Final report with major results

1. Synthesis and preliminary tests of lipid- and plasmamembrane selective water soluble hydrogenation complexes.

Several complexes of the [RuH(O2CR)(mtppms)3] general composition (O2CR = long chain fatty acid, e.g. octanoic acid, mtppms = monosulfonated triphenylphosphine) were synthetised and characterized (1H, 13C, 31P NMR, ESI MS, elementary analysis). These are expected to be incorporated preferentially into the vicinity of specific phospholipid classes within the model- and biomembranes. Although the new complexes were able for activation of molecular hydrogen at higher temperatures (60 °C), however, at room temperature they did not catalyze hydrogenation of simple waters-soluble unsaturated substrates (maleic acid, fumaric acid, crotonic acid, etc.). Further investigations are needed to learn their catalytic properties in hydrogenation of the double bonds of polar lipids (e.g.soy-bean lecithin) at lower temperatures. Our hydrogenation complex candidate molecules were expected to be incorporated preferentially into the vicinity of specific lipid classes within the model- and biomembranes. Investigations presently are in progress to learn more about their catalytic properties in hydrogenation of the double bonds of different lipid molecular species by using lipidomics technology, on live K562 and CHO cells.

2.Establishing the mouse melanoma B16 cellular models possessing different metastatic abilities and HSP70 expression for studying the heterogeneity of heat shock response.

The B16F0 has been described as a weakly metastatic cell line therefore we have set out to express the HSP70 chaperone and a newly discovered metastasis associated phosphatase PRL3 in the B16F0 cell line. Using the isogenic B16F10 cell line isolated from secondary metastatic sites would allow us to compare the differential changes in the metastatic process in the context of HSP70 and its membrane-lipid regulation. To ensure the validity and identity of our cell lines we have freshly ordered our B16F0 and B16F10 stock from the ATCC cell bank at the beginning of the study. In order to establish our cellular system the B16F0 cell line has been transfected with the metastasis associated PRL3 and the HSP70 chaperone separately. The human PRL3 and the mouse HSP70 cDNA was cloned into the pcDNA4/TO vector under the control of the tetracycline repressor dependent promoter (pTOprl, pTOhsp70). The repressor protein has been provided by the pcDNA6/TR plasmid. The pTOprl and the pTOhsp70 construct has been introduced into B16F0 cells and stably transfected cell lines were selected. These cell lines were then transfected with the pcDNA6/TR plasmid allowing the regulation of the HSP70 and the PRL3 genes by doxycycline.

The B16F0, B16F0-PRL3, and B16F10 has been subjected to a comparative analysis regarding their heat shock protein response, and metastatic abilities. At first we had compared the metastatic capabilities of the two B16 cell line in a C57BL/6 mouse model. For *in vivo* monitoring C57BL/6 mice were maintained on a daily 12 hour light/ 12 dark cycle, housed under pathogen free conditions in micro-isolator cages with laboratory chow and water *ad libidum*. The tail vein of female mice were intravenously inoculated with $1x10^5$ B16F0 or B16F10 cells with an inner repetition number of 5 animals in 3 independent experiments.

Control animals has been given physiological salt solution only. After 12 days the mice has been sacrificed according to the current guidelines regarding experimental animals, their lungs has been isolated and the number of tumor metastatic nodules were examined. In parallel to the animal studies we have analyzed the heat shock protein expression upon heat treatment with western blot. B16F0 and B16F10 cells has been subjected to heat shock at 43°C degrees for one hour. Cells were scraped in ice cold lysis buffer and substituted with Laemmli sample buffer. An equal amount of total proteins has been introduced on SDS PAGE gels and blotted to nitrocellulose membranes, incubated with primary antibody to ensure detection selectivity and secondary antibodies for the luminescent detection of the bound proteins of interest.

