summary:

We aim to develop a standard protocol for design of synthetic viruses.
Those viruses should be tissue-specific due to recognition of cell surface markers. Moreover a threshold device as a sensitive promotor system enables defined viral replication and host cell lysis depending on intracellular signals.
The synthetic viruses can be applied to transfect ensemble zinc finger nucleases for targeted gene correction.

The following pages deal with former iGEM approaches in this direction and further precise aspects, which were not already presented.

former iGEM approaches for designing endonucleases or zinc finger proteins:

- Wisconsin (2007): zinc finger transcription factor
  problems: unsuccessful expression of the protein in mouse fibroblasts
- Freiburg (2009): combination of nuclease domain with ssDNA probe for sequence specificity
  problems: cytotoxic effects during expression because of nuclease without immanent sequence specificity, DNA probe binding only in vitro

further aspects on:

I) zinc finger nuclease transfection

- certain means for transfections independent from viruses
- LipofectAMINE 2000 by Invitrogen: quite common and widely applied
  - Urnov, F.D. et al., Highly efficient endogenous human gene correction using designed zinc-finger nucleases. Nature 435 (7042), 646-651 (2005).

II) zinc finger nuclease patenting

- public databases with guiding information (as already presented)
- some indications concerning patenting and required licenses for research
  - Scott, C.T., The zinc finger nuclease monopoly. Nature Biotechnology 23 (8), 915-918 (2005).
- turned out to be no current problem at least for our research purpose

III) zinc finger nuclease "star activity"

- attempts to design chimeric nucleases, which delete own recognition sequences
  - Zhang, P., Bao, Y., Higgins, L., & Xu, S.Y., Rational design of a chimeric endonuclease targeted to NotI recognition site. Protein Eng Des Sel 20 (10), 497-504 (2007).
- still an open question, whether this is realy necessary or not

IV) zinc finger nuclease degradation

- coupling to protein degradation pathways avoids prolonged and thus toxic activity
  - Wu, J., Kandavelou, K., & Chandrasegaran, S., Custom-designed zinc finger nucleases: what is next? *Cell Mol Life Sci* 64 (22), 2933-2944 (2007).
- transient expression reduces immune response
  - Durai, S. *et al.*, Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. *Nucleic Acids Res* 33 (18), 5978-5990 (2005).
- concrete information: N-terminal fusion with ubiquitin moiety to control processing
  - Pruett-Miller, S.M., Reading, D.W., Porter, S.N., & Porteus, M.H., Attenuation of zinc finger nuclease toxicity by small-molecule regulation of protein levels. PLoS Genet 5 (2), e1000376 (2009).

former iGEM approaches dealing with viral applications:

I) Team Caltech (2007)

Engineered viruses to selectively kill or modify specific subpopulations of target cells, based on their RNA or protein expression profiles.

1) engineered lambda viruses
   - lack the native copy of key developmental genes, while containing a second regulated copy, which is only expressed, when the virus infects specific target cells.

2) riboswitches for target specificity
   - viral mRNAs for the regulated developmental genes will be expressed with a stem loop, sequestering ribosome binding sites, preventing translation. Specific mRNA in target *E. coli* will invade the stem loop, freeing the ribosome binding site and allowing proper translation.

problems: Formed plaques do not reveal the expectations, although some riboswitches seemed promising.

II) Team SupBiotech-Paris (2009)

Double Vector System (DVS) to improve intrinsic abilities of vectors:

1) bacterial vector targets the tissue and is resistant to the immune system
   - *Mycobacterium avium* goes to the tissue of interest for which it has a high affinity. It is resistant to the immune system thanks to its cell wall and has a low toxicity for its host. In the target tissue, it creates the second vector

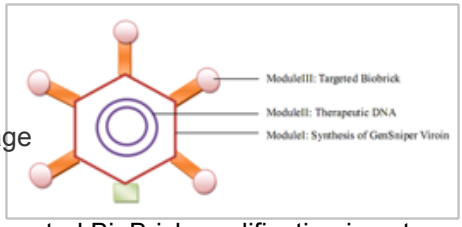
2) cell vector (controlled by the TetON/TetOff system)
   - Cell vector is composed of Lambda phage type recombined with a penton base from the adenovirus serotype 5 fused with its D protein.
     – non-pathogenic, so not toxic
     – cell targeting → 2nd specificity
     – passage of the eukaryotic cells membrane
     – encapsidation and delivery of a therapeutic plasmid

