UCART22: An Allogeneic Adoptive Immunotherapy for Leukemia Targeting CD22 with CAR T-cells

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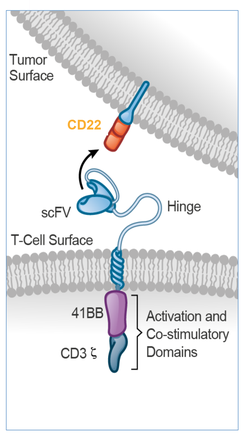
#1 Summary

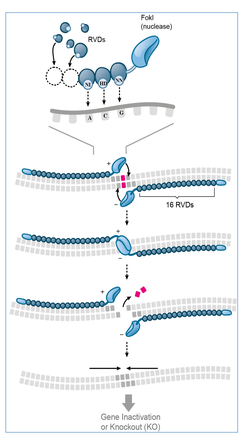
We have developed a standardized platform for manufacturing CAR T-cells from third-party healthy donors to generate allogeneic "off-the-shelf" engineered CAR+ T-cell–based frozen products. Our allogeneic platform utilizes the Transcription Activator-Like Effector Nuclease (TALEN) gene editing technology to inactivate the TCRα constant (*TRAC*) gene, significantly reducing the potential for T-cells bearing alloreactive TCR's to mediate Graft-versus-Host Disease (GvHD). We have previously demonstrated the precise and efficient disruption of the *TRAC* gene by gene editing, yielding up to 85% of TCRαβ-negative cells, and allowing efficient production of TCRαβ-deficient T-cells that no longer mediate alloreactivity in a xeno-GvHD mouse model.
We are now developing allogeneic CAR T-cells targeting the CD22 antigen. CD22 is expressed on tumor cells from the majority of patients with B-cells leukemia.
In a first step, we have screened multiple antigen recognition domains in the context of different CAR architectures to identify effective CAR candidates displaying activity against cells expressing variable levels of the CD22 antigen. In addition, experiments in an orthotopic ALL mouse model demonstrated important anti-tumor activity *in vivo* with both gene edited or non gene edited CD22 CAR T-cells.
As a safety feature, T-cells are engineered to co-express a depletion gene, rendering them sensitive to the monoclonal antibody rituximab. Several constructs of depletion genes are evaluated in the context of the CD22 CAR.
Lastly, we developed a GMP-compatible large scale manufacturing process of allogeneic, non alloreactive CD22 specific CAR T-cells from healthy donor cells in order to manufacture an off-the-shelf CAR T-cell frozen product that would be immediately available for administration to a large number of patients with B-cells leukemia.

#2 Gene-edited CAR T-cells

TALEN gene editing associated with CAR technology: a dual platform to enhance the power of the immune system against cancer.



Chimeric Antigen Receptor
Tumors recognition

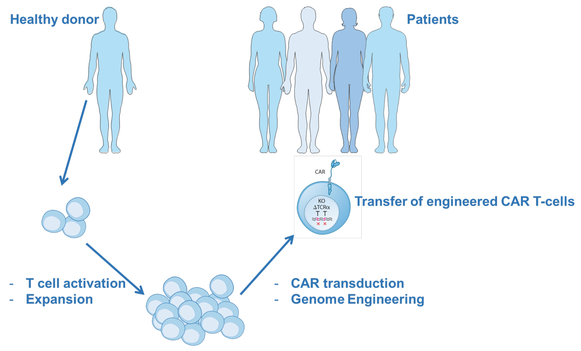


TALEN Nuclease Gene Editing
T-Cell properties

#3 Allogeneic approach to target CD22

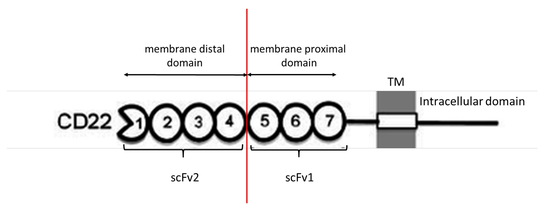
Key barriers to the adoptive transfer of 3rd party CAR T-cells can be overcome via the application of TALEN gene editing technology:

- The deletion of the TCRα gene would allow the use of any donors' T-cells and minimize the risk of GvHD.
- The knock-out of the CD52 gene renders the CAR T-cells resistant to alemtuzumab allowing its potential use as a lymphodepleting drug to increase CAR T-cell persistence.



