Investigating the function and cancer genomics of *FAT3 gene* via human bladder cancer cells

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Introduction

FAT3 gene is one of the human homologs of *Drosophila fat* gene in the Cadherin superfamily, which is involved in tumor suppression and planar cell polarity that might lead to excessive cell proliferation in the imaginal discs or in other tissues.

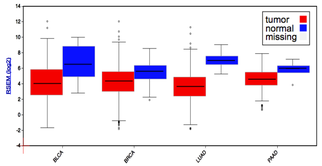


Fig 1. *FAT3* gene expression on different types of tumor cells. Data and graphs retrieved from TGCA Portal and FireBrowse.

Bladder cancer is one of the most common urinary system malignancies that leads to cause of cancer related death of men over the world and the incidence is three to four times greater in male than in female. According to the Cancer Browser Database from the University of California-Santa Cruz, *FAT3* is reported to be mutated in 10% of bladder carcinomas. There is a 1.63-fold decrease in *FAT3* gene expressions in bladder tumor samples compared to those in normal tissue samples (*p=0.00037*) (The UCSC). This led us to question the potential effects of *FAT3* gene during tumor progression on human bladder cell line UM-UC3 in vitro using gene knockout with CRISPR-Cas9 system and MTS cell proliferation assays methods.

Materials and methods

In the first phase, three best sgRNA oligos were cloned into three independent pSpCas9(BB) plasmids. All pSpCas9(BB) plasmids had essential antibiotic resistant genes for survival and could transform *E.coli* using standard protocol. UMUC3 cells were cultured in D10 medium in advance with confluency around 70-80%. The pSpCas9(BB) cloning plasmids were transfected into 24-well plates containing UMUC3 cells and incubated for 4 days at 37°C with 5% CO_2.
In order to validate transfected cells, T7 Endonuclease I Assay and TIDE Analysis were performed to confirm the target mutations. Finally, the MTS assays was used for the measurement of cell proliferation of UMUC3 cells in response to the existence of cloning plasmids with *FAT3* gene.

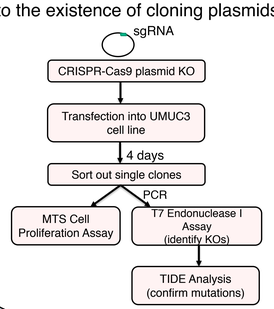


Fig 2. Pathways of designing genome sequence and testing function of *FAT3* gene in human bladder cancer cells. There are three main phases: plasmid engineering, transfection into tumor cells and analysis of cleavage efficiency/cell proliferation.

Results

By using an online CRISPR Design Tool, three target sgRNA oligos were chosen to perform the cloning into pSpCas9(BB) vector for co-expression with Cas9. Double digestion with restriction enzyme *BbsI* and *NcoI* was performed to validate the cloning plasmids by producing smaller DNA fragments. Only two out of three pSpCas9 plasmids were cloned successfully with completed insertions of sgRNA *FAT3* (**Fig 4**).

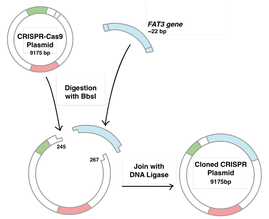


Fig 3. A method to clone sgRNA pairs using restriction enzyme BbsI. *BbsI* enzyme cuts pSpCas9(BB) at site 245 and 267 base pair. A ~20bp *FAT3* gene is inserted into the pSpCas9 backbone and ligated with DNA ligase.

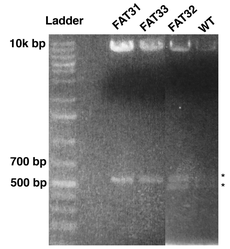


Fig 4. Agarose 1.5% gel electrophoresis of *BbsI/NcoI* double digestion of pSpCas9 knock-out with *FAT3* gene. Lane *FAT31* and *FAT33* show successful engineered pSpCas9 plasmids with 2 different pairs of sgRNA oligos targeting *FAT3* gene (with two fragments of ~530 bp and ~8670bp). Lane *FAT32* and *WT* show the incompleted digestion or unsuccessful engineered pSpCas9 plasmids (with three fragments of ~450bp, ~530bp and ~8180bp)

After the transfection of cloning pSpCas9 plasmids into UMUC3 cells (around 4 days), cells were optimal at 70-75% confluency. **Fig 5** shows a 1.5% agarose gel electrophoresis of DNA sequence fragments (around 600 bp) flanked by a set of Primer3 oligos. Those DNA sequences included the CRISPR target regions or *FAT3* gene. Isolated transfected knockout plasmids were tested for cleavage efficiency using T7 Endonuclease I assay (T7EI). **Fig 6** illustrates that knocked out Cas9 plasmids with two different *FAT3* sgRNAs show the presence of mismatches in the DNA sequence.

