Advance Sequencing Technologies for Identification of Off-Target Mutations Associated with Genome Editing

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Cell Therapy - TRAcking, Circulation, & Safety (CT-TRACS) committee
The promise of curative therapies from genome editing technologies such as CRISPR is becoming a reality.

Current trials are underway in five treatment areas: blood disorders, cancers, eye disease, chronic infections, and protein-folding disorders.
However, there are still safety gaps, including delineating the risk of off target editing\textsuperscript{1,2}

- Several commonly used technologies may not have the required sensitivity (e.g., indel freq of $<0.5\%$ not reproducibly detected\textsuperscript{3})
- There are 33 trillion cells in a human!

We are investigating a new unbiased technology (Induce-Seq) invented by AZ/Cardiff Uni graduate student Felix Dobbs

INDUCE-Seq can identify DNA double strand breaks without the need for DNA amplification, thus greatly increasing sensitivity

\textsuperscript{1} Kenpton and Qi, 2019. \url{https://science.sciencemag.org/content/364/6437/234.summary}
\textsuperscript{2} Han \textit{et al.} 2020. \url{https://link.springer.com/article/10.1007/s00109-020-01893-z}
Project Proposal

Primary proposal
- Utilise novel unbiased NGS technology (INDUCE-seq) to identify CRISPR off-target DNA strand breaks
- Follow-up with secondary analysis (e.g. DUPLEX-seq) to confirm if identified DNA break sites lead to mutation

Secondary proposal
- To utilise the technologies to identify mutational changes under different culture conditions for hPSC
Accurate analysis of off-target CRISPR is essential

Numerous methods to detect genomic DSB events in cells:
**Direct** break labelling: e.g. BLESS, DSBCapture, END-seq, BLISS

- Key limitation to PCR-based library preparation for NGS
  - Break detection by this method is **biased** and **not** quantitative
  - This introduces a **high background** reducing the signal:noise ratio for break detection
INDUCE-seq: a novel PCR-free NGS library preparation enables flow cell enrichment of DSBs*

**DSBs in cells**

**In situ break labelling**

**INDUCE-seq**

- DNA extraction
- Fragmentation

**DSBCapture**

- Streptavidin-biotin
- *In vitro* transcription

**BLISS**

**END-seq**

**Break-end enrichment**

- DNA extraction
- Fragmentation

**PCR-free library preparation**

**Standard PCR based library preparation**

*doi: https://doi.org/10.1101/2020.08.25.266239*
INDUCE-seq

PCR-free library preparation

Sequencing

Break-end enrichment

Standard PCR based library preparation

Sequencing

Mapped DSBs
1 read = 1 break

Number of reads

Reads mapped to Chr

100kb

Mapped DSBs
1 read ≠ 1 break

Number of reads

Reads mapped to Chr

100kb
Genome browser comparison of AsiSI-inducible U2OS cell break capture by INDUCE-seq and alternative PCR-based approaches
Advance sequencing technologies for identification of off-target mutations associated with genome editing. "FIND"

Objectives

1. Understand the frequency and location of CRISPR Cas9 off target editing
2. Understand the mechanisms that underpin the formation of mutations caused by DSBs.
3. “Validate” (assess applicability) the assays in a multisite study and establish their value.

Starting materials
- CRISPR Cas9 edited cells (to a known site)
- Promiscuous and specific guide
- 2 cell lines
- Test in multiple labs

Step-1: Induce-Seq
- Samples to Broken String:
  - Genome-wide, unbiased sequencing for DSB

Current plan is to use INDUCE-seq to ‘FIND’ double strand breaks

Anticipated outcomes

1. Joint publication with all collaborators; joint presentations at conferences.
2. Development of a method to detect unwanted genomic events with increased predictive ability and sensitivity >> de-risk CRISPR Cas9 edited cells for therapeutic use >> contribute to translation of CRISPR Cas9 cell therapies.
Advance sequencing technologies for identification of off-target mutations associated with genome editing. **CONFIRM**

### Objectives
1. Understand the frequency and location of CRISPR Cas9 off target editing
2. Understand the mechanisms that underpin the formation of mutations caused by DSBs.
3. “Validate” (assess applicability) the assays in a multisite study and establish their value.

### Secondary plan is to use to CONFIRM mutations

#### Step-1: Induce-Seq
- **Samples to Broken String:**
  - Genome-wide, unbiased sequencing for DSB

#### Step-2: Duplex-Seq
- Targeted assessment of mutation at the sites identified in step-1

#### Step 3: Functional Assays
- Understanding function consequences of the mutations and translatability risk
- Methods: SACF, clonality, others?

### Anticipated outcomes
1. Joint publication with all collaborators; joint presentations at conferences.
2. Development of a method to detect unwanted genomic events with increased predictive ability and sensitivity >> de-risk CRISPR Cas9 edited cells for therapeutic use >> contribute to translation of CRISPR Cas9 cell therapies.
Secondary objectives: Confirm

- DSB does not equal mutation following repair
- Mutation does not equal toxicological risk

**Step 2: FIND DSB**
- INDUCE-Seq identifies DSB

**CONFIRM MUTATION**
- DUPLEX-Seq
  - Random tagging of double-stranded DNA detect mutations with high accuracy
  - DUPLEX-Seq to confirm if INDUCE-Seq identified DSB generate stable mutations

**Step 3: CONFIRM CONSEQUENCE**
- In vitro
  - Soft Agar Colony Forming Assay (SACF)
  - Identifying potentially carcinogenic cells
  - Links to other CT-TRACS project proposal: ‘SACF assay for tumourigenicity assessment of genome edited adherent cells’
  - Participants welcome?
Opportunity for HESI/Broken String collaboration (accelerator program) at reduced cost if enacted quickly (timeline: May-Sept. 2021).

- 2 company members ready to enroll immediately: AZ & Novartis
  - PoC study instead of larger roll-out in the immediate term
- PoC will generate data to inform next steps;
- “Smaller” format enables funding via CT-TRACS’ budget *in full*.
- Unanimous approval for project launch at May 2021 CT-TRACS Annual Meeting
Project Participants & Study plan

Steering team
- Project scoping
- Study design & protocols

CT/gene editing
- AstraZeneca
- Janssen
- NIST
- Novartis

Regulatory
- FDA
- MEB
- NIHS

Adv. sequencing
- Broken String/Cardiff Univ.
- NIST
- U. Sheffield

2 sites
- Samples generation
  (same cells; same edits with same methods)

June/July 2021

Broken String
- INDUCE-Seq
- Bioinformatics Analysis

August/Sept. 2021

Q4 2021

Steering team
- Results analysis
- Discussion & interpretation
- Scoping step-2 (Duplex-Seq)
Call for participants

- Follow-up work with Duplex-seq (Twin-Strand now joined CT-TRACS, eSTAR, GTTC)
- Interest in secondary proposal for identification of mutational changes under different culture conditions for hPSC

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