

Genetic marking with the Δ LNGFR-gene for tracing goat cells in bone tissue engineering [☆]

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Abstract

The use of bone marrow derived stromal cells (BMSC's) for bone tissue engineering has gained much attention as an alternative for autologous bone grafting. Little is known however, about the survival and differentiation of the cells, especially in the clinical application. The aim of this study was to develop a method to trace goat BMSC's *in vivo*. We investigated retroviral genetic marking, which allows stable expression of the label with cell division.

Goat BMSC's were subjected to an amphotropic envelope containing a MoMuLV-based vector expressing the human low affinity nerve growth factor receptor (Δ LNGFR). Labeling efficiency and effect on the cells were analyzed. Furthermore, transduced cells were seeded onto porous ceramic scaffolds, implanted subcutaneously in nude mice and examined after successive implantation periods.

Flow cytometry indicated a transduction efficiency of 40–60%. Immunohistochemistry showed survival and subsequent bone formation of the gene-marked cells *in vivo*. Besides, marked cells were also found in cartilage and fibrous tissue. These findings indicate the maintenance of the precursor phenotype following gene transfer as well as the ability of the gene to be expressed following differentiation. We conclude that retroviral gene marking with Δ LNGFR is applicable to trace goat BMSC's in bone tissue engineering research.

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Introduction

Tissue engineering (TE), by combining specific cells with an appropriate scaffold has gained much attention during the last decade. The proof of the concept has been shown for many tissue types [25]. Especially the mesenchymal lineage has been investigated extensively, following the identification of putative stem cells in the adult bone marrow [8,10,11]. These Bone Marrow Stromal Cells (BMSC's) can be cultured and directed

towards several lineages of differentiation [6,8,11,33] e.g. bone [24,32], cartilage [11,35], and tendon [15]. Furthermore, these cells have been investigated as a delivery vehicle for gene therapy [1,26,30]. The application of BMSC's in tissue engineering of bone has progressed fast and some investigators have already made steps towards clinical application [34,36].

However, with regard to the clinical application of bone TE, there are two important gaps in the current knowledge of cell performance inside TE constructs. First, the differentiation of BMSC's cannot be predicted, because they constitute a heterogeneous population [6,11,33]. Second, little is known about cell survival. Although survival was shown convincingly in implants of the mm³-volume range in rodents [1,10,12,21], cell

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survival will be compromised in the clinical situation, due to the dramatically increased volume (several cm³), and delayed vascularization [13].

The most reliable method to investigate these two issues will be to monitor labeled BMSC's in an established "large animal" model for bone TE. Recently, we developed such a model for ectopic bone TE in the goat [23,24]. Together with a more challenging, critical size defect model in the same animal [2], this gives the opportunity to extensively evaluate both differentiation and survival of the BMSC's. However, an accurate labeling method to trace the cells inside TE bone, currently does not exist.

To trace cells efficiently, a variety of labeling methods have been developed. The use of fluorescent membrane markers is relatively simple [16,22,35], but inherent to dilution with cell division and the risk of label transfer to neighboring cells [22]. More reliable methods are the use of transgenic cells [21], x–y mismatches [10], or xenogenic transplantation [12]. These approaches however, are not compatible with autologous transplantation. Recently, genetic marking has become a standard tool and it was shown to be compatible with bone histology [1,3,9,14,28]. Although an immune response cannot be excluded, this approach seems to be most optimal at this moment [3,28].

To trace cells after long implantation periods in large bone samples, the label must be compatible with histologic procedures, such as dehydration and decalcification, and not depend on the diffusion of a substrate into the sample for enzymatic conversion. Therefore a retroviral label (which integrates stable in the cell genome), that can be traced with routine immunohistochemistry, is most appropriate. The truncated human nerve growth factor receptor (Δ NGFR) has been applied successfully for tracing many cell types, with both flow cytometry and immunohistochemistry [3,7,19,37]. The purpose of the present study was to develop a method to retrovirally label goat BMSC's with the NGFR label without noticeable effect on cell viability and bone forming capacity. Furthermore, the process of tissue engineered bone formation by constructs of goat BMSC's and ceramic scaffolds in the ectopic nude mice model was investigated.