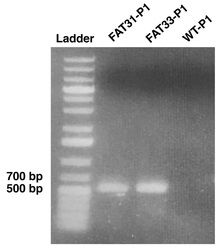


Fig 5. Agarose 1.5% gel electrophoresis of PCR products for T7EI Assay. Designed set of Primers3 oligos flanks the Cas9 knockout gene into a sequence of 600 base pairs. Lane *FAT31-P1* and *FAT33-P1* both show the bands of 600bp. As expected, lane *WT-P1* shows no band since this includes the wild-type pSpCas9 genome without sgRNA targeting *FAT3* gene.

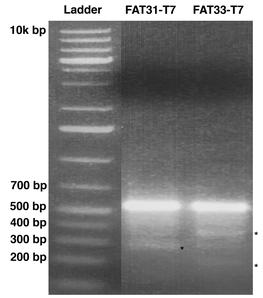


Fig 6. Agarose 1.5% gel electrophoresis of T7EI Assay to validate the indels at the *FAT3* gene locations. In both lane *FAT31-T7* and *FAT33-T7*, the highest bands are produced by the uncleaved PCR amplified DNA of 600 base pairs, whereas cleaved DNA fragments are smaller in size and run faster in the agarose gel. As predicted, in lane *FAT31-T7*, two cleaved fragments are 259 and 258 base pairs; while in lane *FAT33-T7*, two cleaved fragments are 334 and 183 base pairs.

PCR products from CRISPR knockout genome were sent for sequencing using Sanger sequencing. TIDE analysis was then used to verify the mutations resulted from the *FAT3* gene in the Cas9 knockout plasmids. However, contrary to the result from the T7E1 assay, the mutation spectrum from the cloned CRISPR-Cas9 plasmids did not indicate the efficacy of induced mutations (data not shown).

MTS Assay was used to measure knockout cell viability and proliferation. The result shows that after being treated with MTS reagent and incubated up to 4 hours, the higher cell density shows higher absorbance values (**Fig 7**). Comparing between two types of knockout and wild-type UMUC3 cells, there is no significant difference between them.

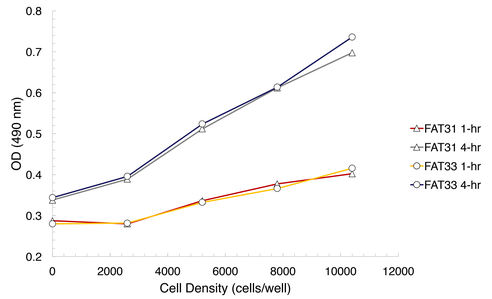


Fig 7. Effect of knockout cell density on absorbance at 490 nm. Two types of knockout UMUC3 cells *FAT31* and *FAT33* were cultures at different densities (0, 2600, 5200, 7800 and 10400 cells) in a final volume of 100μL per well. MTS reagent was added to each well, incubated up to 4 hours at 37°C with 5% CO_2. Absorbance with OD=490 nm at 1-hour and-4 hour incubations were recorded using ELISA plate reader. Each point represents a mean of 4 replicates. There is the positive relationship between cell density and absorbance in each knockout cell types (92-98% confidence). There is no significant differences in the relationship between cell density and absorbance of two types of knockout and wild type UMUC3 cells (p>0.05).

Conclusions

Although *Drosophila* FAT is discovered to function as tumor suppressor gene, specific contribution and expression of mutated *FAT* family genes in the human cancer cells remain challenging, especially in human bladder cancer. Despite tremendous studies in this type of cancer, the molecular mechanisms in signaling pathways involved in the initiation and progression of bladder cancer, especially with the existence of mutated tumor suppressor, are poorly understood.
Our study shows the potential in testing the function of *FAT3* gene in UMUC3 human bladder cell line. In the first phase of genome modification, the result demonstrated successful cloning pSpCas9(BB) plasmids with pairs of sgRNAs targeting *FAT3* gene. This suggests that CRISPR-Cas9 system can serve as a potential method for genome editing in studying *FAT3* gene in mammalian cells. The result of T7EI assay also demonstrated that cloned Cas9 plasmids actually existed; however, TIDE analysis cannot detect mutations in knockout UMUC3 cells, which may be due to low efficiency, degradation of DNA or a potential difference in repair activities in UMUC3 cells. Finally, MTS assay shows positive relationship between cell density and absorbance; however, due to the low efficiency of knockout cells, the effect of *FAT3* gene on cell proliferation is not guaranteed in this study.
For future approach, there are processes that we might consider to work on in order to get better results:

- Increasing efficiency in transfection into UMUC3 cells by increasing knockout DNA concentration and purity using ethanol precipitation.
- The concentration of MTS reagents may be need to be adjusted for optimal performance of knockout cells

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Acknowledgments

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