Pharmacogenetic heterogeneity of transgene expression in muscle and tumours

Pierre Lefesvre*, Joline Attema and Dirk van Bekkum

Address: Crucell Holland BV, PO BOX 2048, 2301CA, Leiden The Netherlands
Email: Pierre Lefesvre* - p.lefesvre@crucell.com; Joline Attema - j.attema@crucell.com; Dirk van Bekkum - bekkum@crucell.com
* Corresponding author

Abstract

Background: Recombinant adenoviruses are employed to deliver a therapeutic transgene in the liver, muscle or tumour tissue. However, to rationalise this delivery approach, the factors of variation between individuals need to be identified. It is assumed that differences between inbred strains of laboratory animals are considered to reflect differences between patients. Previously we showed that transgene expression in the liver of different rat strains was dependent on the transcription efficiency of the transgene. In the present paper we investigated if transfection of muscle and tumour tissue were also subject to such variations.

Methods: Variation, in transgene expression, after intramuscular gene delivery was determined in different rodent strains and gene expression in tumours was investigated in different human and rodent cell lines as well as in subcutaneously implanted rodent tumours. The molecular mechanisms involved in transgene expression were dissected using an adenovirus encoding luciferase. The luciferase activity, the viral DNA copies and the luciferase transcripts were assessed in cultured cells as well as in the tissues.

Results: Large differences of luciferase activity, up to 2 logs, were observed between different rodent strains after intramuscular injection of Ad Luciferase. This inter-strain variation of transgene expression was due to a difference in transcription efficiency. The transgene expression level in tumour cell lines of different tissue origin could be explained largely by the difference of infectibility to the adenovirus. In contrast, the main step responsible for luciferase activity variation, between six human breast cancer cell lines with similar phenotype, was at the transcriptional level.

Conclusion: Differences in transcriptional efficiency in muscles as observed between different inbred strains and between human breast cancer cell lines may be expected to occur between individual patients. This might have important consequences for clinical gene therapy. The variation between tumour types and tissues within a species are mainly at the levels of infectivity.

Background

In an attempt to increase efficacy and decrease toxicity, new drugs are designed to interact with unique or specific cellular pathways. Therefore the expected responders are likely to be a subpopulation of patients falling under one diagnosis but with a specific targeted pathway; e.g. Herceptin in the HER2-positive patients with a breast cancer. However, this subpopulation bearing the targeted receptor or the specific pathological pathway still includes patients that are poor responders to the treatment. One of
the reasons for that may be the inter-individual variation of susceptibility to the tested drug. For example, many xenobiotics are metabolised at different rates in the liver from one individual to the other. The targeted pathway, through the receptor of the drug or the cofactors involved, may also vary in the population. The new molecular technologies enable to screen the population for genotypic variation of the gene or genes involved in the control of those pathways. When these parameters are elucidated, the responsiveness of individual patients to the drug can be predicted. On the one hand this knowledge might be used to adapt the dose to the individual patient in order to increase the drug efficacy and to avoid adverse effects. On the other hand this pharmacogenetic knowledge can be used to identify a subpopulation of patients that would respond better to the treatment without adverse effects. There is evidence that not only responses to xenobiotics, but also to non-viral and viral vectors, are subject to significant inter-individual variation. Indeed, clinical trials using gene therapy were hindered by inter-individual variation leading either to lack of therapeutic effect or to unexpected toxicity [1,2]. In the case of adenoviral gene therapy, the causes of these inter patient variation are only partially clarified. Several studies showed the crucial role of neutralising antibody (nab) against viral vectors [3–5]. Anti-Ad5 nab titers vary greatly in the population and its determination is now generally accepted as obligatory to adjust the Ad5 dose [6–8]. However, this is probably not the only factor that influences gene transfer efficiency between individuals. The present study is an attempt to determine where the variation occurs after the gene transfer, from the penetration of the virus in the cell to the synthesis of the encoded protein. The magnitude of variation between the response of patients to gene therapy is difficult to establish because it requires large numbers of individuals and because this type of gene transfer studies with healthy volunteers are problematic. The genetic differences observed between inbred strains of animals assumedly reflect differences between individuals of an outbred strain. Accordingly, we decided to explore the issue using inbred strains of rodents and human cell lines.

Several investigators have reported large variations of transgene expression between inbred mouse strains after intravenous administration of a recombinant adenovirus to naive animals [9–13]. The main tissue that is transfected after intravenous administration of a recombinant adenovirus is the liver. In a previous paper, we have described differences between three rat strains in transgene expression efficiency in the liver [14]. In order to determine which step was involved in this variation, the transfection machinery of the liver was dissected. It was demonstrated that the differences of transgene expression occur not at the level of penetration of the virus into the cell, but at the transcriptional level. Indeed the transcription efficiency in the liver, which comprises both mRNA synthesis and stability, differed up to one log between the rat strains. Many clinical application of gene therapy involve tumour transfection or intramuscular gene transfer. However variations of transgene expression were poorly documented in such clinical investigations, principally because these trials are mainly focused on the toxicity and the therapeutic efficacy of the gene transfer. Nevertheless, large variations of transgene expression between cultured tumour cells were reported by several investigators [8]. Such variations were also observed between mouse strains after muscle transfection [15,16]. Therefore, the present study attempts to determine the causes of such variation after transfection of tumour and muscle tissue.

