Einführung in die Grundlagen des Genome Editing am Beispiel von Punktmutationen

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Genome Editing in mice (pre-GE era)

Finding the needle in the haystack!!
Dolly breaks a dogma

Gurdon 1962

Sir John Gurdon, Nobel laureate 2012 (together with Shinya Yamanaka)

Dolly 1996-2003, with Sir Ian Wilmut
Genome Editing in farm animals (pre-GE era)

Efficiency of successful targeting: $1 \times 10^{-6}$

Efficiency of pig cloning: $3-5 \times 10^{-2}$

Feasible, but very inefficient!!

Only heterozygous KO/KI achievable
Genome editing tools

[Foto: Gernot Krautberger - Fotolia.com]
Promises of Genome Editors

Promise
Precise gene alterations

Hope
Cure of deadly diseases

Yaoyong Chen, Xingcheng Gao, Xiaofang Sun,
Genome Editing Publications

The number of papers about CRISPR has outstripped the numbers mentioning the gene-editing technologies known as TALENs and zinc fingers.

Papers mentioning induced pluripotent stem (iPS) cells, another rapidly adopted technique, are shown for comparison.

Nature 2015
Classes of genome editing tools

Meganucleases

Zinc-Finger Nucleases

Transcription activator-like effector nucleases (TALENs)

CRISPR/CAS
General mechanisms of genome editors

Genome editing begins with dsDNA cleavage
General mechanisms of genome editors

BfR-Symposium, Berlin, 06.12.16
General mechanisms of genome editors

NHEJ

HDR

CAGTGGACATGGGGA

GTCACTGCGAGGCACGAT

GTCACTGCTACCCCT

CAGTCACTCCGCTA
General mechanisms of genome editors

Knockout  Knock-in  Knock-in  Knockout

Modifiziert nach Maeder & Gersbach, Molecular Therapy 2016, (24), 3, 430-446
Differences between Genome Editors

- **Zink-Finger-Nucleases (ZFN)**
  - Recognition domain: ~24-30 bp
  - In use since: seit 2003

- **TAL-Effektor-Nucleases (TALEN)**
  - Recognition domain: ~36 bp
  - In use since: seit 2011

- **CRISPR/Cas**
  - Recognition domain: ~22 bp
  - In use since: seit 2013
Classes of genome editing tools

- Meganucleases
- Zinc-Finger Nucleases
- Transcription activator-like effector nucleases (TALENs)
- CRISPR/CAS

BfR-Symposium, Berlin, 06.12.16
Genome Editing by CRISPR/Cas
CRISPR/Cas in action

TARGETED LOCUS:
ATTGCAGGTTGCATTAGCCCTGAACCTACAAGGCTCGGCAGGTCACCAA
TAACGTCCAACGTAAAGCCTTGAGGACTTGATGTTCCGAGGCTCCAGTGGTT

20nt
PAM

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Universal application of Genome Editors
Advantage: Gene correction and Gene inactivation

Gene-correction

Donor-DNA ($A_{\text{functional}}$)

Gene $A_{\text{mutated}}$

Gene $A_{\text{functional}}$

Gene-Knockout

Gene $A_{\text{functional}}$

Gene $A_{\text{mutated}}$
Genome Editing

- Use of Genome Editors to correct a genetic information

Es kann der Dümmste nicht in Frieden leben, wenn es dem bösen Nachbar nicht gefällt.

Es kann der Dümmste nicht in Frieden leben, wenn es dem bösen Nachbar nicht gefällt.

x          x
kann der Frömmste nicht in Frieden

Es kann der Frömmste nicht in Frieden leben, wenn es dem bösen Nachbar nicht gefällt.

