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TITLE: Nanoparticle-Based CRISPR Screen to Identify Genetic Regulators of Chemoresistance

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14. ABSTRACT

Lung cancer is principal cause of cancer death in United States. Although many currently employed treatments are initially effective, the acquisition of chemoresistance in the majority of lung cancer patients frequently leads to eventual patient mortality. Recently, pooled CRISPR/Cas9 screenings strategies have enabled unbiased and massively parallel analysis of molecular determinants. In some cases, the delivery of Cas9 and sgRNA were conducted in vitro, and then followed by in vivo validation to modulate acquired drug resistance in cancer. However, most CRISPR screens have been performed in vitro, and the small number of such screens conducted in vivo have utilized in vitro viral delivery of sgRNA libraries. In such cases, the identified targets are relevant to tumor initiation but may lack relevance to the acquisition or persistence of chemoresistance. In addition, viral delivery lacks specificity in vivo and, if repeated, may demonstrate immunogenicity. Given these unfavorable features, we here propose to use fully in vivo CRISPR screens utilizing lipid-polymer hybrid NPs to a deliver pooled sgRNA libraries into growing chemoresistant tumors in vivo, to identify genes that modulate chemoresistance in a clinically relevant setting. The LCRP Area(s) of Emphasis that will be addressed would be "Identify innovative strategies for the treatment of lung cancer." and "Understand mechanisms of resistance to treatment (primary and secondary)."

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

We propose to use a fully *in vivo* CRISPR screens utilizing lipid-polymer hybrid NPs to a deliver pooled sgRNA library into growing tumors *in vivo*, followed by *in vivo* selection of chemotherapy (paclitaxel) resistant cells, to identify genes that modulate chemoresistance in a clinically relevant setting.

2. KEYWORDS:

Lung cancer, Nanoparticle, CRISPR screens

3. ACCOMPLISHMENTS:

What were the major goals of the project?

1) Generation of NP-mediated platform to deliver integrable sgRNA pooled library
2) Optimization of NP-mediated platform for sgRNA library delivery in *vitro*
3) *In vivo* studies of toxicity and biodistribution (BioD) of NP
4) *In vivo* NP-mediated CRISPR knock-out screens in lung cancer xenografts

What was the accomplished under these goals?

1) To generate nanoparticle (NP)-mediated sgRNA library delivery platform, we first determined the minimum components that are required for the sgRNA to be intergrade to the genome. We cloned gag-pol fraction from lentiviral plasmid, which is essential for lentiviral packaging and production. Gag is a polyprotein and is an acronym for Group Antigens (ag). Pol is the reverse transcriptase. sgRNA library (sgRNA) is obtained from Feng Zhang's validated and commercially available sgRNA pooled library vectors (Addgene #1000000049). Gag-pol is amplified from pCMV delta R8.2 lentiviral packaging plasmid (Addgene #12263). In **Figure 1**, equal amount of sgRNA library and gag-pol were transfected into 293T cells via lipofectamine. Different amount of gag-pol from 0.05ug to 0.5ug was used to examine the integration efficiency. The integration was confirmed by qPCR amplifying a region from sgRNA sequence after 3 weeks. Interestingly, the sgRNA alone (0.5ug) without gag-pol can also be integrated with less efficiency shown in green bar of **Figure 1**.

   To confirm this, we repeated the experiment with a wider range of sgRNA (0.00625ug-0.5ug). The sgRNAs with and without gag-pol were transfected into 293T cells via lipofectamine. Puromycin selection were treated in both groups with 0.5ug sgRNA as a positive control. As shown in **Figure 2**, even without gag-pol, the sgRNAs can still be integrated in the genome in a dose dependent manner, although the efficiency is lower than the ones with co-transfection of gag-pol. Puromycin selection significantly increased the efficiency in both groups.

   After successfully integrating sgRNA into genomic DNA with just gag-pol, we attempt to use NP to deliver the sgRNA plus gag-pol into 293T cells. Based on the does we previously tested, we used 0.125ug to 1ug sgRNA. qPCR were performed to measure the integration efficiency. As shown in Figure 3, the sgRNA is successfully integrated into cell genome and puromycin selection significantly increased the efficiency for about 4 fold. Lipofectamine delivery method was used as a positive control.