Results

Gene transfer in muscle

An impressive difference in muscular expression between rat strains was first observed with adenoviral vectors that contain the transgenes for endostatin, ATF-BPTI and ATF (Fig. 1). After intra muscular injection of the vector, the plasma levels of the encoded proteins rose to one log higher in Wag/Rij rats than in Brown Norway rats. To investigate if this phenomenon was indeed due to a difference in muscular production of the encoded protein the marker gene luciferase was employed. In order to expand this strain specificity of transgene expression a third strain, the Fisher rat, was also included in the experiments. At all 3 vector doses a difference in luciferase expression was noted between the strains (Fig. 2). At 1010iu Ad luciferase, as for the preceding transgenes, the difference was also one log between the Wag/Rij and the Brown Norway rats. Thus, the difference of plasma levels of the secreted encoded-proteins is most likely explained by differences in their rate of synthesis in the muscle.

In order to determine if the infection of the myocytes differs from one strain to the other, the number of Ad DNA copies was determined in the muscle after injection with the luciferase vector. The amount of Ad DNA copies detected by real time PCR in the gastrocnemius was about 3 to 9 % of the dose injected (Fig 3). According to Scheffe’s test the differences between the three rat strains were not significant. The muscle is almost exclusively composed of myocytes and the adenoviral DNA detected in the muscle tissue should reflect infection of myocytes. This strongly suggests that a similar amount of adenovirus transfected the muscle in the three rat strains. However, adenovirus particles in apoptotic myocytes or phagocytosed by immune cells would not contribute to the luciferase production. Therefore, to exclude that a massive rhabdomyolysis or a severe inflammatory infiltrate in the gastrocnemius is causing the difference of luciferase expression, the histology of the muscles and the serum
levels of creatine phosphokinases (CPK) were assessed at 2 days after injection. In all 3 strains a mild inflammatory infiltrate was observed in the muscle, but almost no cell necrosis (Table 1 and 2). Scoring of the damage by a pathologist did not reveal differences between the rat strains. The CPK were only two times the normal value. Seemingly, the difference in luciferase expression is due to processes that occur somewhere after the penetration of the virus into the cells.

Figure 1
Transgene expression in the plasma after intra-muscular gene delivery. Plasma levels of endostatin (yellow bars), mhATF-BPTI (blue bars) and ATF (red bars) in Wag/Rij rats (A) and in Brown Norway rats (B) after intra muscular injection of 10^{10} iu Ad5 Adapt encoding the respective transgenes. Ten animals per group, data are expressed as mean ± SD. The higher levels of endostatin compared to the ATF and ATF-BPTI is due to the pharmacokinetic characteristics of these molecules.

Figure 2
Luciferase expression in the gastrocnemius muscle of 3 rat strains after intra-muscular delivery. Luciferase activity was determined 48 hours after intra-muscular injection of 10^{6} iu (white bars, n = 6), 3 \times 10^{9} iu (striped bars, n = 4), and 10^{10} iu (black bars, n = 9) Ad5 Adapt Luc. The mass of the gastrocnemius is similar in the 3 strains. Data are expressed as mean ± SD. The value of the mean is shown above the bars.

Figure 3
Infectibility of the gastrocnemius muscle to the adenoviral vector in the 3 rat strains. The same muscles were used as in Fig. 2. The number of Adenoviral DNA copies per muscle was determined by duplex real time PCR. The number of DNA copies was determined in a sample of a homogenate of the whole gastrocnemius muscle and then extrapolated to the total mass of the gastrocnemius. Data are expressed as mean ± SD. The value of the mean is shown above the bars.
The next step to investigate was the levels of luciferase mRNA. For this purpose the gastrocnemius of the rat is too large to be processed in one time and the extraction of mRNA would be cumbersome and subject to error. To avoid these inconveniences, we decided to switch to mice. The gastrocnemius muscle of a mouse is large enough to be injected with the required volume of adenoviral suspension and small enough to be processed as a whole for RNA extraction. We studied 6 different mouse strains with different genetic background. The animals were injected with Ad luciferase in the gastrocnemius and the muscle was harvested 2 days later for luciferase measurement, DNA and RNA extraction. Large differences in luciferase activity were measured between the mouse strains and the range of values was even greater than that observed in the rats (Fig 4A). DBA mice showed the lowest transgene expression whereas the luciferase activity was 60 times higher in C57BL/6 mice. The creatine phosphokinases (CPK) level in the plasma is correlated to the degree of muscle fiber damage [17,18]. Thus, the plasma CPK was measured at the time of sacrifice, in order to verify that the low luciferase values were not caused by massive rhabdomyolysis. The CPK plasma levels were only slightly elevated and roughly similar in all strains (Table 3). Similar to the situation observed in rats, the number of adenoviral DNA copies in the injected gastrocnemius was also not

Table 1: Criteria for scoring muscle damage. The pathological changes were graded on 4 transversal sections of the right gastrocnemius. The total damage score is a compilation of scores of apoptosis, hyaline degeneration, myoblasts regeneration, and inflammation. All scores are ranging from 0 to 3.

| Score | 0  | 1 | 2 | 3 |
|-------|----|---|---|---|
| Apoptosis | Number of apoptotic cells / bodies in 10 random 20 × magnification fields | 0–1 | <5 | 5–13 | >13 |
| Hyaline degeneration | Percentage of fibers affected. | 0–1% | 1–10% | 10–50% | >50% |
| Myoblasts | Number of myoblasts in 10 random 20 × magnification fields | 0–1 | <5 | 5–13 | >13 |
| Inflammation | Diffuse or multifocal inflammation in muscle fibers | Normal background | Increase of inflammatory cells | Aggregates of inflammatory cells | Aggregates of inflammatory cells obscuring the muscle architecture |