(Friedrich Schiller, Wilhelm Tell)
HDR (10%)

NHEJ (90%)

BfR-Symposium, Berlin, 06.12.16
Asymmetric donor design facilitates HDR

Asymmetric PAM or Non-PAM strand ssDNA annealing

Base substitution

PAM 5' Non-PAM3'   3' 5'   0 +1 DSB Resection ssDNA annealing

PAM ssDNA is defined as the PAM-containing strand

Asymmetric donor design

Base substitution

5' 3' 3' PAM 5' Non-PAM

3' 3' PAM 5' Non-PAM

HDR efficiencies

Asymmetric donor design facilitates HDR

Correlation between cut-to-mutation distance

Paquet et al., Nature (2016), Vol 533, 125-29
Correlation between cut-to-mutation distance

Paquet et al., Nature (2016), Vol 533, 125-29
Ways to a “Correct” HDR

Paquet et al., Nature (2016), Vol 533, 125-29
2015 NOBEL PRIZE in Chemistry

for mechanistic studies of DNA REPAIR

Tomas Lindahl
Aziz Sancar
Paul Modrich

Image by Abigail Malate
Inhibition of key molecules of DNA repair by NHEJ

- Suppression of KU70 and DNA ligase IV promotes HDR efficiency 4-5-fold
- Co-expression of AD4 proteins improved HDR up to 8-fold

(Chu et al. 2015, Nature biotechnol. 33 (5), 543-48)

Image credits:
David Gohara, Saint Louis University, and Tom Ellenberger, Washington University School of Medicine in St. Louis
Programmable Editing without DNA cleavage

Programmable Editing without DNA cleavage

protospacer and PAM sequence: 5'-TTCCCCCCCCGATTTATTTATGG-3'

<table>
<thead>
<tr>
<th>sequence</th>
<th>% of total reads</th>
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<tbody>
<tr>
<td>...CCCCCCC...</td>
<td>62.4</td>
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<tr>
<td>...TTTTTTTCC...</td>
<td>18.2</td>
</tr>
<tr>
<td>...TTTTTTTTC...</td>
<td>13.4</td>
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<tr>
<td>...TTTTTTTTT...</td>
<td>3.3</td>
</tr>
<tr>
<td>...TCCCCCC...</td>
<td>0.8</td>
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<tr>
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<td>0.3</td>
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<tr>
<td>...TTTTTCCC...</td>
<td>0.3</td>
</tr>
<tr>
<td>...CCCCCTCCC...</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Genetic variants from ClinVar, that could be corrected by Base editing

- entries in ClinVar
- unique SNPs in ClinVar
- pathogenic SNPs in ClinVar
- pathogenic→normal is C→T or G→A
- proper distance from NGG PAM
- single C in base-editing window
Familial Hypertrophic Cardiomyopathy (FHC)

FHC: - Prevalence 1:500, most frequent disease of the myocard
- frequent cause of sudden death, particularly in young adults including athletes
- inherited as a mendelian autosomal dominant trait, caused by mutations in any 1 of 10 genes

**Typical symptoms:**
- asymmetric left ventricular hypertrophy
- cardiac arrhythmias
- fainting
- ventricular fibrillation

→ frequent cause of sudden death, particularly in young athletes
FHC is mainly caused by mutations in genes encoding proteins of the cardiac sarcomere.
Myosin head domain with some mutations studied in biopsies from FHC patients

Adapted from Rayment et al., Science 261, 1993
Known proteins with mutations and the frequency of the mutations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Number of mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYH7</td>
<td>β-Myosin heavy chain</td>
<td>193</td>
</tr>
<tr>
<td>MYBPC3</td>
<td>Cardiac myosin binding protein C</td>
<td>138</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Cardiac troponin T</td>
<td>33</td>
</tr>
<tr>
<td>TNNI3</td>
<td>Cardiac troponin I</td>
<td>32</td>
</tr>
<tr>
<td>CSRP3</td>
<td>Cardiac muscle Lim protein</td>
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<tr>
<td>TPM1</td>
<td>α-Tropomyosin</td>
<td>11</td>
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<tr>
<td>MYL2</td>
<td>Regulatory myosin light chain</td>
<td>10</td>
</tr>
<tr>
<td>ACTC</td>
<td>Cardiac actin</td>
<td>7</td>
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<tr>
<td>MYL3</td>
<td>Essential myosin light chain</td>
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<tr>
<td>PRKAG2</td>
<td>AMP-activated protein kinase</td>
<td>4</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
<td>2</td>
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<tr>
<td>TNNC1</td>
<td>Cardiac troponin C</td>
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</tr>
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<td>MYH6</td>
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<tr>
<td>TCAP</td>
<td>Telethonin</td>
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</tr>
<tr>
<td>CAV3</td>
<td>Caveolin-3</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>455</td>
</tr>
</tbody>
</table>

Note: According to HGMD (www.hgmd.cf.ac.uk/ac/index.php).
Gene sequences placed on the Geneva HCM resequencing chip.