Materials and methods

Study design

A key consideration in retroviral transduction is the choice of the viral surface envelope—pseudotype—which determines the vector tropism. In a first experiment we therefore compared amphotropic, GALV, RD114 and VSV-G vector pseudotyped virions and selected the best vector pseudotype for further studies with the NGFR marker gene.

The transduction efficiency, i.e. the percentage of NGFR-positive goat BMSC's, was determined using flow cytometry. Long-term sta-

bility of NGFR expression in vitro was determined by weekly analysis for up to 6 weeks. In addition, a population of transduced cells was co-cultured with mock-transduced cells to determine relative differences in cell proliferation.

To analyze in vivo traceability, transduced and mock-transduced cells were seeded on porous ceramic scaffolds and implanted subcutaneously in nude mice. The implants were evaluated at different time points.

Cell cultures

Cryopreserved goat BMSC's (2nd passage $P = 2$), that had shown to be osteogenic in a previous study [24] were thawed and replated in culture medium containing 30% Fetal Bovine Serum (FBS, Gibco, Paisly, Scotland, lot# 3030960S) [24]. When confluent ($P = 3$), the cells were detached and replated at 5000 cells/cm² in standard culture medium containing 15% FBS.

Phoenix-Ampho retrovirus packaging cells were cultured in DMEM (Gibco), supplemented with 10% FBS, penicillin (100 U/ml) streptomycin (100 μ g/ml) and 2 mM L-glutamine (Gibco). Cultures were passaged twice a week and selected for gag, pol, env expression every 8 weeks using Hygromycin B (300 μ g/ml) (Roche Diagnostics, Mannheim, Germany) and Diphtheria Toxin A (1 μ g/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands).

Construction and packaging of retroviral vector containing NGFR

The construction of the retroviral vector, pLZRS-TK (Fig. 1) has been described previously [37]. The Δ NGFR marker gene is under the control of Simian Virus 40 early promoter (SV40), flanked by the Moloney Murine Leukaemia Virus (MoMuLV) long-terminal repeats. All experiments for evaluation of the NGFR label were done with amphotropic pseudotyped viral particles, generated in Phoenix-Ampho packaging cell line as follows: 20 μ g DNA of a retroviral plasmid construct was transfected into Phoenix-Ampho packaging cells that had grown to 70% confluence by using calcium phosphate precipitation [18]. Twenty-four hours after transfection, medium was replaced with fresh culture medium. The following day, retroviral supernatant was collected, filtered through a 0.45 μ m filter and stored at -80°C . For an additional harvest of retroviral supernatant, transfected Phoenix-Ampho cells were cultured for 3 days in the presence of puromycin (1 μ g/ml Sigma) followed by 2 days in culture medium without puromycin.

Selection of vector pseudotype

To select an optimal vector pseudotype, we used the pSFFV-EGFP-vector created and characterized by Baum et al. [5] containing the Enhanced Green Fluorescent Protein (EGFP) under the control of a hybrid promoter of Spleen Focus Forming virus and Stem Cell Virus. EGFP was chosen as the transgene because of the ease of detection and quantification of expression by flow cytometry. Two goat BMSC batches were transduced with vectors from four different retrovirus producing cell lines (see also Fig. 2): (1) The Phoenix-Ampho packaging cell line expressed the amphotropic envelope [18]; (2) the PG 13 packaging cell line expressed the Gibbon-Ape Leukemia Virus envelope (GALV) [27]; (3) the 293 GPG expressed the vesicular stomatitis

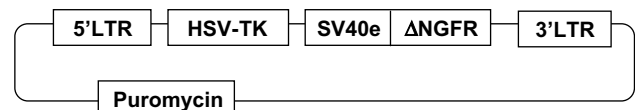


Fig. 1. The retroviral construct. The retroviral vector LZRS-TK as described by Weijtens et al. [37], contains the HSV-TK suicide gene under the transcriptional control of the 5'LTR and the truncated nerve growth factor receptor gene (Δ NGFR) under the control of the SV40 early promoter that is constitutively switched on. The puromycin resistance gene which is driven by the PGK-1 promoter is present in the nonretroviral portions of the plasmid to allow selection of packaging cells.

