tested to reverse osteopenia in ovariectomized mice.[19] An alternative treatment strategy has been to transduce cells *in vitro*, expand and implant them at the desired site.[20–23]

Most gene transfer studies conducted to date have used the so-called 'first-generation' adenoviruses.[2,24,25] Their application has been limited by the inflammatory response they elicit *in vivo*.[6,16,26,27] Lentiviruses, derived from feline (FIV) and the human immunodeficiency virus (HIV), have been developed as alternative vectors as they are capable of stably transducing both dividing and quiescent cells, and integrate into the host genome for prolonged transgene expression.[26] In addition, they are less likely to induce an inflammatory reaction.[28] Efficient lentivirus-mediated gene transfer has been demonstrated in several cells and tissues, including skeletal muscle,[29] salivary glands,[30] liver,[29] neural tissues[31,32] and keratinocytes.[33] There are only few studies on the administration of lentiviral vectors (LVs) at calcified tissue sites and these dealt with the periosseous implantation of cells infected *ex vivo*[21,34] and local injection of the vector into the synovium.[10,35]

Local delivery of viral vectors into bone remains problematic because it has so far been limited to readily accessible anatomical sites such as the synovial cavity and bone defects, and relatively large amounts of vector and its local persistence are required to successfully infect a sufficient number of cells.[36] The latter issues are particularly problematic for small animal models, where size greatly limits the amount of virus that can be administered locally. The laboratory has been using 'bony windows' coupled to osmotic minipumps to continuously deliver to calcified tissues bioactive agents.[37–39] Whereas this approach allows administration of small quantities that respect the local tissue physiology, it also permits to locally attain relatively high

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concentrations and, most importantly, to continuously replenish any material that would be rapidly cleared away.

The objective of the present study was to first demonstrate that LVs can efficiently transduce osteogenic cells *in vitro* and then determine the feasibility of directly targeting cells constituting bone tissue. For *in vitro* analyses, we have used both the mouse MC3T3-transformed cell line and primary rat calvaria-derived cells, both of which are well-established models of *in vitro* osteogenesis. The 'bony window' approach was applied to continuously infuse LVs in the rat hemimandible and tibia. Two vectors were tested, one encoding for the cytoplasmic green fluorescent protein (GFP) reporter molecule and the other for ameloblastin (AMBN), a matrix protein secreted by the epithelial tooth ameloblasts.[40] AMBN was selected as it is implicated in biological mineralization but has been found in bone, and thus could easily be distinguished from other products manufactured by osteoblasts. The results demonstrate that LVs efficiently transduce both transformed and primary osteogenic cells *in vitro*, and that their continuous infusion is an advantageous strategy to locally transfer DNA to bone cells.

Results

Construction of LVs and in vitro *transduction efficiency in osteogenic cells*

The lentiviral maps of the vectors encoding for GFP (GFP-LV) and for AMBN (AMBN-LV) are illustrated in Figure 1. Each LV was pseudotyped with vesicular stomatitis virus G (VSVG) glycoprotein, packaged, concentrated by ultracentrifugation, and then titered in human embryonic kidney (HEK) 293 cells. Titers were estimated in the range between 7.5×10^8 and 1×10^9 infectious units (IU)/ml. The relative efficiency of the generated lentiviruses was evaluated *in vitro* on osteogenic cells from the mouse MC3T3 cell line and primary cultures (Figure 2). GFP was distributed throughout the cell (Figure 2a and b), including the nucleus, whereas AMBN was immunolocalized to a perinuclear region where the Golgi apparatus is found (Figure 2d and e). The percentage of infected MC3T3 cells showed little variation (almost all cells were infected) as the number of IU/well increased (data not shown). However, the intensity of the fluorescence signals, at 72 h post-infection, increased dramatically with the number of IU/well (Figure 2c and f). Overall, there was no significant difference in the transduction efficiency between MC3T3 and primary calvaria-derived cells (Figure 2c and f).

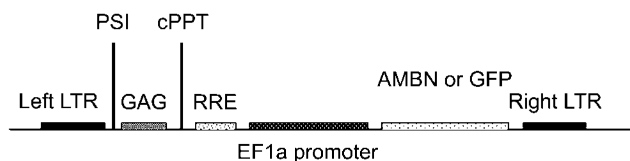

Figure 1 Plasmid maps for GFP-LV and AMBN-LV vectors under the control of the EF-1 α promoter. AMBN, ameloblastin; GAG, viral helper gene; GFP, green fluorescent protein; LTR, long-term repeats; PSI, packaging signal; cPPT, central polypurine tract; RRE, Rev-responsive element.

