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Role of the MEK/ERK pathway in chondrogenic differentiation: Establishment of a protocol for the generation of MEK1-knockout hTERT ASCs and assessment of their differentiation potential

104 – Biomedizin Innovativ – patientInnenfokussierte, anwendungsorientierte sowie interdisziplinäre Forschung am Puls der Zeit

Abstract

With a population growing older every year, development of new treatment approaches for osteoarthritis and other degenerative joint diseases is called for. Next to conventional treatments, tissue engineering in combination with the use of adipose-derived mesenchymal stem cells (ASCs) is a promising candidate for future treatment approaches. However, the signalling events involved in the differentiation processes are not yet fully understood. One prominent signalling pathway is the MEK-ERK signalling cascade, regulating cell proliferation, differentiation and survival. This pathway was reported to play a significant role in the differentiation of mesenchymal stem cells into the adipogenic, osteogenic and adipogenic lineage, respectively. However, results are contradictory. A new tool that will help to elucidate the role of MEK in chondrogenesis has emerged recently: The clustered regularly interspaced palindromic repeat (CRISPR) technology. This technology uses an RNA-guided nuclease to target a specific site of the genome. It can be used to introduce double strand breaks, which can lead to a frameshift mutation and gene knockout. Thus, it proves to be a versatile tool in the field of genomic engineering. Our aim was to establish a reliable and easy to adjust protocol for the generation of knockout human telomerase reversed transcriptase (hTERT) immortalized ASC lines. Furthermore, with these knockout cell lines, we want to investigate the role of different proteins involved in the MEK/ERK signalling cascade in chondrogenic differentiation. So far, this method allowed us to generate 2 MEK1 ablated cell lines. In the future, we plan to investigate the differences between MEK isotypes and study the impact of MEK1 deletion on mechanotransduction.

Keywords:

Human adipose-derived mesenchymal stem cells, CRISPR/Cas9, MEK1, gene knockout, differentiation

Introduction

Tissue Engineering is a promising approach to treat a wide range of disease or trauma related conditions. Use of mature autologous cells, however, exhibits major drawbacks like enhanced donor site

morbidity and limited proliferation potential (Makris et al. 2014). In contrast, the use of mesenchymal stem cells (MSCs) is thought to have major advantages, including increased accessibility, low donor site morbidity and higher proliferation potential. Thus, MSCs are most commonly used in cartilage and bone regeneration, and cellular therapies for bone and cartilage related diseases are emerging. In particular, high abundance of adipose-derived mesenchymal stem cells (ASCs) makes them an attractive source of adult stem cells for further use in regenerative medicine applications. They are capable of differentiation into multiple lineages including adipogenic, osteogenic, chondrogenic, neuronal and myogenic (Zuk et al. 2001; Dominici et al. 2006). The signalling events involved in differentiation of ASCs, however, are not fully understood yet.

One prominent signalling pathway governing stem cell fate is the MEK-ERK signalling cascade, regulating fundamental processes like cell proliferation, differentiation and survival. In short, signals from diverse membrane receptors are passed on by different adaptor proteins to A-Raf, B-Raf or C-Raf. Subsequently, activated by Raf, MEK1 and 2 signal to ERK 1 and 2. ERK activation then regulates a plethora of cellular processes (Chang et al. 2003; Stanton et al. 2003). Interestingly, both the Raf and the MEK isoforms are not functionally redundant, further contributing to complexity (Nowacka et al. 2016; Papaioannou et al. 2017; Zmajkovicova 2013).

It has to be pointed out that MEK/ERK signalling seems to play an essential role in the differentiation of ASCs into the chondrogenic, osteogenic, and adipogenic lineage, respectively. The reports published on this role of the MEK/ERK pathway are, however, contradictory (Arita et al. 2011; Ge et al. 2016; Pelaez et al. 2012).

A new tool to investigate the role of different signal transducers in cellular processes has come up recently and has revolutionized the field of genomic engineering: The clustered regularly interspaced short palindromic repeat (CRISPR) technology uses an RNA-guided nuclease to target specific sites of the genome. Although, it has its origin in bacteria, this system can be used to introduce double-strand-breaks in eukaryotes, providing an easily adaptable genome editing tool for the generation of various mutant cell lines and/or organisms (Ran et al. 2013 / Hsu et al. 2014). Thus, also knockout stem cell lines can be generated to study stem cell fate, providing us with a more detailed understanding of the individual role of certain proteins during differentiation processes.

Whilst the ultimate goal would be to understand the impact of MEK-ERK signalling on differentiation of ASCs into any lineage to its full extent, in this project we want to focus on the chondrogenic lineage. Tissue engineering of cartilage is of great interest. Our population grows older every year, and development of new treatment approaches for osteoarthritis and other degenerative joint diseases are urgently needed. Strikingly, in contrast to other *in vitro* engineered tissues like muscle or bone, cartilage is a non-vascularized tissue and therefore makes it a promising candidate for *in vitro* graft formation.

