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FELLOWSHIP FINAL REPORT

Recombinant Intrabodies as Molecular Tools and Potential Therapeutics for Amyotrophic Lateral Sclerosis

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REPORT INFO

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that has no diagnostic marker, prognosis, nor an effective treatment. Numerous physiopathological mechanisms have been described for this disease, such as glutamatergic excitotoxicity, oxidative stress, and the accumulation of protein aggregates in cells of the central nervous system, in particular the aggregation of cytoplasmic TDP-43.Our aim was targeting the protein aggregates containing TDP-43 through fragments of antibodies synthesized by the cell, termed intrabodies. In order to determine the most relevant criteria to test the protective effects of the intrabodies, we searched for different toxicity markers associated with TDP-43aggregates. During the fellowship, the fellow participated of 2 publications of the host laboratory in this field. Besides, at the end of the fellowship, the host Scientist and the Le Studium fellow organized a conference about iPS cells, a powerful tool to model *in vitro* neurodegenerative diseases such as ALS. In addition, the fellow generated preliminary results showing that TDP-43 overexpression in HEK 293 cells does not affect mitochondrial respiration, but causes an increase in cytoplasmic calcium levels, while impairs the mitochondrial capacity to buffer the excessive cytoplasmic calcium. Moreover, preliminary patch clamp data showed alterations in spontaneous currents in primary hippocampal and motor neurons overexpressing TDP-43. If these results are further confirmed, calcium signaling and spontaneous currents could be used as parameters to measure the efficacy of anti-TDP-43 intrabodies.

1- Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal, neurodegenerative disease causing progressive loss of motor neurons and death only 3-5 years after diagnosis[1,2]. The incidence is 2-3 out of 100,000 individuals per year. There is no cure for ALS, and the only disease-modifying drug available to date is riluzole, which presents limited efficacy and severe side effects. The pathophysiology of ALS includes glutamatergic excitoxicity, oxidative stress, mitochondrial malfunctioning, axonal transport impairment, aberrant axonal transport, inflammation, and cell death[2-7]. Identifying primary targets is of utmost importance for the development of

effective treatments for ALS. In this context, mutations in the TARDBP gene, associated with the abnormal presence of aggregates of hyper-phosphorylated TAR DNA-binding protein 43 (TDP-43) in the cytosol of affected motor neurons, are present in 5% of familial and 1% of sporadic ALS cases. Importantly, TDP-43 aggregates are a hallmark of the disease, being present in most of the ALS patients, even in sporadic ALS cases[8-10]. TDP-43 is a nuclear protein with RNA processing and splicing function, but its overexpression results in cytosolic aggregates and provokes toxic effects in yeast, primary cultures of rodent neurons, and in in vivo models[11]. The development of cellular models of TDP-43 proteinopathy enabled to confirm a correlation between cell death and TDP-43 aggregates quantity and cytosolic localization in cultured neurons[8,11,12]. Notably, primary neurons submitted to TDP-43 overexpression presented cytosolic TDP-43 aggregates co-localized with mitochondria and a decrease in the length and density of dendritic mitochondria, whereas TDP-43 suppression caused an opposite effect. Interestingly, both overexpression and suppression of TDP-43 caused impairment in axonal mitochondrial transport, which is typical of ALS[13]. TDP-43 overexpression in human mutant fibroblasts, mouse cultured neurons, and in a mouse model causes cytosolic accumulation of TDP-43, particularly in the inner mitochondrial membrane, provoking a TDP-43 gain-of-function which results in mitochondrial fragmentation, decrease in ATP levels, defects in respiratory chain complex I translation and assembly and function, and cell death. These deleterious effects were decreased by suppression of cytosolic TDP-43, underscoring the exploitation of TPD-43 blockage as an attractive method to treat ALS[14]. Recently TDP-43 has been shown able of spreading from cell to cell similarly to prion diseases[15,16]. Recently, a number of approaches targeting TDP-43 proteinopathy, such as gene therapy[17-19], RNAi[20, 21], and treatments with methylene blue[22] or protein kinase inhibitors[23] have been employed in an attempt to avoid TDP-43 aggregates formation and pathophysiological features in ALS models, but none so far has been based on anti-TDP-43 protein antibodies.