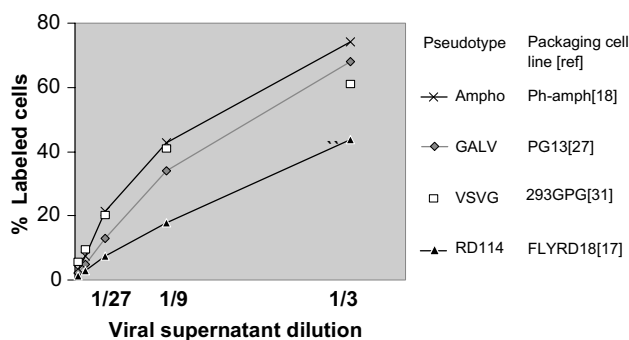


Fig. 2. Selection of optimal envelope pseudotype. The transduction efficiency of the four different envelope pseudotypes as a function of the dilution of viral supernatant. With a 3-fold diluted supernatant about 80% of the goat BMSC's was transduced successfully with the EGFP gene packaged with the amphotropic packaging cell line.

virus G envelope (VSVG) [31] and (4) the FLYRD18 packaging cell line expressed the Feline Endogenous Virus envelope (RD114) [17]. Viral supernatant containing the different pseudotyped vector particles was generated according to standard procedures. The viral titers were determined on 293T cells, calculated from the linear part of the curve in which the percentage of fluorescent cells (transduction efficiency) was plotted against the dilution of supernatant. The viral titre was adjusted to 1.0×10^5 Transduction Units (TU) per ml.

Retroviral transduction of goat BMSC's

For comparative goat BMSC transduction studies, the ($P = 3$) BMSC's were plated in six-well plates (Nalge Nunc, Roskilde, Denmark) at 10^5 cells per well the day before transduction. Transduction was performed by replacing the standard culture medium with a 3-fold dilution of the retroviral supernatant in culture medium supplemented with 6 $\mu\text{g/ml}$ polybrene (Sigma). Culture medium supplemented with polybrene was used for mock-transductions. The plates were cultured for another 24 h, after which the medium was refreshed. Two days later the cells were harvested and analyzed.

Flow cytometry

After transduction, aliquots of cells were withheld for flow cytometry to determine the transduction efficiency. The cells were labeled with primary mouse αNGFR monoclonal antibody (20.4 culture supernatant) for 20 min at 4 °C, washed and then incubated with goat anti-mouse Phycoerythrin (PE) conjugated IgG₁ (Southern Biotechnologies, Birmingham, US). Cells were washed in PBS–1%FBS and resuspended in standard culture medium, immediately before analysis of 10,000 events on a flow cytometer (FACS Calibur, Becton and Dickinson, San Jose, US).

Immunomagnetic purification of the NGFR-positive BMSC's

The presence of NGFR on the cell surface allows in vitro selection of transduced cells by the use of immunomagnetic microbeads. Transduced goat BMSC's were incubated with diluted αNGFR Mab 20.4 (20 $\mu\text{l}/1 \times 10^6$ cells) and subsequently incubated with goat anti-mouse IgG microbeads (20 $\mu\text{l}/10^7$ cells) for 10 min at 4 °C. Then, cells were washed and separated using a miniMACS separation column according to the manufacturers protocol (Miltenyi Biotec, Bergisch Gladbach, Germany).

Long-term evaluation and co-culture assay

To investigate long-term label expression, two populations of transduced cells were plated in 25 cm² flasks, cultured for 6 weeks and analyzed by FACS at each passage. Mock-transduced populations were analyzed parallel. To determine a potential difference in prolif-

eration between transduced and mock-transduced cells, a 50/50% mixture was also investigated.