Aim

In this project, we aim at elucidating the role of MEK-ERK signalling in the differentiation of adipose-derived mesenchymal stem cells into chondrocytes. We established a protocol (Figure 2) which uses the novel CRISPR technology to generate knockout human telomerase reverse transcriptase (hTERT) immortalized ASC lines. With the help of these knock out cell lines, we want to investigate the role of different proteins involved in the MEK/ERK signalling pathway in chondrogenic differentiation and contrast it to its impact on adipogenesis and osteogenesis. Given that the therapeutic control of cartilage formation requires an understanding of signals governing cell fate, this study will contribute to the enhancement of future treatment approaches.

Methodology/Results

Using the CRISPR/Cas9 technology for gene knockout in hTERT ASCs

We used the pSpCas9(BB)-2A-GFP plasmid kindly provided by M. Baccarini and J. Nowacka (Nowacka et al. 2016), to generate a knockout cell line using the CRISPR/Cas9 technology. This vector inherits multiple components that meet different requirements (Figure 1). Amplification of this vector is performed in *E. coli,* requiring an origin of replication (ORI) as well as an antibiotic resistance for selection. After nucleofection of the human ASCs with this vector, *Cas9* is expressed in the cells and forms together with a 20 bp long guide sequence, which targets the first exon of *mek1,* a functional restriction endonuclease that will cut specifically at the target site within *mek1*. A green fluorescent protein (GFP) is used as reporter.

Cells that are successfully transfected showed fluorescence approximately 48 h after nucleofection. In theory, all of them should have a double strand break at the site of interest, resulting in a frameshift and thus a gene knockout. In reality, the efficiency is significantly lower, and not all cells have MEK1 ablation. Additionally, alterations due to repair mechanisms are unique in every cell. Therefore, single cell isolation and subsequent expansion to multiple clonal cell lines was necessary to generate a homogenous MEK1 ablated cell population. This was performed by a separation using fluorescence-activated cell sorting (FACS) resulting in 1 cell per well of a 96-well-plate (performed at the University of Natural Resources and Life Sciences, Vienna).

Next, MEK1 ablation was analysed using Western blotting. In order to confirm an isotype specific knockout, total protein lysates were stained with antibodies against MEK1 and MEK2, respectively. Clone lines lacking MEK1, yet still expressing MEK2 were identified and further tested for their stem cell characteristics and changes in behaviour due to the knockout.