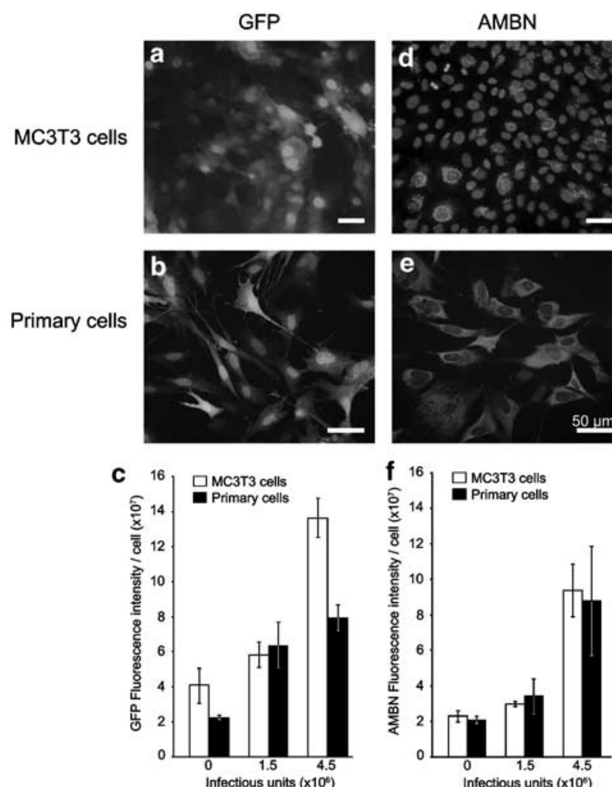

Figure 2 Transduction efficiency of GFP-LV and AMBN-LV in MC3T3 cells (**a**, **d**) and in primary, rat calvaria-derived osteogenic cells (**b**, **e**), at 72 h after infection, with an infectious unit of 4.5×10^6 IU/ml. Expression of GFP is revealed by direct epifluorescence imaging and of AMBN by immunofluorescence labeling. Note the diffuse cytoplasmic distribution of GFP (**a**, **b**) and the perinuclear localization of AMBN (**d**, **e**). The intensity of expression of the transgene increases with viral dosage (**c**, **f**). Nuclei (blue) in (**b**, **d**, **e**) are stained with Hoechst. (For colour figure see online version)

In primary osteogenic cells, immunoblotting revealed no major difference in the expression of bone sialoprotein (BSP) and, osteopontin (OPN) between cells expressing the AMBN transgene and control, uninfected cells at 96 h post-infection (Figure 3a). Double immunofluorescence labeling confirmed the coexpression of AMBN and OPN in infected cells (Figure 3b and c). Transgene expression persisted for almost 2 weeks without any apparent change in intensity (Figure 3c). Measurements of the intensity of fluorescence signals after passage (Figures 4a and b), indicated that the level of GFP expression was not diminished, however, it was reduced by about half for AMBN (Figure 4c). Subculturing the cells did not significantly affect the percentage of infected cells for both the GFP and AMBN transgenes (Figure 4d). Uninfected cell cultures, and infected cultures under control immunolabeling conditions exhibited only background fluorescence (data not shown).

In vivo *expression of lentiviruses delivered with an osmotic minipump*

Having demonstrated that lentiviruses can effectively transduce osteogenic cells *in vitro*, we evaluated their