Intrabodies are antibody fragments expressed intracellularly to block specific proteins inside the cell. They present highest affinity to the target protein as compared to small molecules, an advantage that enables the elimination of side effects. Intrabodies are able to access cytoplasmic proteins that parentally injected antibodies cannot reach. Moreover, specific types of intrabodies are capable of crossing the blood-brain barrier and reaching nervous tissue, enabling its application in neurological diseases[1,24,25]. Intrabodies against huntingtin, Aβ, α-synuclein, and prion proteins have been engineered, and introduced by means of vectors into cellular and rodent models of Huntington's, Alzheimer's, Parkinson's, and Prion diseases[24-30]. Intrabodies have been also successfully applied in a motor neuron cell line expressing mutant SOD1, an aberrant protein forming intracellular aggregates in 25% of familial cases of ALS[1]. Intrabodies hold a huge potential for clinical trials, since they require gene therapy, a practice approved in 2014, and a combination of new methods of antibodies engineering and gene delivery into the nervous system may emerge[30]. Thus, our hypothesis postulates that overexpression of TDP-43 in motor neurons and fibroblasts from ALS patients can constitute a cellular model of TDP-43 proteinopathy, recapitulating cytosolic TDP-43 aggregation and pathological alterations observed in ALS motor neurons and fibroblasts. Finally, we hypothesize that gene delivery of anti-TDP-43 intrabodies to these cells or the use of biochemical mimetic compounds may neutralize TDP-43 aggregates, rescuing altered pathological markers. Among the different steps of this global project, we focus on an axis related to the one-year fellowship. The specific objectives are the following: (1) to induce the formation of cytosolic TDP-43 aggregates in Human Embryonic Kidney (HEK) cells and in primary cultures of neurons; (2) to identify specific endophenotypes linked to TDP-43 aggregates, such as mitochondrial and calcium signalling parameters, (3) to test the efficacy of a panel of different anti-TDP-43 intrabodies in

suppressing cytosolic accumulation of TDP-43 aggregates and in rescuing the markers selected in (2).

2- Experimental details

Cell cultures and transfection

All mouse experiments were performed according to protocols approved by the University of Tours and the INSERM. Hippocampi were dissected from E17.5 C57BL/6J mouse embryos and were manually dispersed in phosphate-buffered saline (PBS) and triturated with papain (10 U per ml) (Worthington, Lakewood, USA) for 22 min at 37 °C. Cells were resuspended in Dulbecco's modified Eagle's medium/F12 with 10% fetal bovine serum (Invitrogen), centrifuged at 400 g for 3.5 min, and the final pellet was resuspended in Neurobasal/B27/0.5 mM Gln (Invitrogen). Dissociated cells were then plated onto poly-;D-lysine and laminin (Sigma)-coated substrates at a density of 400 cells per mm2. For culture of primary motor neurons, dissected spinal cords of E14 rat embryos were first dissociated (trypsin and mechanically). Motor neurons in the suspension were then isolated using metrizamide (Santa Cruz Biotechnology, sc-211905) and plated on poly-ornithine/laminin-coated wells in neurobasal medium (Invitrogen) with BDNF 1 ng/ml, GDNF 100 pg/ml, CNTF 10 ng/ml, 2% horse serum, B27, L-glutamine 0.05 mM, L-glutamate 25 mM, and b-mercaptoethanol 25 mM. HEK 293 cells were grown in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS and continuously passaged when confluent Neurons were transfected at day in vitro (DIV) 14–16, and HEK 293 cells when at 70% comfluent with the appropriate plasmids (EGFP-tagged wt TDP-43) using Lipofectamine 2000 (Invitrogen) in serum-free medium. Respirometry, calcium and patch clamp analyses were carried out 24–48 h after transfection.

