Cancer therapy by lentivector based 19S RP depletion

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We have reported that the aggressive and drug resistant tumor cell lines are addicted to the maximal level of the 26S proteasome complex. Even a minor reduction at the level of the 26S proteasome causes cancer cell death without a noticeable effect on the normal cells (see doi: 10.1038/s41419-018-0806-4). Based on these findings we have formulated a new approach in cancer therapy by depleting the 19S regulatory (19S RP) particles, a component of the 26S proteasomes. We found that shRNA against one of the 19S components, namely PSMD1, PSMD6 and PSMD11, reduces the level of the 26S with simultaneous increase at the 20S proteasome level. We generated lentivector (LV)-based shRNA expression that are highly effective in depleting the 19S RP. Two different LV were generated; silent **LV-tuki** (for tumor killer) which can be activated by doxycycline treatment, and a constitutively active LV-tuki. Remarkably these vectors efficiently kills cancer cells but not the normal cells. Furthermore, the more aggressive the tumor cells are, the better they are killed. Drug resistant triple negative breast cancer (TNBC) cells and ovarian cancer cells are killed with about 100% efficiency. Using xenograft mice models we demonstrated that the LV-tuki is very effective in eliminating TNBC cells outgrowth. The description and in vitro utilization of LV-shRNA against the component of the 19S RP are described in the attached paper (see doi: 10.1038/s41419-018-0806-4), below described the results on the xerograph model experiments. Two different experimental models were used. In one the tumor cells bear the silent LV-tki. The expression of the resident shRNA was induced by doxycycline after tumors reached certain size. In the second model naïve MDA-MB-231 cells were first injected and after reaching a reasonable tumor size, they were transduced by intratumoral injection with the active LV-tuki.

Treating MDA-MB-231-Luc-LV-tuki xenograft mice by doxycycline induction.

The TNBC cell line MDA-MB-231 was obtained from the National Cancer Institute collection (NCI-60). In order to follow tumor progression and the efficacy of LV-tuki treatment we generated a lentiviral vector for constitutive expression of firefly luciferase in MDA-MB-231 cells (named MDA-MB-231-Luc).
Female nude mice HsdHli:CD1-Foxn1nu were obtained from Envigo (Harlan) at 6-7 weeks of age. All mice were maintained in specific pathogen-free conditions and allowed to acclimate for a while. MDA-MB-231-Luc cells (50 µl cell suspension per mouse) was subcutaneously injected under sterile conditions using 27G needle equipped syringes. Growth status of tumors was examined by digital caliper measurements. Tumor volume was calculated as $X^2Y/2$ (X is the smallest tumor dimension).
Bioluminescent in vivo imaging: Growth status of the subcutaneously implanted cells was also followed twice a week by bioluminescent image analysis using the IVIS SPECTRUM (Caliper Life Sciences) imaging system. The exposure time was set to 1 sec. Image signals were analyzed using the Living Image® software (Xenogen). The growth status of the

subcutaneously implanted cells was depicted by a tumor-growth curve (average radiance [p/s/cm²/sr]) vs. time in days post-implantation).

Fluorescent imaging: We also inserted inducible RFP in the inducible LV-tuki cassette expression. The efficiency of the doxycycline induction in vivo was monitored by RFP.

We first found that subcutaneous tumors were formed at the injection site in all the mice inoculated with either 4 or 8 x 10^6 cells. Some mice inoculated with 2 x 10^6 cells did not develop tumors. On the other hand, tumors inoculated with 8 x 10^6 cells developed too rapidly. Therefore, the amount of 4 x 10^6 cells was chosen as optimal for further experiments. In addition, these results demonstrated that tumors originating from both naïve or LV-tuki cassette harboring cells grew with similar kinetics, meaning that there is no significant leakiness from the inducible LV-tuki cassette without doxycycline treatment.

For the following experiments 4 x 10^6 of either naïve or MDA-MB-231-Luc (harboring an inducible LV-tuki cassette) cells were injected subcutaneously into right back of each mouse at day 1. To activate LV-tuki, we used doxycycline (Glentham Life Sciences, UK) (1 mg/ml) in drinking water, starting from day 5 after injection of the tumor cells. Due to the bitter taste of doxycycline, drinking water was also supplemented with 1% sucrose. The drinking water was changed every three days. Three groups of ten mice were used for the study: 1) mice injected with naïve cells; 2) mice injected with LV-tuki cassette harboring cells, and maintained without doxycycline supplementation (= no shRNA expression); and 3) mice injected with LV-tuki cassette harboring cells, and maintained with doxycycline supplementation (= induction of shRNA expression). Growth status of the tumors was monitored by caliper measurements and by *in vivo* optical bioluminescent imaging.
The obtained results demonstrates that LV-tuki is induced *in vivo* by doxycycline administration. Both bioluminescent imaging and physical measurements of tumor volumes demonstrated that induction of **LV-tuki markedly reduced the tumor size in the xenograft mice** (Figs 1,2, 3, 4).