3) therapeutic vector
     – a sequence ensuring its delivery to the nucleus
     – one or moretherapeutic genes
     – a resistance cassette used for selection of transformed bacteria

Problems: Tissue specificity did already NOT work!
But: TetON activation of cell vector encapsidation worked as the usage of a recombined lambda phage to insert a therapeutic gene. Construct: a cosmid (a molecular cloning with a cos site) encoding the conceptual therapeutic genes, because the structural proteins of bacteriophage lambda will only package the circular DNA with cos sites and 40-50 kb in size.

III) Team Tsinghua (2009)

Construction of a targeted gene therapy vector with high cellular specificity, considerable capacity and the potential for mass production and universal modification.



1) GenSnipper virion
   - module and generate lambda virion

2) therapeutic DNA
   - cosmid production in regard to the capacity of the lambda phage

3) target specificity
   - modification of lambda protein C by fiber shaft at its N-termini

Problems: Production of GenSniper with both Therapeutic DNA and Targeted BioBrick modification is not done and activity could NOT be tested!

<u>further convincing reasons for:</u>

I) **virus synthesis** (synthesis and re-design of adenoviral genome)

Why Adenovirus? *(Nettelbeck, 2008)*
- best investigated and most often used for virotherapy
- small genome: 35-40 genes, nevertheless long foreign elements can be tolerated

Which serotype? (serotype = sub-cassification of viruses)
- serotype 2: it can be used for virotherapy, but is not yet fully characterized
- serotype 5: is already modified and characterized in many different ways and there are all kinds of vectors available, so there is no sense in synthesizing and re-designing it

What kind of modifications? *(Weber, 2008)*
- modification of genetic elements to achieve higher expression levels
- stronger promoters (SFFV promoter) and enhancer elements (retroviral enhancer)
- elimination of genes encoding viral proteins → more space for insertion of desired sequences

Can a virus synthesis be successful? *(Müller, 2009)*
- already successful virus synthesis with small modifications: poxvirus, bacteriophage T7 re-design
- also larger genomes were successfully synthesized: *Mycoplasma genitalium*

II) **cell surface targeting** (virus-antibody-interaction through His6-tag and bi-specific antibody)

Why His_6-tag and not direct fusion? *(Dmitriev, 2009)*
- no fibre-antibody fusion proteins can be created, since the fibre is synthesized in the cytosol and the antibody is synthesized in the ER
- the fibre and the antibody can attach to each other later through the His_6-tag and the bi-specificity of the antibody
- a direct interaction of the antibody to the fibre is not compatible, so a linker is required (there are other possibilities of linkages, but the His_6-tag is the most simple one)

What if the antibody gets out of the cell and blocks cell surface receptors after more viral replication cycles?
- a weaker promoter for the antibody synthesis would avoid the exocytosis of excessive antibodies

III) **promoter system** (establishment of a standard measurement system for threshold devices)

Which experimental approach?
- *in vivo* dual luciferase assay with microplate reader

How to set up a standard?
- creation of a calibration curve by measuring the final luminescence of single synthetic promoters with increasing expression activity (e.g. 5% - 200% activity of NF-KB)

How to measure the threshold switch activity?
- cells to be measured are transferred with a synthetic promoter of e.g. 200% NF-KB activity and a threshold promoter, each promoter expresses a different luciferase, thus both promoter activities correspond to a luciferase activity in the dual luciferase assay

Which cells to take? *(Heidelberg iGEM team 2009 wiki, Promega protocol)*
- Hela cells: stable cell lines created by Heidelberg iGEM team 2009
- or HEK cells: standard cells for *in vivo* luciferase assay kit

How can controlled induction of NF-KB transcription be achieved? *(Covert, 2004)*
- addition of liposaccharides can trigger precise NF-KB activity