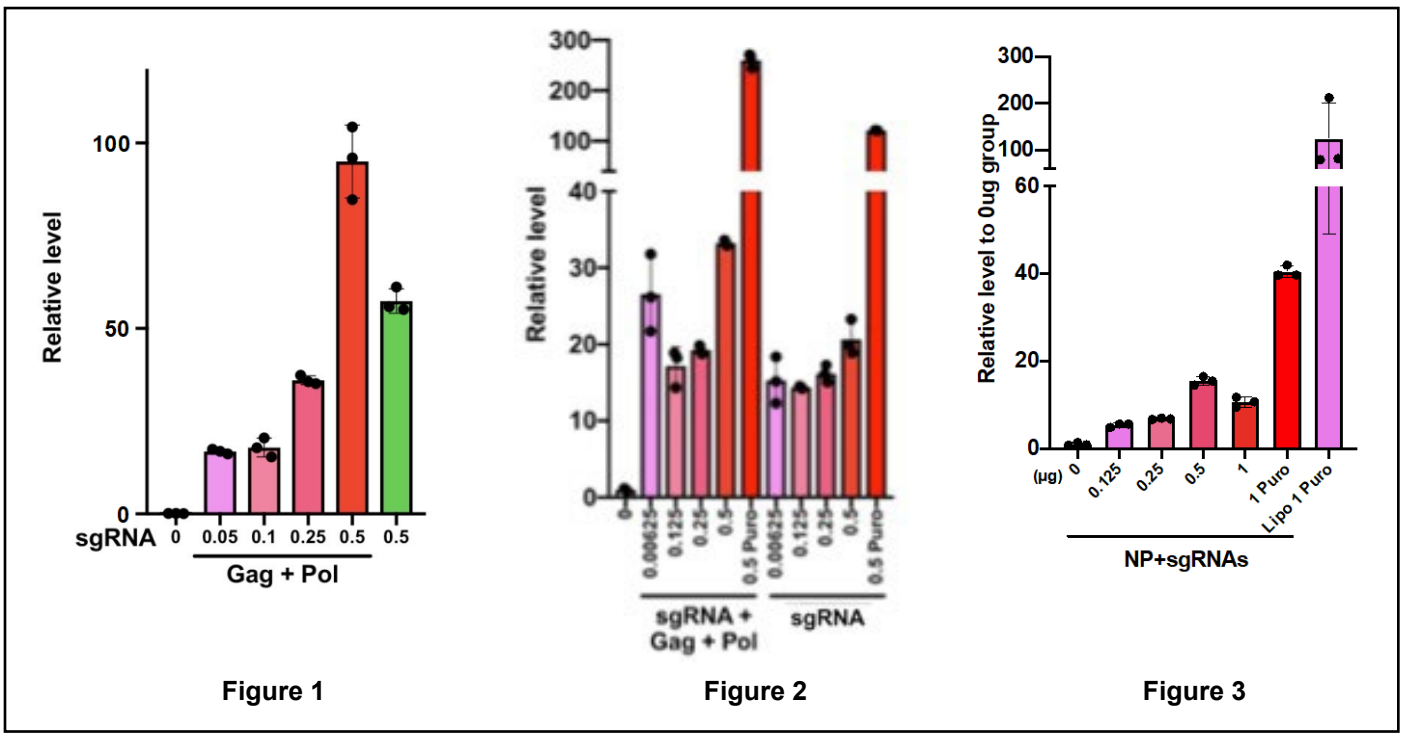

Figure 1. qPCR of sgRNA library (0.05ug to 0.5ug) plus gag-pol transfection via lipofectamine in 293T cells.
Figure 2. qPCR of sgRNA library (0.00625 to 0.5ug) alone and co-delivered with gag-pol via NP in 293T cells. Puromycin selection were used for 0.5ug sgRNA (0.5 Puro).
Figure 3. qPCR of sgRNA library (0.125 to 1ug) plus gag-pol delivery via NP in 293T cells.

