Utilizing Targeted Nucleases for Gene Knockout and Validation of Candidate Cancer Genes

Natalie K. Wolf[1,2], Branden S. Moriarity[1,2,3], Eric P. Rahrmann[1,2,3], Dominic A. Beckmann[1,2,3], and David A. Largaespada[1,2,3]

[1]Department of Genetics, Cell Biology and Development, [2]Masonic Cancer Center, and [3]Center for Genome Engineering and Institute of Human Genetics, University of Minnesota, Minneapolis, MN, USA.

Abstract

Cancer is a genetically heterogeneous disease in which numerous genes have altered expression by a variety of mechanisms compared to the normal somatic cell counterpart. A recent technology for genome modification, Transcription Activator-Like Effector Nucleases (TALENs), has been successfully utilized to knockout genes and allow for a way to study the effects of these knockouts on tumorigenesis. TALENs are designed to specifically target DNA sequences and cut the DNA, leaving a double stranded DNA break, which can be repaired by non-homologous end joining leaving insertions and deletions (termed indels) that will disrupt gene function. To generate TALENs, TALEN target sites are determined using the TALE-nt software. TALENs are assembled using the golden gate cloning method based on a plasmid. This method uses type IIS restriction endonucleases to cleave library segments, which allows for 10 specific modular segments to be assembled together into a single plasmid backbone. The resulting arrays can be further assembled together into a final TALEN backbone to produce a fully functional TALEN. Validation of the TALEN is done after assembly to ensure the TALEN functions properly and to assess the amount of nuclease activity. TALENs can be utilized to knockout genes in cell lines to validate candidate cancer genes identified in *Sleeping Beauty* (SB) forward genetic screens in mice that have been previously published by our lab. In this study, TALENs were designed and validated for *Fah* and *G6PD*. Validation of the TALEN nuclease activity with a CEL-1 assay showed that *Fah* had gene modification of rate 35% and 39% respectively, while *G6PD* had gene modification of 8%. Moreover, we implemented previously designed and validated *FOXR2* TALENs for cancer gene validation experiments (alterations in proliferation, anchorage independent growth, xenografts). The results from these experiments will contribute to our knowledge on the function of these genes, their role in cancer, and may lead to new insights into therapeutic treatment.

TALEN Background

Transcription Activator-Like (TAL) Effectors

- TAL effectors are a class of DNA binding proteins discovered in the bacterial plant pathogen *Xanthamonas*
- TAL effectors act as transcriptional activators in the plant cell nucleus
- TAL proteins contain an array of nearly identical 34-Amino Acid (AA) DNA binding domains, termed effector binding elements (EBEs)
- EBE domains are distinguished only by the 12th and 13th AA, termed repeat variable di-residue (RVD), that specifies nucleotide binding in a predictable fashion

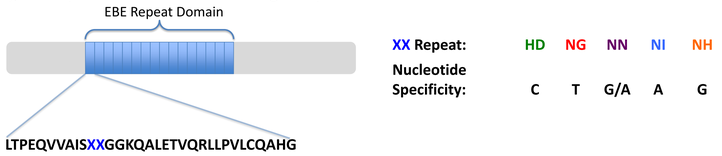

Figure 1: Transcription Activator-Like Effectors. Effector binding elements (EBEs) have a nearly identical amino acid sequence except for the 12th and 13th amino acids. The XX repeat corresponds to the RVD that allows for specificity of the TALE with any given DNA sequence.

TAL Effector Nucleases (TALENs)

- The Fok-I endonuclease domain was fused to the C-terminus of TALs to produce TAL Effector Nucleases (TALENs) for targeted genome modification
  - TALENs provide the capability to create targeted double strand breaks (DSB) in DNA
  - Double stranded breaks (DSB) induced by TALENs can lead to non-homologous end joining (NHEJ) or homologous recombination (HR)
  - When a double stranded break occurs, knock-out of the gene can occur by NHEJ

