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Establishing a screening system in iPSC derived myotubes for imaging calcium dynamics

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Bachelor thesis, molecular life sciences, molecular bioanalytics

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ABSTRACT

Calcium (Ca^{2+}) plays an essential role in muscle contraction. Ca^{2+} is released from the sarcoplasmic reticulum (SR) upon neuronal stimulation, which results in muscle contraction. Sarcoplasmic reticulum calcium ATPase (SERCA) is responsible for transporting Ca^{2+} from the cytosol back into the SR. Dysfunction of SERCA is involved in the pathogenesis of several muscle diseases such as Brody myopathy, Duchenne muscular dystrophy (DMD) as well as heart failure. Therefore it exists a common interest in stimulating SERCA pharmacologically. Induced pluripotent stem (iPS) cells open up new possibilities since their pluripotency gives them the ability to differentiate to all cell types of the body including skeletal and heart muscle cells. Being ethically accepted and susceptible to genetic modifications makes iPS cells a very compelling instrument. GCaMP is a genetically encoded calcium indicator (GECI), which can be used to image calcium dynamics in real time. GCaMP reporter gene can be used for compound screening by targeting transporters involved in Ca^{2+}-flow.

The goal of this bachelor thesis was to establish a screening system in iPS cell derived myotubes for imaging Ca^{2+} dynamics in real time in the SR. Indeed 16 clones could be isolated, 1 homo- and 4 heterozygous clones were characterized through PCR and FACS. Selected clones were then differentiated to myotubes. Through Immunofluorescence (IF) GCaMP could be localized within iPS cells as well as in myotubes. With an FDSS assay fluorescence was measured after stimulating the myotubes. However fluorescence signal was at the lower limit of detection.

INTRODUCTION

Since SERCA plays an important role in the pathogenesis of muscle diseases, observing and measuring influx and efflux of Ca^{2+} in the SR is a key aim of research and pharmacology. Goal of this bachelor thesis was to establish an iPS cell line, which can be used as a tool to measure Ca^{2+} flow of the SR in real time. For this a modified GCaMP expressing in the ER was aimed to be introduced into an iPS cell line. When differentiating iPS cells to myotubes, this tool would be very useful to screen for compounds which modify Ca^{2+} flux in skeletal muscle.

RESULTS

Around 100 clones resulted from the transfection with CAG-GCaMPer-2A-Puro, from which 16 were picked. Since some clones did not continue to grow, only 5 clones were analyzed by PCR. Tab.1 provides an overview about the PCR results

Characterization of clones through PCR

Table 1: Results overview characterization of the clones by PCR

Clone/Cells	PCR -random integration	PCR – 3' / 5' integration		PCR – Homoz. / Heteroz.	
		5'	3'	Hom.	Het.
#2B	✓	?	✓	-	✓
#2C	✓	✓	✓	✓	-
#2E	-	✓	✓	-	✓
#2G	-	?	?	-	✓
#2H	✓	✓	✓	-	✓
MyoD-GCaMP6f	-	✓	n.d.	-	✓
hDFa90/1.2	-	-	-	-	-

Differentiation of iPS derived myotubes

Differentiation of iPS cells to myotubes *in vitro* occurs over 4 stages(Fig. 1).