2) To construct NP-mediated *in vitro* delivery of sgRNA library, we tested in two different paclitaxel resistant lung cancer cell lines A549-TR and PC14-TR. Different amount of sgRNA (0.5ug-5ug) were delivered into A549 TR cells via NP followed by puromycin selection for one week. As shown in **Figure 4**, sgRNA were successfully delivered into the cells in a dose dependent manner. Similarly, different amount of sgRNA (0.25ug to 5ug) was delivered into PC14-TR cells via NP and the integration efficiency was measured by qPCR shown in **Figure 5.**

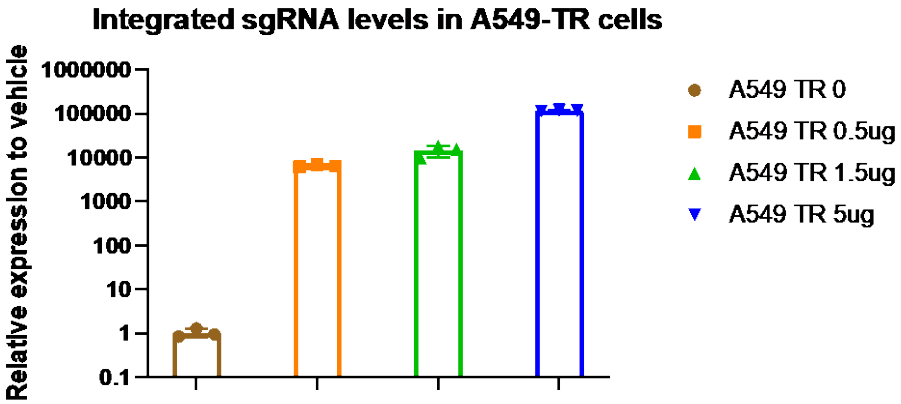

Figure 4

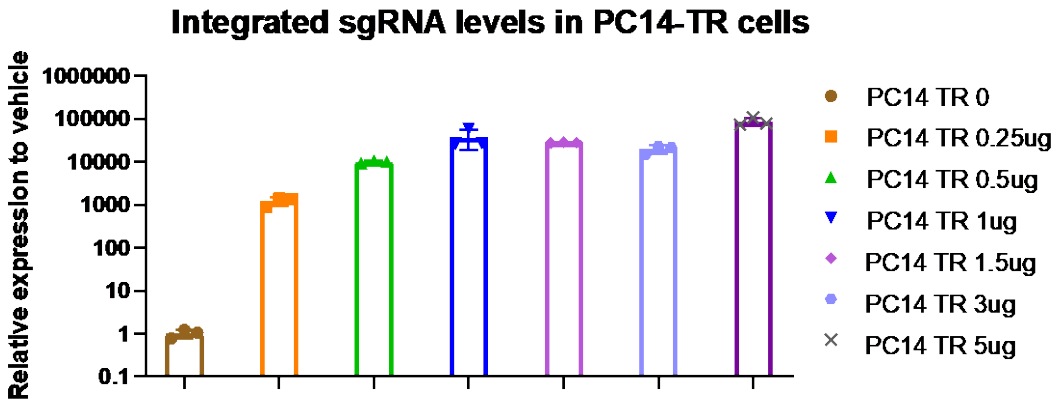

Figure 5

Figure 4. qPCR of sgRNA library (0.5ug to 5ug) delivered into A549-TR cells via NP
Figure 5. qPCR of sgRNA library (0.25ug to 5ug) delivered into PC14-TR cells via NP

3) To perform *in vivo* CRISPR knockout, we first established Cas9 expressing A549-TR and PC14-TR cell via lentivirus transduction. By western blot shown in **Figure 6**, the expression of Cas9 was confirmed in both cell lines.