Table 2: Muscle damage caused by injection of adenovirus in rats. The muscle lesions were assessed, as described in table 1, two days after the intramuscular injection of the adenoviral vector. The total muscle inflammation and damage scores range from 0–3 and 0–12 respectively. 4 animals per group. The CPK were measured 2 days after the vector injection. Values show the mean ± SD.

|           | Brown Norway | High dose (10¹⁰iu) | Wag / Rij |
|-----------|--------------|-------------------|-----------|
| Total muscle inflammation | 1.2 ± 0.5 | 1.0 ± 0.8 |
| Total muscle damage | 2.2 ± 0.5 | 2.4 ± 0.8 |
| Total muscle proteins (mg/g tissue) | 85 ± 22 | 77 ± 33 |
| CPK in plasma (IU) | 950 ± 205 | 1096 ± 173 |

Table 3: Creatine phosphokinase (CPK) concentrations in the plasma of mice. The CPK were determined in the plasma of the mice 2 days after viral administration in the muscle (N = 4) and in untreated control animals (N = 3). Data were derived from the same experiment as depicted Fig 4.

| Strains: | BALB/c | C57BL/6 | CBA | DBA | NOD/scid | C3H |
|----------|--------|---------|-----|-----|----------|-----|
| Treated mice | 1570 ± 427 | 1248 ± 391 | 1427 ± 342 | 1291 ± 225 | 1332 ± 258 | 1343 ± 217 |
| Control mice | 405 ± 72 | 455 ± 143 | 316 ± 15 | 443 ± 83 | 401 ± 78 | 405 ± 72 |
The quantification of luciferase transcripts was performed by Northern blotting (Fig 5). All the mouse strains showed similar amounts of total RNA and β-actin mRNA in the gastrocnemius, but different amounts of luciferase mRNA. The amount of luciferase transcript was proportional to the luciferase activity. Therefore, the differences in encoded protein expression in the muscle are due to differences in transcription efficiency, including mRNA synthesis or and stability.

**Gene transfer in tumours**

Tumours are composed of tumour cells and of various infiltrating stroma cells. It was shown that mainly tumour cells are infected after intratumoral delivery of a recombinant adenovirus encoding the β-galactosidase gene, and that the transfection of the stroma cells seems not to be statistically different between the mouse strains (Fig 4B). The quantification of luciferase transcripts was performed by Northern blotting (Fig 5). All the mouse strains showed similar amounts of total RNA and β-actin mRNA in the gastrocnemius, but different amounts of luciferase mRNA. The amount of luciferase transcript was proportional to the luciferase activity. Therefore, the differences in encoded protein expression in the muscle are due to differences in transcription efficiency, including mRNA synthesis or and stability.
significant (unpublished data). Nonetheless we studied 8 rodents tumours with less than 20% of stroma. We showed that the transgene expression in these tumours in vivo merely corresponds with that of the tumour cell lines in culture (Fig 6). This finding suggests that information collected with human tumour cell lines is likely to reflect the transduction of the corresponding human tumours in vivo. Therefore the variation observed with cultured cell lines may give us some insight in the variation among patient tumours. We studied the transduction in cell lines of different tissue types and within a certain tissue type, namely mammary cancer.

**Figure 6**
Comparison of in vitro and in vivo gene transfer in tumours.
A: Luciferase activity in rats and mouse tumours 48 hours after intratumoral injection of 10^10 IU Ad5 Adapt Luc (n = 5). The tumours are growing subcutaneously, and were about 8 x 8 mm at the time of injection. Values indicate means ± SD.
B: Luciferase activity measured in lysate of cell cultures 48 hours after in vitro infection with Ad Adapt Luc at different MOI. Values are mean of a triplicate ± SD.

**Figure 7**
Luciferase activity, viral DNA and luciferase mRNA in different human cancer cell lines after adenoviral transfection.
A: Luciferase activity measured in cell lysate 48 hours after infection with different MOI. B: Number of Adenoviral DNA copies per cell determined by duplex real time PCR. The number of cells was determined by microscopic counting and the number of genome by real time PCR of the GAPDH gene. All cell lines had similar ploidy. C: Quantification of the luciferase mRNA in the cell lines after infection at different MOI by Northern blotting. The density of the bands was determined with a phosphorimager. The β-actin blotting was also performed afterwards to control the quality of the RNA extraction and the quantity of RNA loaded. The density of the β-actin bands was similar in all the cell lines (data not shown). Values indicate mean of a triplicate ± SD.
Human and rodent tumour cell lines of different tissue origins were employed to study the molecular steps leading to encoded protein synthesis. Huge differences in transgene expression, up to a magnitude of 2 logs, were measured between human and rat tumour cell lines (Fig 7A and 8A). Among the human tumour cells, the U251 Glioma showed the highest expression, whereas the LS 174T colon adenocarcinoma expressed the lowest luciferase activity. To roughly determine at which steps these differences reside, the amount of adenoviral DNA and luciferase transcripts were quantified. As depicted in Fig 7B and 8B a wide range of infectibility to the adenovirus, defined as the proportion of adenoviral copies that entered the cell, was observed in this panel of cell lines. The Glioma U 251 was very permissive to infection by adenovirus as nearly all the infectious units that were added to the culture were retrieved in the cells 2 days later. In contrast, only 0.5–1% of the seeded adenoviruses infected the LS 174T cells. The luciferase mRNA was determined (Fig 7C and 8C) and the results showed that the RNA levels corresponded with the DNA levels and luciferase activity and consequently that the transcription efficiency was very similar for all cell lines except for the A459 which showed a 5 time lower efficiency (Table 4). The translation efficiency (ratio of luciferase per transcript), even if statistically different between the cell lines, did not vary in the same proportion as the transgene activity (Table 4). The A549 had the lowest translation efficiency but the U251 had only a 4 time higher efficiency. In all cases, except for the A549, the variation in transgene expression between the cell lines can be attributed to a difference in infectibility. The A459 cell line produced slightly more luciferase (3 times) than the LS 174T but had a much higher infectibility (36 times) and a 5 and 2 times lower transcription and translation efficiency, respectively.

