MULTIPLEX STRATEGY FOR GENE EDITING AND GENE INDUCTION BASED ON CRISPR/CAS9 TECHNOLOGY

DEIS HAXHOLLI., BENEDICT RAUSER., OSKAR SANCHEZ., WOLFGANG WURST.

Department of Neurodegenerative Diseases, at Helmholtz Research Centre Technical, University Munich

e-mail: deis.haxholli@live.it

Abstract

Today, the most promising tool which has caught the attention of many researchers from basic research to translational medicine is CRISPR/Cas9 technology. The aim of the study is to be able to generate a two-fronts multiplexing strategy through CRISPR/Cas9 toolkit for genome editing and gene induction. The multiplexing is based on a single polycistronic tRNA gene, which in between the tRNAs, there are the sgRNAs designed and then processed by the same endogenous mechanism as the one of the tRNAs by producing an array of sgRNAs targeting specific genes simultaneously. The multiplex strategy was planned for gene induction of three important genes, MASH1, Nurr1 and Lmx1a, which are known to be important transcription factors. They were shown to be involved in direct convertion with transdifferentiation from fibroblasts to dopaminergic neurons. Parallel to this multiplexing strategic plan, in the meantime were tested SAM and SpyCatch-SpyTag activation systems, multiplexed together, understanding which was the most robust and strong for gene induction. Combining the two multiplexing strategies and specially for transcriptional activation of the genes through CRISPR/Cas9 technology seems promising in the future for in-vivo experiments with special focus on gene therapy for Parkinson's neurodegenerative disease and not only.

Key words: Crispr/Cas system, multiplexing, cell direct differentiation.

Përmbledhje:

Sot instrumenti më i rëndësishëm biologjik që ka tërhequr vemendjen e shkencëtarëve është teknologjia CRISPR/Cas9. Qëllimi i këtij studimi është ndërtimi i një strategjie multiplekse me dy fronte për të ndryshuar gjenomen në aktivizimin ose jo të gjeneve. Kjo strategji bazohet në gjenerimin e gARNve duke shfrytëzuar sistemin endogjen të prodhimit të tARNve të veçanta nga ajo policistronike. gARN e prodhuara do të synojnë tri gjene të ndryshme njëkohësisht, MASH1, Nurr1 dhe Lmx1a, të rëndësishme për transdiferencimin direkt nga fibroblaste në neurone prodhuese të dopaminës. Paralelisht me këtë strategji, u testuan sistemet aktivatore për induktimin e gjeneve të lartpërmendura, SAM dhe SpyCatch-Spytag në strategji multiplekse së bashku, duke përcaktuar sistemin më të mirë për shprehjen e gjeneve. Kombinimi i të dy strategjive multiplekse me anën e teknologjisë së instrumentit biologjik CRISPR/Cas9 është premtues në të ardhmen në eksperimente in-vivo me fokus në terapinë e personalizuar gjenetike për sëmundjet neurodegjenerative si Parkinsoni etj.

Fjalëkyçe: Sistemi Crispr/Cas9, strategjia multiplekse, diferencimi direkt qelizor.

Introduction

Since 1970, when the era of genetic engineering started to revolutionise the field of biotechnology, studying and understanding the role of genes and their function became one of the main goals which should be achieved for the future prospect in human medicine. Clustered regularly interspaced short palindromic repeats (CRISPR), CRISPR associate proteins (Cas) is an adaptive immune system in bacteria and archea toward the prophage infection, which now has been widely adopted in an engineered form for genome editing and gene transcription modulation (Jinek *et al.,* 2012). Functional CRISPR/Cas9 system comprises the CRISPR locus with an array of identical palindromic repeats intercalated by spacer sequences deriving from the DNA-targeting invader cleaved genome, which are responsible for the transcription of the loci into crRNA, and cas genes encoding for cas proteins (Rath *et al*., 2015). The applications of CRISPR/Cas9 system nowadays are gene editing to study the function of a specific gene, transcription activation and repression through the tethering of effector molecules, functional screening with gRNA libraries, inducible regulation, DNA labelling and multiplexing.

Interestingly a question was raised asking whether the possible applications of CRISPR/Cas9 toolkit could be used as an engine in reprogramming cells. For direct reprogramming from mouse fibroblasts to neural cells it is required the ectopic expression of three cell-lineage specific transcription factors as cell fate determinants, and these are Ascl1, Brn2 and Myt1I known also as BAM factors (Vierbuchen *et al.,* 2010). Neuron-specific transcription factors ectopically expressed first initiate reprogramming of the cells and secondly they activate the neuron-specific downstream target genes. The resulting cells, called induced neuronal cells (iNCs) are shown to undertake action potentials from synapses and to express many neuronal specific proteins. The aim of the study is to be able to generate a two-fronts multiplexing strategy through CRISPR/Cas9 toolkit for genome editing and gene induction for direct reprogramming from mouse embryonic fibroblast to neurons, especially dopaminergic neurons, since mesencephalic neuronal dopamine deficiency is known to be the main cause of Parkinson's degenerative disease (Lindvall *et al*., 2004, Politis *et al*., 2010, Caiazzo *at al.,* 2011)

Materials and methods

Culture cells and bacterial strains

The cell lines Neuro-2a WT (murine albino neuroblastoma cell line) and human embryonic kidney 293 cells (HEK-293T) cells were used. As a primary cell culture there used mouse embryonic fibroblasts (MEFs) of E 6.5 and Astrocytes isolated from 7 days newborn mice. In both cases they were WT mice housed in the mouse facility of the Institute of Developmental Genetics, Helmhotlz Research Centre, Munich Germany. E. coli K12 strain DH5α are used for transformation of electro - competent bacteria. For transformation of chemically competent cells Invitrogen Kit was used.