#4 CD22 CAR constructs

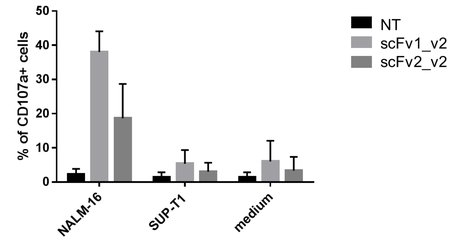
CAR constructs contain a scFv directed against CD22 fused to a 4-1BB co-stimulatory domain and the CD3ζ activation domain with different lengths of hinges (v1 or v2).
Several scFv targeting either the membrane distal or the membrane proximal part of the extracellular domain of the human CD22 antigen were screened.



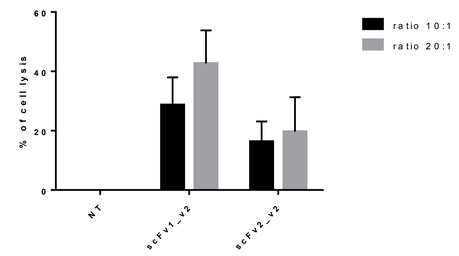
Schema of the CD22 antigen representing the domains targeted by scFv1 and scFv2.

#5 CD22 CAR T-cells activity *in vitro*

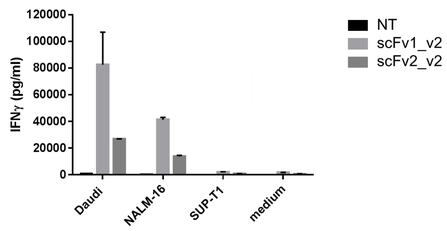
The CD22 CARs were screened using *in vitro* assays for expression, activity and specificity.
Two lead candidates were identified.



% degranulation of CD22 CAR T-cells after 6h co-culture with NALM-16 (CD22+) or Sup-T1 (CD22-) cells. NT: non transduced T-cells.



Cytotoxic activity of CD22 CAR T-cells after 4h co-culture with NALM-16 or Sup-T1 cells.
Specific cell lysis = 1- (% viable target CD22+ cells after co-culture with CAR T-cells or NT T-cells / % control CD22- cells after co-culture with CAR T-cells or NT T-cells) / (% viable target CD22+ cells after co-culture with NT T-cells / % control CD22- cells after co-culture with NT T-cells)



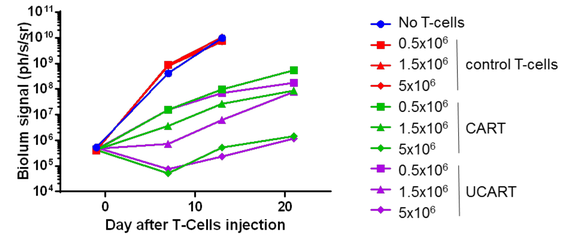
Interferon γ production by CD22 CAR T-cells after 24h co-culture with Daudi, NALM-16 (CD22+) or Sup-T1 (CD22-) cells.

#6 CD22 CAR T-cells activity *in vivo*

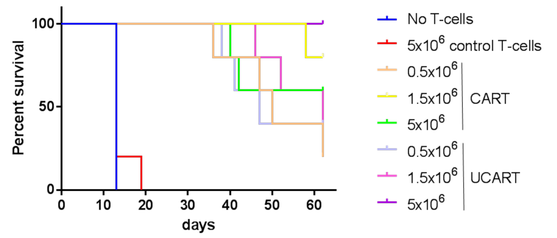
The activity of the T-cells expressing the scFv1_v2 CD22 CAR (with [UCART] or without [CART] inactivation of the *TRAC* gene and purification of TCRαβ- cells) was assessed *in vivo*.