= 42% of the genetically characterized cases

Nach: Fokstuen et al., Hum Mutat 29, 2008
**Sequence analysis of porcine MYH7 gene**

**Codons 717- 744**

**DNA-Alignment**

<table>
<thead>
<tr>
<th>Codon Position</th>
<th>porcine</th>
<th>german</th>
<th>landrace</th>
<th>human</th>
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<tbody>
<tr>
<td>R719V</td>
<td>GACTTC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R723G</td>
<td>CCGCCAGAG</td>
<td>GTATGCATC</td>
<td>CTGAACCTG</td>
<td>CGGCCATCCC</td>
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<tr>
<td>I736T</td>
<td>CGAGGAGCAG</td>
<td>TTCAAGAGCA</td>
<td>GCAAGAAGG</td>
<td>AGCAGAGAAG</td>
</tr>
<tr>
<td>G741R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Amino Acid-Alignment**

- porcine: DFRQRYRILN
- human: ************

**FLI**

Bundesforschungsinstitut für Tiergesundheit

Federal Research Institute for Animal Health
The project: Generation of transgenic pigs that carry FHC-related *MYH7*-mutations

**Codon 723:** Wildtyp: CGC  
**R723G:** GGC
Summary and Conclusions

- Genome Editors offer sophisticated new opportunities to manipulate the mammalian genome with a previously unknown precision and efficiency.

- HDR can be used to integrate point mutations, short insertions, or longer sequences at a desired locus within the genome.

- HDR efficiency can be increased by the use of an asymmetric donor DNA (>50%).

- The mutation should be within the range of 0-5 bp to the cut to ensure integration of the mutation.

- NHEJ can be prevented by the use of specific inhibitors of DNA Ligase IV.

- Additional NHEJ events can be repaired by a second round of genome editing or can be prevented by modified genome editors that lead to a direct base editing without cutting the DNA.

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Biology´s IT toolbox

- DNA structure/sequencing
- Restriction enzymes
- PCR
- Genome editing
Acknowledgements

Heiner Niemann
Andrea Lucas-Hahn
Stoyan Petkov
Monika Nowak-Imialek
Antje Frenzel
Janet Hauschild-Quintern
Doris Herrmann
Klaus-Gerd Hadeler
Hendrik Sake
Eva Mall
SCNT Team
SVA Team

BfR-Symposium, Berlin, 06.12.16
Thank you for your attention

„Success is the ability to go from one failure to another with no loss of enthusiasm“ (Winston Churchill)
Specificity of Genome Editors

Activity and Specificity (Precision)

- **High Activity** at targeted locus → *On-Target*
- **No Activity** at non-targeted loci → *Off-Target*

*Activity Profile*
CRISPR/Cas9-Nuclease targeting *VEGF-A*

*Quelle: Tsai et al., Nature Biotechnology 2015*
Asymmetric donor design facilitates HDR
Target 2: CMAH (Neu5GC epitopes)

- Humans can’t produce Neu5GC
- Uptake by animal products
- Preformed antibodies
- Necessary to knockout the porcine CMAH
Target epitopes on the pig endothelium

Alpha1,3Gal:
- major antigen
- 1% of all preformed antibodies
- Locus: GGTA1

Neu5GC:
- not present in humans
- role in xenotransplantation to be determined
- Locus: CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase)

B4GalNT2:
- not present on human endothelium
- 5% lack functional gene
- role in xenotransplantation to be determined
- Locus: B4GaNT2 (beta-1,4-N-acetyl-galactosaminytransferase 2)
CRISPR/Cas in action

10th Spring Meeting Working Group Transplant Immunology, Würzburg 08./09.05.2015
CRISPRs: Hallmarks of acquired immunity in bacteria

CRISPR:
Clusters of regularly interspaced short palindromic repeats

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