Respirometry

For oxygen consumption measurements, tripsinizated HEK 293 cells were numbered with hemocytometer (Malassez, Marienfiled, Germany), centrifuged 3 min at 700*g*, and then suspended in a respiratory buffer without BSA (10 mM KH2PO4, 300 mM mannitol, 10 mM KCl, and 5 mM MgCl2, pH 7.4). After a second 2 min centrifugation at 800*g*, 1 x 10^6 cells were resuspended in 2 ml of respiratory buffer with BSA. Then, respiration of the intact cells was measured by a high resolution respirometer (Oroboros Instruments, Innsbruck, Austria). After stabilization of routine respiration, the ATP-synthase inhibitor oligomycin (2 µg/mL) was added to obtain a measure of leak respiration, followed by titration of FCCCP to maximum oxygen flux (ETS capacity). Finally, rotenone was added.

Cytosolic ($[Ca^{2+}]i$) and mitochondrial ($[Ca^{2+}]mi$) calcium

Changes in the $[Ca^{2+}]$i were monitored using Fura-2/AM dye and in the $[Ca^{2+}]$mi by Rhod-2/AM, using a FLEXstation III (Molecular Devices). HEK 293 cells were seeded onto 96-well assay plates (Greiner Bio-one) at a density of 2-5x10^4 cells per well and incubated in culture medium overnight. In the next day cells were transfected with cDNA codifying TDP-43-GFP. After 48 hs cells were loaded with either 2 µM Fura-2/AM or 5 µM Rhod-2 (Molecular Probes) in standard buffer solution (SBS: 147 mM NaCl, 2 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 10 mM HEPES and 13 mM glucose 13, pH 7.3) at 37°C for 30 min, and after washing, maintained in SBS at 37°C for 30 min. The ratio of the fluorescence intensity F340/F380 was used to indicate the $[Ca^{2+}]$I, and F552/F581 was used to indicate $[Ca^{2+}]$mi. Both $[Ca^{2+}]$i and $[Ca^{2+}]$mi were stimulated by the automatic addition of 50 µM ATP to each well. Data fit was carried out using Origin software.

Patch clamp

A whole cell patch clamp technique was used for recording the sodium channel activity in primary hippocampal and motor neurons. Na^+ currents were recorded under voltage-

clamp mode using an Axopatch 200B patch clamp amplifier (Axon Instruments, USA), compensating for cell capacitance and series resistance by 60 %. The P/2 sub pulse protocol was applied to correct the linear component of capacitance and cell leak. Borosilicate glass was used to pull the patch pipettes at a resistance of 5–8 MΩ. Analog signals were filtered at 5 kHz and sampled at 10 kHz via a 1440A Digidata converter and analyzed using pCLAMP software (v10.4, Axon Instruments). Cells were studied in ruptured, whole-cell, voltage-clamp mode of the patch-clamp technique. Before starting the experiment growth medium was removed and replaced with physiological saline solution (PSS, composition given below). In this experiment the tip of the micro capillary (perfusion system) delivering the PSS or the test compound was placed almost on top of the cell, so that the cells were continuously perfused with the respective solutions. The physiological saline solution (PSS) contained (in mM): NaCl 140, KCl 4, $MgCl_2$1, $CaCl_2$ 2, NaH_2PO4 0.33, D-Glucose 11.1, and HEPES 10, and was adjusted to pH 7.4. The intrapipette solution (in mM) contained: KCl 130, NaCl 15, $CaCl_2$ 0.37, $MgCl_2$ 1, Mg-ATP 1, EGTA 1, and HEPES 10, and was adjusted to pH 7.2.

3- Results and discussion

We first examined the influence of TDP-43 overexpression in HEK 293 cells on O2 consumption. For this purpose, we transfected cells with TDP-43-GFP cDNA using Lipofectamine 2000. For our surprise, this transfection agent increased mitochondrial cellular respiration by itself in a dose-dependent manner from 4 µL/well (Fig 1).