0.5, 1.5 and 5x10⁶ CD22 CAR T-cells were intravenously injected in NOG mice 7 days after tumor cell injection (0.25x10⁶ Raji cells expressing the luciferase). Non transduced (CAR-) T-cells were used in control.
Activity was assessed by A. serial measurement of luminescence and B. Survival of the mice.

A.



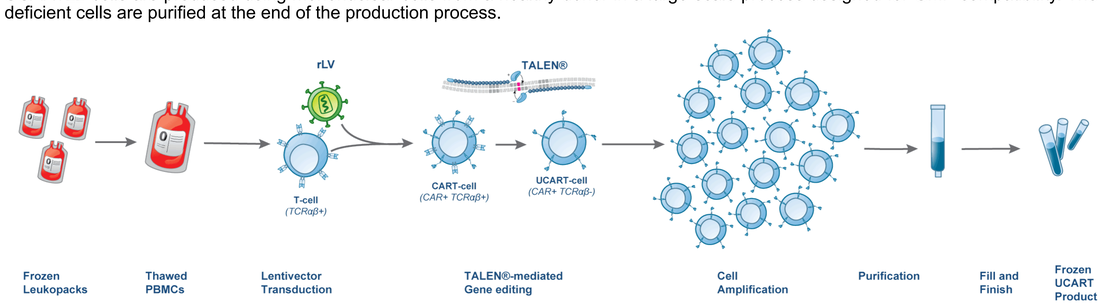
B.



Both TCRαβ+ and TCRαβ- CD22 CAR T-cells display anti-tumor activity *in vivo*.
The absence of the TCRαβ does not influence the *in vivo* activity of CD22 CAR T-cells.

#7 UCART22 manufacturing

UCART22 cells are produced using mononuclear cells from a healthy donor in a large-scale process designed for GMP compatibility. The TCRαβ-deficient cells are purified at the end of the production process.



#8 Conclusions

1) **We have identified effective CAR candidates displaying activity against CD22+ tumor cells *in vitro* and *in vivo*.**
2) **TCRαβ-deficient CD22 CAR T-cells are as active as non gene-edited CD22 CAR T-cells.**
3) **We have developed a GMP-compatible large scale process for the manufacturing of gene-edited CD22 specific T-cells.**

Studies are ongoing in collaboration of the MD Anderson Cancer Center (Pr. Marina Konopleva) to evaluate the activity of UCART22 against primary ALL samples.

Abstract number 372

Manufacturing and study of gene-modified mouse CAR T-cells

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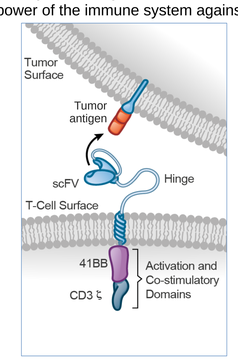
#1 Summary

The study of the human immune response to cancer is largely performed in a xenograft model in which human tumor and immune cells are transplanted into immunocompromised mice. This model is beneficial for assessing pre-clinical activity and safety of adoptive human T cell therapies. However, as the stromal components of the host are mouse, cellular communication is between mouse and human, and the host is severely immunocompromised, both tumor growth and immune cell responsiveness may not accurately represent disease progression and therapeutic response in immunocompetent mice. To address these issues, we developed a mouse model in which mouse chimeric antigen receptor (CAR) T-cells are adoptively-transferred that target murine antigens. Further, to model our human "off-the-shelf" CAR T-cell based therapies which use TALEN® nuclease targeting the human T cell receptor alpha constant (TRAC) gene to prevent the potential of graft-versus-host disease of the transferred allogeneic CAR T-cells, we eliminate T cell receptor expression in mouse T cells with TALEN® to the mouse TRAC gene which will permit the adoptive transfer of these CAR T-cells to allogeneic hosts. In total, we describe the in vitro culture, transfection and transduction conditions for mouse CAR T-cell manufacturing leading to robust in vitro expansion of functional TALEN®-edited mouse CAR T-cells. Here we use these manufacturing conditions to characterize different CAR constructions and the role of inactivating mutations in various signaling domains in the functional responses of CAR T-cells against a hematological tumor target cell. In conclusion, we describe the conditions for the large-scale production of functional allogeneic mouse CAR T-cells that mimics the human CAR T-cell manufacturing process. Adoptive transfer of these cells will provide a platform to interrogate genetic influences that impact anti-tumor activity of CAR T-cells as well as on-target/off-tumor toxicities of novel CAR T-cell therapies.

