Use of CRISPR - Status update

New technical developments
  - Tim Ott, Kris Vleminckx, Annie Godwin

CRISPR alternatives – Jaques Robert, Kris Vleminckx

Pitfalls – Tim Ott

Missing resources
CRISPR in *Xenopus* – status update

- State of the Morpholino vs. CRISPR debate?

- Effect on grant-reviewer panels? – Martin Blum

- Use of CRISPR in the Xenopus community?
  
  - Experience in paper reviews / grant reviews?
  - CRISPant vs. Morphant?
New CRISPR developments:

- optimized protocol – Tim Ott (Blum lab)

- Disease modelling, gRNA Design, genotyping – Kris Vleminckx

- Knock in – Annie Godwin (EXRC)

- ...

Protocol recommendations

Tim Ott
Try CRISPRscan for your sgRNA design

<table>
<thead>
<tr>
<th>Gene</th>
<th>Submit sequence</th>
<th>Browser tracks</th>
<th>Protocol</th>
<th>Citing</th>
<th>Help</th>
<th>CRISPRscan</th>
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<td>GAGCTCCACAACTCCAGGTCATGTTCTTACGATGTGCTGAGC</td>
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</table>

But add additional bases 5' (efficient transcription) and 3' (efficient annealing)!

```
GCAGCTAATACGACTCACTATAGgNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG
```

Moreno-Mateos et al., 2015 & Nakayama et al., 2014
Use Cas9 protein instead of mRNA

Bhattacharya et al., 2015
Wet lab: Buy the "expensive" stuff

- Proofreading DNA-polymerase (Pfu, Phusion ...)

- MEGAshortscript™ T7 Transcription Kit (478 € for 25 prep)
- MEGAclear™ Transcription Clean-Up Kit (167 € for 20 prep)
  → 650 experiments

- PNA Bio Cas9 Protein with NLS (310 € for 200 µg)
  → 400 experiments

In total only 0,8 € per experiment
in vitro RNP formation and injection

- Heat sgRNA stock at 70 °C for 2 min and immediately chill on ice

- Mix sgRNA with Cas9 and heat at 37 °C for 5 min

- Inject 1 ng Cas9 with 300 pg sgRNA (1-4 cell stage)
Successful genome editing in ~ 3 out of 4 cases

<table>
<thead>
<tr>
<th>gene</th>
<th>guides</th>
<th>indels</th>
<th>efficiency (%)</th>
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<tbody>
<tr>
<td>A (cilia related)</td>
<td>sgRNA 1</td>
<td>Yes</td>
<td>86</td>
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<tr>
<td>A</td>
<td>sgRNA 2</td>
<td>Yes</td>
<td>83</td>
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<tr>
<td>A</td>
<td>sgRNA 3</td>
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<td>Yes</td>
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<tr>
<td>B</td>
<td>sgRNA 2</td>
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<td>C (cilia related)</td>
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<td>Yes</td>
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<tr>
<td>C</td>
<td>sgRNA 2</td>
<td>No</td>
<td>0</td>
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<tr>
<td>D (cilia related)</td>
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<td>D</td>
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<td>F (enzyme)</td>
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<tr>
<td>F</td>
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<td>No</td>
<td>0</td>
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<tr>
<td>G (scaffolding)</td>
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<td>31</td>
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<tr>
<td>G</td>
<td>sgRNA 2</td>
<td>Yes</td>
<td>92</td>
</tr>
</tbody>
</table>
High reproducibility of phenotype frequencies
Xenopus at your (= human clinical geneticists) service

Needs - functional annotation

- novel disease genes
- known - Variants Uncertain Significance (VUS)
- non-coding variants
- therapy
- ....

Present Status
- KO models (mosaics / lines)
- ....

To do’s
- knock-in methods
- non-coding genome
- spatio-temporal control KO
- ....
Novel disease genes
Predicting the outcomes of template-free Cas9 editing

Predictable and precise template-free CRISPR editing of pathogenic variants
Max W. Shen, Manda Arbab, Jonathan Y. Hua, David Worstell, Sannie J. Calberson, Olga Knebel, Christopher A. Cusan, David R. Liu, David R. Giffard, & Richard I. Sedgewick
Nature, 2018

CRISPR/Cas9 editing outcomes are either:

a) Frameshift
b) In-frame

Double-stranded break repair is much less random than previously assumed.
Editing outcomes can be predicted by machine learning tools: InDelphi
Validating the oracle of InDelphi

Select guide RNAs with high in-frame prediction for structure/function studies

Pearson correlation 0.819, \( p < 0.001 \)
Methods for genotyping

**F0 mosaics**

- Heteroduplex Mobility Assay (**HMA**)
- TA-cloning and Sanger sequences
- NGS + **Batch-GE** analysis