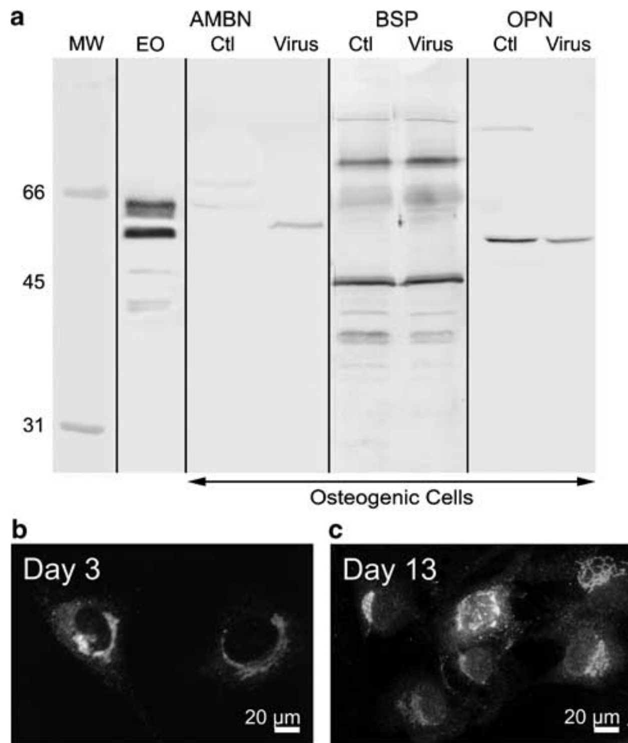

Figure 3 (**a**) Western blot analysis of AMBN expression in rat incisor enamel organ sample from the secretory stage (EO) and uninfected (Ctl) and AMBN-LV infected (virus) rat calvaria-derived osteogenic cells. The osteogenic cells were also probed for the production of BSP and OPN. Expression of the AMBN transgene by osteogenic cells does not significantly alter that of these two representative bone matrix proteins. The first lane contains standard broad-range molecular weight markers (MW in kDa) stained with Ponceau red. (**b**, **c**) Double-labeled immunofluorescence preparations for OPN (green) and AMBN (red) in osteogenic cells at day 3 (**b**) and day 13 (**c**) post-infection with AMBN-LV. In cells expressing both proteins, the green and red fluorescence signals overlap generating a yellowish-orange color over the Golgi apparatus. (For colour figure see online version)

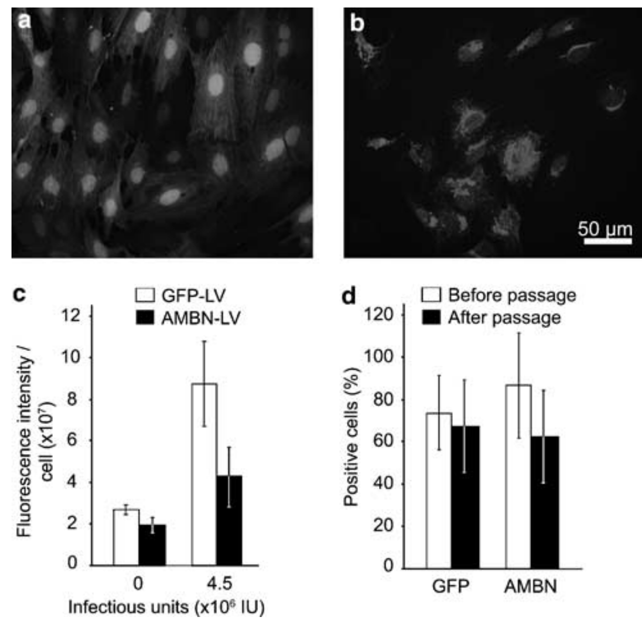

Figure 4 Lentivirus-infected rat calvaria osteogenic cells. As seen at 48 h after subculturing, second-generation cells maintain expression of GFP (**a**) and AMBN (**b**). When comparing before and after passage, the intensity of GFP labeling was not significantly reduced, whereas AMBN expression decreased (**c**). However, the proportion of infected cells was maintained after passage (**d**). Nuclei are stained blue with Hoechst. (For colour figure see online version)

capacity to infect bone cells in the hemimandible and tibia (Figures 5 and 6). GFP was used to assess vector delivery by minipumps ($\sim 30 \times 10^6$ IU over 5 h) and the resultant macroscopic distribution of infected cells. Observation under epifluorescent light revealed GFP expression mainly at the site of infusion and its immediate periphery (Figures 5b and 6b). It was estimated that about 3–8% of cells on a tissue section, within a radius of 200 μm from the drilling site, were positive for GFP (Figure 5b). However, some infected cells were detected several millimeters away along the incisor tooth (Figures 5c and d). Occasionally, the GFP signal extended outward of the 'bony window' along the surface of the hemimandible and tibia (data not shown), suggesting that some of the vector may have leaked out during infusion.