Strikingly our results had revealed no or a low level differences either in the metastatic abilities or in the HSP70 protein induction by heat in the B16F0-F10 system in contrast to our primary hypothesis based on pioneering studies from the 1970s. Therefore to have a wider spectra of view on this phenomena we have introduced the B16F1 another cell line from the B16 family to our studies. Furthermore we have designed an unbiased study in cooperation with an external research group to ensure our results. These second round of studies has strengthened our previous findings. As such convincing data occurred at our hands we had to acknowledge the de facto similarity of the B16 cell lines regarding their ability to form secondary tumors in the lung of the mice. Therefore despite the fact that we have cloned the hsp70 gene into our expression vector and successfully selected B16F0 cells expressing the protein in a regulatory manner we have decided not to use it for further studies. Since in the light of our results the use of an HSP70 expressing cell line or either an HSP70 silenced one has lost its purpose as a “calibrating device” for the role of HSP70 in tumorigenesis in a background which is isogenic but differently aggressive.

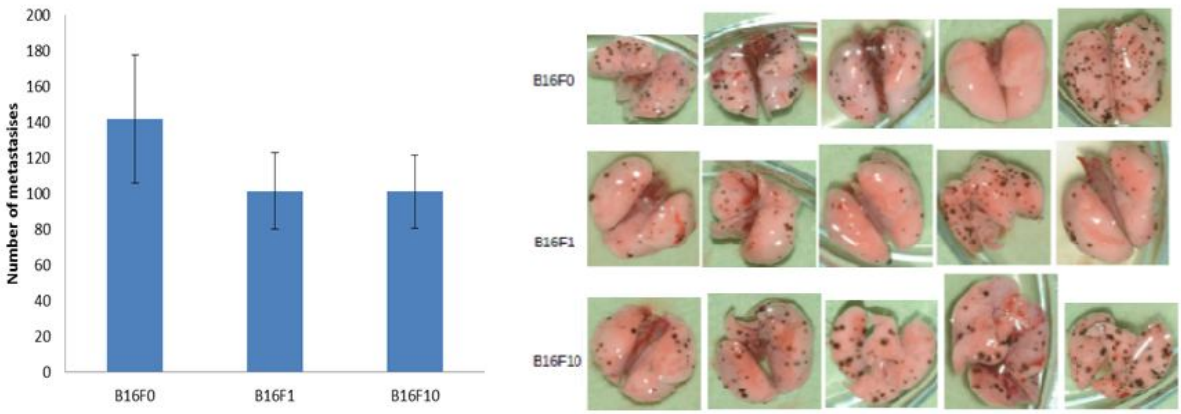

Figure 1. Comparative analysis of the metastatic nodules arisen from the separate B16 lines.

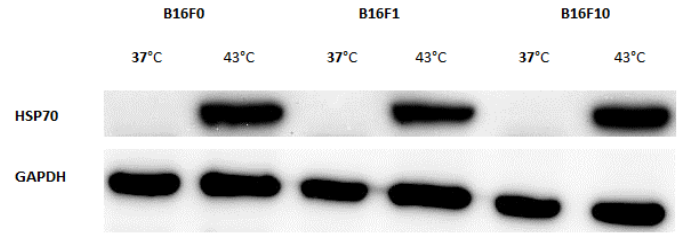

Figure 2. Heat shock response of the B16 cell lines analyzed by the induction of HSP70 by heat

Proceeding with the schedule proposed in our work plan we have sought to investigate the nature of PRL3 action. As described previously B16F0 cells with constitutive and regulated expression of the PRL3 protein has been selected. These cells has been subjected to a comparative analysis similar we have executed with the B16 lines. $1x10^5$ B16F0 and B16F0-PRL3 cells were intravenously inoculated to the tail vein of female mice and heat shocked to asses HSP70 expression upon heat as described earlier. Most surprisingly the direct metastatic ability of the B16F0-PRL3 cells has been greatly reduced compared to the control B16F0 line. To investigate this surprising phenomena we have felt the need to investigate other tumorigenic properties of the B16F0-PRL3 cells to tackle the molecular mechanism which could resolve the contradiction between our results and the observed high expression of the PRL3 phosphatase in secondary tumor sites described in the literature. Therefore we set up an experiment to assess the growth abilities of these cells. For this reason the B16F0 and the B16F0-PRL3 has been inoculated subcutaneously into the backs of female mice. Control animals are given physiological salt solution only. Tumor volumes are measured twice per week from day 19 with a caliper and expressed in nm^3 by the formula for the volume of a prolate ellipsoid. Tumors were removed from mice after 21 days of growth and were frozen in liquid nitrogen for further analysis. The results of the tumor growth analysis revealed a significantly greater growth rate for the PRL3 expressing cells in contrast with their lowered ability to migrate within the animal. As a next step we subjected these cells to heat shock in order to examine HSP70 expression as described earlier to investigate if the observed phenotypic changes could be a derivative of altered heat stress sensitivity. Our experiments on the heat stress response has not revealed a difference in HSP70 expression. Therefore we set out to further analyze the lipidome and proteome of these cells to gain an omics-based deeper understanding of the molecular happenings in order to identify the molecular change behind their cellular behavior.

