EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research,
Development and Education

MINISTRY OF EDUCATION, YOUTH AND SPORTS

Realizováno v rámci dotačního programu
„Operační program výzkum, vývoj a vzdělávání“, program
Ministerstva školství, mládeže a tělovýchovy,
Výzvy č. 02_18_056 ESF výzva pro vysoké školy II

Název projektu: ESF pro VŠ II na UK reg. č.:
CZ.02.2.69/0.0/0.0/18_056/0013322

Studijní podpora, předmět Precision medicine
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The Cas9 endonuclease expression observed using GFP as a reporter gene

Theoretical part:

Genome engineering

The era of genome engineering began in late 1970s, when multiple research groups realized that exogenous DNA could be taken up by yeast or bacteria and randomly integrated into the genome. Subsequently, these processes were engineered for targeted use. *In 1989, molecular geneticists Mario Capecchi, Martin Evans and Oliver Smithies created the first knockout mouse, a milestone in genome engineering. In 2007, they were awarded the Nobel Prize in Physiology and Medicine.*

In late 1980s, **Cre-Lox recombination** was introduced. This system was derived from P1 bacteriophage and is widely used to control gene expression in mice, with several thousands of Cre transgenic mouse strains already available. Despite the introduction of Cre-lox allowed for the first time to overcome embryonic lethality of constitutive deletions of essential, multifunctional genes, the preparation of Cre-lox transgenes was laborious and costly.

The first step towards efficient, targeted nucleases consists of **zinc finger nucleases** (ZNFs), the use of which was, however, limited by complicated design and synthesis. In 2011, **TALENs** represented a huge step in genome engineering. This system is based on TAL effector DNA binding proteins, isolated from *Xanthomonas* spp., fused to FokI endonuclease. A year later, in 2012, **CRISPR/Cas9** system was engineered and introduced as a breakthrough in genome editing. This system derived from a bacterial adaptive immunity is much less expensive and time-consuming, as well as more precise and scalable than ZFNs and TALENS. Since 2013, the CRISPR/Cas technology has been upgraded and now represents the strongest tool for genome engineering.

Welcome to the Golden Age of genome engineering ☺

CRISPR/Cas9 technology

The first evidence of the CRISPR/Cas employment in RNA-programmable genome editing has been published by the Czech scientist **Martin Jinek**, at that time he was a postdoc in the group of Jennifer Doudna at UC Berkeley (Jinek M, et al. *Science* 2012;337:816-821). They proved that the Cas9 protein

(the RNA-guided DNA endonuclease enzyme) needs a base-paired structure located between the transactivating crRNA (tracrRNA)[1] and the targeting crRNA in order to cleave the targeted DNA sequence. The cleavage of the Cas9 endonuclease is directed by both complementarity between the crRNA and the target protospacer DNA, and a short motif, which is known as the protospacer adjacent motif (PAM). Moreover, Martin Jinek and collaborators revealed the cleavage mechanism of the Cas9 endonuclease.

The cleavage of plasmid DNA by Cas9 produces blunt ends at a position 3 bp upstream of the PAM sequence. In contrast, the non-complementary DNA strand is cleaved at one or more sites within 3 to 8 bp upstream of the PAM. The PAM sequence is recognized specifically by Cas9 as a prerequisite for DNA binding and following strand separation prior to Cas9 cleavage. The PAM sequence and position is varying according to a CRISPR/Cas system type. The turnover number[2] of Cas9 is comparable to that of restriction endonucleases and ranges from 0.3 to 1.0 min^{-1}. The endonuclease Cas9 from *Streptococcus pyogenes* (SpCas9) can be navigated by a sgRNA to any genomic locus followed by a 5'NGG PAM sequence and a 20-nucleotide guide sequence within the sgRNA, responsible for genome targeting, thus SpCas9 can be easily engineered according to a gene of interest.

Transfection

Transfection, originally meant 'infection by transformation', is a process when nucleic acids are introduced intentionally into eukaryotic cells. The term '**transformation**' is preferred to describe nucleic acids transfer into bacterial and non-animal eukaryotic cells, including plant cells. **Transduction** is used to describe gene transfer into eukaryotic cells by viruses. The principles of transfection is the opening transient pores in the cell membrane in order to allow the uptake of nucleic acids. Transfection can be performed by non-chemical methods (e.g., electroporation[3]) or by chemical methods (e.g., using cationic lipids in order to produce liposomes[4], which fuse with the cell membrane, also referred as lipofection).

Flow cytometry

Flow cytometry serves for determination of physical and chemical features of a population of cells or particles. A sample containing cells is injected into a flow cytometer and cells are carried by a fluid and focused to an ideally single-cell flow when it flows (in FACS Verse at a speed of 5.4 m s^{-1}) in a series of droplets through a laser beam or a series of laser beams. The light scattering is characteristic to the cells and their components (FSC and SSC = forward and side scattered light). Cells can be labeled with fluorescent markers that use the principle of Stokes shift – the irradiation of fluorescent markers with

[1] The tracrRNA is pronounced "tracer RNA". In the CRISPR-Cas9 system, the tracrRNA base pairs with the crRNA to form a functional guide RNA. Cas9 uses the tracrRNA portion of the guide as a handle, while the crRNA spacer sequence directs the complex to a matching sequence.