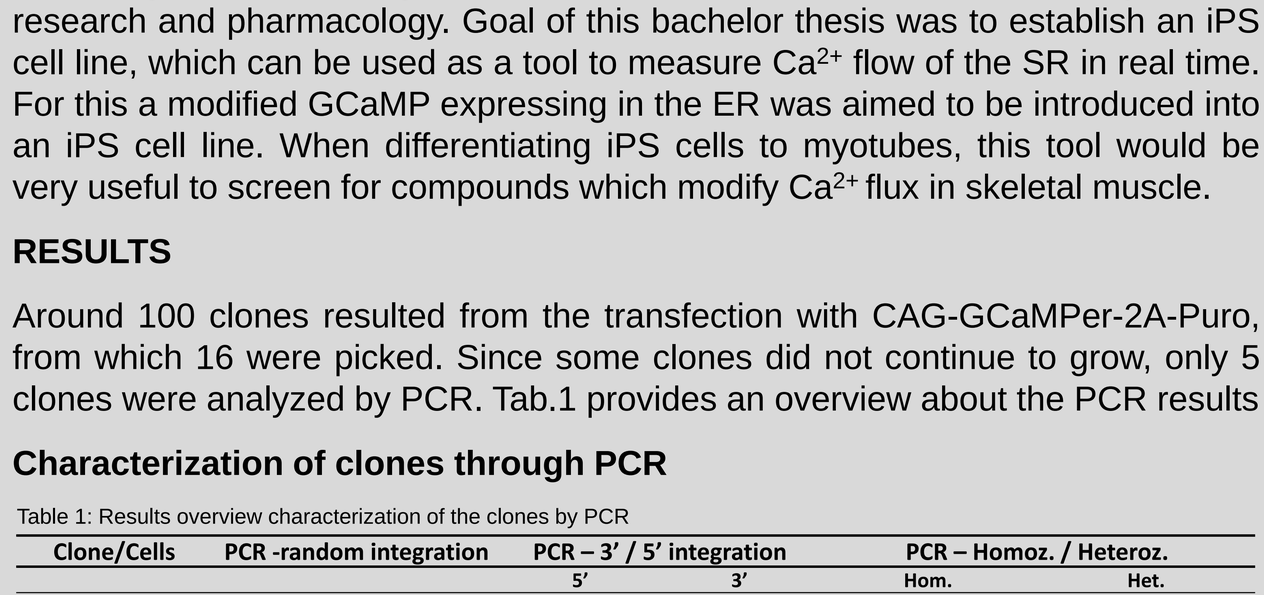

Figure 1: Overview differentiation protocol of iPSC to myotubes with pictures of the corresponding cell stages.

Localization of GCaMP within the cell

Since GCaMP was emitting GFP signal only very faintly, there was need to localize GCaMP in the cells to asses if GCaMP is expressed in the SR.

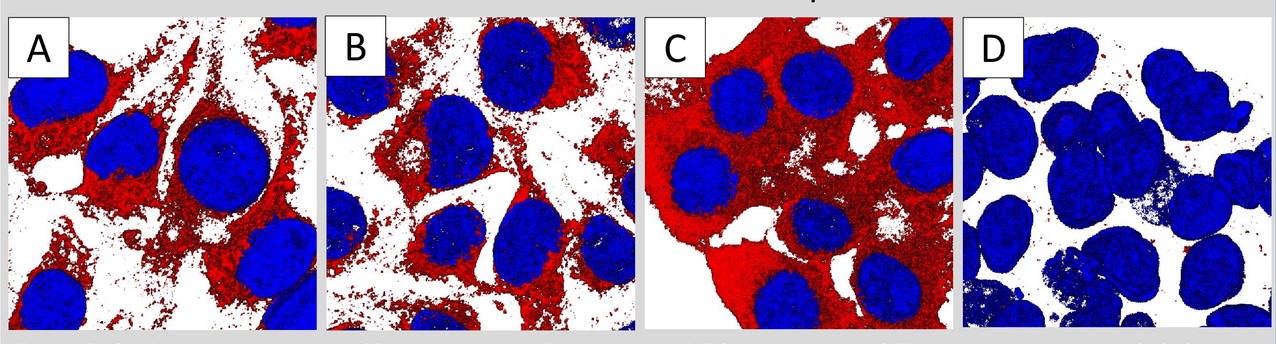

Figure 2: Confocal z-stack images (63 x) from immunofluorescence of iPS cells with anti-GFP antibody and hoechst33342 nucleic acid marker. (A) clone #2C; (B) clone #2E; (C) iPS-hDFa90/1.2-MyoD-CAG-GCaMP6f-2A-Puro (D) iPS WT hDFA90/1.2

(A and B) GCaMP in the clones is expressed in a different pattern than the cell line expressing GCaMP in the cytosol (C).

