Allele specific inhibition. A novel approach to cancer therapy

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ABSTRACT

Loss of heterozygosity (LOH) reduces many genes to hemizygosity in cancer cells and presents an absolute difference between normal and cancer cells. The regions of LOH are usually much larger than the tumor suppressor gene that is lost, and are expected to contain genes that are essential for cell survival. Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation in man, often giving rise to two or more allelic forms of most genes. SNPs of essential genes that are frequently affected by LOH can be used as a target for a novel therapy against cancer cells with LOH. The SNPs can be targeted by antisense oligonucleotides (ODNs) that will discriminate between two alleles. We have designed allele specific phosphorothioate ODNs against the gene of the large subunit of RNA polymerase II (POLR2A), a gene located in close proximity to the tumor suppressor gene p53, which frequently shows LOH in cancer cells. This report shows that phosphorothioate antisense ODNs directed against POLR2A can inhibit tumor growth in vivo as efficiently as a well described anti-tumor antisense ODN directed against Ha-ras. In addition, we show that a single base pair mismatch can be sufficient to obtain allele specific inhibition of tumor growth, demonstrating that the effects observed are true antisense effects.
INTRODUCTION

The central problem in cancer therapy is to kill cancer cells without damaging normal cells. In order to successfully solve this problem it is crucial to identify a consistent and absolute difference between cancer cells and normal cells and to have a therapeutic approach that exploits this difference to selectively target and kill cancer cells. In many cases cancer cells differ from normal cells because they have lost large segments of DNA. The loss of large chromosomal regions, or even whole chromosomes, is an early event in the clonal evolution of cancers. Loss of heterozygosity (LOH) can involve >20% of the total genome in certain cancers (1). Hence, the genetic difference between normal cells and cancer cells extends beyond the loss of a tumor suppressor gene. Many genes will be reduced to hemizygosity in cancer cells due to LOH, and some of these genes may be essential for cell survival. This irreversible difference between normal and tumor cells forms the basis of a new approach for anti-cancer drug development, called allele specific inhibition (ASI).

We have identified a large number of single nucleotide polymorphisms (SNPs) in genes that are essential for cell survival and are localized in chromosomal regions often involved in LOH in various cancer types (2). Many of these SNPs have a high degree of heterozygous expression in a panel of normal individuals. In principle these polymorphisms can be selectively targeted by antisense oligonucleotides (ODNs) that will discriminate the two alleles. Allele-specific inhibition (ASI) of the allele of an essential gene left as the only allele in the cancer cells as a result of the LOH will lead to cell death. The heterozygous normal cells, however, will only lose 50% of the expression of the gene by this ODN and, provided that 50% gene activity is enough for cell survival, will not be damaged by this antisense ODN. This novel approach to cancer treatment has been described in greater detail by Basilion et al. (3).

The large subunit of RNA polymerase II (POLR2A) is a potential target gene for ASI since it fulfils three important criteria (4). First, POLR2A encodes a 220-kDa protein that is the heart of the cellular transcription machinery. The POLR2A gene product is essential for cell survival as has been demonstrated by the use of the mushroom toxin α-amanitin, which binds specifically to the POLR2A 220kDa protein (5,6). Second, POLR2A contains many SNPs with a high frequency of heterozygous expression in normal individuals (2). Third, POLR2A is localized on chromosome 17p13.1 in close proximity to p53 (7), a region frequently involved in LOH in many tumor types (8).

We have described the development of a set of antisense phosphorothioate ODNs that can discriminate between the two alleles of a polymorphic site in POLR2A (4). These ODNs were designed to selectively target the matched allele, whereas the mRNA level of the mismatched allele, that differs in one nucleotide, is only slightly affected. However, a major question left unanswered in our previous in vitro study was whether these phosphorothioate ODNs could
arrest tumor cell growth in vivo. We, therefore, setup an in vivo model in which a continuous dose of antisense ODN can be administered systemically to tumor xenografts using osmotic mini-pumps. We show here that phosphorothioate antisense ODNs directed against POLR2A can inhibit tumor growth in vivo as efficiently as a well described anti-tumor antisense ODN against Ha-ras. In addition, we show that single base pair mismatches can be sufficient to obtain allele specific inhibition of tumor growth, giving proof of principle for allele specific targeting as cancer therapy, and demonstrating that the observed effects are true antisense effects.