At 4 days following the infusion of AMBN-LV in the hemimandible, the protein was detected in mononuclear cells, including some osteoblasts and multinucleated cells bordering the 'bony window' (Figure 5e). In the tibia, there was an accumulation of mesenchymal cells in relation to the surgical site and cells with a rounded profile and others with a fibroblast-like appearance expressed AMBN (Figure 6c and d). After 7 days at both infusion sites, osteoblasts and osteocytes in newly formed bone were seen to express the transgene (Figures 5f, g and 6e). In the tibia, disturbance of the periosteum by drilling frequently caused focal areas of endochondral ossification on the nearby bone surface (Figure 7a). Chondrocytes forming the cartilage callus expressed AMBN (Figure 7b), whereas distant chondrocytes within the epiphysal growth plate were unlabeled (Figure 7c).

No obvious side effects as well as no significant difference in bone formation between animals receiving GFP-LV or AMBN-LV were apparent from routine histological observation.

Discussion

Gene transfer has been applied successfully in various animal models to locally express growth factors and stimulate bone repair.[2,41] The majority of these studies have used adenoviral vectors to infect cells either *ex vivo* or *in vivo*. These vectors, however, elicit an immune response and have a limited duration of expression.[4,26] New generations of vectors have been generated to address these limitations. Among these, adeno-associated viruses appear promising as they do not encode for inflammatory viral proteins[26,42–44] and have successfully been applied to stimulate osteogenesis *in vivo*.[8,9] However, they do not integrate the host genome and are difficult to generate at high titers.[42,43] We have opted to use an HIV-derived vector because of the following well-established characteristics of lentiviruses: (1) they are capable of infecting both dividing and non-dividing