Media and antibiotics

All media and solutions designated for works with cells, proteins or DNA were autoclaved or sterile filtered. Media were prepared with deionised water, all other solutions used MilliQ water. Media were stored at 4 °C, solutions at room temperature. Antibiotics were added just prior to use.

T7 endonuclease I assay

The T7 endonuclease I assay was performed and quantified as described below by Reyon et al. [91]The isolated genomic DNA was amplified for 35 cycles using specifically designed primers at the modified gene locus (Rosa26 and Rab38, respectively). The amplified product was purified using the QIAprep PCR Purification Kit (QIAGEN) and 200 ng DNA was used together with NEBuffer 2 (New England Biolabs) was diluted in 20 µL of ddH2O. The DNA was denatured and reannealed with the following protocol: 95 °C

5 min; 95-85 °C at -2 °C s-1; 85- 25 °C at -0.1 °C s-1; hold at 4 °C. The hybridised products were processed by 10 U of T7 endonuclease I at 37 °C for 60 min and the reaction was then stopped with 2 µL of 0.5 M EDTA. The products were then quantified using the 2100 Bioanalyzer system (Agilent Technologies) [94]. The number of cleaved fragments corresponds to mismatched DNA duplexes. The primers were designed with Genious Software and Vector NTI software.

Total RNA isolation from multi-well plates.

For the RNA isolation from 24 well plate, it has been followed the procedure as it was described according to the manufacturer protocol of RNeasy Plus Mini Kit. For better lysis, the cells collected from each well in each RNAse free eppendorf tube were freezed in -80 °C immediately after lysis and the RNA isolation procedure followed the next day with one modification in the protocol. The RW1 washing buffer solution has been used subsequently for the next two washing steps, instead of RPE solution, resulting in a better yield and purer isolated RNA.

Reverse Transcriptase Quantitative Real-Time PCR.

SuperScript® VILO cDNA Synthesis Kit and Master Mix is used for qRT PCR according to the protocol. It improves the yield and the dynamic range of qRT PCR by obtaining the same relative representation of the cDNA independently from gene abundance. The Taqman fluorescent probes with the specific primers were used for quantification and normalised to B- actin and GapDH

Transfections

The X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics) was used according to the manufacturer's protocol. One day prior to transfection, the adherent cells were passaged and 5×104 cells in 300 µL medium per well

were seeded on an 48-well dish. Directly prior to lipofection, the medium was replaced with fresh complete DMEM medium. The plasmids were mixed and volume corresponding to total of 300 ng was mixed with OptiMEM to a total of 30 µL.

Dual-luciferase assay

After 48 h, the cells were lysed measured using the Dual-Luciferase Reporter Assay System (Promega) after manufacturer's protocol. Values were normalised to the negative control. Error bars in the dual-luciferase assay rep- resent SEM of the biological triplicates. MicroWin 2000 was the software used for luciferase measurements.

Results and discussion

Multiplexing in genome editing

Based on the focus of the study, first multiplexing strategy was to test three polycistronic constructs, which are based on the tRNA genes intermediated by gRNA, each gRNA after one tRNA sequence. It is based on the endogenous mechanism of the primary transcript cleavage by two enzymes, RNAse P and RNAse Z. They cleave the tRNAs in the 5' leader and 3' trailer end respectively, resulting in the processing of the gRNAs, which are ready to be delivered to the target sequence through the mediation of Cas9 protein. By the moment the guides are "free" and ready to be loaded in the Cas9 scafold, the system is ready to undertake the targeted DNA double strand break and introducing the desired indels. Our two guides are targeting two safe loci, Rosa26#3 and Rab38#2, which have a restriction enzyme specific recognition site for cleavage, XbaI and XcmI respectively. After the genomic DNA extraction, the restriction enzyme analyses of the PCR products of the two loci has been analysed in a 2,5% agarose gel electrophoresis, showing that the wild type band, which have the restriction site, are cleaved by the enzyme after the restriction digestion analyses. in contrary the mutants have a deletion in the restriction recognition site because of the CRISPR/ Cas9 system which caused the mutation. Thus the mutants cannot be cleaved by the specific restriction enzyme, and the migrating DNA in the agarose gel has a bigger size than the cleaved DNA sequence. The gRNAs in the polycistronic gene were of the order where the first gRNA was targeting Rab38#2 and the second guide after the tRNA is targeting Rosa26#3. The three constructs tested were:

1. U6: tRNA -gRab38 -tRNA -gRosa26 2.
2. U6: gRab38 -tRNA -gRosa26
3. psT -gRab38 -tRNA -gRosa26 (without promoter, tRNA is a promoter itself)