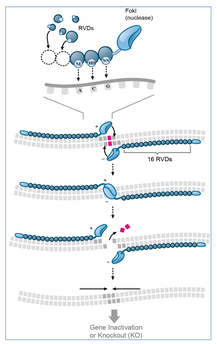
#2 Gene-edited CAR T-cells for allogenic adoptive therapy

TALEN® gene editing associated with CAR technology: a dual platform to enhance the power of the immune system against cancer.



Chimeric Antigen Receptor

Tumor recognition

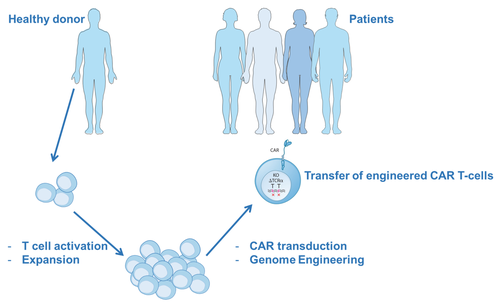


TALEN® Nuclease Gene Editing

T-Cell properties

Key barriers to the adoptive transfer of 3rd party CAR T-cells can be overcome via the application of TALEN® gene editing technology:

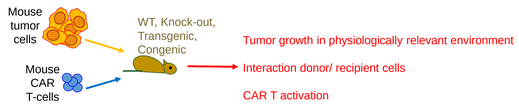
- The deletion of the TCRα gene would allow the use of any donors' T-cells and minimize the risk of GvHD.
- The use a lymphodepleting drug allows the persistence of allogenic CART-cells.



#3 Relevance of a syngeneic mouse model

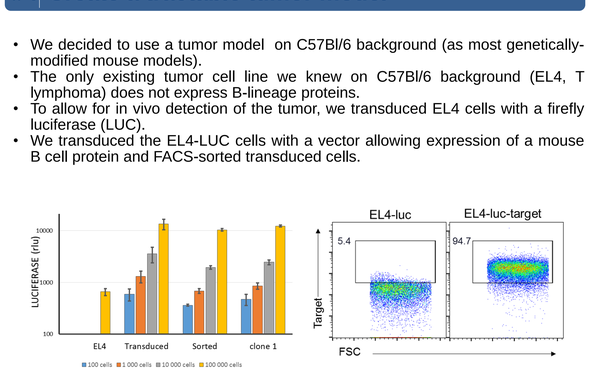
An "all mouse" model is particular relevant:

- To interrogate the function of CAR T-cells and the factors that influence anti-tumor activity without the confounding effects of human cells in a mouse environment.
- To recapitulate the engineering steps of clinical human CAR T-cells (transduction, TALEN®-mediated gene KO) on mouse T-cells.
- To take advantage of available genetic models (KO, congenic mouse strains) and study individual factors (MHC, cytokines, etc.).
- To model anti-tumor activity and on-target/off-tumor toxicities of CARs against tumor-associated antigens for both liquid and solid tumors.



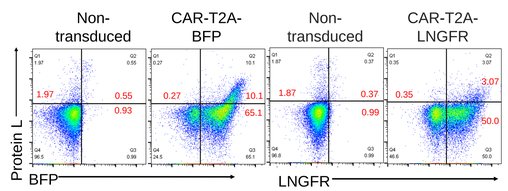
#4 Create a tractable tumor model

- We decided to use a tumor model on C57Bl/6 background (as most genetically-modified mouse models).
- The only existing tumor cell line we knew on C57Bl/6 background (EL4, T lymphoma) does not express B-lineage proteins.
- To allow for in vivo detection of the tumor, we transduced EL4 cells with a firefly luciferase (LUC).
- We transduced the EL4-LUC cells with a vector allowing expression of a mouse B cell protein and FACS-sorted transduced cells.