Considering the farnesyl modification present on the PRL3 protein our approach were specifically targeted to the lipids involved in the structure of the detergent resistant membrane micro-domains generally termed as lipid rafts. Data gained from the comparative lipid investigations of possible lipid changes regarding the PRL3 expression gave us a clue in the higher level of a sphingolipid glycosylceramide in the PRL3 cells. Since the abundance of ceramide lipids in lipid rafts this data has pointed out our next step to concentrate our efforts in a membrane raft proteomic studies. Distinct membrane sub-fractions were harvested then their protein content were fractionated with SDS-PAGE. The protein lane were divided into

12 sections and each part were reduced alkylated and trypsin digested. The resulting digests were analyzed by LC/MS/MS. Protein identification was accomplished from the CID data by a database search. Spectra counting is used for quantitative comparisons, the spectral counts were normalized using the number of identified components in each sample. Our analysis were in line with the data obtained from the earlier lipidomic studies. We have observed a great change of expression in key organizing proteins connected to lipid rafts especially in the downregulation of the Caveolin 1 protein, a central organizer of the caveolae. The validation of our proteomic data regarding the lipid raft proteins has been done by western blot. Further analysis of the possible protein-protein and protein-lipid interactions to identify the role of lipid rafts in such cellular phenotypic change is in progress.