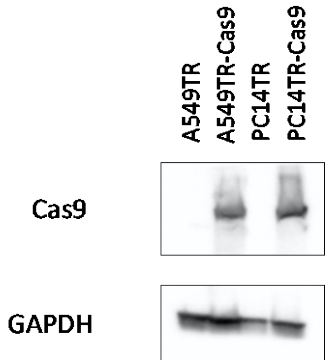

Figure 6

Figure 6. Western blot of Cas9 and GAPDH expression in A549-TR and PC14-TR cells.

We then conducted *in vivo* CRISPR library screen by introducing sgRNA library (5ug) into the tumor site via NP delivery. In details, the A549 TR cells were injected subcutaneously in nude mice. Upon treatment by paclitaxel (20mg/kg, every other day), tumors were established at with different size due to the knockdown by sgRNA. Finally, the tumors were collected and the genomic DNA were collected for CRIPSR library sequencing. The tumor volume of both saline control group and NP-mediated sgRNA group was shown in **Figure 7**. However, there was some technique problems in the process of extracting genomic DNA so that we repeated the tumor injection and paclitaxel treatment again. **Figure 8** shows the tumor volume from the repeated experiment. The growth of the tumor was also monitored by caliper measurement throughout the experiment. The tumor growth curve is shown in **Figure 9**. The genomic DNA of each tumor were extracted for amplicon sequencing and the potential tumor suppressor candidates were listed in **Table1.**

Unfortunately, we did not yet have the opportunity to validate and follow up on the candidate genes to further understand their function in chemoresistance. However, we have successfully developed a NP-mediated sgRNA delivery platform to perform *in vivo* screening for identifying innovative therapeutic targets.

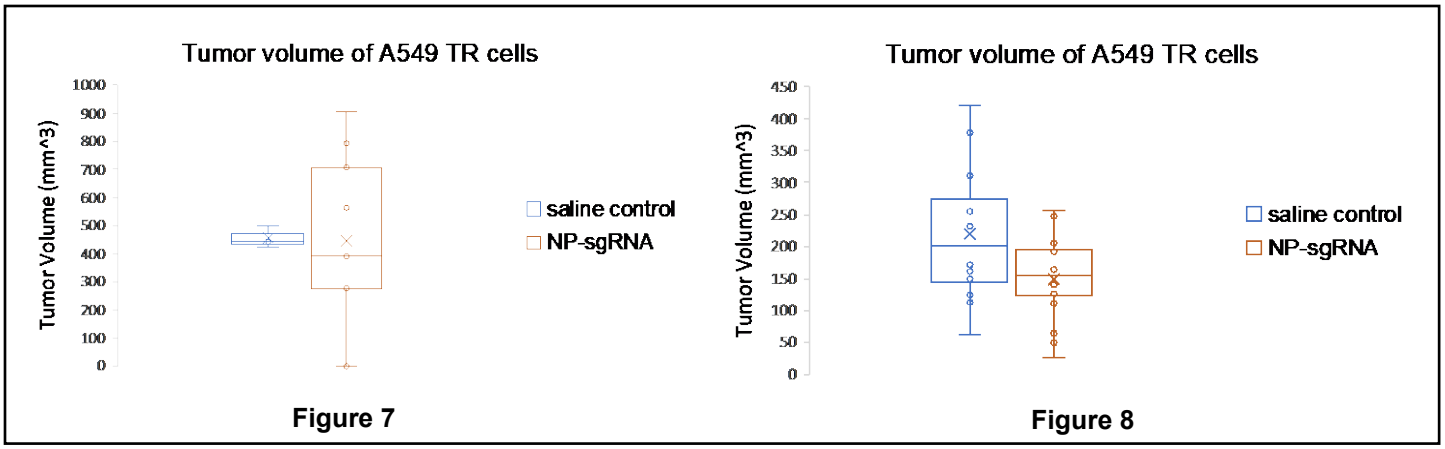

Figure 7. Tumor volume of A549TR cells after 16 days treatment with paclitaxel of the mice in the saline group and NP-sgRNA delivery group.
Figure 8. Tumor volume of A549TR cells after 16 days treatment with paclitaxel of the mice in the saline group and NP-sgRNA delivery group.