To address the issue of variation within a single tumour type, we used 6 human breast cancer cell lines. All these cell lines showed a similar phenotype. Indeed, the cytological examination revealed glandular formation typical of ductal adenocarcinomas in all of them (Fig 9). These cell lines were infected in vitro with the Ad Adapt Luc using a range of MOIs. The different steps of gene expression were investigated as described before. The luciferase measurements showed a very large variation between the cell lines with differences up to 2 logs (Fig 10). All the breast cancer cell lines were very susceptible to adenoviral infection and contained similar amounts of viral DNA (Fig 10 Table 5A). In contrast to cells of different tissue origin, the variation of transgene expression among the breast cancer cell lines is not explained by differences of infectibility. Furthermore the luciferase mRNA content of the cells was proportional to the luciferase activity (Fig 10) and accordingly the translation efficiency was similar in all cell lines (Table 5C). However, these adenocarcinoma cell lines with similar infectibility and translation efficiency have different transcription efficiency (Table 5).
Thus, in case of tumour cell line of similar histological origin (in our study breast cancer cell lines), the difference of transgene expression might be due to different transcriptional efficiency and not to difference in susceptibility to infection.

### Discussion

Adenoviruses are attractive vectors for gene therapy due to their high transfection efficiency in a large number of tissues and their relatively easy production at a large scale. However, there are still some drawbacks that hinder their clinical application. The first factor that was recognised to

| MOI | U251 | NCI H460 | Cell lines | A549 | LS 174T | Average | SD  |
|-----|------|----------|------------|------|---------|---------|-----|
| 1   | 72   | 82       | 13         | 15   | 12      | 14      | 34  |
| 10  | 55   | 104      | 7          | 13   | 10      | 18      | 21  |
| 100 | 78   | 101      | 10         | 13   | 9       | 12      | 22  |
| Average | 96  | 14       | 15         | 36   | 8       |

**Table 4:** Transgene expression in different human cancer cell lines. These values are determined in the same experiments as depicted in figures 7 and 8. A: Infectibility of the cell lines as defined by the percentage of adenovirus DNA seeded that was detected in the cells 2 days after infection. B: the transcription efficiency is defined by the ratio of the mRNA values divided by the number of adenoviral copies in the cells. C: The translation efficiency is the ratio of the luciferase activity / luciferase mRNA. D: Luciferase activity in 10^3 RLU / mg protein. The absolute values are shown in italic. The values relative to the LS174T, which was arbitrary set as reference are indicated in plain text.
limit adenoviral gene therapy was the presence of neutralising antibodies. Measures have been implemented to overcome this limitation, such as developing vectors that evade the immune system. In this report we highlight other factors that should be taken into account for the clinical application of gene therapy. The first factor is the variable transcription efficiency (level of transcript accumulation) between individuals after transfection of a specific tissue like the liver or muscles. There is no clear indication in the literature whether this variation is specific to one promoter or is this phenomenon general for all promoters. However, viral promoters are usually sensitive to cytokines and it is known that they influence the transcription efficiency. Furthermore, it was recently shown that transcriptional repression of the CMV pro-

Figure 9
Cytology of the different human breast cancer cell lines. The photographs show cell cultures of the different breast cancer cell lines (magnification × 200). All cell lines develop a glandular phenotype: cuboid cell shape and clusters of few cell layers around a canalicule formation (black arrows).

Figure 10
Luciferase activity and viral DNA in human breast cancer cell lines. A: Luciferase activity measured in cell lysate 48 hours after infection at different MOI. B: Number of Adenoviral DNA copies per cell determined by duplex real time PCR. The number of cells was determined by microscopic counting and the number of genome by real time PCR of the GAPDH gene. All the cell lines had similar ploidy. C: Quantification of the luciferase mRNA in the cell lines after infection at different MOI by Northern blotting. The density of the bands was determined with a phosphorimager. The β-actin blotting was also performed afterwards to control the quality of the RNA extraction and the quantity of RNA loaded. The density of the β-actin bands was similar in all the cell lines (data not shown). Values are means of a triplicate ± SD.
moter induces interspecies variation of the transgene expression [19]. However this poorly understood phenomenon, known as promoter interference, seems to be specific for retroviral vectors and was only observed between species. In addition we identified a tissue-specific variation, which occurs at the level of infection only as well as in addition at the transcriptional level (Table 6).