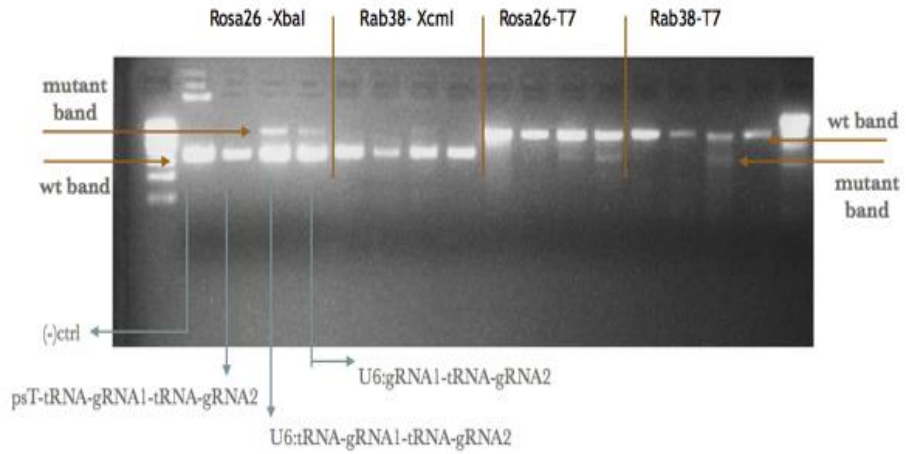


Figure 1. Restriction enzyme analysis of Rosa26#3 locus with XbaI and Rab38#2 with XcmI in 2,5% agarose gel. There is an evident mutant band in both Rosa26#3 and Rab38#2 loci with U6:tRNA-gRab38-tRNA-gRosa26 and U6:gRab38-tRNA-gRosa26 contracts tested. T7 endonuclease assays reveal the same results, where the mutants are the lower band showing the cleaved hydride duplexes of one mutant DNA strand and the other one the wild type DNA strand.

Although in an agarose gel electrophoresis the intensity of the band is proportional to the amount of DNA migrated in that band, in this case it is important to quantify the amount of DNA. For this purpose the same replicates that were analysed by gel in the restriction enzyme analyses of Rosa26#3 with XbaI and T7 nuclease assay of Rab38#2, were later on analysed in Agilent Bioanalyser 2100 for quantification. The results of the Bioanalyser are shown below:

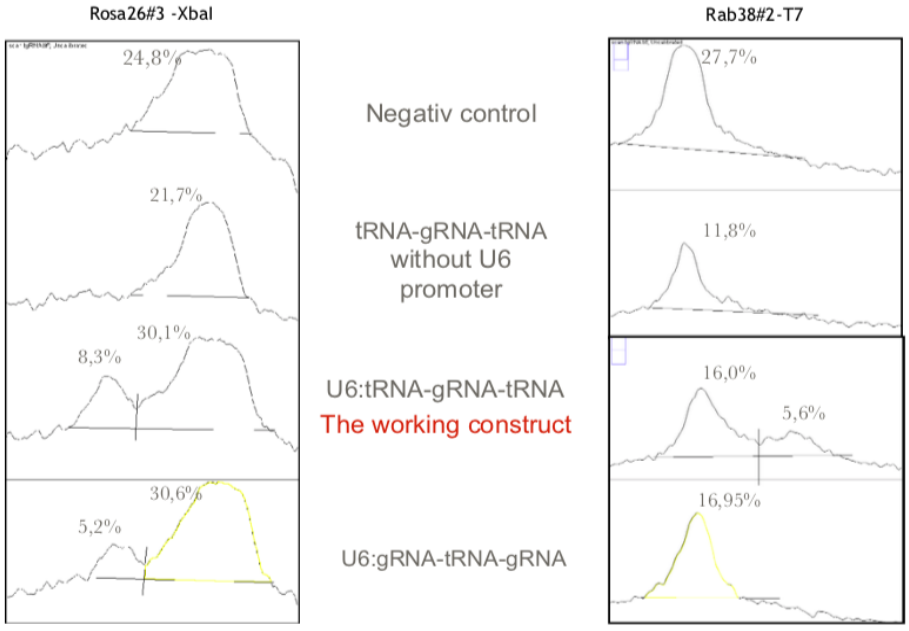


Figure 2. Results of Bioanalyser 2100 Agilent Technologies (above). These are the treated PCR products of the targets loci from the extracted genomic DNA. a) The first panel is the analysis of Rosa26#3 locus treated with XbaI restriction enzyme. The negative controls are the wild type cells, not transfected with none of the constructs, only with Cas9. As a result from the tested constructs, the one with higher mutation efficiency is **U6: tRNA-gRab38-tRNA-gRosa26** for targeting for two or more specific loci.

Multiplexing in gene induction

Investigating multiplexing in gene induction is the other side of the coin of CRISPR/Cas9 system. Multiplexing in this case is more complex in its composition and it requires many perquisites to reach the goal. This is because the system itself is based on critical features to obtain gene or genes activation. The major purpose is to achieve gene activation by targeting more than two loci with the minimal lentiviral system with one single gRNA, precisely the Ascl1 (M15), Lmx1a (L24) and Nurr1 (N3), important for direct cell differentiation into neurons. To achieve our goal we started to switch the inducible system into a non-inducible one under the expression of Ef1a promoter by using two other different activator systems, SpyCatch_12xSpyTag System and Synergic Activator Mediators (SAM) system and both of these in only two viral systems.

Multiplexing with two viral systems with multiple gRNAs and one activator

The first multiplexing strategy for gene induction is by introducing all the three guides, M15, L24 and N3 (MNL) expressed with different promoters in ne lentiviral vector with SpyCatch under the expression of Ef1a promoter.