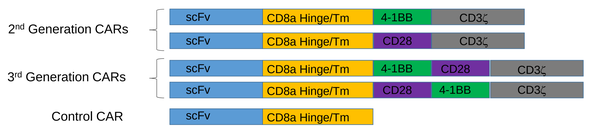
#5 Transducing CARs into mouseT-cells

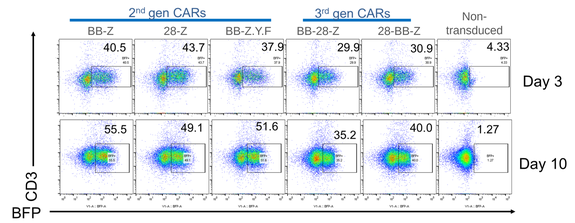
5.1 Constructs and methods were developed to produce vectors and transduce CARs into mouse T cells robustly and with high efficiency.



1D3 CAR is not readily detectable using protein L but bicistronic constructs using a fluorescent reporter (BFP) or a surface reporter (LNGFR) facilitated the evaluation of transduction efficiency.

5.2 CARs were designed using murine signaling and costimulatory domains.

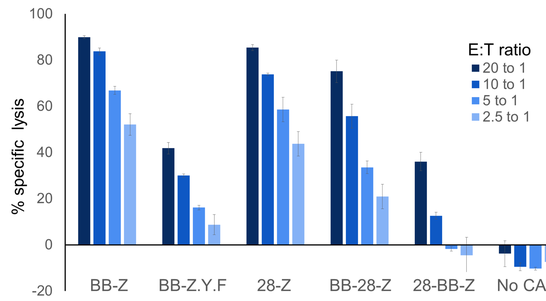




Efficient and stable transduction was observed for all CARs tested

#6 Mouse CAR T-cell activity

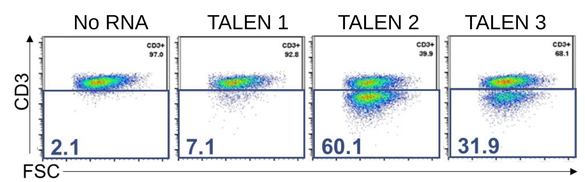
Mouse splenocytes were activated, transduced with vectors carrying different CAR constructs (including one with ITAM mutations reported to support cell viability) and exposed to target cells at different ratios (10 days post transduction).



Second generation CARs confer more robust cytolytic activity than third generations CARs. Mutated ITAMs strongly diminish activity

#7 TALEN®-mediated gene editing in mouse T cells

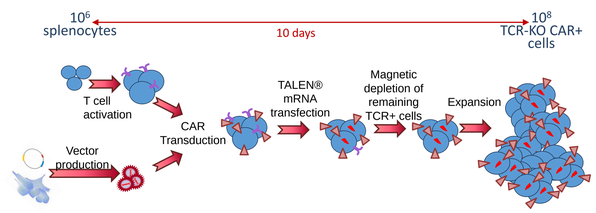
TALEN® were designed to target mouse TCR-alpha constant chain (TRAC). Mouse splenocytes were activated and electroporated with mRNA encoding 3 different TALEN®. Cells were surface-stained with anti-CD3 antibodies and analyzed by flow cytometry



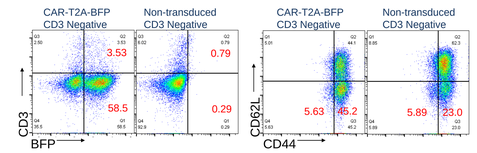
Two TALEN® induce high levels of TRAC gene inactivation. Insertion/deletion formation at TRAC gene was confirmed by T7-endonuclease assay.

#8 TCR KO mouse CAR T-cells activity

8.1 Gene-edited mouse CAR T-cell production platform:

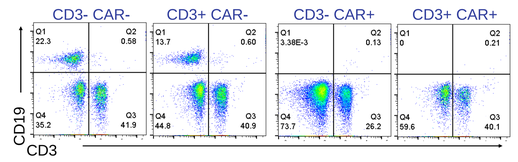


8.2 Phenotype at day 9 post transduction: cells were analyzed by flow cytometry for expression of CD3, CAR-associated reporter and naive/memory markers



TALEN®-treated CAR T-cells show efficient TCR-KO, high transduction efficiency and are composed of central memory and effector/effector memory cells

8.3 10^6 TCR-KO and TCR+ CAR T-cells were injected into B6 mice one day after treatment with 100 mg/kg cyclophosphamide. 6 days later, blood cells were analyzed by flow cytometry.