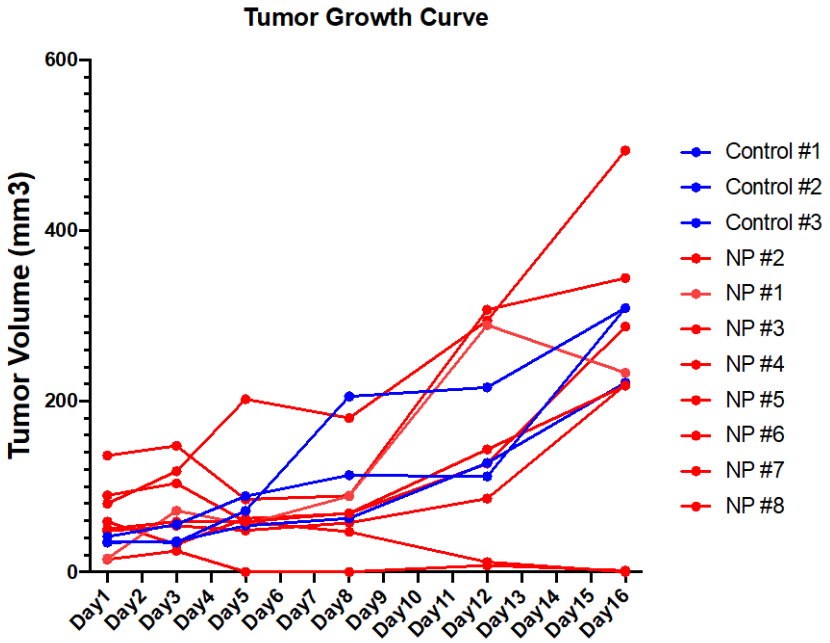

Figure 9. Tumor growth curve of control mice and NP mediated CRISPR library delivered mice.

Table1.

	Cancer Types	Functions

FLNC	GC and prostate cancer	Critical for normal myogenesis and for maintaining the structural integrity of the muscle fibers
GRIN2C	malignant melanoma	This gene encodes a subunit of the N-methyl-D-aspartate (NMDA) receptor
KCNAB1	breast cancer	encodes subunit of Potassium channels
EHD3	Multiple types of cancer	Ehd3, a regulator of vesicular trafficking, is silenced in gliomas and functions as a tumor suppressor by controlling cell cycle arrest and apoptosis
NRP2		Neuropilin 2
TRIM24		Transcriptional coactivator that interacts with numerous nuclear receptors and coactivators and modulates the transcription of target genes. Tripartite motif 24 (Trim24/Tif1α) tumor suppressor protein is a novel negative regulator of interferon (IFN)/signal transducers and activators of transcription (STAT) signaling pathway acting through retinoic acid receptor α (Rarα) inhibition.
PCYOX1	expressed higher in normal breast	Prenylcysteine oxidase 1
ENOX1		involved in plasma membrane electron transport pathways
GABBR1		This gene encodes a receptor for gamma-aminobutyric acid (GABA), which is the main inhibitory neurotransmitter in the mammalian central nervous system
SPINK2	colon cancer	This gene encodes a member of the family of serine protease inhibitors of the Kazal type (SPINK). The encoded protein acts as a trypsin and acrosin inhibitor in the genital tract and is localized in the spermatozoa. The protein has been associated with the progression of lymphomas. Alternative splicing results in multiple transcript variants
PCYOX1		Prenylcysteine is released during the degradation of prenylated proteins. PCYOX1 catalyzes the degradation of prenylcysteine to yield free cysteines and a hydrophobic isoprenoid product
PROKR2		Prokineticins are secreted proteins that can promote angiogenesis and induce strong gastrointestinal smooth muscle contraction. The protein encoded by this gene is an integral membrane protein and G protein-coupled receptor for prokineticins.
RING1		This gene belongs to the RING finger family, members of which encode proteins characterized by a RING domain, a zinc-binding motif related to the zinc finger domain. The gene product can bind DNA and can act as a transcriptional repressor. Notch Signaling Pathway and SUMOylation.
MED4		Mediator is recruited to promoters by direct interactions with regulatory proteins and serves as a scaffold for the assembly of a functional preinitiation complex with RNA polymerase II and the general transcription factors
ZNF106		RNA-binding protein. Specifically binds to 5'-GGGGCC-3' sequence repeats in RNA. Essential for maintenance of peripheral motor neuron and skeletal muscle function.
CSRNP2		The protein encoded by this gene belongs to the CSRNP family of nuclear proteins that share conserved regions, including cysteine- and serine- rich regions, a basic domain, a transcriptional activation domain, and bind the sequence 'AGAGTG', thus have the hallmark of transcription factors
SHBG	expressed higher in normal liver	This gene encodes a steroid binding protein that was first described as a plasma protein secreted by the liver but is now thought to participate in the regulation of steroid responses. Novel target of TP53
ONECUT2		Transcriptional activator. Activates the transcription of a number of liver genes such as HNF3B
RXFP1	expressed much higher in normal lung	Receptor for relaxins. The activity of this receptor is mediated by G proteins leading to stimulation of adenylate cyclase and an increase of cAMP. Binding of the ligand may also activate a tyrosine kinase pathway that inhibits the activity of a phosphodiesterase that degrades cAMP