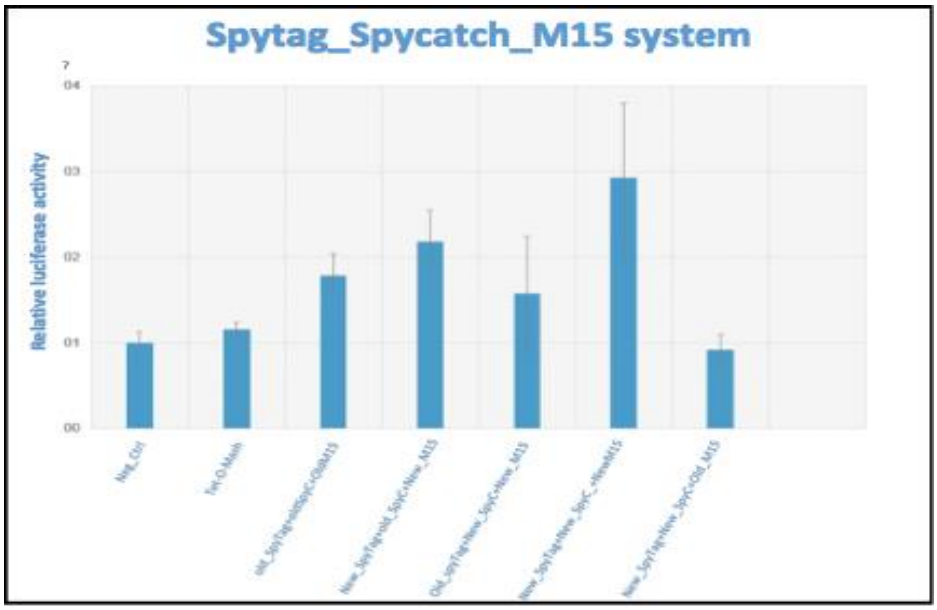


Figure 3. The graphic above are the results of luciferase assay of lipofected HEK 293T cell line. The graphic shows the comparison of the plasmid version of SpyCatch-SpyTag system with the lentiviral one after transferring all the system into viral vectors. Here what is termed "old" indicates the plasmid version which is the previous one used and as "new" the lentiviral version as the latest one.

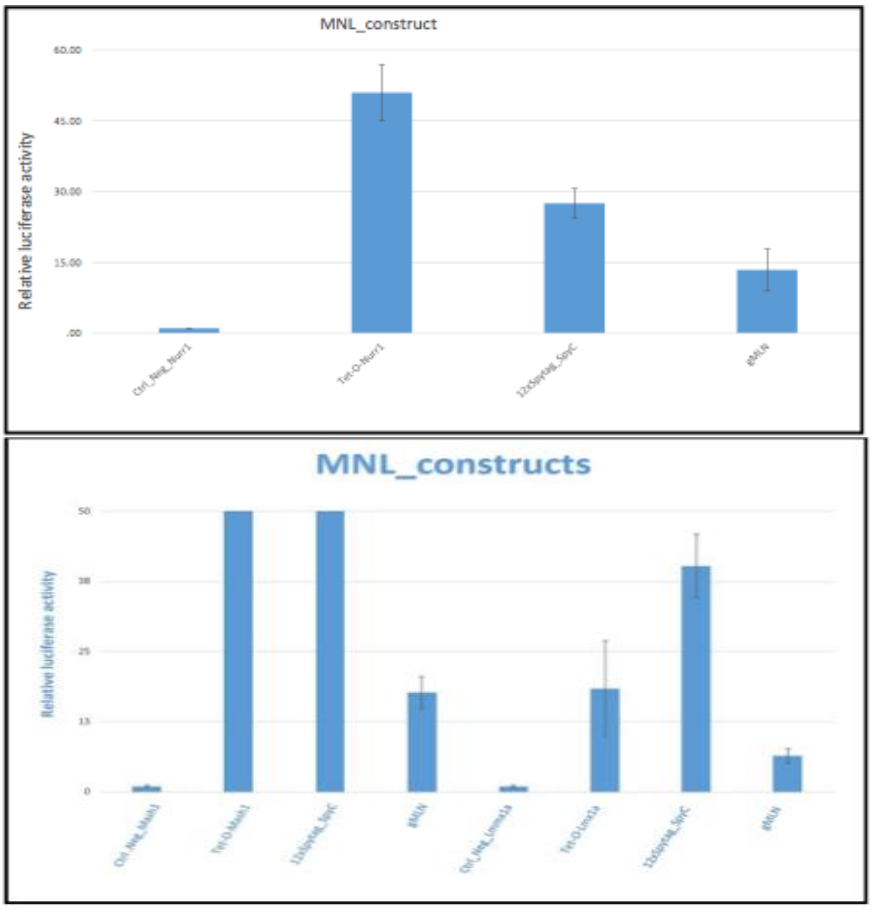


Figure 4. (a): Luciferase assay (below) of the lipofected HEK 293T cell lines. a) The graphics show the relative luciferase activity for the multiplexing gMNL construct in comparison with the single vector guides constructs using the same activation system, SpyCatch-12xSpyTag under EF1a promoter expression. b) The second graphic is the luciferase assay for comparing Nurr1 (N3) gRNA with the same multiplexing construct syste.. Tet-O-Nurr1 as a positive control.

The lentiviral constructs for multiplexing is based using one activation system, our Spycatch-12xSpyTag one with three different guides, M15, L24 and N3, which are of our focus for direct neuronal reprogramming from mouse embryonic fibroblast and astrocytes. dCas9-12xSpyTag is expressed under Ef1a promoter in one expression lentiviral vector and SpyCatch with the three guides on the other expression vector again under the same constitutive promoter.