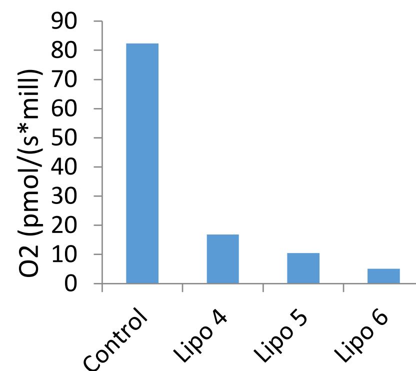


Figure 1. Effect of Lipofectamine 2000 on basal O_2 consumption in HEK 293 cells.

Then, we choose another transfection agent, Fugene HD, in order to test this parameter. We verified that TDP-43 overexpression did not affect oxygen consumption in] Patient mutation ells (Fig 2).

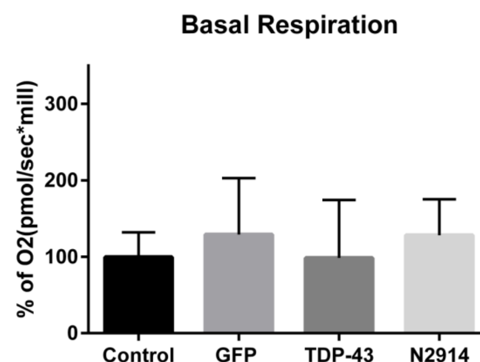


Figure 2. Basal O_2 consumption in HEK 293 cells. HEK 293 cells expressing TDP-43 or patient mutation do not present alterations in mitochondrial respiration

This result is discrepant with previous data showing that TDP-43 would damage mitochondrial respiration by affecting respiratory chain complex I assembly[31]. However, our results are consonant with a study showing no difference between mitochondrial respiration of controls and cells overexpressing TDP-43 or ALS patient mutations[32]. One possible explanation lies in the differences of transfection protocols, which could interfere in the mitochondrial respiration, as we demonstrated here for Lipofectamine 2000.

Next, we tested whether TDP-43 overexpression affects calcium homeostasis in HEK 293 cells. We first tested the ideal conditions for the calcium assays, and found that plating 10,000 cells 24 h before cell transfection and adding 50 µM of ATP at the moment of the experiments would give a robust measurable amount of increase in $[Ca^{2+}]i$ (Fig 3). Under these conditions we compared the levels of $[Ca^{2+}]i$ between TDP-43-GFP- and GFP-transfected HEK 293 cells. In all experiments it was verified that the levels of $[Ca^{2+}]i$ were increased in cells transfected with TDP-43-GFP and patient-mutation-GFP as ared to cells transfected only with GFP, and that there was no difference between $[Ca^{2+}]i$ transfected with GFP and non-transfected cells (Fig 4).

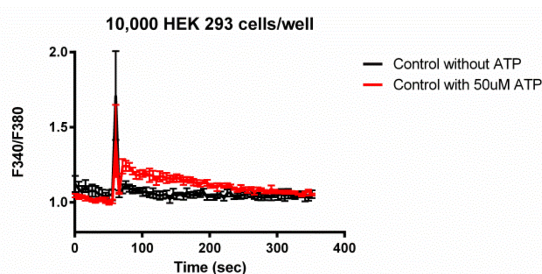


Figure 3. Optimization of Ca^{2+} imaging in live HEK 293 cells. Traces of basal intracellular Ca^{2+} fluorescence ratio (F340/F380) (black), and Ca^{2+} fluorescence ratio stimulated by 50 µM ATP (red) in wells cointaining 10,000 HEK 293 cells

