Adenovirus-mediated CRISPR/Cas9 gene editing in patient primary fibroblasts to validate potential drug targets in lung fibrosis

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1 INTRODUCTION

Targeted gene editing has become a key tool in deciphering the molecular mechanisms underlying disease processes and for target validation in the drug discovery process. For research into new drug targets and compounds for treatment of pulmonary fibrosis, we previously developed a cell-based assay with primary lung fibroblasts derived from idiopathic pulmonary fibrosis (IPF) patients. In this in vitro model, the transition of fibroblast into myofibroblast (FMT) is triggered by TGFβ. Myofibroblasts are thought to play a major role in fibrosis through excessive deposition of extracellular matrix. A key feature of myofibroblasts is the production of α-smooth muscle actin (αSMA).

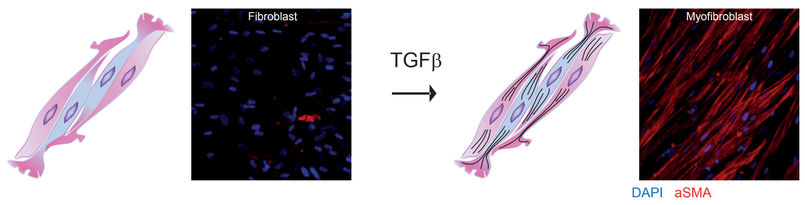

Figure 1: Cell-based phenotypic assay for pulmonary fibrosis

In this study, we have evaluated the feasibility to validate potential drug targets in fibrosis by CRISPR/Cas9-mediated targeted gene knockout in this assay. For delivery of Cas9 and gRNA the adenoviral system was chosen that is known for its ability to transduce a wide range of cells with high efficiency, including human primary lung fibroblasts that are difficult to transfect. The ACTA2 gene encoding αSMA was selected as target to demonstrate feasibility of CRISPR/Cas9-mediated knockout in lung fibroblasts. The setup was validated using a small gRNA library with putative drug targets for FMT/IPF emerging from literature.

2 EXPERIMENTAL DESIGN

An adenoviral system for CRISPR/Cas9 delivery was constructed with Cas9 and the gRNA being expressed from two different adenoviruses (AdV). Four gRNAs targeting ACTA2 and ≥2 gRNAs per putative drug target were designed and cloned into the adapter vector of the adenoviral system. High titer stocks of adenoviruses expressing either Cas9 or ACTA2 gRNAs were produced and used in the transduction of primary fibroblasts derived from four IPF patients. CRISPR/Cas9-mediated knockout of ACTA2 was measured by high content analysis (HCA).

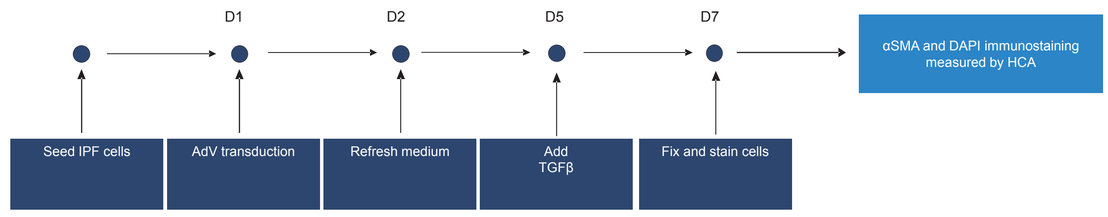

Figure 2. Timeline for a cell-based phenotypic assay for lung fibrosis. Fibroblasts derived from IPF patients were transduced with AdV-Cas9 and AdV-gRNA one day after seeding. The fibrotic process was triggered by adding 2 ng/µl TGFβ 4 days after adenoviral transduction. The fibroblast to myofibroblast transition (FMT) was quantified by measuring αSMA production by high content analysis (HCA) 2 days after adding the TGFβ trigger.

3 RESULTS

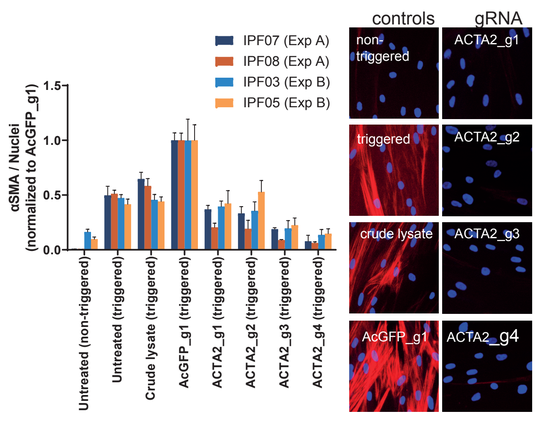

Figure 3. Impaired αSMA production in FMT assay due to CRISPR/Cas9-mediated *ATCA2* knockout. IPF patient-derived fibroblasts from four donors (IPF03, -05, -07 and -08) were transduced at a MOI of 24 each for AdV-Cas9 and AdV-gRNA_ACTA2 (ACTA2 _g). TGFβ was added 4 days after transduction and αSMA production was quantified by HCA 2 days after TGFβ trigger (day 7). A non-targeting control adenovirus (AcGFP_g1) was used to normalize the data as adenoviral transduction is known to stimulate αSMA production. All four gRNAs were effective in *ACTA2* gene knockout in two independent experiments, however gene editing efficiency was donor-dependent. Typical high content images are shown.