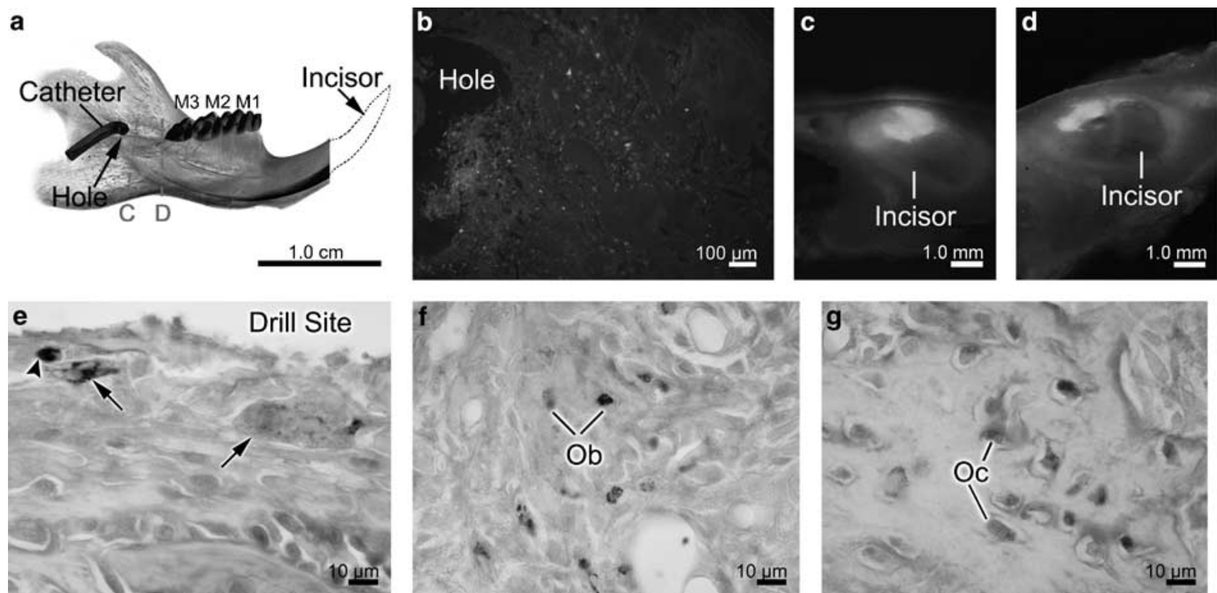

Figure 5 Infusion of LVs through a 'bony window' in the rat hemimandible. (**a**) Computed tomography scan image illustrating the position of the metal catheter linking the vinyl tubing of the osmotic minipump to a hole on the buccal aspect of the hemimandible. (**b**) Low magnification epifluorescence micrograph from a section passing through the 'bony window' illustrating expression of GFP by cells near the site of infusion. (**c**, **d**) Whole-mount cross-sectional profiles at position of the red lines in (**a**). The viral vector can diffuse for several millimeters along the periodontal space surrounding the incisor tooth. (**e**) Immunolabeling showing expression of AMBN by mononuclear (arrowhead) and multinucleated (arrows) cells at 4 days following infusion of AMBN-LV. (**f**, **g**) At 7 days post-infusion, numerous osteoblasts (Ob) and osteocytes (Oc) in bone near the drill site are immunoreactive. M1, 2, 3, molars. (For colour figure see online version)

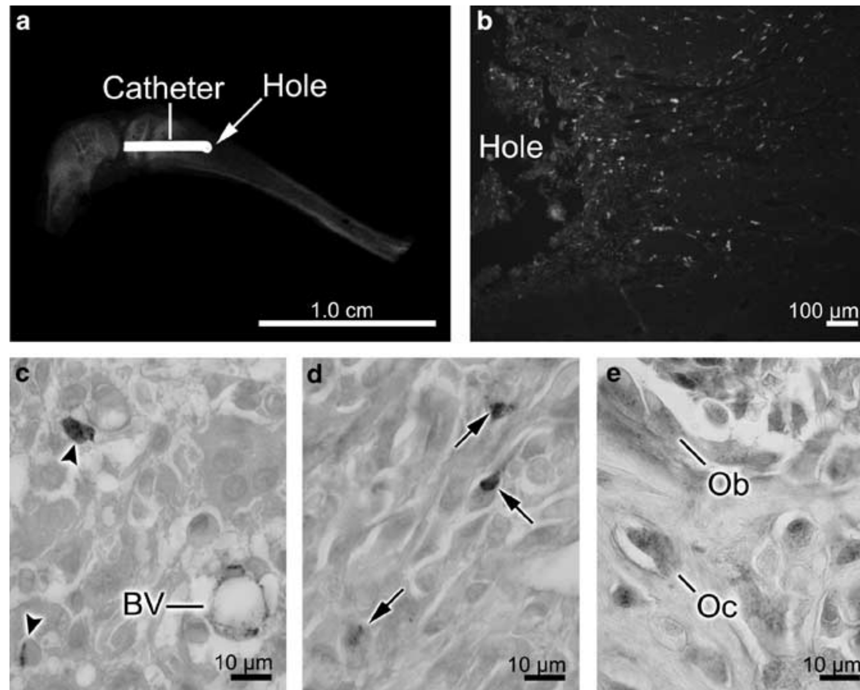

Figure 6 Infusion of LVs through a 'bony window' in the rat tibia. (**a**) X-ray showing the insertion position of the minipump catheter into the cortical bone of the in tibia. (**b**) Transgene expression, here illustrated following infection with GFP-LV, is generally restricted to the site of infusion. (**c**, **d**) At 4 days following infusion of AMBN-LV, AMBN was observed in some mononucleated (arrowheads), endothelial (BV) and fibroblast-like (arrows) cells within the mesenchymal condensation that occurred in neighboring marrow. (**e**) At 7 days, some osteoblasts (Ob) and osteocytes (Oc) associated with newly formed bone express the transgene. (**c**, **d**, **e**) Sections were counterstained with methyl green. (For colour figure see online version)

cells, (2) they are less immunogenic because no viral proteins are produced, (3) they have a wide tropism, (4) they can be concentrated to high titers and (5) they incorporate into the host genome.[28,45]

Results from the present study show that LVs can efficiently be used to drive expression of exogenous proteins in osteogenic cells, both *in vitro* and *in vivo*. The transduction efficiency of our vectors was evaluated