From intra-muscular protein vaccination it is known that the amount of antigen used influences the type of immune response. A low level of antigen will elicit only

| MOI | EVSA-T | CAMA-1 | MCF-7 | T47 D | SKBR-3 | ZR 75.1 |
|-----|--------|--------|-------|-------|--------|---------|
| 1   | 7      | 1.1    | 6     | 1.0   | 6      | 1.0     | 6.7     | 1.1    | 6.0   | 1 |
| 10  | 6      | 1.2    | 5     | 1.1   | 6      | 1.2     | 4.0     | 0.8    | 4.8   | 1.0   | 4.9   | 1 |
| 100 | 9      | 1.2    | 9     | 1.2   | 9      | 1.3     | 8.0     | 1.0    | 7.9   | 1.1   | 7.3   | 1 |
| Average | 1.2 | 1.1   | 1.2   | 1.0   | 1.1   | 1.0     | 1.1     | 1.1    | 1.1   | 1.1   | 1.1   | 1 |

| MOI | EVSA-T | CAMA-1 | MCF-7 | T47 D | SKBR-3 | ZR 75.1 |
|-----|--------|--------|-------|-------|--------|---------|
| 1   | 213    | 55     | 71    | 18    | 27     | 6.8     | 9       | 2.2    | 9.5   | 2.4   | 3.9   | 1 |
| 10  | 176    | 61     | 49    | 17    | 19     | 6.6     | 9       | 3.2    | 8.0   | 2.8   | 2.9   | 1 |
| 100 | 137    | 56     | 41    | 17    | 12     | 5.0     | 6       | 2.4    | 6.0   | 2.5   | 2.4   | 1 |
| Average | 57.2 | 17.4   | 6.1   | 2.6   | 2.6   | 1       | 1       | 0.5    | 0.2   |       |

| MOI | EVSA-T | CAMA-1 | MCF-7 | T47 D | SKBR-3 | ZR 75.1 |
|-----|--------|--------|-------|-------|--------|---------|
| 1   | 2      | 1      | 2     | 1     | 3      | 1.2     | 4       | 1.6    | 4     | 1.6   | 2.4   | 1 |
| 10  | 6      | 1      | 6     | 1     | 6      | 1.0     | 8       | 1.4    | 6     | 1.0   | 6.0   | 1 |
| 100 | 26     | 2      | 38    | 2     | 33     | 2.0     | 15      | 0.9    | 13    | 0.8   | 16.2  | 1 |
| Average | 1.2 | 1.4   | 1.4   | 1.3   | 1.1   | 1       | 1       | 0.8    | 0.4   | 0.4   |      |

| MOI | EVSA-T | CAMA-1 | MCF-7 | T47 D | SKBR-3 | ZR 75.1 |
|-----|--------|--------|-------|-------|--------|---------|
| 1   | 28     | 50     | 10    | 18    | 5      | 8       | 2       | 4      | 2     | 4.4   | 1     | 1 |
| 10  | 620    | 74     | 167   | 20    | 65     | 8       | 32      | 4      | 22    | 2.7   | 8     | 1 |
| 100 | 30473  | 105    | 13171 | 46    | 3790   | 13      | 648     | 2      | 616   | 2.1   | 289   | 1 |
| Average | 76.4 | 28    | 9.7   | 3.2   | 3.0   | 1       | 1       | 0.8    | 1.2   |       |      |
Table 6: Assumptions concerning the origin of the variation of transgene expression after adeno viral transfection.

| Transfection          | Variation                |
|-----------------------|--------------------------|
| Different Tissues     | Infection                |
|                       | w/ wo                    |
|                       | Transcription            |
| Different Individuals | Transfection             |

The difference of transgene expression between tissues of different histological origin is mainly due to differences in infectivity coupled in some cases with differences in transfection efficiency. The infectivity of a specific tissue is similar between individuals. However, the transcription efficiency may vary greatly with subsequent large difference of transgene expression between two individuals.

cellular immunity but no neutralising antibodies. Therefore, it is of importance for DNA vaccination approaches to control the quantity of antigen that is produced in the muscle after gene transfer. The study of adeno viral gene delivery in the muscle is more cumbersome than for the liver. The principal reason is that the transfection is very inhomogeneous after intra-muscular injection. Some parts of the muscle are very poorly infected if at all, whereas the majority of the fibers around the needle track are transfected. Therefore the whole of the injected muscle has to be homogenised to get an indication of the degree of transfection. Secondly, the different parameters (Luciferase activity, DNA or mRNA amount) cannot not be determined per muscle cell. Indeed, the number of myocytes per mg muscle cannot be accurately determined as the cellular volume and the number of nuclei varies greatly between muscle fibers. Therefore, the parameters have to be expressed per whole muscle. It was demonstrated that the huge difference in transgene expression between the different strains was explained by difference of transcription efficiency. Extrapolated to the clinical situation this would mean that antigen production and therefore the expected immune response after intra-muscular vaccination with adeno virus might also differ widely between patients. In humans and in large animals the transfection efficiency cannot not be assessed by muscle biopsy. Due to the inhomogeneous distribution of the transfection, one cannot perform the biopsy in such a way that all of the transfected muscle is recovered. One possible way to circumvent this obstacle is to identify a circulating surrogate marker of transfection.