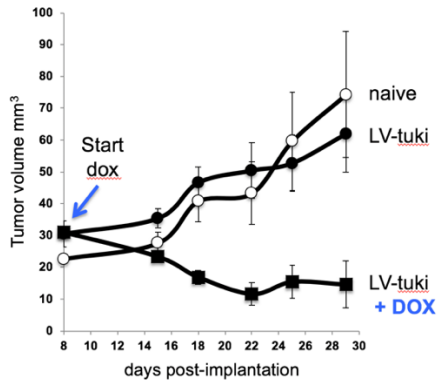

Fig 1: LV-tuki effectively reduced the tumor size by caliper measurements.
Either naïve or an inducible LV-tuki cassette cells (4 x 10^6) were injected subcutaneously into right back of each mice at day 1. To activate LV-tuki, we used doxycycline supplementation (1 mg/ml) in drinking water starting from day 5 after injection. Growth status of the tumors was examined by caliper measurements. Tumor volume was calculated as $X^2Y/2$ (X is the smallest tumor dimension).

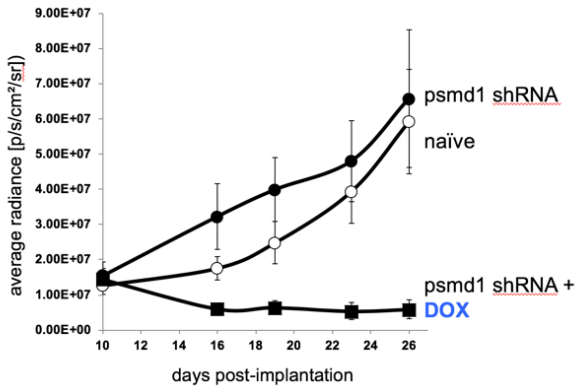

Fig 2: induction of LV-tuki effectively reduced the tumor size by bioluminescent image analysis. The experiments were conducted as in Fig 1 but growth status of the subcutaneously implanted cells was examined by bioluminescent image analysis as described above. D-luciferin was injected into the abdominal cavities of the mice. Mice were completely anesthetized with a mixture of oxygen and isoflurane following imaging. Image signals were analyzed using the Living Image® software (Xenogen). The growth status of the subcutaneously implanted cells was depicted by a tumor-growth curve (average radiance [p/s/cm²/sr]) vs. time in days post-implantation)

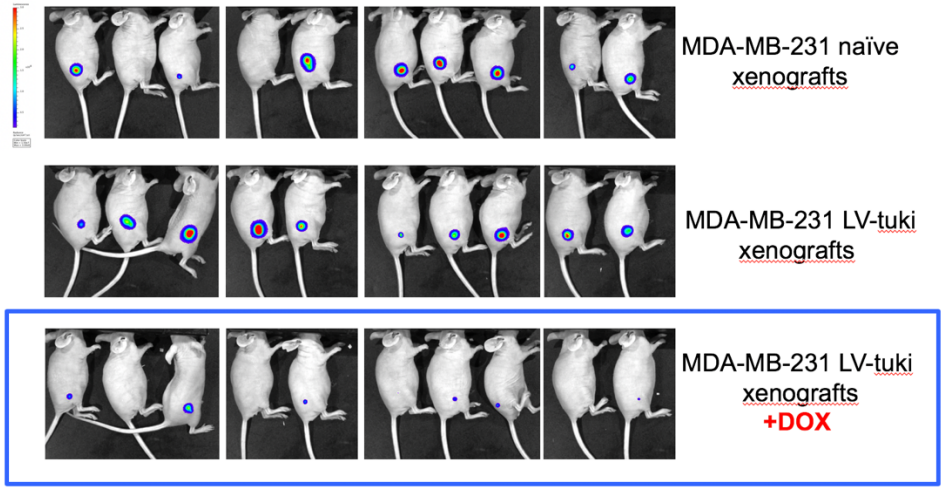

Fig 3: active LV-tuki inhibited MDA-MB-231 tumors in xenograft mice. Live imaging of mice at the end of the experiment (day 26 after injection of tumor cells). Luciferase activity in s.c tumors was measured by IVIS Spectra.