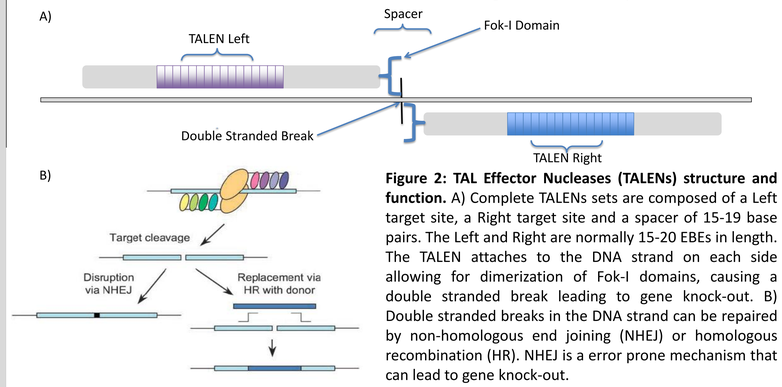

Figure 2: TAL Effector Nucleases (TALENs) structure and function. A) Complete TALENs sets are composed of a Left target site, a Right target site and a spacer of 15-19 base pairs. The Left and Right are normally 15-20 EBEs in length. The TALEN attaches to the DNA strand on each side allowing for dimerization of Fok-I domains, causing a double stranded break leading to gene knock-out. B) Double stranded breaks in the DNA strand can be repaired by non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ is a error prone mechanism that can lead to gene knock-out.

Objectives

1. Create *Fah* and *G6PD* TALENs and validate their nuclease activity.
2. Utilize TALENs to knock-out genes in cell lines to validate candidate cancer genes identified in *Sleeping Beauty* forward genetic screens in mice that have been previously published by our lab.

TALEN Construction through Golden Gate Cloning

Golden Gate Cloning Method

- Golden Gate Cloning Method uses type IIS restriction endonucleases to cleave library segments, which allows for 10 specific modular segments to be assembled together into a single plasmid backbone
  - The highly repetitive nature of EBEs has made assembly difficult without Golden Gate Cloning
  - Cleaving of the library allows for precise excision of fragments and 'sticky ends'
  - RVDs combined with optimal ligase and BsaI in a single reaction are activated by working temperature for 10 cycles followed by heat inactivation
  - This process is highly efficient for assembly of up to 10 RVD fragments with close to 100% correct construction

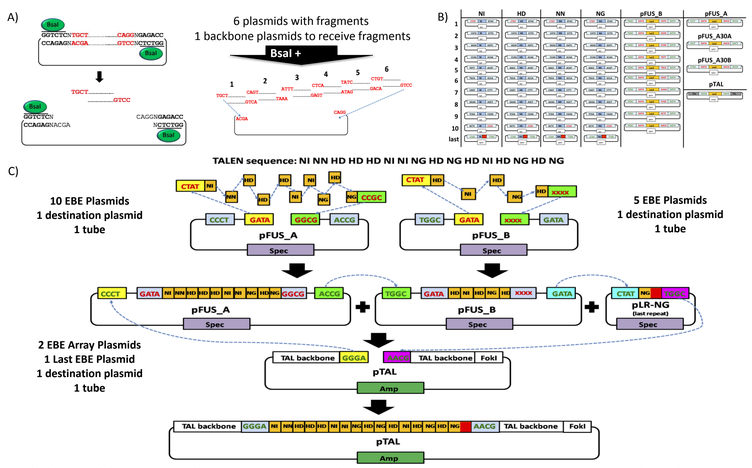

Figure 3: Golden Gate Cloning Method for TALEN construction. A) BsaI binds a specific sequence and cleave at a distance away from the recognition site creating 'sticky ends'. In combination with ligase, these fragments can be pieced together into one plasmid. B) Library of RVDs and backbones which can be assembled in a single reaction using golden gate cloning. C) The full process of TALEN assemble. All RVDs are assembled into a backbone in a single reaction. The resulting arrays can be further assembled into a backbone to produce a fully functional TALEN. The picture shows the construction of a TALEN that is 15 RVDs in length. This process is repeated in order to create the left and right TALENs (a TALEN pair).