Tumours are composed of cancer cells and of a variety of infiltrating cells. The latter non-neoplastic cells include endothelial cells, fibroblasts, and inflammatory cells. The composition and the proportion of these infiltrating cells vary greatly from one tumour to the other. Some tumour types, like glioma’s, contain few infiltrating cells whereas epithelioma’s are usually infiltrated by a large amount of fibroblasts. This variation is also found between patients with histologically identical tumours. After local gene delivery, the contribution of these cells to the production of the encoded protein has not yet been evaluated. Thus, different tumour compositions between patients might also be a factor of variability of intra tumoral gene transfer. Therefore in vitro cell culture appeared to be more representative to study the variation of tumour cell transfection per se. However it is claimed that the membrane receptors for adeno virus might be down regulated in culture and gene transfer would consequently be lower. However, this would interfere only with the penetration of the vector into the cells. We have shown that breast cancer cell lines with similar numbers of transgene copies may present large differences of transgene expression and that this was due to different transcriptional efficiency.

In the case of intra-tumoral delivery, preexisting immunity against the viral vector may be of less importance than it is for muscle gene transfer. It was shown in animal models that the presence of neutralising antibodies did not completely impair gene transfection after intratumoral delivery (unpublished data, [20,21]). In patients the situation is also not clear, as therapeutic efficacy of gene therapy has been reported in patients with titers of nab, high enough to completely neutralise the viruses if administrated intravenously. Thus, apart from the variation in neutralizing antibodies titers it is suggested that different transcriptional efficiency might be the cause of large inter-patient differences in responsiveness to intratumoral gene therapy. Therefore, future clinical studies should not only include the determination of the transfection of the targeted organs by measuring the viral DNA but also an assessment of the locally encoded protein production.

Methods

Animals

Pathogen-free inbred male Wag/Rij and Brown Norway rats, weighing 300 to 350 gr and male C57BL/6, Balb/c, C3H, CDA, CBA, and NOD/scid mice weighing about 20g were purchased from Harlan, The Netherlands. All animals were fed ad libitum with laboratory chow and water and were kept under standard laboratory conditions. The intra muscular injections were performed in the right gastrocnemius of the animals. The volume injected was 50 µl for the mice and 100 µl for the rats. It was verified that 98% of the transgene expression occurs in the gastrocnemius and the rest in the surrounding muscles.
All animal procedures were performed in accordance with the official guidelines after obtaining permission of the animal welfare committee.

**Tumours**

The tumours in the Wag /Rij rats are the L42 bronchial squamous carcinoma, the L37 bronchial adenocarcinoma, and the CC531 colon adenocarcinoma [22,23]. The L44 is an anaplastic lung tumour in Brown Norway rats [24]. The IL-45 is a malignant mesothelioma in the Fisher 344 rats [25]. The B16F10 (ATCC#: CRL-6475) is a melanoma in the C57BL/6 mice. The C26 coloncarcinoma (ATCC#: CRL-2638) and the TSA breast adenocarcinoma are growing in the BALB/c mice. The TSA was a kind gift of Dr. T de Vries (University of Leiden, The Netherlands).

The U251 human glioma cell line was obtained from the American Type Culture Collection. The NCI-H460 (ATCC#: HTB-177) is a human non-small cell lung carcinoma cell line derived from the pleural fluid of a patient with large cell cancer of the lung [26]. The A549 cell line (ATCC#: CL-185) is derived from an alveolar lung carcinoma with properties of type II pneumocytes [27]. The HepG2 (ATCC#: HB-8065) is a human hepatocellular carcinoma cell line. The LS 174T (ATCC#: CL-188) is a human colon adenocarcinoma cell line [28].

All the human breast cancer cell lines were kindly provided by Dr A. Sieuwerts (University Hospital Rotterdam, The Netherlands). The EVSA-T cells were derived from malignant ascitic effusion from a female patient with metastatic infiltrating ductal breast carcinoma [29]. The SKBR-3, MCF-7, SK-BR-3, ZR75-1, and the CAMA-1 cell lines were established from pleural effusions of patients suffering from a breast cancer [30–33,32].

All the cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. When the cells reached 80% of confluence, they were trypsinised, washed, resuspended in PBS and used for further in vitro or in vivo experiments.

**Adenoviral vectors**

Recombinant adenovirus vectors were generated in PER.C6™ cells by homologous recombination between an adapter plasmid (pAdapt) and the E1 deleted Ad 5 DNA plasmid as described elsewhere [34]. The expression cassette contains a CMV promoter and SV40 poly A signal. As a result of the absence of sequence overlap between the Adapt plasmid and the Ad5 E1 sequences integrated into the genome of PER.C6, the vector stocks used in this study did not contain replicative competent adenovirus (RCA) [35].

The Ad5 Adapt mhAB encodes for a murinised form of the human ATF-BPTI (mhAB) [36]. In this construct the mhATF-BPTI, is preceded by the native secretion signal peptide of the human urokinase. The human endostatin coding sequence (InvivoGen, CA, USA) was cloned in the Ad5 Adapt shuttle vector. The encoded endostatin corresponds to the 183 residue of the human endostatin described by O’Reilly et al (1997) with an intact N-terminus (HSHRDFQ…), preceded by the secretion signal peptide of the human IL-2. The Ad.Adapt.Luc is a recombinant adenoviral vector in which the E3 region of Ad5 is retained and the luciferase gene replaces the E1 region. The Ad5 Adapt empty is identical to Ad5 Adapt luc except that it does not encode any transgene.

All vectors were produced on PER.C6™ using standard procedures [35]. Infectious units (iu)/ml were determined by end point cytopathogenic effect (CPE) assay on 911 cells [37]. Viral particles were determined by HPLC [38]. The particle to infectious unit ratio was always lower than 10.