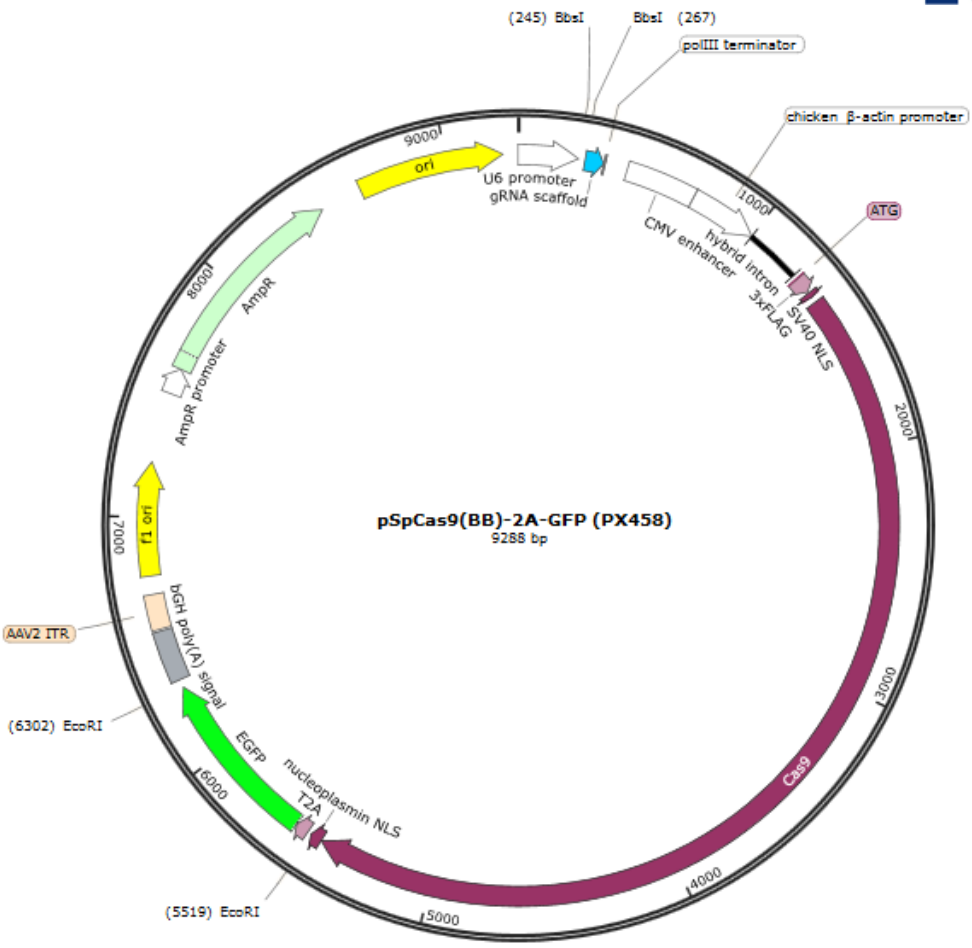

Figure 1 Schematic map of the plasmid used; coloured arrows indicate site of individual components (Cas9 – violet, ampicillin resistance gene – mint, EGFP – green), lines point at restriction enzyme cutting sites, image from www.snapgene.com (GSL Biotech LLC 2017)

Analysis of stem cell characteristics and differentiation potential

According to Dominici et al. (Dominici et al. 2006), stem cells have to fulfil three minimal requirements to be considered as mesenchymal stem cells :

1. Plastic adherence
2. Stem cell surface marker expression
3. Differentiation into adipogenic, osteogenic and chondrogenic lineage

First, the generated knockout lines were analysed for their surface marker profile. The markers of interest included CD105, CD90 and CD73, which are related to multipotency. Additionally, lineage specific surface protein expression of CD14 (macrophages, monocytes), CD34 (hematopoietic progenitors) and CD45 (leukocytes) were assessed using fluorescence-activated cell sorting (FACS).

Further, the differentiation potential of the clones into adipose and osteogenic lineages was assessed with basic assays. Differentiation was performed by seeding cells at suitable concentrations in a 2D