**RESULTS**

We have described a complete set of ODNs around the SNP at position 2673 of POLR2A \(^{(4)}\). The ODNs L5Tas17 and L5Cas16 could discriminate between the two alleles of the targeted SNP in vitro. Figure 1 shows the sequence around the polymorphic site and the two antisense ODNs tested. A tritiated phosphorothioate ODN was used to monitor the delivery of the ODNs in vivo to the tissues of nude mice bearing 15PC3 or MiaPacII tumor xenografts, 48 hours after implantation of the osmotic minipumps. Both liver and kidneys were found to take up most of the recovered amount of radioactivity (15.7 ± 5.7 % and 8.0 ± 0.1% respectively for the complete organs). Less then 1% of the radioactivity could be recovered in either the 15PC3 or MiaPacII xenografts. The specific uptake per gram tissue is shown in table 1. The liver, kidneys, and to a lesser extent the lungs, were found to have a relatively

<table>
<thead>
<tr>
<th>2673</th>
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<tr>
<td>U</td>
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<tr>
<td>human mRNA: 5'GGUGGUGCGAGCGGCUAGGCAGCCUGGCAG 3'</td>
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<tr>
<td>ODNs (3'-5')</td>
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<tr>
<td>L5Tas17: GTCGAGCGATACCGCTT</td>
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<tr>
<td>L5Cas16: TCGACGCGATGCCGCTT</td>
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<tr>
<td>mouse mRNA: 5'GGUGGUAACAGCGCUAGAGAGAGGACGCCUGGCAG 3'</td>
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Figure 1: Schematic representation of the antisense ODNs L5Tas17 and L5Cas16 and the target sequence surrounding the polymorphic C/T site at position 2673 relative to the startcodon of the POLR2A mRNA sequence (Genbank accession no. X63564). The corresponding polr2a sequence of the mouse is also depicted. * denotes single base differences between the human and the mouse sequence. The NMRI mouse strain used in this study is T at the targeted position.
Table 1. Tissue distribution of a [\textsuperscript{3}H]-phosphorothioate ODN

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific radioactivity</th>
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<tbody>
<tr>
<td>liver</td>
<td>1.1 ± 0.5</td>
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<tr>
<td>lung</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>kidneys</td>
<td>3.6 ± 0.1</td>
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<tr>
<td>small intestines</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>large intestines</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>stomach</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>skin</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>MiaPacall xenograft</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>15PC3 xenograft</td>
<td>0.2 ± 0.1</td>
</tr>
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Nude mice (n=2) received an osmotic mini pump filled with 10^6 dpm [\textsuperscript{3}H]-ISIS 12790, a 20-mer phosphorothioate ODN. The osmotic mini pumps were implanted after overnight incubation in PBS at 37°C to start up the pumps. After 48 hours the mice was killed and the distribution of radioactivity was determined. The radioactivity in the different organs was corrected for serum present at the time of sampling as determined by the distribution of \textsuperscript{125}I-BSA. The results are expressed as specific radioactivity (% of total administered radioactivity divided by tissue weight) ± S.E.M.

High specific uptake per gram tissue. The specific uptake of radioactive oligonucleotide by tumor xenografts is similar to the uptake by the skin, muscles and intestinal tissues.

To test whether the amount of ODN found within the tumor xenografts was sufficient to deliver an effective dose, a well-described and potent antisense ODN against Ha-ras \textsuperscript{(9)} was administered to the tumor xenograft bearing nude mice. One week after injection of 10^6 MiaPacall or 15PC3 cells the pumps were implanted dorsally delivering a dose of 5 mg/kg/day of the anti Ha-ras phosphorothioate ODN (ISIS 2503). Both MiaPacall and 15PC3 tumor growth was inhibited by the treatment with ISIS 2503 as compared with saline and sense (5 mg/kg/day) ODN controls (Figure 2).