The results of dual luciferase assays make evident in each of the cases that the relative luciferase activity is of much higher level when the targeted gene is activated with SpyCatch-12xSpyTag system and an individual gRNA lentiviral vector either than in multiplexing with three different gRNAs in one single construct. This might be due to the fact of not achieving good expression of gRNAs altogether.

3.2.2 Multiplexing in two viral systems with multiple activators and one gRNA.

Keeping the focus in multiplex strategy, parallel to the previous described strategy, but in an inversive way of problem solving, it was thought to achieve multiplexing by boosting an adequate transcriptional activation system to achieve gene induction of multiple genes. The new activation system was going to be generated and thereafter optimized. Comparing and combining was the strategic plan for optimization and establishing which activation system could be the more promising one. For this reason the system was simplified by using first only one gRNA for gene induction. Mash1 (M15) was the chosen gRNA for testing the system since it is considered as a master regulator amidst other transcription activators responsible for neuronal reprogramming.

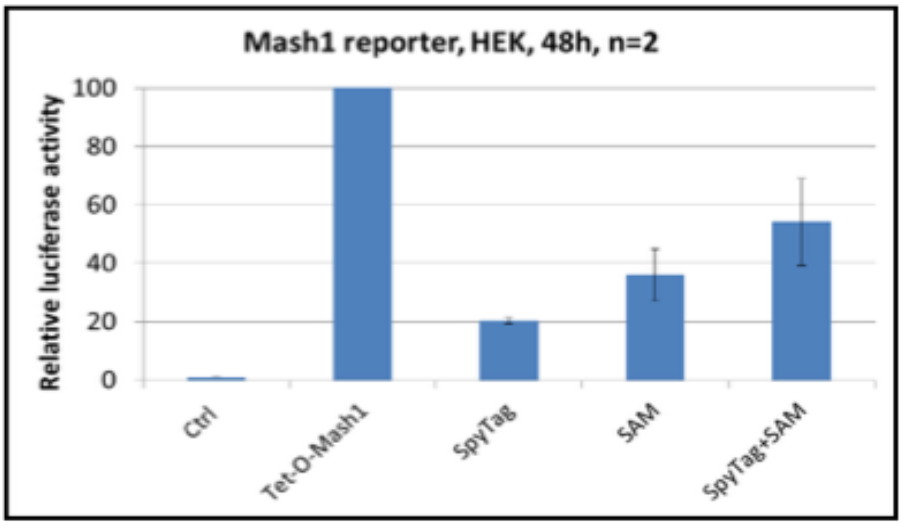


Figure 4. (b): Luciferase assay of lipofected HEK 293T cell lines, 48 hours post transfection. The relative luciferease activity shows the difference in comparing the activation systems SpyCatch-12xSpyTag, SAM and both of them together in inducing the targeted gene Mash1 (M15). The Mash1 is used as a reporter of the system and all normalised to renilla reporter. It is needed to emphasise that as termed SAM+SpyTag is the combination of both activation systems SAM and SpyCatch (which activates by covalently binding to SpyTag) but as individual constructs, different from the SAM/SpyCatch/gRNA vector. This result suggests that probably combining the two systems together, could possibly have additional effect

in mediating gene induction. Taking advantage of these results, it was designed the multiplexing project with two lentiviral constructs which in synergy induce high level of activation of the target genes by one or more gRNAs, SAM+SpyCatch+gRNA and dCa9_12xSpyTag.

3.3. Gene induction for MEFs and astrocytes reprogramming.

The main reason for exploiting the potential of CRISPR/dCas9 activation systems for boosting gene induction is to be able to reprogram mouse embryonic fibroblasts (MEFs) and astrocytes into neurons by direct conversion. This can be possible by stimulating the activation of specific target genes and in our system, the master regulator is Mash1. As a transcript factor, after its high expression, it is able to induce the expression of a cascade of downstream genes which are responsible for neuronal differentiation, and more specifically Glutamatergic and GABAergic neurons. As our goal is to generate dopaminergic neurons, for system simplification we are initially redirecting the focus first only in inducing neuronal conversion of a non-specific subpopulation, until the system is completely optimized for targeting several genes simultaneously by multiplexing.