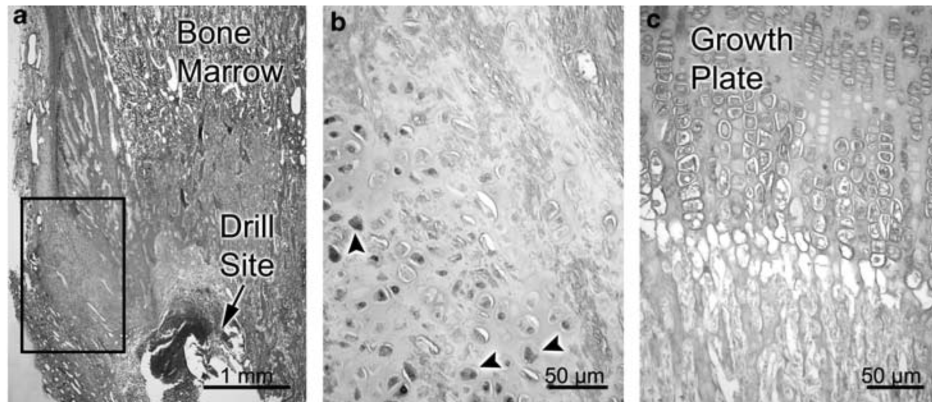

Figure 7 Infusion of AMBN-LV in the tibia. (**a**) Disturbance of the periosteum during surgery can cause formation of a cartilaginous callus (boxed), which undergoes endochondral ossification. Section stained with hematoxylin and eosin. (**b**) Immunodetection performed on an adjacent section shows chondrocytes (arrowheads) constituting the callus (boxed area in a) at day 7 post-infusion express AMBN. (**c**) Growth plate chondrocytes on the same section are not immunoreactive for AMBN. Sections (**b**, **c**) were counterstained with methyl green. (For colour figure see online version)

in vitro on both MC3T3 and primary osteogenic cells. With both the GFP-LV and AMBN-LV, it was possible to achieve 100% infectivity even at the lowest viral concentration used. Expression of the transgenes was sustained for almost 2 weeks and, in the case of primary osteogenic cells, it was maintained after passage. Taken together, the persistence of expression and its retention after subculturing suggest that the viral DNA integrated the host cell genome, allowing constitutive expression of the exogenous proteins. To our knowledge, this is the first demonstration of the use of lentiviruses to over-express a desired protein in primary calvaria-derived osteogenic cells. Although it is difficult to extrapolate the *in vitro* behavior to the *in vivo* situation, clearly the vectors are highly infectious and have a high propensity to infect cells of the osteogenic lineage. It should also be pointed out that expression of AMBN did not significantly alter the production of two representative endogenous matrix proteins (BSP and OPN), suggesting that it has no short-term detrimental effect on the cells.

Although there is abundant literature on the experimental use of various gene therapy vectors for skeletal applications, these studies essentially aimed at releasing mediators in proximity to the site of administration that would diffuse locally to influence repair and regeneration of cartilage/bone. Our objective was to target directly the cells that constitute bone tissue. At the two anatomical sites tested, that is, the hemimandible and tibia, osteoblasts, osteocytes, osteoclasts and fibroblast-like cells that could represent osteoprogenitor cells were infected and expressed AMBN. These cells were generally found in proximity to the site of infusion; however, transduced cells were sometimes also present some distance away. In the hemimandible, GFP-expressing cells were detected along the periphery of the incisor, suggesting diffusion of the vector. However, the complexity of the mineralized tissues of the tooth would make such diffusion unlikely. An alternative explanation is that cells in the apical portion of the tooth, which is situated nearby the 'bony window',[37] were infected. As these cells differentiated, they passively migrated away with the incisor, which continuously erupts at a rate of $\sim 650\ \mu$m/day).[46] In the tibia, cells expressing AMBN were found in a cartilage callus near the surgical site. It is known that disturbing the periosteum of long bones stimulates the formation of cartilage.[47] Some vector may have leaked out of the 'bony window' during infusion and transduced some precursor cells in the periosteum. Noteworthy, these cells retained the transgene throughout the differentiation sequence into chondrocytes. As the callus results from several cell divisions, the presence of chondrocytes expressing AMBN in them suggests that the viral DNA integrates the host cell genome also *in vivo*.