The results with the ISIS 2503 antisense ODN showed that the osmotic minipumps delivered enough antisense ODN to arrest the growth of both the 15PC3 and MiaPacall tumor xenografts. Next, the anti-POLR2A ODNs (Figure 1) were tested in this model to determine whether POLR2A is a suitable target for allele specific inhibition in vivo. Nude mice with either MiaPacall xenografts, which are T at position 2673, or 15PC3 tumor xenografts, which are C at position 2673, received via the osmotic mini pumps either the matched L5Tas17 ODN (5mg/kg/day) or the one base mismatched L5Cas16 (5 mg/kg/day). Figure 3 shows the effects of the anti-POLR2A ODNs on tumor growth. The matched L5Tas17 ODN significantly reduced the MiaPacall tumor growth as compared with the mismatched L5Cas16 and sense (5 mg/kg/day) and saline controls (P <0.01, two-way ANOVA). In contrast, the ODN L5Tas17 did not inhibit the mismatched 15PC3 xenograft growth at all,
Figure 2: The effect of Ha-ras antisense ODN (ISIS 2503) on the growth of human tumor xenografts in NMRI nu/nu mice. NMRI nu/nu mice were injected subcutaneously in the flank with (A) $10^6$ MiaPacaII cells or (B) $10^6$ 15PC3 cells from culture in 300 µl Matrigel. After one week of tumor growth the mice received an Osmotic mini-pump (Alzet model 1002), which was implanted dorsally. The Osmotic mini-pumps were filled with either antisense ODN (5mg/kg bodyweight) directed against Ha-ras (filled circles) and sense (5 mg/kg/day) (open triangles) and saline controls (open circles) (n=4 for each group). The tumor growth was monitored following the implantation of the osmotic pump. The results, expressed as tumor volume are given as means ± S.E.M.

while L5Cas16 inhibited 15PC3 growth significantly ($P <0.01$, two-way ANOVA). These results indicate that the ODNs against POLR2A are also allele specific in vivo and that POLR2A is a suitable target for tumor growth inhibition. Furthermore, these results indicate that the observed effects are true antisense effects, since a single base mismatch determines the efficacy of the antisense ODN.

To exclude the possibility that the external measurements during the experiment of the tumor xenografts were biased or affected by the co-injection of Matrigel or to subcutaneous fluid depositions, fluorescent GFP-expressing 15PC3 cells were created. When injected subcutaneously into nude mice the resulting xenograft is fluorescent and can be imaged externally (15). Only the tumor cells are fluorescent and can be distinguished from other cells and tissues of the mouse. The fluorescence remained localized in the tumor xenograft and the growth characteristics were similar to the non-fluorescent 15PC3 xenografts (data not shown). Nude mice with fluorescent 15PC3 (C) tumors were treated with the anti POLR2A ODNs L5Tas17 and L5Cas16 using the osmotic minipumps. The xenografts were imaged after 10 days of treatment. In Figure 4 two representative fluorescent 15PC3 tumors are shown. The fluorescent 15PC3 tumors treated with the matched L5Cas16 ODN were markedly smaller ($90 ± 10 \text{ mm}^3$, n=5) and less fluorescent as compared to tumors treated with
The effect of POLR2A antisense ODNs; L5Tas17 and L5Cas16 on the growth of human tumor xenografts in NMRI nu/nu mice. NMRI nu/nu mice were injected subcutaneously in the flank with (A) 10^6 MiaPaca II cells (T for the targeted SNP) or (B) 10^6 15PC3 cells (C for the targeted SNP) from culture in 300 μl Matrigel. After one week of tumor growth the mice received an Osmotic mini-pump (Alzet model 1002), which was implanted dorsally. The Osmotic mini-pumps were filled with L5Tas17 (filled circles) and L5Cas16 (filled squares) antisense ODN (5mg/kg body weight) and sense (5 mg/kg/day) (open triangles) and saline (open circles) controls (n=5 for each group). The tumor growth was monitored following the implantation of the osmotic pump. The results, expressed as tumor volume are given as means ±S.E.M.

the mismatched L5Tas17 ODN (200 ± 20 mm^3, n=5).