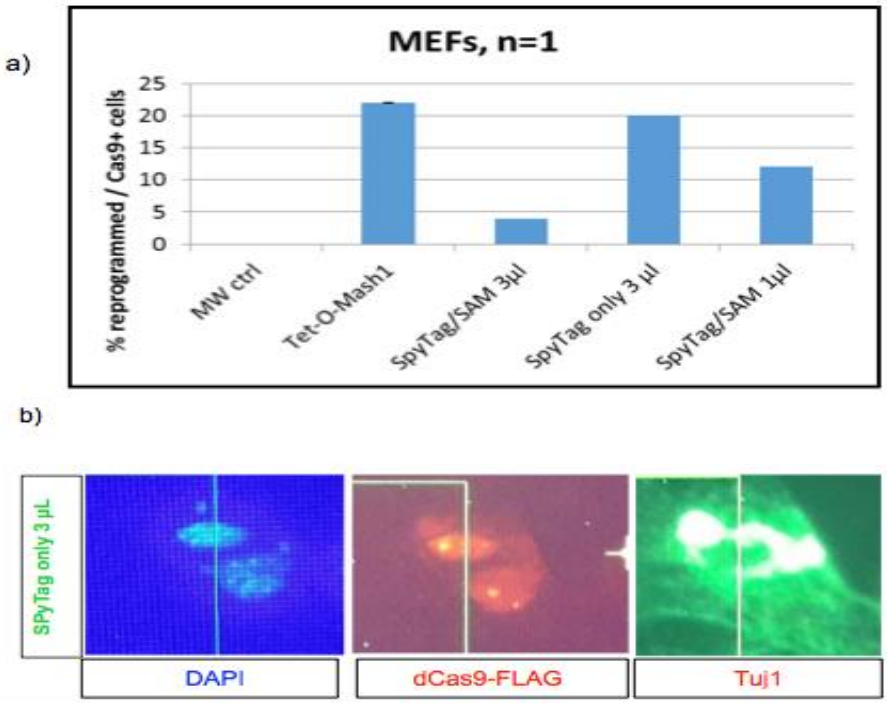


Figure 5. a) The preliminary results of reprogrammed MEFs. The graphic shows the % of reprogrammed cells per dCas9 expressing cells. Viral transduction with 4 viral constructs: dCas9 (SpyTag/VP-160), Ef1a_SpyCatch, gRNA and SAM complexes. b) The counted cells were treated with immunocytochemical techniques. The cells were DAPI stained (blue), dCas9_FLAG stained (red) and for Tuj1 as a early

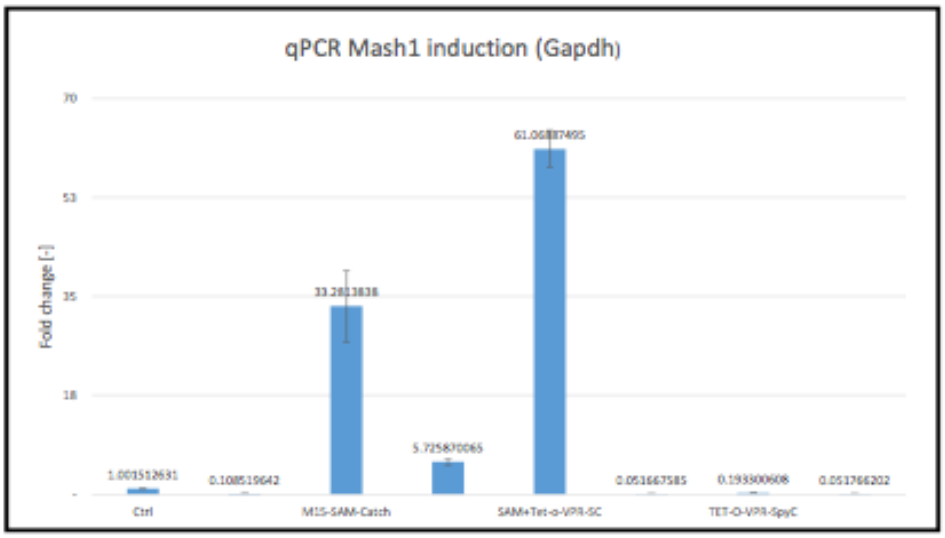
neuronal marker (green). In the pictures above, captured from Stereo Investigator software, is shown a reprogrammed MEFs in intermediate differential state and stained with the aforementioned antibodies in the same sequential flow

The results of the first attempt for MEFs reprogramming with the our activator systems of SAM and SpyCatch-SpyTag, alone and also together could not lead to any conclusive result. But what it is important to mention is the fact that in these preliminary results, in cell reprogramming SpyCatch-SpyTag system alone generates better results than the SpyTag/SAM together, which is in contrary to the results of the previously shown lipofection. This might be because of many reasons, the cells used which are different up the virus production optimization. The low titter yield is partially because of the procedure followed for ecotropic virus production, which is significantly different from the second generation of lentivirus production. The low percentage of reprogrammed cells can be related to the fact the previous result of qRT PCR, in which strongly indicates the importance of gRNA designing and their multiplicity for sequence targeting and gene induction.

3.4 Screening for debugging the system

Regarding to the results already presented, as we have emphasized there is a discordance related to the activation systems and target Mash1 gene induction. Of course this might be due the use of two different cells types used, one for luciferase assay and the other one for reprogramming, which most probably might influence the results. We planed two parallel experiments which had the purpose to understand and correct the mistakes of the system in general. One experiment was focused in testing the activators used and here we included a new activator which was already shown and verified for its high activation efficiency. VPR is the new EBV transactivation system, the same that used for generating neurone from iPSCs by CRISPR/dCas9 mediated transdifferentiation (Chavez et al., 2015). It has been cloned in a Tet-On inducible system. The second experiment was designed to test the gRNA M15 targeting efficiency which tremendously affect especially the gene activation results. The experiments were performed with Quantitative Reverse Transcriptase Real-Time PCR with Taqman probes for Ascl1(M15) primers. The results are shown below.

a)



b)