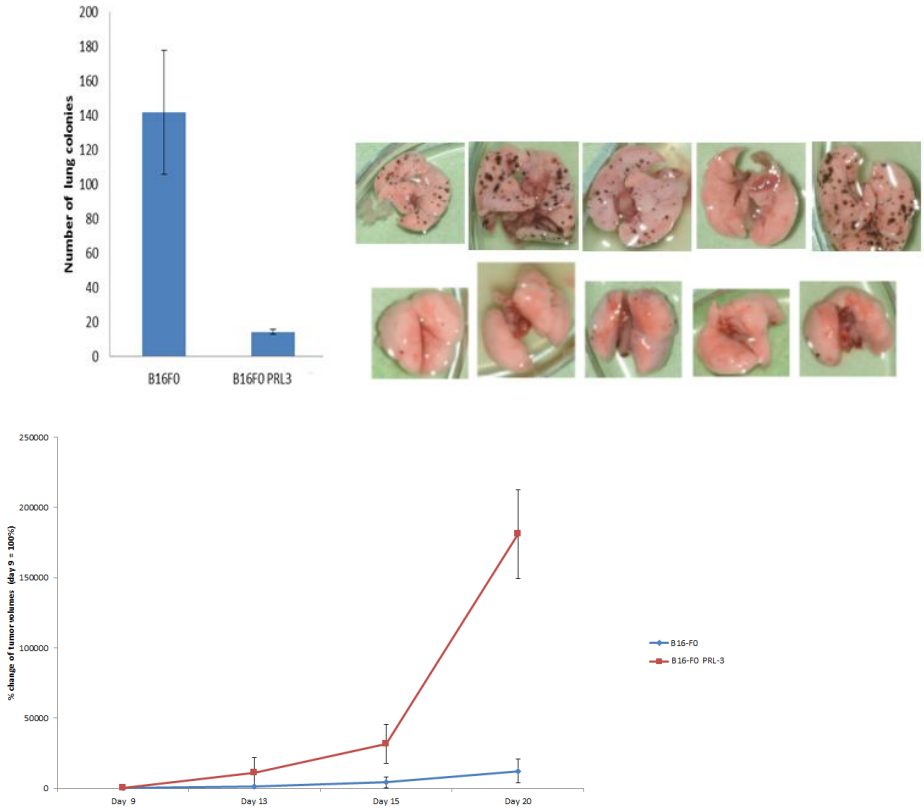

Fig 3. The tumorigenic action of the PRL3 phosphatase

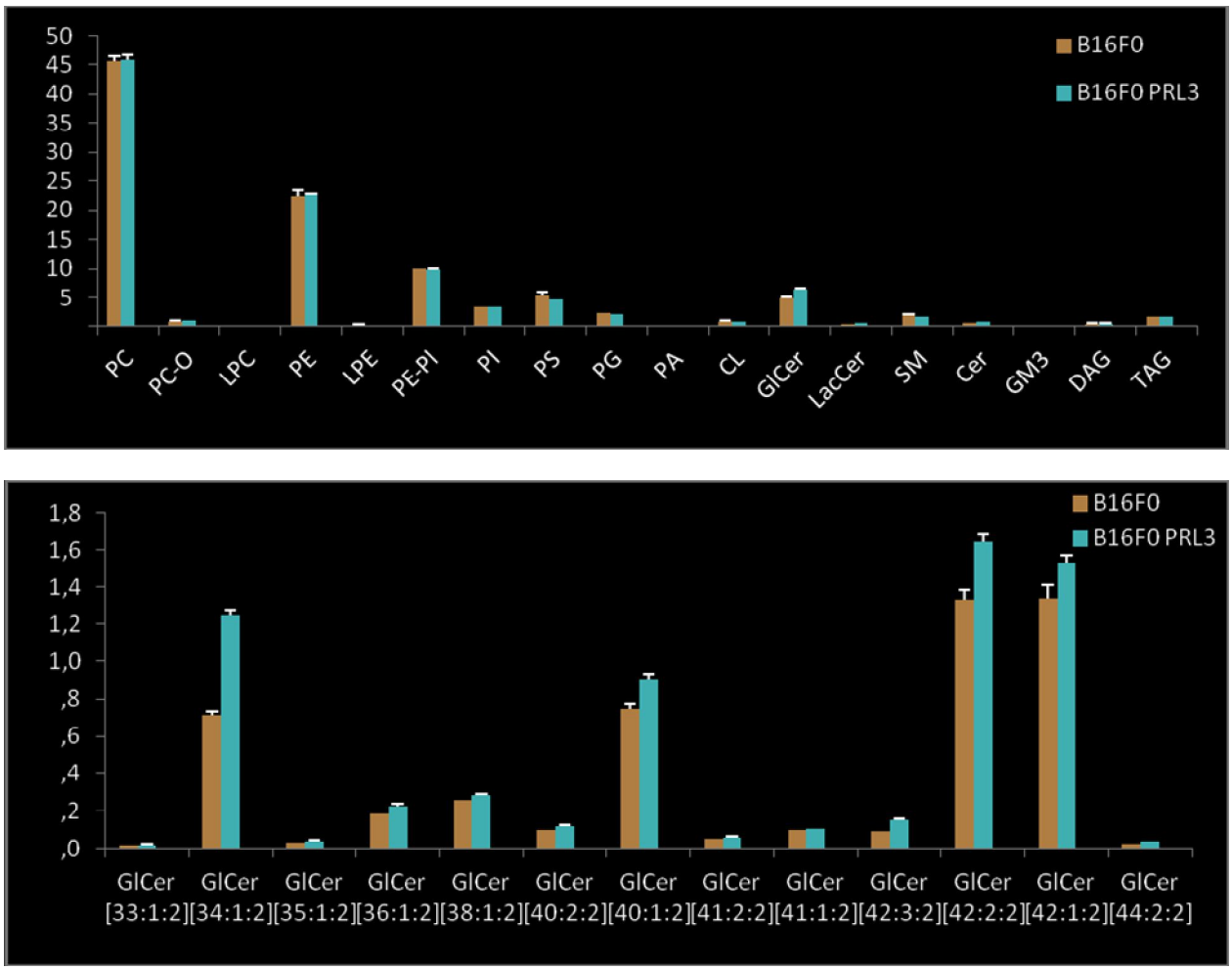

Fig 4. Lipid analysis of B16F0 and B16F0-PRL3 cells

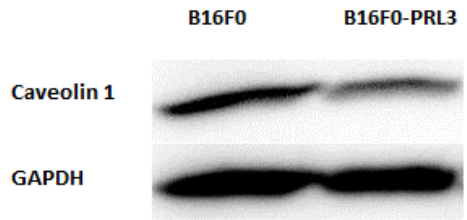

Fig 5. Caveolin 1 expression changes in PRL3 cells

3. Providing evidences on the operation of Rac1 signaling cascade in heat and membrane perturbation/stress using specific Rac1 inhibitors.