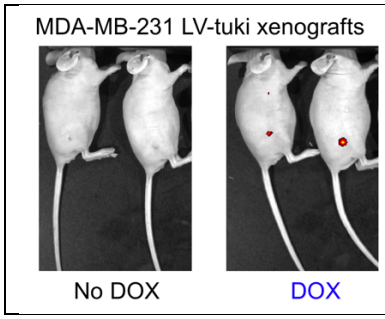

Fig 4: In vivo RFP fluorescence imaging (marker of LV-tuki cassette expression) in representative mice injected with LV-tuki cassette bearing cells treated or not with doxycycline. RFP positive signal reports expression LV-tuki cassette in response to doxycycline

Treating TNBC xenograft mice with LV-tuki transduction

Next we used xenograft mice and treated the tumors by intratumoral injection of the active LV-tuki. As control we used LV- with irrelevant sequence.

MDA-MB-231 (4 x 10^6 cells per mouse) were subcutaneously implanted in female nude mice HsdHli:CD1-Foxn1nu (obtained from Envigo) at 7 weeks of age. On day 10 after tumor cell injection the tumors were LV-tuki infected. The tumors were successfully transduced as

monitored by GFP expression (Fig 5). This was repeated 5 times (Fig 6). The results show that the tumors were significantly reduced in size.

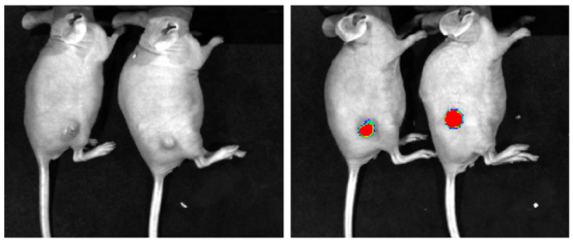

Fig 5: monitoring LV transduction.
In vivo GFP fluorescence imaging (marker of infection with LV-tuki or the control virions) in representative mice at day 31. GFP positive signal reports that tumors were successfully transduced upon injection of the LV virions directly into the tumors. At left are the non-LV-tuki injected controls.

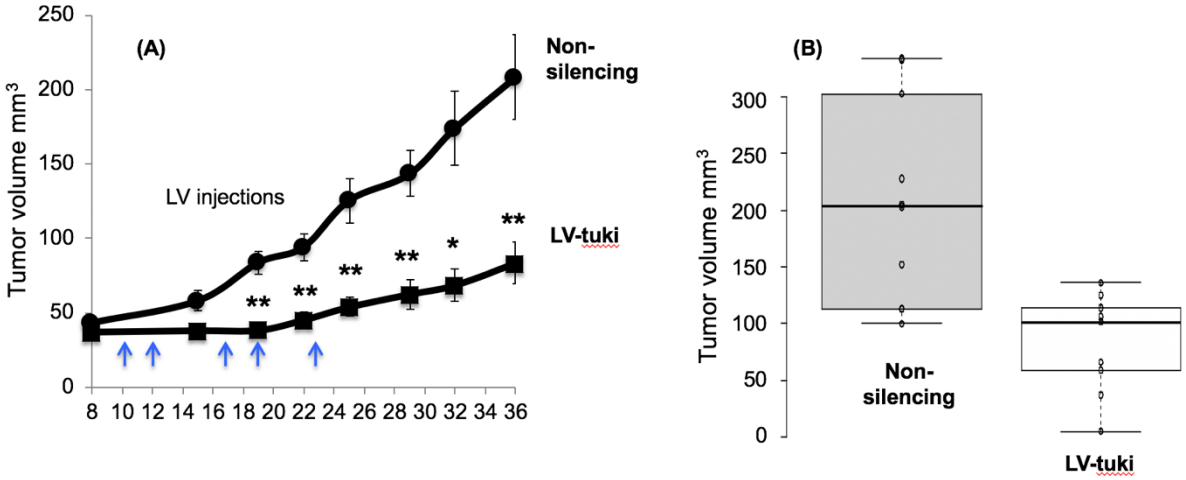

Fig 6: treating TNBC xenografts with LV-tuki transduction.
(A) MDA-MB-231 cells (4 x 10^6) were injected subcutaneously into right back of each mice at day 1. Either active LV-tuki or irrelevant sequence-based virions (LV-non-silencing) were injected intratumorally (2-10 x 10^6TU) at the indicated days by arrow. Growth status of the tumors was examined by caliper measurements. Tumor volume was calculated as $X^2Y/2$ (X is the smallest tumor dimension) and used to plot the tumor volume fold change over time from the initial measurement (day 8).
(B) Tumor volume fold change was shown as box plot (day 32).
* $p < 0.05$, ** $p < 0.01$

Conclusion: the LV mediated expression of shRNA to knockdown PSMD1, a 19S RP subunit is very effective in treating a TNBC cell lines. Preliminary data indicate that this is the case also with the drug resistant ovarian cancer ovcar3 cell line.