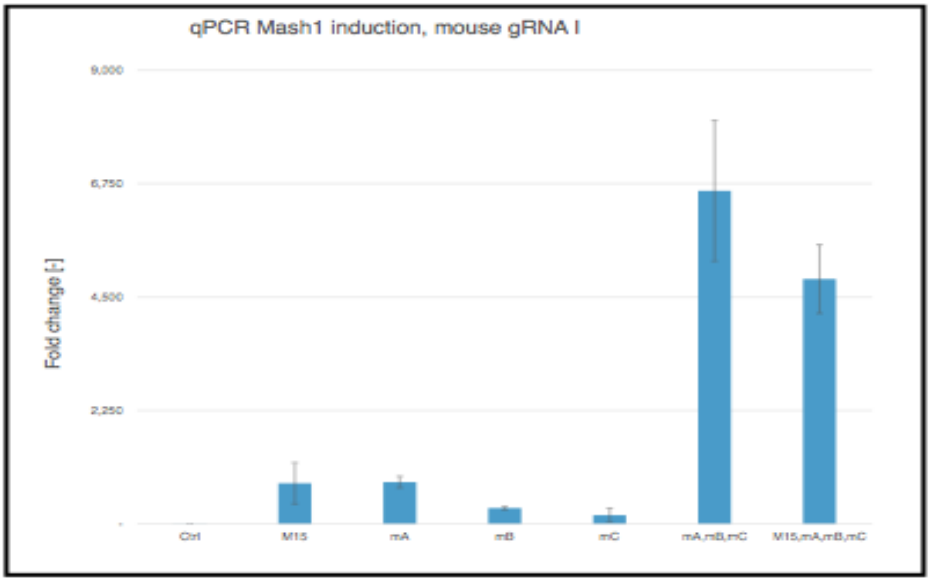


Figure 6. qRT-PCR analysis of: a) transcriptional activators systems. Comparing the activation systems of SAM, SpyTag/SAM/M15, SpyCatch-SpyTag, Tet-O-VPR, in complex and individually. normalised to GapDh. b) Testing gRNAs of mouse gM15 first contract designed by us, this is the one used in all the introduced experiments. And the new mouse gM15, those are indicated as mA, mB and mC. The above results of both experiments conducted in parallel show that the gRNA design is crucial for gene activation. Our previous gM15 was not so efficient in targeting, which most probably have a major influence in gene induction, but not in transactivation systems efficiency.

The results of the first attempt for MEFs reprogramming with the our activator systems of SAM and SpyCatch-SpyTag, alone and also together could not lead to any conclusive result. But what it is important to mention is the fact that in these preliminary results, in cell reprogramming SpyCatch-SpyTag system alone generates better results than the SpyTag/SAM together, which is in contrary to the results of the previously shown lipofection. This might be because of many reasons, the cells used which are different up the virus production optimization. The low titter yield is partially because of the procedure followed for ecotropic virus production, which is significantly different from the second generation of lentivirus production. The low percentage of reprogrammed cells can be related to the fact the previous result of qRT PCR, in which strongly indicates the importance of gRNA designing and their multiplicity for sequence targeting and gene induction. Below are shown the results of another reprogramming experiment. This is based on astrocyte reprogramming via lipofection in the same testing conditions as the previous reprogramming experiment, with the

only difference of an additional componet, Smarca1. The results are measured 14 days post transfection.

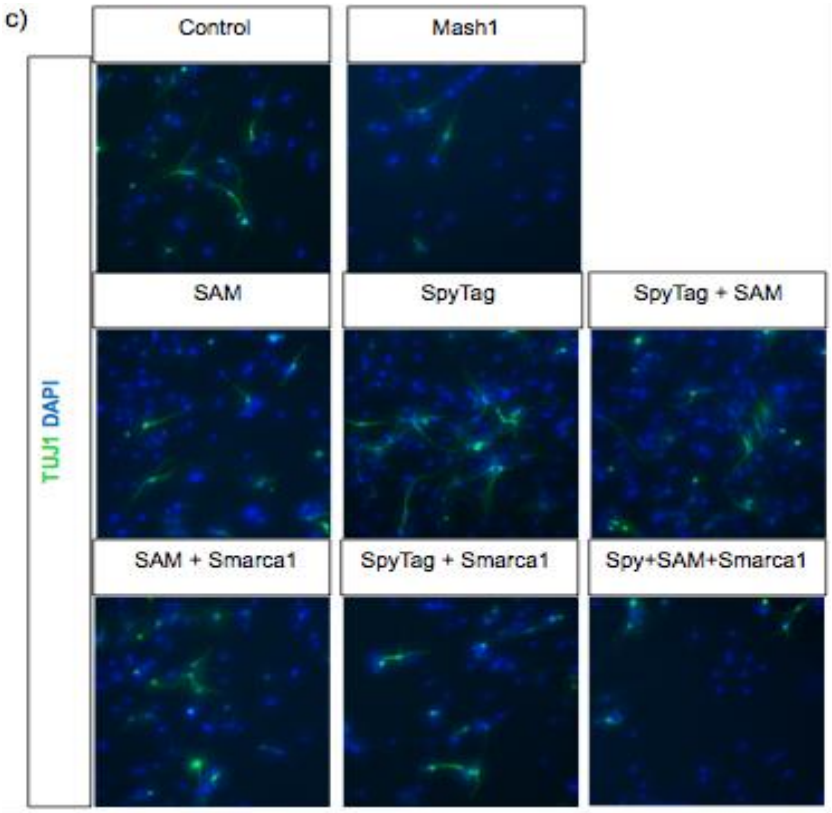


Figure 7. Reprogrammed astrocytes transfected with lentiviral vectors of the activator systems, SAM, SpyTag-SpyCatch, SpyTag/SAM, individually and together as in the previous experiments targeting Mash1. In this experiment is included Smarca1. It is chromatin remodeller of SWI/SNF family, which enables gene induction via its interaction with VP16. Immunocytochemical staining of cells with DAPI (blue) and Tuj1 which is a neuronal marker (green). There is no conclusive result in astrocyte reprogramming. Beside the fact that here it was slightly shown to obtain the same evidence as in reprogrammed MEFs for higher expression of Mash1 by Spycatch-SpyTag system alone, contrary to HEK cells induction with the same activation systems.