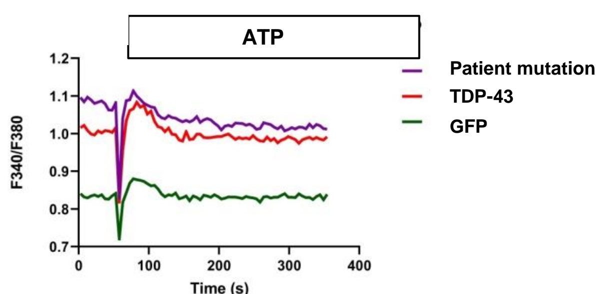
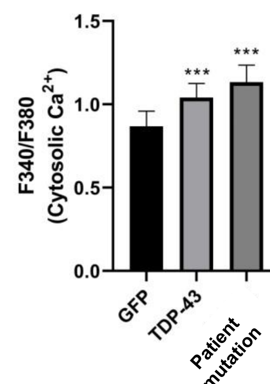


Figure 4. Ca^{2+} imaging in live HEK 293 cells transfected with TDP-43 or the ALS patient mutation. Notice that the intracellular Ca^{2+} fluorescence ratio (F340/F380) of TDP-43 and patient mutation expressing cells (red and purple, respectively) is largely increased as compared to GFP controls (green) before and after adding ATP.

Analyzing in more detail our data, we could identify that the $[Ca^{2+}]i$ was significantly increased both before and after ATP stimulation (Fig 5), suggesting that TDP-43 and patient mutation overexpression may cause endoplasmic reticulum stress.

However, when we analyzed the $[Ca^{2+}]mi$, for our surprise, cells transfected with TDP-43 had a significant decrease whereas cells transfected with the patient mutation presented a complete depletion of $[Ca^{2+}]mi$ (Fig 6), despite the increase they presented in $[Ca^{2+}]i$. This is strong evidence that the cells overexpressing both TDP-43 and the patient mutation probably lost their mitochondrial capacity of buffering excessive cytosolic calcium. These are in agreement with an association of TDP-43 overexpression with the decrease of the contacts between the endoplasmic reticulum and mitochondria[33], and with an increased sensitivity to toxicity upon calcium overload[34]. Therefore, increased $[Ca^{2+}]i$ and decreased $[Ca^{2+}]mi$ can be used as altered parameters in cells overexpressing TDP-43 or specific mutations obtained from samples of ALS and FTD patients in order to test new drugs capable of preventing these effects.



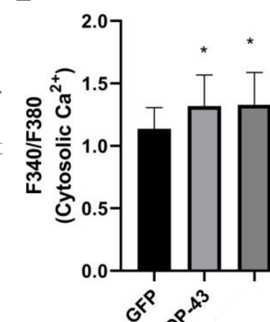


Figure 5. Intracellular Ca^{2+} fluorescence ratio of TDP-43 and patient mutation expressing cells before (A) and after stimulatrion by ATP (B). Both TDP-43 and patient mutation expression increase cytosolic calcium before and after ATP stimulus. N = 4, *p<0.05, **p<0.001.

In order to test the effect of TDP-43 and of the patient mutation on electrophysiological parameters we employed the patch clamp technique in primary culture of mouse hippocampal and motor neurons, the cells affected in ALS. Our preliminary data suggests that hippocampal neurons expressing both TDP-43 and the patient mutation appear to present altered spontaneous currents regards the intensity and frequency as compared to non-transfected or GFP-transfected neurons (Fig 7). Finally, we could observe similar pattern in preliminary experiments also with primary neurons (Fig 8). The confirmation of these data by increasing the N of experiments will enable to confirm that TDP-43 and the patient mutation provoke electrophysiological change in spontaneous currents as well as better characterizing the mechanisms behind this effect in more detail.

4- Conclusion

This project provided preliminary data supporting that the protein TDP-43, found in the form of cytoplasmic aggregates in the motor neurons of most ALS patients, does not affect mitochondrial respiration, but causes a disruption in the calcium signaling between endoplasmic reticulum and mitochondria. In addition, we found preliminary evidence that TDP-43 and the patient mutation could potentially affect spontaneous currents when expressed in excitable cells such as hippocampal and motor neurons. However, more experimental data is needed in order to confirm these results. If confirmed, these data have the potential to be applied as parameters altered in *in vitro* models of ALS based on TDP-43 and specific mutations from ALS patients. In the practical terms, calcium assays and patch clamp can be used for the evaluation of the efficacy of alternative treatments for ALS, such as anti-TDP-43-intrabodies in preventing calcium dyshomeostasis and alterations in spontaneous currents in HEK 293 cells and neurons respectively.