In favor of ‘‘membrane stress sensor’’ model, earlier we suggested that one of the potential stress-sensing mechanism of mild, non-denaturing heat stress (or heat analogous membrane stress, typically induced by the administration of benzyl alcohol) can be based on membrane

fluidization and remodeling. We assumed, that such membrane alterations may activate growth factor receptor tyrosine kinases by causing their non-specific clustering, followed by the activation PI3K, which in turn activates the small GTPase Rac1. In the absence of stress a Rho-GDI protein keeps Rac1 solubilized by shielding its geranyl-geranyl group from the solvent in the cytosol, under stress conditions Ca2+ and PKC-dependent phosphorylation of Rho-GDI promote, however the release of bound Rac1 and subsequent translocation to the surface membrane. Plasma membrane localization of Rac1 is required for activation of its effector, PAK, which mediates also a downstream signaling cascade to MAP kinases. Activation of PAK is known to lead to the activation of HSF1. In full support of the above speculation, using the well established Rac1 specific inhibitor NSC23766 along with 2-bromopalmitate – which blocks Rac1 palmitoylation necessary for the interaction of Rac1 with the liquid ordered surface membrane domains – have been investigated. Both Rac1 inhibitors tested caused a strongly reduced heat shock protein response (HSR). Moreover, upregulated HSR initiated by the administration of the HSP co-inducer BGP15 was also diminished by NSC23766. We have shown, that palmitoylation affects Rac1 relocalization under stress conditions and, the p38 MAPK pathway has a key role in the Rac1 actions. Not only the relocalization of Rac1 to the plasma membrane but also its capacity for microdomain remodelling have been documented.

4.Investigation of the effect of the ectopic overexpression of different mutant Rac1 isoforms on the lipid composition, membrane microdomain organization and stress response of B16 mouse melanoma cells.

We have decided to investigate the effect of the ectopic overexpression of different mutant Rac1 isoforms on the cell mebrane physiology of B16 mouse melanoma cells. The concept that the B16 cell line is a relevant model in this regard is supported by the fact that *RAC1* is an important oncogene frequently mutated in human melanoma [see:Krauthammer, M., et al., *Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma.* NatGenet,012]. The chosen B16 melanoma cell line does not contain endogenous *Rac1* mutations [see: Castle, J.C., et al., *Exploiting the mutanome for tumor vaccination.* Cancer Res, 2012.]. Therefore, we decided to silence the endogenous wild type copy of the *Rac1* gene in this cell line with a parallel overexpression of different mutant Rac1 isoforms. However, according to the scientific literature and to our preliminary results, the efficient silencing of the *Rac1* gene and the overexpression of some mutant Rac1 protein isoforms are potentially cytotoxic [see:Halaban, R., *RAC1 and melanoma.* Clin Ther, 2015. **37**(3): p. 682-5.)
Being aware of these, we designed a well-controlled site directed chromosomal transgene delivery approach and an inducible gene expression system to achieve our goal. The new arrangement better supports the further investigations in large populations of modified B16 cells and also allows side by side comparisons of cell populations expressing different Rac1 isoforms.