TCR-KO and TCR+ CAR T-cells show equivalent cytotoxic activity in vivo towards mouse B cells

#9 Conclusions

A syngeneic in vivo mouse model is a valuable tool for the study of engineered CAR T-cells and their anti-tumor activity.

We have developed a platform for the production of TALEN®-modified mouse CAR T-cells. We obtain high levels of TALEN®-mediated gene KO as well as robust and stable transduction of CAR constructs.

Mouse CAR T-cells show robust cytotoxicity activity.

Second generation CARs perform better that third generation CARs and ITAM mutations previously reported to increase cell recovery strongly impair function.

Mouse CAR T-cells show in vivo target-specific cytotoxicity which is not impaired by inactivation of TCRα using TALEN®

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Genome-wide Analysis of TALEN® Activity in Primary Cells

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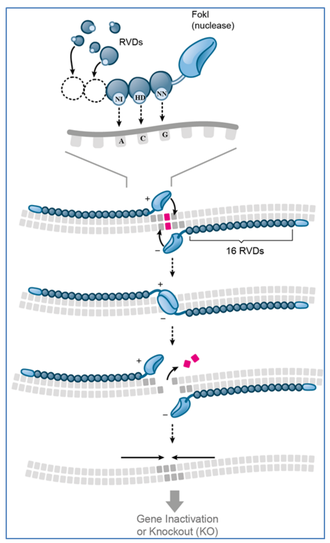
#1 Summary

Genome engineering with programmable nucleases such as CRISPR-Cas or TALEN has revolutionized biological research and has broad-based therapeutic applications. However, for clinical use, it is essential to understand the totality of genome-modifying effects of these nucleases as deleterious off-target mutations may create cells with oncogenic potential or impaired function. Genome-wide, unbiased identification of double-stranded breaks (DSBs) enabled by sequencing (GUIDE-seq) is an unbiased approach to identify the on- and off-target effects of programmable nucleases as it marks DSBs as they occur in living cells by the integration of a small, blunt double-stranded oligodeoxynucleotide (dsODN). These labeled DSBs are then amplified by PCR and mapped by deep sequencing. GUIDE-seq was developed using CRISPR-Cas nuclease which creates blunt-ended DSBs and it was unknown if the procedure could be adapted to assess the genome-modifying effects of nucleases such as TALEN that create 5' overhangs. Furthermore, GUIDE-seq is routinely applied in cell lines which may not accurately reflect the genome-modifying potential of programmable nucleases due to differences in repair mechanisms inherent to primary cells and immortalized cell lines. Here we adapted the GUIDE-seq procedure to measure the genome-modifying effects of TALEN in primary human T-cells. We show that the procedure is highly sensitive as combining samples treated with TALEN with non-treated samples allowed us to define the percentage of TALEN-treated cells necessary in the sample to identify the on-target effects of the TALEN. In addition, the procedure is transferrable as the genome-modifying effects of multiple TALEN were characterized by GUIDE-seq in primary human T-cells. In conclusion, GUIDE-seq can be used to assay the genome-modifying effects of TALEN in primary cells. These results suggest that the procedure is widely applicable to measuring the genome-wide specificities of programmable nucleases and can be readily applied to primary human cells permitting off-target identification in the actual edited cell type.