In vivo traceability of gene-marked BMSC's

Tissue engineered constructs with transduced ($n = 14$) and mock-transduced ($n = 14$) cells (both $P = 4$) were prepared as described before [22]. Briefly, 10^5 cells were seeded on 50%-porous, $4 \times 4 \times 3$ mm ceramic scaffolds (OsSaturaBCPTM, IsoTis, Bilthoven, The Netherlands). The hybrid constructs were cultured for 1 week before implantation in separate subcutaneous pockets on the back of 14 NMRI nude mice [22] (two mice per evaluation period). Additional constructs with transduced cells ($n = 7$) were devitalized by freezing in liquid nitrogen before implantation (one mouse per evaluation period), to investigate the specificity of the label for viable cells [22]. The implants were retrieved after 2, 4, 7 and 10 days, and 2, 4 and 6 weeks. All animal experiments were according to the regulations of the local committee for animal research.

Immunohistochemistry

Samples were fixed in a 4% formaldehyde solution and decalcified for 1–2 days in 50% formic acid. Dehydration to allow paraffin embedding, was performed by graded alcohol series. The decalcification procedure was based on series of experiments with increasing concentrations of formic acid or EDTA (data not shown). Sections of 5 μm were dewaxed, rehydrated and endogenous peroxidase activity was blocked with 1.5% H₂O₂ in phosphate-citrate buffer. To prevent nonspecific staining due to reactivity of the secondary anti-mouse IgG antibodies with the surrounding murine tissue, the DAKO ARKTM kit was applied according to manufacturers' recommendations (DAKO Corporation, Carpinteria, USA). For counterstaining we used hematoxylin and Giemsa (Merck, Darmstadt, Germany) which specifically colors the bone bright pink [4]. Samples were analyzed with light microscopy (E600 Nikon eclipse, Japan).

Results

Selection of vector envelope (Fig. 2)

Fluorescence microscopy showed effective EGFP transduction of both goat BMSC batches with all vector pseudotypes. Although these different pseudotyped viruses were used at identical MOI's, transduction with retroviral particles derived from the Phoenix-Ampho packaging cell line resulted in the highest transduction efficiency i.e. nearly 80% when using a 3-fold dilution of the virus. While the GALV and VSV-G pseudotyped viral vectors gave only slightly lower values, the efficiency with RD114 was substantially lower. Because of the ample experience with the Phoenix-Ampho system and the intention to transduce human BMSC's in future studies, the Phoenix-Ampho line was chosen for further experiments.

In vitro analysis of transduced goat BMSC's (Fig. 3)

A single round of transduction of various batches of goat BMSC's with a 3-fold dilution of retroviral supernatant expressing NGFR, resulted in 40–60% transduction efficiency. In subsequent weeks, this percentage dropped to 30–40%. The long-term culture experiment also showed this decrease during the first period after

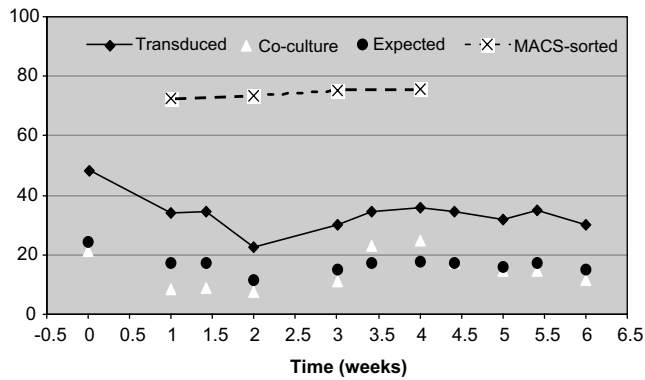


Fig. 3. Long-term label expression. The transduction efficiency of this batch of BMSC's was 50% (squares). After an initial decrease and moderate increase, the percentage stabilized around 35% for up to 6 weeks. The white interrupted line shows the expected percentage labeled cells in the 50/50% mixture. The actual percentage labeled cells in the co-culture (white line) showed to be initially lower, but finally matched the expected percentage. One week after MACS sorting (black interrupted line x-x), the population remained 70–80% positive for 4 weeks.