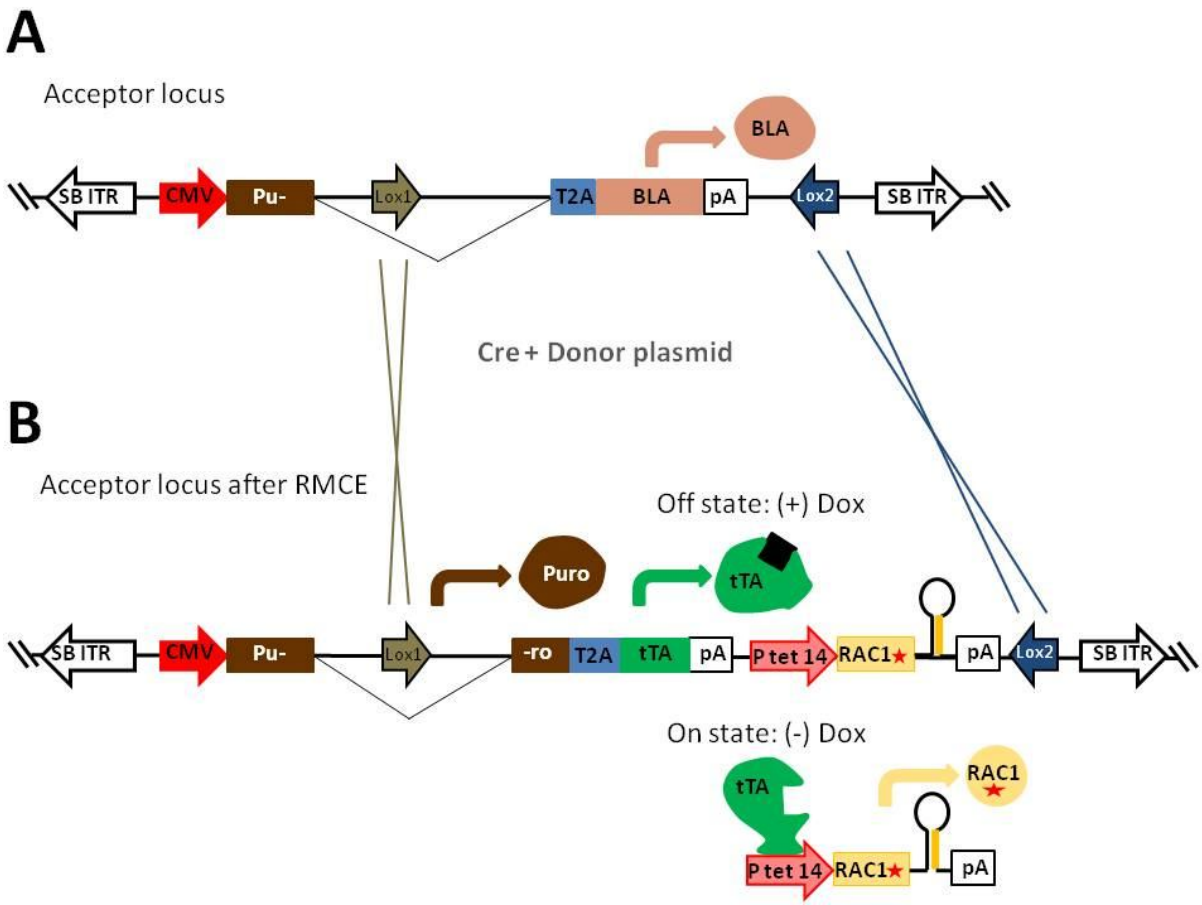

Figure 6. *The site directed chromosomal transgene delivery approach and the inducible gene expression system (A) Schematic representation of the acceptor locus introduced by SB transposition. (B) Schematic representation of the acceptor locus after RMCE. White arrows, SB transposon's inverted terminal repeats (ITRs); red arrow, CMV promoter; light red arrow, Ptet14 inducible promoter; T2A, Thoseaasigna virus 2A peptide; BLA, blasticidin resistance protein coding sequence; Puro, puroycin resistance protein coding sequence; tTA, tetracycline transactivator protein; brown and blue arrows, heterospecific loxp elements; pA, polyA addition signal element; hairpin structure, artificial miR silencing the endogenous mouse Rac1 gene.*

We have created series of plasmid constructs necessary for the establishment of B16 cell lines inducibly expressing different mutant Rac1 proteins. In our transposon coupled Recombinase Mediated Cassette Exchange (RMCE) technology the *Sleeping Beauty* (SB) system is used for the primary chromosomal gene transfer of an "acceptor" construct equipped with a transcription unit expressing an incomplete puromycin resistance protein coding sequence (Pu-), and a complete and functional blasticidin resistance protein (BLA) connected by a T2A peptide. The use of the T2A element allows the separate translation of the BLA protein (**Fig.6A**). An artificial intron has also been inserted into the expression unit, immediately after the incomplete puromycin resistance protein coding sequence. The second exon coding for the BLA resistance protein has been surrounded by heterospecific loxP sites (**Fig.6A**) to allow its later site specific exchange for other functional elements guiding the expression of the different Rac1 isoforms.