[2] Maximum number of chemical conversions of substrate molecules per a unit of time.

[3] Electroporation uses an electrical pulse to allow transfer of polar molecules into cells. When cells experience a controlled electric pulse, the phospholipid layer opens, creating temporary physical channels that allow molecules to enter.

[4] Liposomes are sphere-shaped vesicles that consist of an aqueous core surrounded by one or more phospholipid bilayers, much like cell membranes.

a laser of defined wavelength results in an emission of light at somewhat higher wavelength. The flow cytometer contains detectors of narrowly defined wavelengths, which do not detect the light coming from the lasers but allows the detection of light at wavelengths emitted by the fluorescent markers.

Practical part:

A) Mammalian culture cells seeding

When working with mammalian culture cells, be careful as much as possible, since the cells and their medium are very sensitive to contaminations – wear gloves and a clean lab coat, do not touch nonsterile surfaces. Keep in mind that gloves and a lab coat are not sterile; therefore, spray your gloves with 70% ethanol just before starting work in the laminar flow hood and keep sterile surfaces clean!

1) Spray bottles containing culture medium, PBS and trypsin solution with 70% ethanol and place in the laminar flow hood. Before work, all solutions must be placed for 20 min in a water bath, which is set on 37°C.
2) The assistant gives you one 10-cm plate with ovarian cancer cells growing as a monolayer in a humidified incubator at 37°C, 5% CO_2. Place the plate in the laminar flow hood.
3) Aspirate medium from the plate and wash the cells with **4 mL of PBS**.
4) Aspirate PBS and add **1 mL of trypsin solution** on the cells dropwise.
5) Place the plate for **5 min** in the humidified incubator.
6) Take the plate back to the laminar flow hood, add **4 mL of culture medium** and pipette the trypsinized cells up and down in order to tear the cells off from the plate and have a solution of single cells.
7) Place the cells into the 50 mL tube.
8) **Cell counting:** Mix **10 µL of trypan blue** with **10 µL of cell suspension** in a 1.5 mL tube. Load 10 µL of the mixture onto a slide and insert it into the Bio-Rad Cell Counter to obtain estimates of cell count in your cell suspension.
9) Seed **3 x 10^5 cells/well** into each well in a 6-well plate.

B) Transfection

You use the cells seeded a day before for chemical transfection – lipofection using **polyethylenimine (PEI)**. You transfect a vector coding the endonuclease Cas9 and green fluorescent protein GFP serving as a reporter gene. This vector pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138; http://n2t.net/addgene:48138 ; RRID:Addgene_48138). You prepare **control cells** (without the vector) and **transfected cells with two different concentrations of the vector**.

1) Prepare **four 1.5 mL tubes** – two for the control cells (**tube 1 and 2**), two for the different concentrations of the vector (**tube 3 and 4**). The tube 1 and 2 are controls for the tubes 3 and 4, respectively. The tubes 3 and 4 are prepared in duplicate.

2) Pipette as follows:

	Vector (0.5 µg/µL)	**PEI (1 µg/µL)**
Control cells (tube **1**)	0 µL	3 µL
Control cells (tube **2**)	0 µL	12 µL
Transfected cells (tube **3**)	2 µL	6 µL
Transfected cells (tube **4**)	8 µL	24 µL

3) Add **OptiMEM I** as follows:

	OptiMEM I
Control cells (tube **1**)	197 µL
Control cells (tube **2**)	188 µL
Transfected cells (tube **3**)	392 µL
Transfected cells (tube **4**)	368 µL

4) Incubate **20 min, RT**. In the meanwhile, change the complete medium in the 6-well plate with cells for **1.8 mL of DMEM with 2% fetal bovine serum (FBS) and without antibiotics** (ATB) in each well.
5) Add **200 µL of the transfection mixtures** dropwise into respective wells.
6) After three hours, add **160 µL of FBS** into each well. Keep the cells in the humidified CO_2 incubator overnight.

C) GFP expression

A day after transfection, you must check transfection efficiency – how many cells are positive for GFP expression.

1) Check the cells under the **fluorescent microscope (a tentative method)**.
2) Check the cells by **flow cytometry (a quantitative method)**: a) Aspirate medium and wash the cells with **1 mL of PBS**.

b) Add **200 µL of trypsin solution** into each well and incubate **5 min** in the humidified incubator.

c) Add **800 µL of PBS** into each well and pipette up and down in order to remove cells from the plate. Transfer the cells into a 1.5 mL tube and centrifuge them at **2500 rpm for 5 min**.

d) Aspirate the solution and resuspend the pelleted cells in **500 µL of PBS**. Keep on ice.

e) Under supervision of the assistant, you measure levels of GFP expression by a flow cytometer. You measure cell viability using DAPI (intensely stains dead cells[5]), and GFP expression.

[5] DAPI (4',6-diamino-2-phenylindole, dihydrochloride) is a fluorescent nucleic acid stain that binds to minor grove A-T rich regions of double-stranded DNA. It is excluded from viable cells, but can penetrate cell membranes of dead or dying cells.