**Organ lysate and Luciferase activity assay**

Animals were sacrificed by an overdose of isoflurane and whole organs were removed, frozen in liquid nitrogen and stored at -80°C. The whole gastrocnemius muscle or a piece of the right lobe of the liver were homogenised in phosphate buffered saline pH 7.8 using a blender. To lyse the cells, DTT (SIGMA, The Netherlands) (1 mM) and Triton x-100 (0.1%) (Merck, The Netherlands) were added. After centrifugation at 10,000 rpm for 10 min, 20 µl of the supernatant was added to 100 µl of luciferase assay substrate (Promega, The Netherlands). Relative light units (RLU) were determined for 30 s using a luminometer (Lumat 951, Wallac, Belgium). The amount of protein in the extracts was determined using a commercial kit (Bio-Rad laboratories, The Netherlands) based on the Coomasie brilliant blue G250 binding assay developed by Bradford [39]. The level of luciferase activity in the tissue homogenates was expressed in RLU / mg protein. The background level of this assay is <1000 RLU/mg protein and was defined by measuring luciferase activity in organs of non treated rats.

**Plasma assays**

For assay of plasma creatine phosphokinase (CPK), hEndostatin, ATF and mhATF-BPTI, animals were anaesthetised with isoflurane, bled by tail vein cut and the blood was collected in EDTA tubes.

An mhATF-BPTI enzyme-linked immunoabsorbent assay (ELISA) was developed by Dr. P. Quax (Gaubius Institut, TNO-PG, Leiden), using a monoclonal antibody specific for the ATF as the capture antibody and a polyclonal antibody directed against BPTI as the detector antibody [36].
As a standard we used medium of Ad5 Adapt mhAB infected CHO cell culture in which the mhATF-BPTI concentration was determined by an urokinase ELISA. Secretion of human endostatin in plasma was routinely determined using a commercial ELISA kit (InvivoGen, CA, USA) according to the manufacturer’s procedure.

**Real Time PCR analysis for viral DNA**

The amount of adenoviral genomes per cell was determined by a multiplex real-time polymerase chain reaction [40]. Real-time PCR is based on the 5'-3' nuclease activity of AmpliTaq Gold polymerase, which allows it to cleave fluorogenic probes resulting in fluorescence. The amount of fluorescence obtained during the PCR reaction is representative to the amount of amplified DNA.

Total DNA of transduced rat organs was extracted by using a DNeasy Tissue Kit (Qiagen). The kit efficiency was verified by measuring the recovery of plasmid DNA mixed from a liver lysate. We confirmed that 80% of the total DNA can be extracted from liver samples. To amplify the adenoviral DNA, specific primers (Ad5Clip-F: 5’CGACGGATGTGGCAAAAGT3’ and Ad5Clip-R: 5’CCTAAAACCGCGAAAAT3’) were designed by using the Primer Express Software (Perkin-Elmer, Foster City, CA, USA). A fluorogenic probe (Ad5Clip-Pr: 5’-VIC-CACCGGCCGACACCACAAAAACG-TAMRA-3’) was also designed by the Primer Express Software. To determine the amount of cellular DNA present in the sample a second pair of primers and a FAM-probe specific for 18S rDNA [40] were used. The PCR reaction mixture consisted of 1x buffer A (Perkin-Elmer), 3 mM MgCl2, 200 µM dNTPs, 90 nM of each adenovirus primer, 100 nM of each 18S rDNA primer, 200 nM of each probe, 0.6 U AmpliTaq Gold polymerase (Perkin-Elmer) and 5 µl of total DNA sample. As a standard, to determine the amount of adenoviral genomes and cellular DNA, a plasmid containing approximately 5000 bp of the left part of the Ad5 genome (pAdapt) was mixed with cellular DNA extracted from A549 cells. The PCR reaction was initiated with a hot start at 95°C for 10 min and involved 45 cycles of 15 s at 95°C and 1 min at 60°C.

**Northern Blot**

In case of intramuscular administration, the animals were sacrificed 2 days after vector administration and the whole gastrocnemius muscle was immediately collected and snap frozen in liquid nitrogen. The complete muscle was cut and homogenised in Trizol reagent (Gibco Life Technologies) using a blender. In case of cell cultures, the cells were harvested 2 days after the infection and directly homogenised in Trizol reagent. Total RNA was isolated from the muscle tissue or the cell lysate homogenate by using the method developed by Chomczynski and Sacchi [41]. Briefly, chloroform was added and the aqueous phase containing the RNA was recovered. RNA was precipitated with isopropyl alcohol and the pellet was resuspended in RNase free water. The RNA and DNA content were measured by spectrophotometric analysis.

The quantity of RNA loaded was 20 µg as determined by spectrophotometry. Both in the muscles as in the cell lines, the RNA loaded represents a similar proportion of the total RNA extracted. The RNA was run on a 1% agarose gel and transferred onto Hybond N+ membrane by overnight transfer. The RNA was then fixed by UV irradiation. The hybridisation was performed overnight with one stranded [32P]α-dATF Luciferase probe (1800 bp). A X-ray film (Kodak) was exposed to the membrane for 24 h and afterwards the membrane was scanned in a phosphorimager for quantification. Then the membrane was stripped 15 min at 65°C and prehybridised with salmon sperm DNA. Then a second hybridisation procedure was performed with the [32P]α-dATF β-actin probe.