However, increasing the dose of ODNs to 10 mg/kg/day resulted in a loss of allele specificity. L5Tas17 and L5Cas16 reduced tumor growth independent of the genotype (data not shown), indicating that allele specificity of these ODNs is dose dependent. Similar observations were done in vitro (4). The POLR2A antisense treatment did not result in any overt visible problems for the mice. During the antisense treatments the mice were weighted daily. No weight loss occurred during the ODN treatment. Although the POLR2A sequence is highly conserved, the polr2a sequence of the mice differs at one nucleotide position from the human sequence within the targeted mRNA stretch (Figure 1). Thus for L5Tas17 there is one mismatch and for L5Cas16, two mismatches occur with the mouse sequence. Since the biodistribution studies (Table 1) clearly indicated that most ODN is taken up by both the liver and the kidneys, and since we have seen that an increase in ODN dosage resulted in loss of the allele specificity, it is feasible that at high concentrations, the ODNs also affect the mouse polr2a mRNA. To investigate this possibility we examined the effects of ODN treatment on the mouse liver, one of the principle sites of uptake of phosphorothioates in the mouse. No effects could be found on the levels of polr2a mRNA in the mouse liver due to treatment with ODNs L5Tas17 and L5Cas16 (Figure 5). We also examined the histology of the liver
after ODN treatment. Only minor and diffuse mixed mononuclear cell infiltrates were discovered within the liver in all mice treated with phosphorothioate ODNs including the sense controls. These effects are non-sequence specific and probably caused by the phosphorothioate chemistry of the ODNs as they are also described previously (21). In addition, we also examined the effects of antisense treatment on general liver function by measuring the levels of bilirubin and alkaline phosphatase in the serum of treated mice. Also in this case, no sequence specific effect could be found after ODN treatment as compared to mice treated with saline and sense controls (data not shown). We therefore conclude that the treatment with antisense against the human POLR2A sequence is not overtly toxic for the mice and can effectively inhibit xenograft tumor growth in a genotype selective manner.

**Figure 4:** External images of the effect of POLR2A antisense oligodeoxyxynucleotides L5Tas17 and L5Cas16 on the growth of fluorescent 15PC3 tumor xenografts in NMRI nu/nu mice. NMRI nu/nu mice were injected subcutaneously in the flank with $10^6$ GFP expressing 15PC3 cells (C for the targeted SNP on position 2673) from culture in 300 μl Matrigel. After one week of tumor growth the mice received an Osmotic mini pump (Alzet model 1002), which was implanted dorsally. The Osmotic mini-pumps were filled with L5Tas17 or L5Cas16 antisense ODN (5mg/kg body weight). The tumors were imaged using laser light excitation and a CCD camera.

**Figure 5:** Effect of 14 day treatment with antisense (5mg/kg/day) against human POLR2A on mouse liver polr2a mRNA.  
**Left:** Northern blot, upper panel: mouse liver polr2a message, lower panel: gapdh message.  
**Right:** graph showing normalized polr2a levels as quantified from Northern Blots using AIDA 2.41 software (Raytest benelux, Tilburg, The Netherlands).
DISCUSSION

Previous studies by Basilion et al (3) and ten Asbroek et al (4) have shown that allele specific inhibition (ASI) of gene expression is possible in vitro. POLR2A is one of the target genes for which allele specific ODNs were designed, which discriminate between the two alleles differing only in a single nucleotide polymorphism (SNP) (4). We now demonstrate that these ODNs can inhibit the growth of human tumor xenografts in nude mice.

The ODNs were administered continuously through osmotic mini-pumps. Tissue distribution studies indicate that only a limited amount of the administered ODN reached the tumors. This is in accordance with previous data, which show rapid clearance of phosphorothioate ODNs mainly through high uptake in liver and kidney (12,18).