In the first step, "acceptor" locus bearing transgenic cell lines were established by SB transposition and BLA selection. Those lines were selected for further investigations where

the BLA resistance was provided by a single unit of the SB-based “acceptor” construct, preferably situated in an intergenic region.

Subsequently, the RMCE technology has been used for the targeted integration of our transgene series expressing the different Rac1 isoforms (**Fig.6B**). Targeted exchange of the exon coding for the BLA resistance protein for another one, coding for the second half of the puromycin resistance protein, provided the possibility to screen for correct site specific transgene integrations by puromycin (Puro) selection (**Fig.6B**). The puromycin resistant clones with successful Cre recombinase-mediated exchange events also carried the coding sequence for the tetracycline transactivator protein (tTA) connected to the puromycin coding sequence by a T2A peptide and the expression unit for the mutant Rac1 isoforms (**Fig.6B**). To drive the expression of Rac1 isoforms we used the inducible Ptet14 promoter [4] containing tandem binding sites for the tTA transactivator protein. The tTA transactivator protein is able to bind and activate the Ptet14 promoter in the absence of doxycycline (Tet-Off) (**Fig.6B**). In addition to the expression of mutant Rac1 isoforms, the Ptet14 promoter driven unit also expresses artificial microRNA (miR) structures silencing the endogenous mouse *Rac1* gene (**Fig.6B**).

For the development of the artificial miRs we harness the structures of the endogenous miR155 and miR30a elements. While we maintain the structural elements necessary for miR processig, we exchange the 22 nucleotide long target gene specific regions of these endogenous miRs to target the mouse Rac1 mRNA.

Following the initiation of RMCE events by introducing the “donor” DNA construct and the Cre recombinase protein we administer puromycin to select for the correctly modified cells and doxycycline to keep the Ptet14 promoter in off state. After finishing puromycin selection, doxycycline may be removed from the medium of any arbitrary number of cells, to study the effect of Ptet14 promoter activation and in consequence endogenous *Rac1* silencing and parallel mutant Rac1 isoform overexpression (**Fig.6B**).

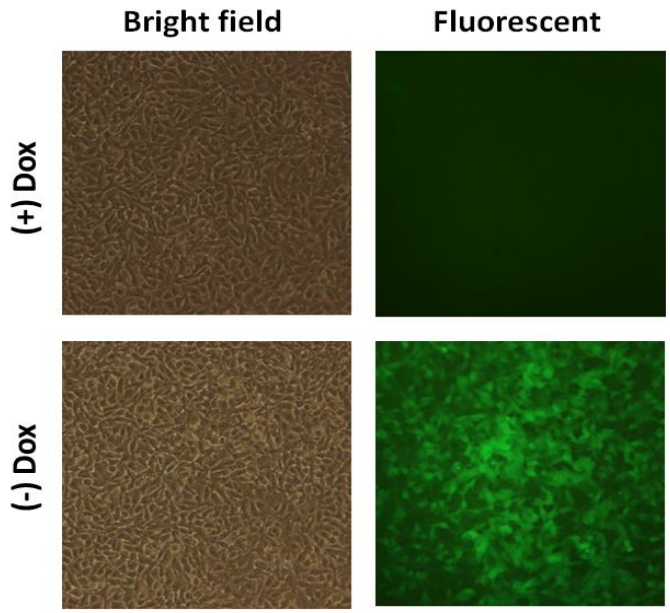

Figure 7. *Inducibility of the Ptet14 promoter upon doxycycline (Dox) withdrawal.*

First to test the inducibility of our new gene expression system, we drove the expression of the green fluorescent protein (GFP) with the Ptet14 promoter, instead of the Rac1 isoforms. We introduced the GFP containing “donor” construct to previously established “acceptor”

locus bearing B16 cell lines by RMCE. After removing doxycycline from the medium we experienced good inducibility of the Ptet14 promoter (**Fig.7**).
We also checked the amount of the GFP mRNA produced by the Ptet14 promoter by quantitative reverse transcription PCR (qRT-PCR) and found that it is similar to the amount of the endogenous Rac1 mRNA. This observation further supports that the precise exchange of the endogenous Rac1 protein for mutant Rac1 protein isoforms is possible using the inducible Ptet14 promoter.