Gene	Full name	Rationale
ACTA2	Actin alpha 2, smooth muscle	Involved in vascular contractility, is used as a FMT marker
IRF5	Interferon regulatory factor 5	Transcription factor modulating cell growth and differentiation
ITGA5	Integrin subunit alpha 5	Involved in the formation of the fibronectin receptor, cell surface adhesion and signaling
ITGB7	Integrin subunit beta 7	Adhesion receptor involved in cellular signaling with the extracellular matrix and MAPK-ERK pathway
ITGB1	Integrin subunit beta 1	Involved in the formation of the fibronectin receptor and tissue repair processes
COL3A1	Collagen type III alpha 1 chain	Fibrillary collagen found in extensible connective tissue, is used as a FMT marker
TGFBR3	Transforming growth factor beta receptor 3	Membrane proteoglycan often functioning as a co-receptor with other TGFβ receptor superfamily members
SMAD4	SMAD family member 4	Signal transduction protein involved in TGFβ signaling regulating transcription processes
MAPK8	Mitogen-activated protein kinase 8	Regulation of kinases signaling processes involved with cell proliferation and differentiation associated with renal fibrosis and fatty liver disease
CXCL10	C-X-C motif chemokine ligand 10	Ligand for the receptor CXCR3 and inflammation marker, involved with adhesion molecule expression
S100A8	S100 calcium binding protein A8	Involved in the regulation cell cycle progression and differentiation associated with cystic fibrosis

Table 1: Selection of putative drug targets for modulation of αSMA protein levels in the FMT process. Candidate genes are selected that are presumed to be involved in IPF based on available literature. The *ACTA2* gene is selected as an assay control. For each target gene ≥2 gRNAs were designed and cloned into the adapter vector of the adenoviral system.

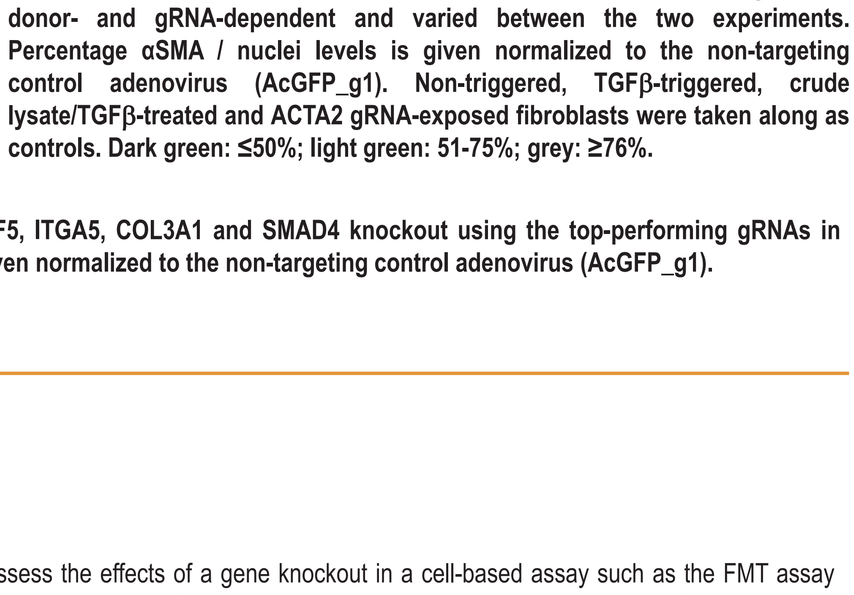

Figure 4: CRISPR/Cas9-mediated downregulation of αSMA protein in FMT assay was observed using gRNA directed against IRIF5, ITGA5, COL3A1 and SMAD4. The relative effect size for modulation of each of these targets was donor- and gRNA-dependent and varied between the two experiments. Percentage αSMA / nuclei levels is given normalized to the non-targeting control adenovirus (AcGFP_g1). Non-triggered, TGFβ-triggered, crude lysate/TGFβ-treated and ACTA2 gRNA-exposed fibroblasts were taken along as controls. Dark green: ≤50%; light green: 51-75%; grey: ≥76%.

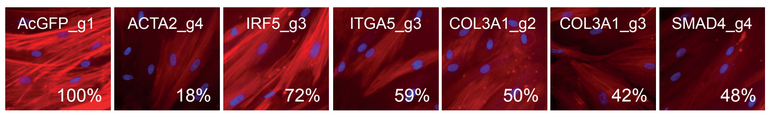

Figure 5. Representative high content images of CRISPR/Cas9-mediated ATCA2, IRIF5, ITGA5, COL3A1 and SMAD4 knockout using the top-performing gRNAs in donor IPF07. Relative percentage αSMA / nuclei levels from high content analysis is given normalized to the non-targeting control adenovirus (AcGFP_g1).

4 CONCLUSION

We conclude that adenoviral transduction of primary fibroblasts for targeting gene editing using CRISPR/Cas9 technology is sufficiently efficient to assess the effects of a gene knockout in a cell-based assay such as the FMT assay without the need for selection or cloning. This was validated not only by targeting the *ACTA2* gene encoding for αSMA protein directly, but also using a small library of gRNAs targeting genes that are associated with the fibrosis process and/or IPF phenotypes. Using this approach *IRIF5, ITGA5, COL3A1* and *SMAD4* are identified as modulators of myofibroblast transition in this assay. Such effective gene targeting in a functional assay is a valuable asset for target validation in drug discovery.

CRISPR-Cas9 was used under license to granted and pending US and international patents from the Broad Institute and ERS Genomics Limited