To demonstrate the validity of both our tumor model and our antisense ODN delivery system, we targeted tumors of both test cell lines with a well known anti-tumor Ha-ras antisense ODN (9). The antisense ODNs directed against POLR2A mediated a similar reduction in growth rate when compared to the Ha-ras antisense ODN in these tumors. Furthermore, the growth of the tumors was only affected by the respective matched ODNs. MiaPaCaII tumors, that are homozygous for a T in the target region, were only inhibited in their growth rate by the matched ODN L5Tas17, and not by the single base mismatched L5Cas16. Similarly, 15PC3 tumors, homozygous for a C in the target region, are only affected by the matched L5Cas16 and not by the single base mismatched L5Tas17. Therefore, the antisense ODNs can discriminate between the two alleles of a SNP in POLR2A, and cause genotype-specific reduction of tumor growth in vivo. These results indicate that ASI therapy is a potentially viable approach to develop a tumor-specific anti-cancer therapy, and that POLR2A is a good target gene for antisense ODN-mediated inhibition of tumor growth. However, the allele specificity of the ODNs was reduced when the daily dose of ODNs was increased to 10 mg/kg. At this higher dosage, both L5Tas17 and L5Cas16 could inhibit tumor growth similarly, irrespective of the tumor genotype. This is in accordance to our previously published in vitro experiments where allele specificity was shown to be dependent on the ODN concentration (4). The differences in affinity for the targeted sequence as compared to the mismatched sequence are dependent on only a single nucleotide difference.

In view of the variation in tissue uptake, we analyzed the effect of ODNs on the mouse tissue. The mouse polr2a sequence differs in one base from the human sequence in the targeted region of the mRNA. Thus, depending of the human genotype, one or two mismatches are present. We observed no effect of the antisense oligonucleotides on the mice. The liver, which takes up a much larger amount of the phosphorothioate ODN, was not affected in a sequence-specific manner. This may bode well for when ASI is to be used in an authentic therapeutic setting, when there is only a single base mismatch between the tumor and the organism in which it resides. In our model we also have shown that two different
homozygous tumor types are protected by a single base mismatch at the effective concentration. In addition, it must be noted that at least a large fraction of the ODNs taken up by the liver and kidneys might be sequestered from the body in such a way that they are not effective anymore (i.e. degraded or rapidly sequestered to the urine and bile for excretion). Future studies will have to incorporate heterozygous tumor xenograft controls, as well as syngeneic mouse tumor models to obtain full proof of principle for ASI as therapy.

Essential genes, like *POLR2A*, which are highly conserved through evolution, have been shown to posses single nucleotide polymorphisms (2) that occur frequently in the human population. Hence, there are enough SNPs within essential genes that are affected by LOH in cancers that may serve as target for ASI. Most of these polymorphisms are silent and do not cause changes in the peptide sequence of the protein. This makes it difficult to find allele specific inhibitors that act on protein polymorphisms that occur frequently enough within the human population to be of any therapeutic use. We, therefore, chose to use antisense ODNs to target SNPs. Antisense ODNs have been proven to inhibit gene expression specifically both *in vitro* and *in vivo*, and have entered clinical trials or are already registered as a drug (16). The most important problem for ASI remains the effective discrimination of the two alleles of a SNP. For each target SNP this will be a process of trial and error, since both the antisense ODN and the target mRNA will influence this process. Shen et al. (17) showed that SNPs can cause marked changes in the secondary structure of an mRNA. This may lead to an altered accessibility of the mRNA for antisense ODNs, and therefore affect the allele specificity of an ODN. For the *POLR2A* target SNP presented in this study we found that shortening the ODN, as well as shifting the SNP position within the ODN, were beneficial for the allele specificity (4). The number of antisense ODNs that can be used for ASI is limited, since the target is limited to a specific region containing the SNP. We, therefore, envisage that ASI might not be feasible for every potential target SNP. Our studies were performed using simple phosphorothioate antisense ODNs. Novel antisense chemistries have recently become available (19,20). These advanced chemistries might further increase the allele specificity and efficacy of ODNs. In the future, ASI therapy might be an attractive addition to current chemotherapies, since an absolute genetic difference between cancer cells and normal cells is targeted. The fact that essential genes are targeted in this approach may even further increase the efficacy of the treatment. Cancer cells cannot retrieve the allele that is lost by LOH during tumorigenesis; the targeted allele can therefore not be replaced.
MATERIALS AND METHODS