**Pathology**

The gastrocnemius muscle was removed 2 days after the vector injection. The formalin-fixed muscle was then cut in ten transversal pieces through its all length. A 10 µm section was performed on all pieces and was stained with haematoxylin and eosin. All sections were examined but the quantification of the rhabdomyolysis was determined only on 3 sections, 1 mm apart from each other, that are corresponding to the middle of the length of the muscle where the injection actually occurred. The total damage score is a compilation of scores of apoptosis, hyaline degeneration, myoblast proliferation, and inflammation (Table 1).

**List of abbreviations**

ATCC: American Type Culture Collection

ATF: Amino Terminal Fragment

BPTI: Bovine Pancreatic Trypsine Inhibitor

iu: Infectious units

IU: International Units

iv: Intra venous

mhATF-BPTI: Murinised human ATF-BPTI

**Authors’ Contributions**

PL and JA participated in the all aspects of the study and DvB is the principal investigator.
Acknowledgment
We thank Angelique Lemkert and Bjorn Koel for technical assistance, Dr Menzo Havenga for critical review of the manuscript and Dr. A. Sieuwert for providing the breast cancer cell lines.

References
1. Sterman DH, Kaiser LR and Albelda SM: Gene therapy for malignant pleural mesothelioma. *Hematol Oncol Clin North Am* 1998, 12:553-568.
2. Swisher SG and Roth JA: *pS3 Gene therapy for lung cancer*. *Curr Oncol Rep* 2002, 4:334-340.
3. Molnar-Kimber KL, Sterman DH, Chang M, Kang EH, Ebash M, Lanuti M, Elshami A, Gelfand K, Wilson JM, Kaiser LR and Albelda SM: Impact of preexisting and induced humoral and cellular immune responses in an adenovirus-based gene therapy phase I clinical trial for localized mesothelioma. *Hum Gene Ther* 1998, 9:2121-2133.
4. Blackwell JL, Li H, Gomez-Navarro J, Dmitriev I, Krasnykh V, Richter and Fawell SE: Observations of adenovirus infections. *Am J Epidemiol* 1995, 141:37-45.
5. Craighead JE, Akley NJ, Gould LB and Libbus BL: Characteristics of tumors and tumor cells cultured from experimental asbestos-induced mesotheliomas in rats. *Am J Pathol* 1987, 129:448-462.
6. Fox JP, Hall CE and Cooney MK: *The Seattle Virus Watch. VII. Observations of adenoviruses in infections*. *Am J Epidiol* 1977, 105:362-386.
7. Havenga MJ, Lemkert AA, Ophorst OJ, van Meijnders PJ, Van Berkel AH and Van Bekkum DW: Impact of preimmunization on adenovirus-mediated gene expression in adult murine skeletal muscle. *Acta Pharmacol Toxicol (Copenh)* 2003, 93:227-234.
8. Kal HB, Zurcher C and van Bekkum DW: Induction of lung tumors by radioactive isotopes implanted in the rat lung. *J Natl Cancer Inst* 1986, 76:943-949.
9. Minna JD, Bunn P. A. Jr., Carney DN, Cohen MH, Cuttita F, Fosieck B. E., Jr., Gazdar AF, Ihde DC, Johnston-Early A, Matthews MJ, Makuch R, Oie H, Rosen S, Lichter A and Glaisttein E. Experience of the National Cancer Institute (USA) in the treatment and biology of small cell lung cancer. *Bull Cancer* 1982, 69:83-93.
10. Lieber M, Smith B, Szakal A, Nelson-Rees W and Todaro G: A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int J Cancer* 1976, 17:62-70.
11. Soule HD, Vazquez J, Long A, Albert S and Brennan M: A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 1974, 53:661-674.
12. Brinkley BR, Beall PT, Wilibe JL, Mace ML, Turner DS and Cailleau RM: Variations in cell form and cytoskeleton in human breast carcinoma cells in vitro. *Cancer Res* 1980, 40:3118-3129.
13. Trempe GL: *Human breast cancer in culture*. *Recent Results Cancer Res* 1976,33-41.
14. Solove HD, Vazquez J, Long A, Albert S and Brennan: A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 1973, 51:1409-1416.
15. Havenga MJ, Lemkert AA, Grimmerben JM, Vogels R, Huisman LG, Valerio D, Bout A and Quax PH: Improved adenovirus vectors for infection of cardiovascular tissues. *J Virol* 2001, 75:3335-3342.
16. Fallet FJ, Bout A, van der Velde I, van den Wollenberg DJ, Heir KM, Keegan J, Auger C, Cramer S, van Ormond H, van der Bej A, Valerio D and Hoeven RC: New helper cells and matched early region I-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 1998, 9:1909-1917.
17. Lamfers ML, Lardenoye JH, de Vries MR, Aalders MC, Engelse MA, Grimmeren JM, van Hinsbergh VW and Quax PH: In vivo suppression of restenosis in balloon-injured rat carotid artery by adenovirus-mediated gene transfer of the cell surface-directed plasmin inhibitor ATfBPTI. *Gene Ther* 2001, 8:534-541.
37. Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoeben RC and Van Der Eb AJ: Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum Gene Ther 1996, 7:215-222.
38. Shabram PW, Giroux DD, Goudreau AM, Gregory RJ, Horn MT, Huyghe BG, Liu X, Nunnally MH, Sugarman BJ and Sutjipto S: Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. Hum Gene Ther 1997, 8:453-465.
39. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976, 72:248-254.
40. Klein D, Bugl B, Gunzburg WM and Salmons B: Accurate estimation of transduction efficiency necessitates a multiplex real-time PCR. Gene Ther 2000, 7:458-463.
41. Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156-159.