5- Perspectives of future collaborations with the host laboratory

For the future, it is expected that the project will keep ongoing. Prof Blasco`s group will finalize the production of plasmids codifying anti-TDP-43-intrabodies. After this, intrabodies will be selected based on their capacity of binding the TDP-43 protein. Then, the candidate intrabodies will be ready to be tested for parameters such as toxicity, and the parameters of calcium homeostasis and spontaneous currents, throughout double transfection of cells with both TDP-43 (or a patient mutation) and each of the pre-selected intrabody. Dr Denis Reis de Assis remains open for future collaborations and interested in the continuity of the project, so that the data can be publish in a well reputed peer reviewed journal.

6- Articles published in the framework of the fellowship

During the time of the fellowship, Dr Denis Reis de Assis participated of the following review papers from Prof Blasco's group:

- Hergesheimer RC, Chami AA, de Assis DR, Vourc'h P, Andres CR, Corcia P, Lanznaster D, Blasco H. The debated toxic role of aggregated TDP-43 in amyotrophic lateral sclerosis: a resolution in sight? Brain. 2019 Apr 1. pii: awz078. doi: 10.1093/brain/awz078.

- Lanznaster D, de Assis DR, Corcia P, Pradat PF, Blasco H. Metabolomics Biomarkers: A Strategy Toward Therapeutics Improvement in ALS. Front Neurol. 2018 Dec 18;9:1126. doi: 10.3389/fneur.2018.01126. eCollection 2018. Review.

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8- References

1. Neurobiol. Dis, 2013. 56:74-78
2. Br. Med. Bull, 2016. 119:87-98
3. Neurosci. Lett, 2017.
4. Mol. Neurobiol, 2016. 53:6910-6924
5. Can J Neurol Sci, 2016. 44:90-95
6. Front Mol Neurosci, 2017. 10:231
7. Prog. Neurobiol, 2012. 97:54-66
8. Amyotroph Lateral Scler Frontotemporal Degener., 2017. July: 1-3
9. Am. J. Pathol., 2007. 171:227-240
10. Neurobiol. Aging, 2005. 36: e2005.e2013
11. Acta Neuropathol., 2013. 125:777-794
12. J. Neurosci., 2010. 30(2):639-649
13. Hum. Mol. Genet, 2013. 22:4706-4719
14. Nat. Med, 2016. 22:869-878
15. Neurobiol. Dis, 2016. 96:236-247
16. J. Cell Biol, 2015. 211:897-911
17. EMBO Mol Med., 2013. 5:1710-1719
18. J Neurochem, 2014. 129(2):350-361
19. Gene Ther., 2015. 22(1):20-28
20. Nature., 2017. 544(7650):367-371
21. Biol. Chem., 2016. 291(37):19437-19448
22. Neuroscience., 2012. 209:136-143
23. J. Med. Chem, 2014. 57:2755-2772
24. Int. J. Biochem. Cell Biol, 2013. Article 710406:6 p
25. Comput. Struct. Biotechnol, 2016. 14:304-308
26. J. Neuropathol. Exp. Neurol, 2010. 69:1078-1085
27. Mol Ther, 2010. 18:1471-1481
28. Mol Ther, 2004. 10:1023-1031
29. J. Biol. Chem, 2005. 280:685-694
30. Prog. Neurobiol, 2012. 97:190-204
31. Nature Med. 2016. 22: 869-878
32. Mol. Neurodegener. 2017 12, 37
33. Nat Commun, 2014. 5:3996
34. Neurosi.c Lett. 2016 633, 28-32