#2 TALEN® gene editing

Transcription Activator-Like Effector Nucleases (TALEN): programmable nucleases that can be engineered to make precise genetic alterations

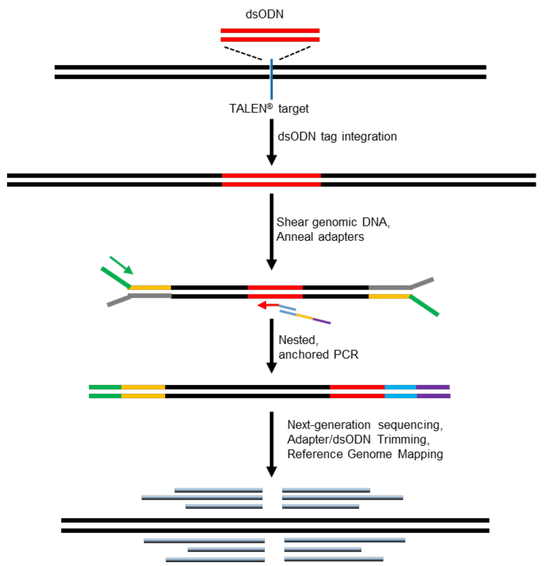


TALEN®

- Transcription Activator-Like Effectors (TALE) are proteins derived from plant pathogens of the *Xanthomonas* genus that bind DNA in a sequence-specific manner
- The DNA binding domain of TALEs contain a highly conserved 33-34 amino acid sequence with a divergent 2 amino acid repeat variable dipeptide (RVD) motif that confers specific nucleotide recognition
- Modular arrangement of these TALE repeats permits tailored DNA specificity
- A custom TALE DNA binding domain is fused to FokI nuclease to cut the DNA. FokI requires dimerization to function which necessitates a pair of TALE-FokI fusions recognize a particular target site in the genome separated by a spacer sequence

#3 Genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq)

GUIDE-seq: a largely unbiased approach to identify the on- and off-target effects of programmable nucleases

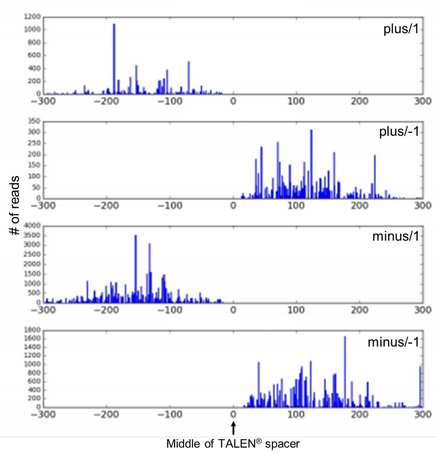


GUIDE-seq

- Identifies the potential genomic effects of programmable nucleases through the incorporation of small duplex oligodeoxynucleotides (dsODNs) into the DSBs induced by the nucleases
- Labeled DSBs are amplified by PCR and mapped by deep sequencing

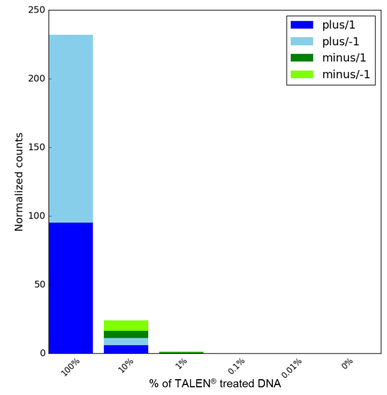
#4 TALEN GUIDE-seq in primary human T-cells: procedure validation and sensitivity

4.1 Primary human T cells were treated with a TALEN to gene A along with dsODN. Genomic DNA was isolated after 6 days of culture and prepared for GUIDE-seq. The size distribution of the sequence reads for the plus and minus strand PCRs associated with the on-target DSB created by TALEN A are shown.



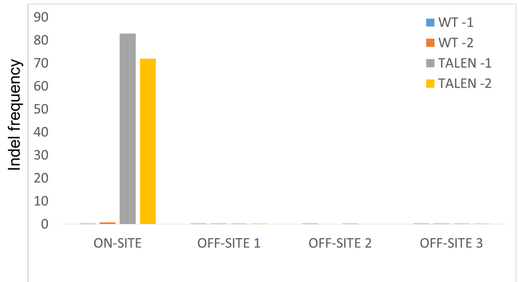
- Read distribution suggests a diverse array of input DNA was amplified

4.2 GUIDE-seq on samples of differing percentages of TALEN A-treated DNA. Genomic DNA was combined from cells transfected with TALEN A plus dsODN with genomic DNA from cells treated with dsODN alone at different ratios. Samples were subjected to GUIDE-seq. Normalized read counts are shown for the on-target DSB for different percentages of TALEN A-treated DNA.