Oligonucleotides
All phosphorothioate ODNs were purchased from IsoGen (The Netherlands) except the ISIS 2503 and 12790 antisense ODNs, which were a kind gift of Variagenics Inc. (Cambridge, Mass. USA). LSTas17: 5’ TCGCCATAGCGCAGCTG 3’, L5Cas16: 5’ TCGCCGTAGCGCAGCT 3’, ISIS 2503 is a 20-mer targeted to the initiation codon (AUG) of c-Ha-ras mRNA: 5’ TCCGTACATCGCTCCTAGGG 3’ (9). ISIS 12790 is a 20-mer targeted to the 70-kDa subunit of replication protein A (3):
5’ TGGTCTGCAGTTAGGGTCAG 3’. Tritium labeling of ODNs was performed using the heat exchange method described by Graham et al. (10).

Cell lines
The pancreatic carcinoma cell line MiaPacaII and the prostate cancer cell line 15PC3 were maintained by serial passage in Dulbecco’s modified Eagle’s medium (DMEM). Cells were grown at 37°C and 5% CO2. Media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

In vivo model
Eight to ten week old NMRI nu/nu mice (Charles River, the Netherlands) were injected subcutaneously in the flank with 10⁶ MiaPacaII cells or 10⁶ 15PC3 cells in 300 μl Matrigel (Collaborative Biomedical products, Bedford, Ma, USA). The cells were injected within one hour after harvesting by trypsin treatment. Before injection the cells were washed with cold PBS, counted with a hemocytometer and subsequently mixed with the Matrigel on ice. One week after tumor cell injection, when tumor take was positive, an osmotic mini pump (Alzet model 1002, Alzet corp., Palo Alto, Ca, USA) was implanted dorsally according to the instructions of the manufacturer. The osmotic minipumps were incubated in PBS 20 hours at 37°C prior to implantation to start up the pump, in order to quickly reach a steady delivery rate after implantation. In vitro testing showed that the Alzet 1002 minipumps reached a steady pumping rate within 24 hours. The osmotic minipumps were filled with phosphorothioate oligonucleotides (5 mg/kg bodyweight) or 0.9% saline. Tumor growth was monitored daily following the implantation of the osmotic mini pump. Tumor volume was measured and calculated as described previously (11). Tissue distribution studies of tritiated ODNs were performed according to Bijsterbosch et al. (12). The radioactivity in the different organs was corrected for serum present at the time of sampling as determined by the distribution of 125I-BSA (personal communication K. Kruijt, University of Leiden, the Netherlands). Liver samples were fixed in 4% formaldehyde in PBS and subsequently embedded in paraffin according to standard procedures. Hematoxylin and eosine (HE) stains were used to visualize the effects of ODN treatment on the liver. Bilirubin and alkaline
phosphatase levels in the serum were determined using standard diagnostic procedures with the H747 (Hitachi/Roche) with the appropriate kits (Roche Diagnostics).

**Green fluorescent protein expressing 15PC3 tumors**

15PC3 tumors expressing GFP were created as described \(^{(13)}\) with the following modifications. Enhanced GFP was cloned into the retroviral LNCX2 vector (Clontech) and 70% confluent Phoenix packaging cells were used to generate the viral supernatant \(^{(14)}\). G418 (Life technologies Inc.) was used to select GFP-expressing 15PC3 cells. The stability of GFP expression was verified by using FACSClone analysis. The GFP-expressing 15PC3 cells were injected into NMRI nude mice as described above and were imaged using diffused laser light excitation (488 nm, argon laser (Spectra-Physics, model 2030) and CCD camera (Telecam SL, Karl Storz GmbH, Tuttlingen, Germany) equipped with a 515 nm longpass filter.

**Acknowledgements**

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