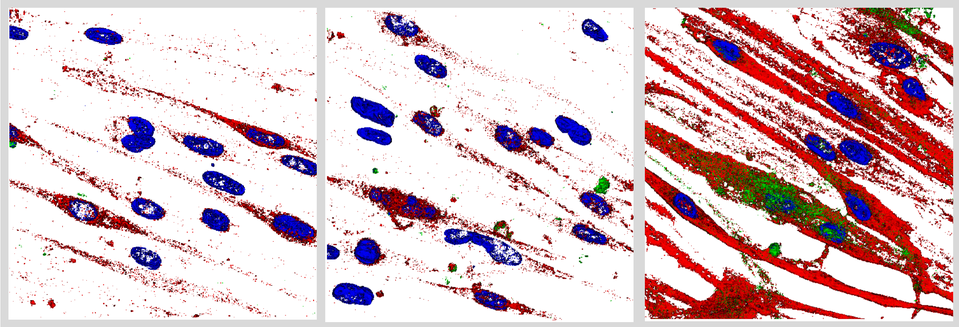

Figure 2: Confocal z-stack images (63 x) from immunofluorescence of iPS cells with anti-GFP antibody and hoechst33342 nucleic acid marker.
(A) clone #2C;
(B) clone #2E;
(C) iPS-hDFa90/1.2-MyoD-CAG-GCaMP6f-2A-Puro
(D) iPS WT hDFA90/1.2

This indicates that indeed GCaMP is expressed in the ER of the cell. When differentiating the cells to myotube, gene expression seems to be less strong. The pattern in myotubes is much diffuser and more dispersed than in iPS.

FDSS assay reveals correlating signal upon stimulation

In order to measure the response of GCaMPer upon stimulation. Ca^{2+} release from the SR, was stimulated by epibatidine (EPI). If GCaMP is expressed in the SR, one would expect a decreasing signal of GFP, since Ca^{2+} streams out of the SR upon stimulation. FDSS analysis revealed that indeed right at the point of injection, GFP signal decreased and comes back up within 1 minute . As predicted the amplitude of the signal was very low (Fig. 3)

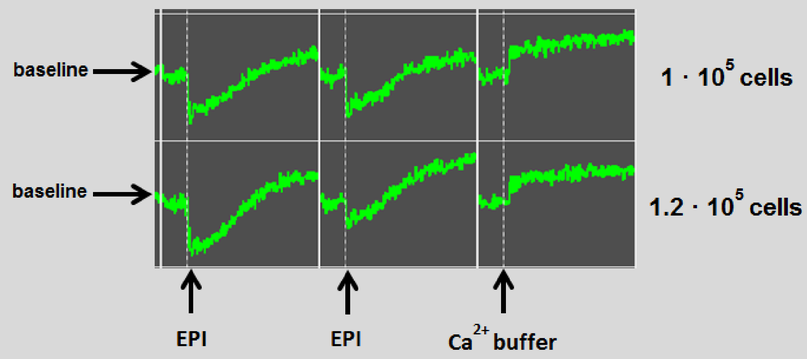

Figure 3: FDSS analysis of myotubes-hDFa90/1.2-MyoD-CAG-GCaMPer-2A-Puro clone #2E injecting EPI.
GFP signal of GCaMPer in 14 days old myotubes-hDFa90/1.2-MyoD-CAG-GCaMPer-2A-Puro clone #2E was measured every 0.53 sec for 2min 38 sec. After 26.5 sec (50. measurement) EPI was injected to induce Ca^{2+} release from the sarcoplasmic reticulum (end concentration of EPI was 1µM).

CONCLUSION

GCaMPer seems not to be strong and is detectable by FDSS and fluorescence microscope only very weakly. Since GCaMPer is thought to be a screening tool for compounds on SR-membrane, this weakness of the signal is not optimal and would cause inconvenience for a screen as well as loss of sensitivity.

To determine successful if GCaMPer is located in the ER (in iPS) and SR (in myotubes), further analyses by immunofluorescence should be performed. Co-localization of the SR and GCaMPer could be shown by co staining GCaMPer with anti-GFP and the ER resp. SR.

REFERENCES

[1] Nakai, J., Ohkura, M., & Imoto, K. (2001). A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nature Biotechnology*, *19*(2), 137–41. [2] Ohkura, M., Sasaki, T., Sadakari, J., Gengyo-Ando, K., Kagawa-Nagamura, Y., Kobayashi, C., … Nakai, J. (2012). Genetically Encoded Green Fluorescent Ca2+ Indicators with Improved Detectability for Neuronal Ca2+ Signals. PLoS ONE, 7(12), 1–10.

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