the transduction, but thereafter stabilized around 35% for up to 6 weeks. The co-culture experiment showed the percentage of labeled cells to be less than expected the first week after transduction, indicating a relative decreased proliferation compared to the nontransduced cell fraction. During the subsequent weeks, the percent NGFR⁺ cells in the co-culture reached the expected percentage, indicating a minimal influence on cell proliferation at the long term. A single round of sorting with immunobeads yielded a stable 70–80% NGFR-positive cells that were cultured for 4 weeks (Fig. 3).

Analysis of the retrieved *in vivo* samples (Fig. 4)

All mice survived the planned implantation periods and all samples were retrieved without signs of infection. Immunohistochemistry did not show aspecific binding of the 20.4 antibody (control samples with mock-transduced cells and secondary antibody stainings were always negative). Analysis of devitalized constructs indicated some residual NGFR label, not associated with viable cells, up to 4 days after transplantation.

The samples implanted for 2 days showed NGFR-positive cells throughout the scaffold that could be well distinguished from surrounding host tissue (Fig. 4a). At 4 and 7 days, small blood vessels were visible inside the scaffolds and NGFR-positive cells were distributed evenly throughout the pores without a preference for the ceramic surface. Ten days after implantation, condensations of fibroblast-like cells on the scaffold surface appeared, partially consisting of gene-marked cells (Fig. 4b). By then, the samples were well vascularized (Fig. 4c). Comparable observations were made for the 2 weeks implanted samples. After 4 weeks, bone that

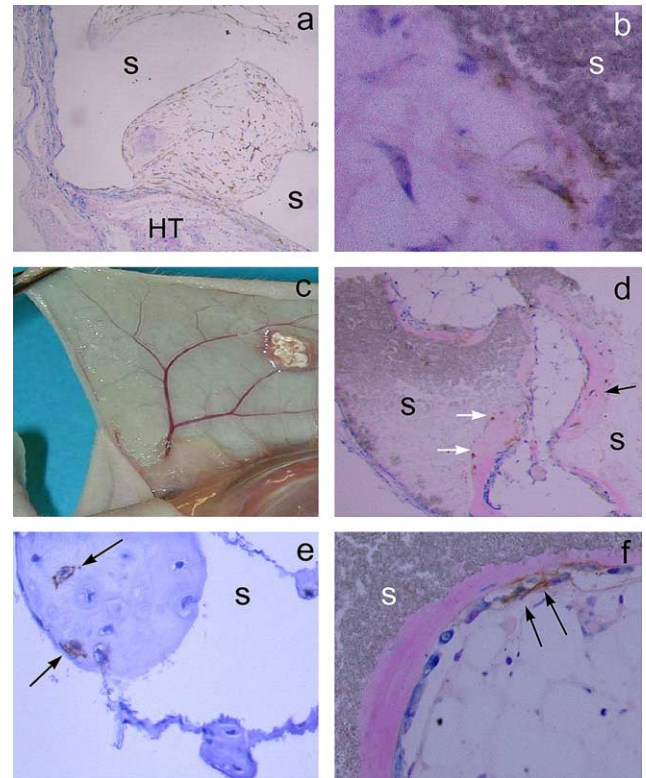


Fig. 4. Samples of *in vivo* hybrid constructs with NGFR labeled cells. (a) Low magnification immunohistochemistry of 2 days implanted sample. The pores surrounded by the ghost of the scaffold (S) were filled with brown (labeled) goat BMSC's, distinctive from the surrounding host tissue (HT). (b) High magnification immunohistochemistry of 10 days implanted sample. Condensations of fibroblast-like cells on the scaffold surface were found including labeled cells. (c) Macroscopic image of sample *in situ* at 10 days. Considerable neo vascularization of the hybrid construct was seen. (d) Low magnification immunohistochemistry of 4 weeks implanted sample. New bone was lining the scaffold ghost. Many osteocyte lacunae were labeled (arrows). (e) High magnification immunohistochemistry of 4 weeks implanted sample. Inside cartilage that occasionally formed, labeled cells were present (arrow). (f) High magnification immunohistochemistry of 6 weeks implanted sample. Labeled cells were present within the osteoblast zone (arrow).