CRX		Transcription factor that binds and transactivates the sequence 5'-TAATC[CA]-3' which is found upstream of several photoreceptor-specific genes, including the opsin genes. Acts synergistically with other transcription factors, such as NRL, RORB and RAX, to regulate photoreceptor cell-specific gene transcription. Essential for the maintenance of mammalian photoreceptors.
RHNO1		Plays a role in DNA damage response (DDR) signaling upon genotoxic stresses such as ionizing radiation (IR) during the S phase. Recruited to sites of DNA damage through interaction with the 9-1-1 cell-cycle checkpoint response complex and TOPBP1 in a ATR-dependent manner.
RNF111	normal lymphoma, leukemia	The protein encoded by this gene is a nuclear RING-domain containing E3 ubiquitin ligase. This protein interacts with the transforming growth factor (TGF) - beta/NODAL signaling pathway by promoting the ubiquitination and proteosomal degradation of negative regulators, like SMAD proteins, and thereby enhances TGF-beta target-gene transcription
ADAMTSL1		ADAMTSL1 encodes a secreted protein that contains a thrombospondin type 1 motif. ADAMTSL1 could play a role in the activation of TGF-β, which is usually stored as a latent inactive complex in the extra-cellular matrix, where it gets activated by thrombospondin.
AMER3		Regulator of the canonical Wnt signaling pathway. Acts by specifically binding phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), translocating to the cell membrane (By similarity).
RAB23		The encoded protein may play a role in central nervous system development by antagonizing sonic hedgehog signaling. Disruption of this gene has been implicated in Carpenter syndrome as well as cancer
ATXN7	expressed higher in normal tissue, melanoma, CRC	The encoded protein is a component of the SPT3/TAF9/GCN5 acetyltransferase (STAGA) and TBP-free TAF-containing (TFTC) chromatin remodeling complexes, and it thus plays a role in transcriptional regulation.
MOB3B		Modulates LATS1 expression in the Hippo signaling pathway which plays a pivotal role in organ size control and tumor suppression by restricting proliferation and promoting apoptosis

What opportunities for training and professional development has the project provided?
I had a deep understanding about NP particle mediated sgRNA both *in vitro* and *in vivo*. In addition, I had a better understanding about NGS amplicon sequencing of tumor genomic DNA, which is a powerfully method for analysis of *in vivo* CRISPR screen.

How were the results disseminated to communities of interest?
The unbiased *in vivo* genome wide CRISPR screen method can be widely used in other tumor models for a more clinically relative screening. The use of NP mediated delivery of pooled sgRNA provides a non-vial and more efficient *in vivo* delivery method with higher specificity, which can be applied for the delivery of other tumor sites with some modification of lipid-polymer hybrid nanoparticles.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

6. PRODUCTS:

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Nothing to report

8. SPECIAL REPORTING REQUIREMENT

None

9. APPENDICES:

None