Next we have tested the efficiency of endogenous *Rac1* gene silencing, mediated by our artificial miR elements. Until now we have investigated 3 different miR155 type artificial miRs targeting Rac1 mRNA. Two of these structures, but especially anti-Rac1miR155 #3 turned to be effectively mediating Rac1 mRNA degradation (**Fig.8**). The most noticeable decrease in endogenous Rac1 mRNA levels (about 50%) has been achieved when two artificial miR structures (anti-Rac1miR155 #2 and anti-Rac1miR155 #3) were co-expressed from the Ptet14 promoter (**Fig.8**). We could also detect similar degree of endogenous Rac1 protein loss in the same anti-Rac1miR co-expressing samples by Western blot. As a spectacular consequence of the *Rac1* silencing, the rate of cell growth was significantly decreased in cell lines co-expressing the two effective anti-Rac1miR structures. This is in accordance with the published observations of others, that silencing of *Rac1* decreases cell proliferation.

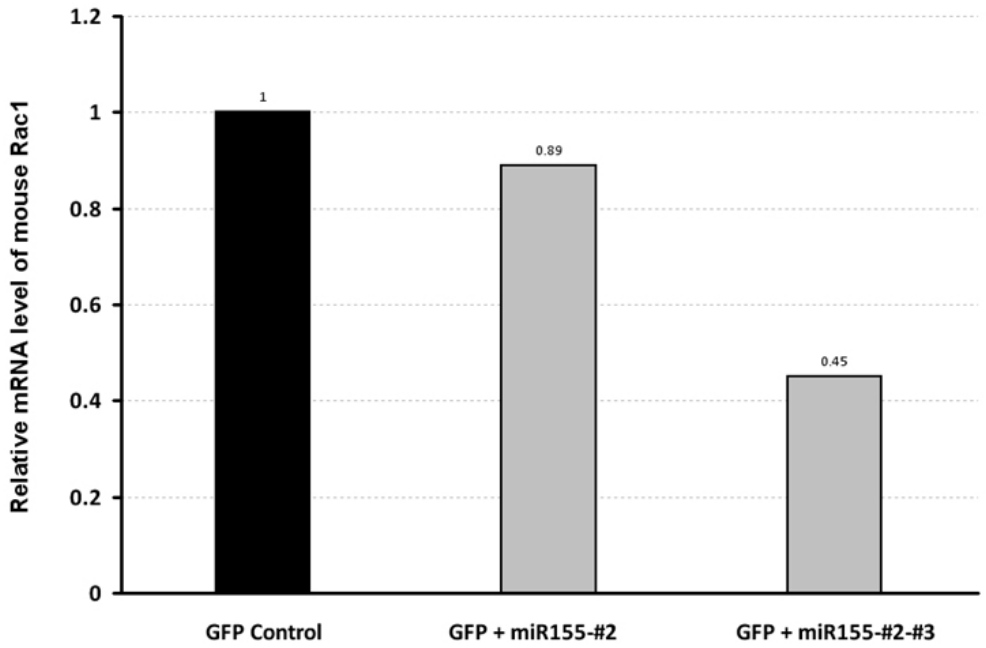

Figure 8. *Silencing of the endogenous Rac1 gene by artificial miR structures expressed from the induced Ptet14 promoter together with GFP. QRT-PCRs to measure the amount of the endogenous Rac1 mRNA were carried out at 240 h following doxycycline removal. GFP Control, only GFP is expressed; GFP+miR155 #2, GFP and anti-Rac1miR155 #2 are expressed; GFP+miR155 #2#3, GFP, anti-Rac1miR155 #2 and anti-Rac1miR155 #3 are expressed.*

We have already started to establish B16 cell lines inducibly expressing the anti-Rac1miR155#2 and anti-Rac1miR155#3 elements together with different mutant Rac1 protein isoforms. Their creation, characterization and further investigation is currently in progress.

Additionally, we will further improve the efficiency of the artificial miR mediated *Rac1* silencing. We will design and test new artificial miRs based on the endogenous miR30a structure and will target new regions of the Rac1 mRNA. We also plan to co-express 3 different artificial miR structures to silence the *Rac1* gene even more efficiently.