harbored labeled cells was present inside all scaffolds that contained transduced cells. In some pores, also cartilage that contained NGFR-positive cells was found (Fig. 4d and e). Six weeks' samples demonstrated lamellar bone in all vital samples. Besides inside the bone, labeled cells were also found within the osteoblast zones (Fig. 4f). No difference in bone forming capacity was observed between transduced and mock-transduced cell constructs (all samples gave bone).

Discussion and conclusions

The aim of the present study was to retrovirally label goat BMSC's, and apply these for experimentation in

bone tissue engineering. We found that 40–60% of the cells were labeled with the NGFR marker gene by means of a relatively simple retroviral transduction protocol, after a single round of transduction. When using retroviruses, the transduction is confined to replicating cells, and therefore a 100% transduction efficiency, was not expected [29]. In a large study, Mosca et al. investigated optimal parameters required to transduce BMSC's from eight different species, including the goat [30]. For goat BMSC's they reported a relatively low transduction efficiency with the amphotropic receptor binding envelope (<10%) compared to 50% when using their xenotropic (ProPak-X) packaging cell line. In our study the amphotropic envelope showed the best results. It is difficult to find an explanation for the discrepancy because different procedures of transduction were followed and the viral titers used in their study might have been different. Retroviral transduction of goat BMSC with xenotropic pseudotyped vector would provide the ultimate method to evaluate whether the results of our experiment would demonstrate the same.

For future studies, a labeled cell percentage close to 100% is preferred to allow quantitative interpretations of the contribution of host and donor cells to colonization of the scaffold and bone formation. We showed this may be accomplished by immunomagnetic sorting and expect even better results from FACS sorting, based on current investigations.

The results from the *in vitro* culture study did not indicate a long-term effect of the marker gene on proliferation, because in the co-culture experiment the expected percentage of labeled cells was matched. After an initial drop in the percentage of labeled cells during the first week from 60% to 35%, the percentage gene-marked cells stabilized at this level. Most likely, the untransduced cells have a slight growth advantage the first week after transduction.

We did not attempt to determine the cell phenotype *in vitro* or to investigate the effect of the marker gene on *in vitro* differentiation, because the frequently used alkaline phosphatase assay has shown to be inadequate for goat and sheep BMSC's [20,23]. Furthermore, the most reliable information on differentiation potential is not provided by the *in vitro* phenotype but by *in vivo* behavior of the transduced cells [6]. The results from the *in vivo* study indicated persistent gene expression following *in vivo* growth and differentiation. Because we do not know what is the detection efficiency of labeled cells *in vivo* and the design of the current study was to primarily investigate the feasibility of the NGFR label, only qualitative interpretations can be made. An interesting observation was the tendency of labeled cells to completely fill the pores the first days after transplantation without a preference for the scaffold surface. Only after 10 days implantation, condensations of cells were observed on the surfaces. This may indicate that as yet

unidentified processes need to precede the cascade of (ceramic) surface related bone formation. These processes can be either cell related (proliferation/differentiation) or scaffold related (ceramic surface changes to provide an optimal substrate for bone apposition) or a combination of these. The presence of labeled cells within osteocyte lacunae illustrated the bone forming capacity of the transduced cells. The presence in the osteoblast linings after 6 weeks suggests that transduced goat BMSC's cells were still actively forming bone. The observations related to *in vivo* tissue engineered bone formation that we made in this study, correlated well with previous findings of CM-Dil labeled goat BMSC's [22]. In that study, we concluded that the CM-Dil label was not applicable for long-term investigation of the process of TE bone formation. With the retroviral NGFR label investigated in this study, long-term investigation of the process of bone TE in the goat model is now feasible.

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