A major limitation associated with injection of viral vectors is the fact that large amounts are required because of local dispersion, clearance out of the injection site and immune inactivation.[36] Precision of the injection may also be an issue; for instance, in the case of bone defects, vector lost to surrounding muscle has been reported to influence the outcome of the treatment.[48] Infusion through a 'bony window' can circumvent these problems by continuously delivering small quantities of vector over time directly into the bone tissue at well-defined locations. Cells at the infusion site and its immediate surroundings are bathed for several hours with the viruses, a situation with similarities to what occurs *in vitro* where it is possible to achieve high levels of infectivity with even very small amounts of vector. Such a sustained exposure may also facilitate incorporation in cells of multiple copies of the transgene, thereby potentially increasing the amount of protein produced.

In conclusion, our study shows that osteogenic cells can be transduced with LVs to express cytoplasmic and secreted proteins, and establishes the feasibility of directly infecting cells in bone tissue using continuous infusion. This proof of principle represents a mandatory first step toward local gene transfer studies in models of bone formation, repair and pathological loss. Expression of matrix proteins, such as AMBN, offers the possibility to study their function and possible application in the development of novel strategies for bone regeneration. In addition, because lentiviruses can sustain expression of a transgene over periods of several months,[29,30,34,49] they can be advantageously exploited for rescue experiments in knockout mice with canonical and mutated proteins.

Materials and methods

LV production and titer determination

Lentivirus encoding for GFP (GFP-LV) or AMBN (AMBN-LV), under the transcriptional control of the EF1-α promoter, were produced in HEK293 cell line. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, Ontario, Canada) with 10% fetal bovine serum (FBS) (Invitrogen). HEK293 cells were plated at a cellular density of 6×10^6, in 175 cm^2 flasks. The following day, the cells were transfected using Fugene 6 (Roche Diagnostics, Quebec, Canada) with 13 μg human papillomavirus (HPV) 275, 1.3 μg P633, 1.3 μg HPV17, 1.3 μg VSVG and 11.7 μg of either GFP-LV or AMBN-LV. The lentiviruses were collected after 48 and 60 h, filtered through 0.45 μm filters and concentrated by ultracentrifugation at 4°C, 2 h, 24 000 r.p.m. The titers of the lentiviruses were determined on HEK293 cells by immunofluorescence detection.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting

Primary-derived osteogenic cells and enamel organ cells prepared from freezed–dried rat incisors, as described previously by Smith and Nanci,[50] were solubilized into sample preparation buffer and applied to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli.[51] Proteins were subsequently transferred onto 0.45 μm nitrocellulose membranes (Mandel, Ontario, Canada) and probed with anti-AMBN antibody (1:1000; courtesy of Dr PH Krebsbach[52]), anti-BSP antibody (1:500; LF-100; courtesy of Dr LW Fisher, NIDCR, NIH, Bethesda, MD, USA) and anti-OPN antibody (1:500; LF-123; courtesy of Dr LW Fisher) as described previously.[53] Antibody binding was revealed with a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (1:1000; Sigma, Oakville, Ontario, Canada) using *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as substrates (Amersham Biosciences, Quebec, Canada).

Cell culture studies with MC3T3 cells

The mineralizing subclone no. 4 of the MC3T3 cell line (ATCC, Manassas, VA, USA) that has been described previously[54] was used in this study. Cells were maintained in α-minimal essential medium (α-MEM) (Invitrogen) with 10% FBS and media changed every 3 days. For infection with lentiviruses, cells were seeded in six-well plates (60 000 cells/well) on glass coverslips.

Cell isolation of primary calvaria-derived osteogenic cells

All animal procedures were in accordance with the guidelines of the Comité de déontologie de l'expérimentation sur les animaux of Université de Montréal. Osteogenic cells were isolated by sequential trypsin/collagenase digestion of calvarial bone from newborn (2–4 days) Wistar rats (Charles River Canada, St-Constant, Quebec, Canada), as described previously.[55,56] Cells were seeded in six-well plates (70 000 cells/well) and 24-well plates (20 000 cells/well) on glass coverslips. Cells were grown for 3 days in MEM with Earle's salts (MEM Earle's) supplemented with 10% FBS and 1% penicillin–streptomycin (Invitrogen).