Conclusions

Our puzzle consists on the questions that we have posed. First, if we can generate a multiplexing strategy with CRISPR/dCas9 to transcriptionally activate three targeted genes, M15, Lmx1a and Nurr1, which are responsible for neuronal differentiation, simultaneously with only one gRNA for each target gene and all-in one system lentiviral vector, which with synergic activation systems can induce neural reprogramming by direct conversion

from either mouse embryonic fibroblasts MEFs or astrocytes into differentiated neurons. Secondly, if we could establish a multiplexing strategy for genome editing via CRISPR/Cas9 which is essentially based on the endogenous molecular biological mechanism of tRNAs processing and which for the first time make evidence in mammalian cells, rather than only in plants as it has been shown from the literature review. Finally, thinking similarly to the convergent-divergent meeting point before mentioned, we deduced from both previous statements if there is the possibility to obtain a generalized multiplexing strategic scheme, which has the flexibility to confer the features either for gene induction or for genome editing, depending on the project plan, thus being compatible to both systems. This idea is the future aspect of our project.

Relating to our data, the results do not show a pronounced activation level as it has been published in other data, but it is still to be emphasized that SpyCatch-12xSpyTag + SAM are a good candidate system, as it was shown in the luciferase assay results of the lipofected HEK cells. But this result was not reproduced in the reprogramming experiments of MEFs and astrocytes. Eventually there was shown the contrary, although they are actually preliminary results of reprogrammed cell counting. I would argue that this results might be many methodological reasons. There are needed to be established: an optimized procedure for virus production. MEFs reprogramming through viral transduction was via ecotropic virus production, different from the third generation virus production procedure, so resulting in a very low titter yield, thus probably effecting the gene integration into the host cells genome, therefore altering the reprogramming results.

Additionally I would like to point out the potential of SpyCatch-12xSpyTag/ SAM as promising system for gene induction based on the luciferase assays. Their combinatorial regulation is still needed to be understood. An overcrowded transcriptional activators system may have the opposite effect rather than inducing gene transcription, taking into consideration the stearic hindrance or possible molecular interference in occasionally repressing each-other when they are in complex.

I would like to bring the attention onto the other side of CRISPR/Cas9 technology which has been part of the study of this work. Genetically engineering the genome has never been so easy and reachable as it is now. This is due to the multifunctional availability of CRISPR/Cas toolkit. Refocusing once again in multiplexing strategy which has been the Gordon Knot of our study, we aimed to achieve multiplexing of two safe loci Rosa26#3 and Rab38#2 by simply exploiting the endogenous tRNA primary process mechanism to generate the two guides for binding in target sequences in complex with the endonuclease for further cleavage by Cas9 and so resulting in detectable indels to the target site. The positive results of the experiment quantified by the Bioanalyser 2001 Agilent Technologies,

gave a new prospective to the project. Since it was not seen to have been used before as a system in mammalian cells except in plants, this strategy was thought to be a good project plan for targeting multiple gRNAs in a row in the same construct intermediated by the tRNA sequences. The construct size is considerably small and this characteristic can be taken as an advantage in gene therapy via adeno associated viruses AAVs. These results also opened the gate towards the future projects of multiplexing with tRNA-gRNA-tRNA-gRNA construct for lentiviral gene therapy.

Acknowledgments

I would like to take the chance and to express all my immense gratitude to all those persons that made possible this study and which helped me during the thesis.

I would like to express my very great appreciation for my supervisors, Prof. Cristina Marchini and Prof. Wolfgang Wurst. Prof Cristina Marchini for her support, help and guidance through all my academic career and specially during the research work despite of the distance and the difficulties that it confers. Absolutely my very special thanks to Prof. Wolfgang Wurst and Dr. Oskar Ortiz Sanchez team with Karin Kuhner, Susane Bourier and also Jeffrey Troung for giving me the opportunity to work with them in Helmhotlz Reseach Centre, Technical University Munich. Their patience, encouragement, persistence and useful critics were of great help in progress.

A lot of sincere gratitude towards Benedict Rauser for his discipline of work, determination in it and his patience of steel in teaching and helping me during all the time of this study.

References

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012): A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821

Rath, D., Amlinger, L., Rath, A., and Lundgren, M. (2015): The CRISPR-Cas immune system: Biology, mechanisms and applications. Biochimie 117 ; 119-128

Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Sudhof, T.C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463, 1035–1041

Lindvall, O. & Bjo¨rklund, A. Cell therapy in Parkinson's disease. NeuroRx 1, 382–393 Politis, M. et al. (2010): Serotonergic neurons mediate dyskinesia side effects in Parkinson's patients with neural transplants. Sci.Transl. Med. 2, 38–46

Caiazzo, M., Dell'Anno, M.T., Dvoretskova, E., Lazarevic, D., Taverna, S., Leo, D., Sotnikova, T.D., Menegon, A., Roncaglia, P., Colciago, G., et al. (2011). Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature 476, 224–227