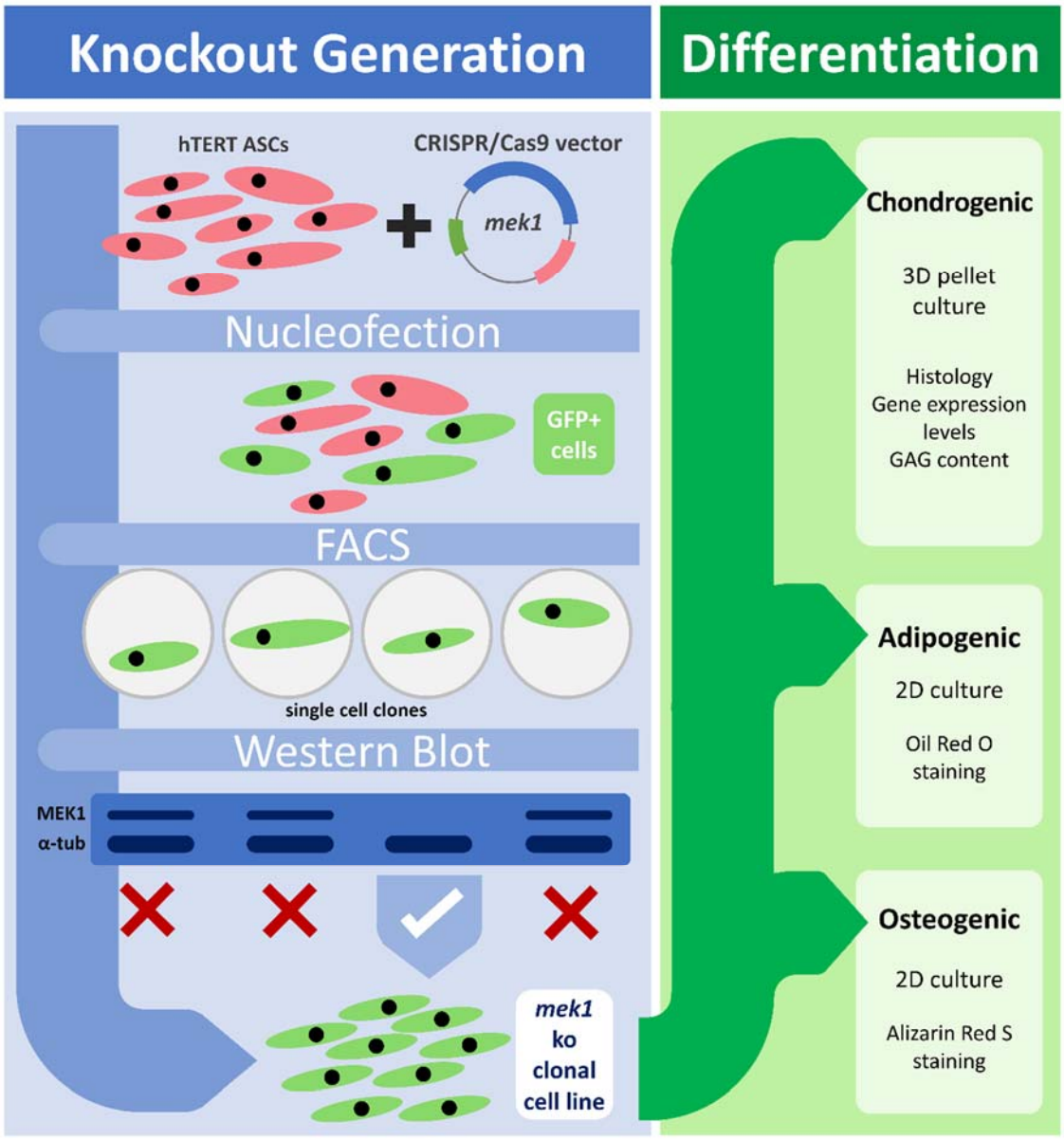

Figure 2 Schematic overview of the established protocol; the first part of the protocol is the generation of the knockout cell line, firstly hTERT ASCs are transfected with a plasmid coding for the Cas9 protein, a 20 bp long guide sequence (targeting mek1) and GFP, after nucleofection GFP positive cells are selected and seeded at 1 cell per well into a 96-well-plate, after expansion protein expression is analysed via western blot (α-tubulin as housekeeper), cell lines lacking MEK1 are then used for further screening of their surface markers and differentiation potential into the chondrogenic (3D pellet culture), adipogenic and osteogenic lineage (2D culture).

monolayer and cultivation in media complemented with supplements favouring differentiation. In the case of osteogenic differentiation, the medium was supplemented with ascorbic acid, dexamethasone and β-glycerophosphate [DMEM LG (Hyclone), 10% FCS (HyClone), 1% Penicillin/Streptomycin (Lonza), 2 mM L-glutamine (Lonza), 0.01 µM dexamethasone (Sigma), 150 µM ascorbate-2-phosphate (Sigma), 10 mM β-glycerophosphate (Stemcell Technologies)]. Differentiation was performed for 4 weeks with sampling days once a week. The cells were fixed and stained with Alizarin Red S to identify calcium deposits. Additionally, the dye was eluted with a 20% methanol – 10% acetic acid solution and quantified via absorbance measurements at 450 nm.

Adipogenic medium was supplemented with dexamethasone, IBMX, hydrocortisone and indomethacin [DMEM HG (Lonza), 10% FCS (HyClone), 1% Penicillin/Streptomycin (Lonza), 2 mM L-glutamine (Lonza), 1 µM dexamethasone (Sigma), 0.5 mM IBMX (Sigma), 0.5 µM hydrocortisone (Stemcell Technologies), 60 µM indomethacin (Sigma)]. Differentiation into adipocytes was performed for 2 weeks, with sample harvest every 2-3 days. The cells were fixed and stained with Oil Red O to visualize lipid vesicles.

Finally, chondrogenic differentiation was performed in a 3D pellet culture with 250 000 cells/pellet. The pellets were cultivated in chondrogenic differentiation medium [DMEM F-12 (Lonza), 10% FCS (HyClone), 1% Penicillin/Streptomycin (Lonza), 2 mM L-glutamine (Lonza), 1 ng/mL bFGF (PeproTech)] for five weeks. Differentiation was assessed by histological analysis and evaluation of glycosaminoglycan to DNA ratio (GAG/DNA assay). Furthermore, gene expression levels of chondrogenic markers *Collagen 1, Collagen 2, Aggrecan* and *Sox9* was assessed by qRT-PCR.

Accomplishments and future goals

The aim of this project was to establish a suitable and easy to adapt protocol for the generation of knockout cell lines in human adipose derived stem cells and the subsequent testing of their differentiation potential. So far, we succeeded in generating at least 2 *mek1* knockout cell lines. The protocol for adipogenic and osteogenic differentiation testing was set up to confirm differentiation capability straightforward into these two lineages. More focus is laid on the chondrogenic differentiation potential, hence a more detailed analysis was employed.

We plan to use this protocol for the generation of multiple different knockout hTERT ASC lines and screen them for their differentiation potential. Particularly, the involvement of the MEK/ERK signalling pathway in chondrogenesis will be examined. Furthermore, the role of mechanical stimulation in these processes will be assessed. Application of shockwaves or perfusion will give insight into the impact of MEK1 deletion on mechanotransduction.

Importantly, although this project focusses on the ablation of the protein MEK1, the established protocol can be easily adapted for CRISPR/Cas9-mediated ablation of any other protein of interest in hTERT ASCs.

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