Transduction of MC3T3 and primary calvaria-derived cells with lentivirus

At confluency (day 3), MC3T3 cells and primary calvaria-derived osteogenic cells were infected with either lentivirus at different doses (1.5×10^6 and 4.5×10^6 IU/ml) in the presence of 8 μg/ml polybrene (Sigma). After 24 h, the medium was replaced with fresh α-MEM and MEM Earle's, respectively, supplemented with 10% FBS and 1% penicillin–streptomycin. After 48 h, the efficiency of transduction was assessed by immunofluorescence. To monitor the expression of the transgenes on a longer basis, primary osteogenic cells were passage and subcultured 7 days after infection. They were seeded in 24-well plates (20 000 cells/well) on glass coverslips and processed after 48 h for immunofluorescence detection.

Detection of transgene expression in cells by immunofluorescence

Cells were fixed and processed for immunofluorescence labeling with a primary antibody against recombinant rat AMBN (1:300/1 h; courtesy of Dr PH Krebsbach[52]) followed by a Alexa fluor 594 (red fluorescence)-conjugated goat secondary antibody (1:500/1 h; Molecular Probes, Eugene, OR, USA) as described previously.[57] Incubation with primary antibody against anti-OPN (1:600; LF-123) followed by a Alexa fluor 488 (green fluorescence)-conjugated goat secondary antibody (1:500/1 h; Molecular Probes, Eugene, OR, USA) was also carried out. The nucleus was stained with Hoescht 33342 (10 μg/ml) (Invitrogen). The cells were examined by epiluminescence under a fluorescence microscope (Axiophot; Carl Zeiss, Oberkochen, Germany). Gene transfer efficiency and intensity of labeling was measured using Version 5.1.2.59 of Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD, USA). Data described in the text and in Figures 2 and 4 are expressed as means$\pm$s.d.'s of five randomly selected fields from two representative experiments.

Surgical procedure

A 'surgical window' was created in the bone of the hemimandible as described by Vu *et al.*[37] and Nanci *et al.*,[39] and in the cortical bone of tibia of male Wistar rats weighting 100$\pm$10 g (Charles River Canada, St-Constant, Quebec, Canada). The lateral aspect of hind limb was shaved and cleaned with 70% ethanol. A 5–10 mm skin incision was made just proximal to the tibial tuberosity over the lateral aspect of the bone. The periosteum at the drilling site was exposed and gently scraped from the bone with a dental spoon excavator over an area of approximately 3 mm^2. A transcortical hole was then drilled using a dental drill size 010 with carbide round burr (Brassler, Montreal, Quebec, Canada), followed by a size 014 (Brassler). One-day Alzet osmotic minipump (model 2001D, 200 μl, 8.0 μl/h; Alza Corporation, Palo Alto, CA, USA) filled with PBS were slipped under the skin of the back of the animal. The vinyl tubing (Scientific Commodities Inc., Lake Havasu City, AZ, USA), connecting the osmotic minipump to the catheter, was filled with 40 μl lentivirus (30×10^6 IU; GFP-LV and AMBN-LV). The animals received an injection of Temgesic (Buprenorphine hydrochloride, Reckitt and Colman, Hull, UK) after surgery, and were fed with soft food

containing Temgesic. The animals were killed after 4 and 7 days.

Tissue processing and detection of transgene expression

The animals were anesthetized and perfusion-fixed as described.[39] Treated and control contralateral hemi-mandibles as well as knees were dissected and further fixed by immersion in the same fixative for 3 h at 4°C. The tissues were decalcified for 14 days at 4°C in 4.13% disodium ethylenediamine tetraacetic acid.[58] Decalcified tissues were washed for 24 h in 0.1 M cacodylate buffer, pH 7.2, and processed for paraffin embedding. Seven to 10-μm-thick sections were mounted on Superfrost/Plus (Fisher Scientific, Whitby, ON, Canada) slides for histology, and stained with hematoxylin and eosin. Briefly, deparaffinized sections were first blocked for 20 min, with a solution consisting of 0.01 M PBS, pH 7.2, containing 0.05% Tween 20 (0.01 M PBS–Tween 20) and 5% skim milk. They were then incubated with a primary antibody against recombinant rat AMBN (1:300/3 h). Sections were then washed with 0.01 M PBS–Tween 20 for 30 min, followed the DakoEnvision+ System, HRP kit (Dako Corporation, Carpinteria, CA, USA) was used as recommended by the manufacturer. Revelation was performed with diaminobenzidine and sections were counterstained with methyl green (Dako Corporation). Gene transfer efficiency and intensity of labeling was measured using Image-Pro Plus software.

Acknowledgements

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