- On-target effects of TALEN A can be detected even after diluting the TALEN treated DNA 100-fold

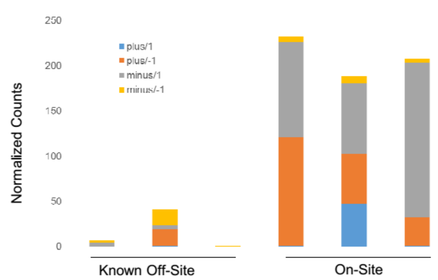
4.3 NHEJ analysis on TALEN A treated DNA. Genomic amplicons which encompass the on-site for TALEN A and three potential off-target sites defined by GUIDE-seq were subjected to deep sequencing validation. Percentage of indels in the indicated locus is shown for two mock-transfected (WT) and two samples treated with TALEN.



- No evidence of off-target effects for TALEN A

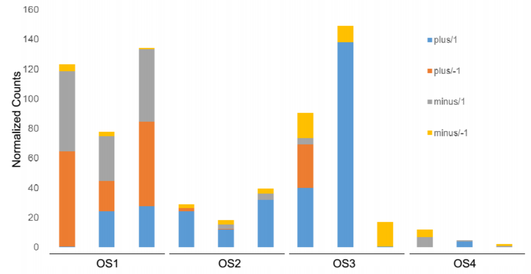
#5 TALEN GUIDE-seq is transferrable: verification of low frequency off-sites

5.1 Primary human T-cells were treated with a TALEN to gene B (which harbors a known off-site) along with dsODN. Genomic DNA was isolated after 6 days of culture and prepared for GUIDE-seq. Distribution of read counts for 3 samples is shown for plus and minus strand PCRs associated with the on-target and off-target DSBs.



- Identification of on-target and off-target effects of TALEN B

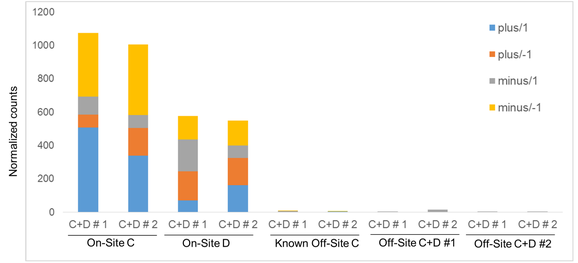
5.2 Off-target effects of TALEN B identified using GUIDE-seq. Normalized read counts for 3 samples are shown for plus and minus strand PCRs for genomic sequences identified in TALEN B treated cells.



- Identification of 4 additional off-sites (OS) for TALEN B
- Validation of new off-sites requires deep sequencing of the loci on TALEN B treated cells

#6 TALEN GUIDE-seq is multiplexable: new TALEN pair combinations can create additional potential off-sites

Primary human T-cells were treated with TALEN against gene C and gene D along with dsODN. Genomic DNA was isolated after 6 days of culture and prepared for GUIDE-seq. Normalized read counts for two samples are shown for plus and minus strand PCRs for the two on-sites, a known off-site and two newly-identified off-sites.



- GUIDE-seq with two TALEN identifies the 2 known on-sites and 1 known off-site
- Combining the two TALEN pairs together creates two additional off-sites created by interactions between one arm of TALEN C and one arm of TALEN D
- Validation by deep sequencing is required on genomic loci for the newly-identified off-sites

#7 Conclusions

- **Developed GUIDE-seq procedure to identify the genome-modifying effects of TALEN**
- **Off-target creation by programmable nucleases can be identified in primary cells by GUIDE-seq**
- **Procedure is transferrable as the genome-modifying potential of multiple TALEN have been interrogated**
- **GUIDE-seq is multiplexable as multiple TALEN can be interrogated in the same cell**
- **Combining two TALEN pairs can create additional off-sites due to additional pairing possibilities between the different TALE**
- **Sensitivity of the methodology is between 0.07 and 0.7%**