Validation of TALEN Activity by CEL-I Assay

- CEL-I assay can determine nuclease activity based on the cutting of the bands observed
- Process:
  - TALENs are electroporated into cells and the genomic DNA is extracted after 3 days and a PCR of the target site is performed
  - The 300-400 base pair PCR product with indels will cause formation of heteroduplex or mismatched DNA when denatured and annealed
  - CEL-I (Surveryor) enzyme identifies and cleaves heteroduplex DNA producing various size bands when run out on an TBE polyacrylaminde gel

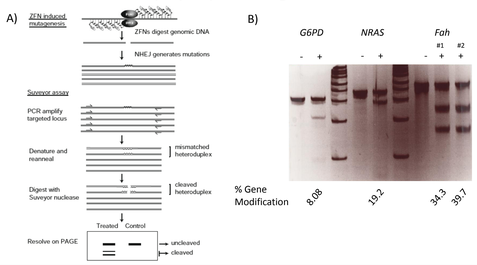

Figure 4: CEL-I Assay validation of nuclease activity. A) This image shows the process of the CEL-I assay and the way that heteroduplex DNA is produced. B) TBE polyacrylamide gel image of CEL-I assay. The cut bands produced from the surveyor enzyme can be measured by densitometry to identify the amount of gene modification rate and nuclease activity.

Candidate Oncogene *FOXR2 V*alidation Experiments

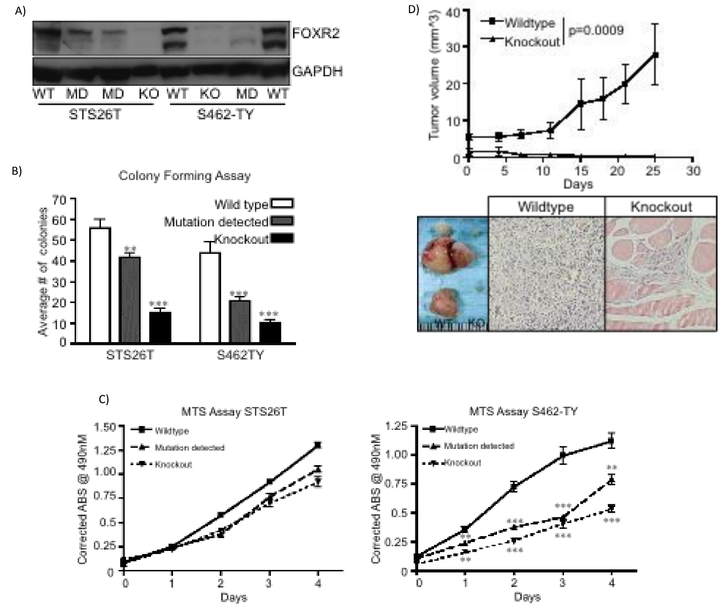

Figure 5: Cancer gene validation experiments performed using *FOXR2* TALENs. A) Western blot for *FOXR2* performed with wild-type (WT) MPNST cell lines (STS26T and S462-TY) and clones that were found to have gene mutations and complete gene knock-out by sequencing . A decrease in protein level is detected in the clones that have been treated with *FOXR2* TALENs. B) Colony forming assays showed a significant decrease in colony formation in clones detected to have a mutation or gene knock-out. C) Proliferation assays show a decrease in cell growth in both STS26T and S462-TY cell lines that have been treated with *FOXR2* TALENs. A significant decrease is observed in the S462-TY clones while a trend is observed in the STS26T clones. D) Knock-out clones injected into skid-beige mice showed significant decrease in tumor volume.

Conclusions and Future Directions

Conclusions:

- Functional TALENs were made for *Fah* and *G6PD*
- *FOXR2* TALENs knock-out clones to validate *FOXR2* as an oncogene in MPNSTs

Future Directions:

- Use the *Fah* and *G6PD* TALENs that have been constructed and validated in future cancer studies
- Make TALENs for genes *PLAA* and *FAF1* to study Schwann cell cancer cell lines (MPNSTs) and their tumorigenic properties
- Increase knowledge on the function of these genes, their role in cancer, which may lead to new insights into therapeutic treatment

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References:

- Boch, J., H. Scholze, S. Schornack, A. Landgraf, S. Hahn, S. Kay, T. Lahaye, A. Nickstadt, and U. Bonas. 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326 (5959): 1509-12.
- Christian, M., T. Cermak, E. L. Doyle, C. Schmidt, F. Zhang, A. Hummel, A. J. Bogdanove, and D. F. Voytas. 2010. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186 (2): 757-61.
- Guschin et al. 2010 *Engineered Zinc Finger Proteins, Methods in Molecular Biology 649*