**F1 offspring**

- High Resolution Melting Analysis (**HRMA**)
- Sanger Sequencing - Tracking of Indels by DEcomposition (**TIDE**) analysis

[https://tide-calculator.nki.nl/](https://tide-calculator.nki.nl/)
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• Heteroduplex Mobility Assay (HMA) (for F0)

• TA-cloning and Sanger sequences
CRISPR/Cas efficiency analysis by NGS

- Speed
- High throughput
- Availability
- Decreasing cost

PCR  NGS (MiSeq)  Data analysis

Slides courtesy of Annekatrien Boel (CMGG)
CRISPR/Cas efficiency analysis by NGS

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PCR  NGS (MiSeq)  Data analysis

BATCH-GE

 CENTRUM MEDISCHE GENETICA GENT
- **Xla Gata2 HA tag insertion**
  - 5' end: sequenced well
  - 3' end: sequence contained mismatches

- Efficiency is currently low (0.1%)
HDR events in *Xenopus* embryos are much less frequent than in *Xenopus* oocytes.

**CRISPR in fertilised eggs**
- CRISPR injection
- Gene editing started after cell division.
- Only some cells contain the edited genome (mosaic).

**CRISPR in oocytes**
- CRISPR injection
- Sperm nuclei injection
- Gene editing started and completed in the single cell oocyte. All cells contain the edited genome (non-mosaic).

**Traditional technique**
- CRISPR injection into oocyte
- Oocytes matured using progesterone treatment
- Matured oocytes transferred into host frog
- Eggs squeezed from host frog and fertilised as normal.
- Oocyte quality
- Sperm nuclei quality
- Timing
Community experience with …

- Alternative Variants (xCas9 / Cpf1 or Cas12) ?

- Inducible systems ?

- Oocyte host transfer ?

- Regulatory authorities ?
CRISPR alternatives

- shRNA transgenes in F0 - Jacques Robert

- TALEN for deletions in non-coding elements - Kris Vleminckx
Reverse genetic loss-of-function combining RNA interference with transgenesis

Advantages:
- $F_0$ tadpoles can be used within a month
- 50% integration efficiency
- Mosaicism minimal
- Screening $F_0$ for GFP

iT cells expressing iVα45-Jα1.14 TCR are critical for host resistance to Mm but not to FV3.

Days post-infection

Percent survival

Dejellied control

Va45Ja1.14 CDR3shRNA

C

iVα45CDR3

**
CRISPR/Cas9-mediated Jα1.14 disruption

5′-GCTTATTCTGGTAGTGGCTGGGAGTTGAACTTGGCAGTGGGACGCAGCTGATTGTACAGCCG-3′

% mutations

> 10 bp deletions
< 10 bp deletions
Insertions
WT

Fold change in expression

P = 0.0645
iT cells expressing iVα45-Jα1.14 TCR are critical for host resistance to *Mm* but not to *FV3*

**Jα1.14 KO (CRISPR-Cas9)**

**FV3**

**Mm**

Days post-infection

300,000 CFU

Edholm et al., PNAS. 2018, 115:E4023
Non-coding genome
Evaluating Non-Coding Genome – *cis* regulatory elements (CREs)

**TALEN (or CRISPR) mediated deletion *cis*-regulatory elements**

1 megabase

**Limb enhancer**

**Shh gene**

Mouse embryo

**Acheiropodia**


Regulation *Shh* expression (limb enhancer (ZRS) in *LMBR1* intron)
Pitfalls

- What are adequate controls?

  Rescue / multiple gRNAs / Efficacy validation / Morpholino
  (suggestions Tim Ott)

- Consistency of phenotype?

  Acceptable knockout frequency?

- off-target effects? Genetic compensation?
Adequate controls
Timm Ott
Check for *in vivo* cutting

- T7 Endonuclease I
- High resolution melting analysis
- Fragment analysis
- Clone and sequence single PCR products
- Direkt sequenzenz + ICE/TIDE analysis

NEB, Thermo Fisher Scientific, Bhattacharya et al., 2015; Nakayama et al., 2014; Synthego
Phenotype specificity

- Second sgRNA (CRISPR or CRISPRi) → phenocopy
- Translation blocking morpholino → phenocopy
- mRNA/DNA rescue
- Immunological methods?
- Check for NMD (in absence of any phenotype) → compensation
Missing community resources?

- What do we need?
- Platform for sharing information? (also failed experiments)
- Agreed upon QC guidelines?
- Helpful hints for dealing with regulatory authorities?
- EXRC resources in light of Brexit?
- ...

THANK YOU

Jaques Robert
Kris Vleminckx
Timm Ott
Annie Godwin

Martin Blum
Thomas Hollemann