The Michael J. Fox Foundation's Strategy to Generate, Characterize, and Distribute Preclinical Alpha-Synuclein Research Tools for Molecular Biology

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Introduction

Mutations in the gene SNCA that encodes the protein alpha-synuclein as well as postmortem pathological studies strongly implicate a general role for alpha-synuclein in Parkinson's disease (PD) pathogenesis. Thus, SNCA and alpha-synuclein are attractive targets for developing novel therapeutic approaches for PD patients. A field-wide challenge in PD research however, is a general lack of availability for high-quality, reproducible, and readily accessible preclinical research tools. To address these challenges, The Michael J. Fox Foundation for Parkinson's Research (MJFF) has developed a growing resource of preclinical tools for the PD research and drug development communities that endeavors to provide researchers with easy access to rigorously validated, research-enabling preclinical tools for molecular biology studies. Here we summarize our characterization and validation data for our anti-alpha-synuclein monoclonal antibody which was recently launched for commercial use. This monoclonal antibody is conformation specific for aggregate alpha-synuclein and binds with very high affinity and exquisite specificity for aggregate over monomeric versions of the protein. Both linear and cyclic epitope mapping data for this antibody are also provided. In addition, we describe our recently developed and commercially launched alpha-synuclein protein library, which contains multiple versions of recombinant alpha-synuclein proteins and a pS129 phospho-mimetic peptide. Moreover, we introduce new alpha-synuclein molecular biology reagents that are currently in development within our preclinical tools pipeline, including an alpha-synuclein aggregation assay. Ultimately, these MJFF-sponsored alpha-synuclein research tools aim to address field-wide challenges in the preclinical tools and reagents space and overall accelerate PD research.

Alpha-Synuclein Filament-Specific Antibody MJFR14

MJFR14 is a rabbit monocloncal, conformation-specific antibody that binds with high affinity to filamentous and oligomeric alpha-synuclein. This antibody was produced by Abcam with validation by Dr. Poul Henning Jensen, Luminex data by Dr. Jing Zhang at the University of Washington, human tissue immunostaining by Dr. Janice Holton at University College London, epitope mapping by PEPperPRINT, and ReNcell immunocytochemistry by Charles River Discovery. MJFR14 is distributed by Abcam.

Figure 1. MJFR14 is specific for alpha-synuclein filaments and soluble oligomers

Description

A) Dot blot analysis of MJFR14 binding affinity to monomeric and filamentous alpha-synuclein protein. The first and second lanes contain recombinant alpha-synuclein monomer or filament, respectively. The third and fourth lanes contain monomer or filament preabsorbed with 100ug/ml monomeric alpha-synuclein protein, respectively. B) Luminex assay results for MJFR14 using alpha-synuclein oligomers, fibrils, and monomers.

Results

A) MJFR14 recognizes alpha-synuclein filaments and soluble oligomers that spontaneously form in equilibrium with the monomer in solution. B) Luminex data confirms high binding affinity of MJFR14 to the oligomeric form of alpha-synuclein.

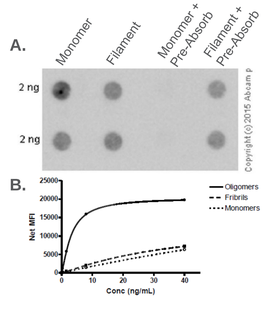
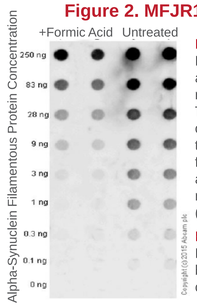


Figure 2. MFJR14 is conformation specific



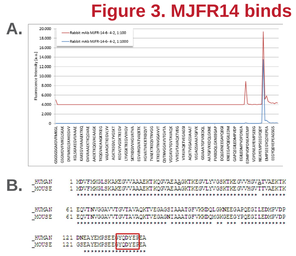
Description

Dot blot analysis of MJFR14 binding affinity to denatured and untreated recombinant alpha-synuclein filaments. The first and second lanes are samples of recombinant alpha-synuclein filament treated with 70% formic acid to denature filaments (spotted in duplicate). The third and fourth lanes are samples of untreated recombinant alpha-synuclein fibrils (spotted in duplicate).

Results

Denaturation of filaments reduces binding between 30-100 times, demonstrating the conformation-specificity of MJFR14.

Figure 3. MJFR14 binds to the linear epitope YQDYEP hexapeptide



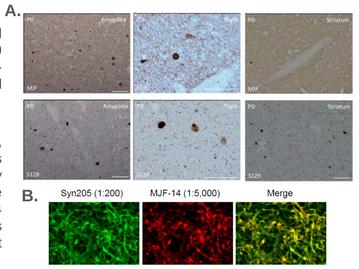
Description

A) Linear high resolution epitope mapping of MJFR14 at 1:100 (red) and 1:1000 (blue) dilutions. B) Alpha-synuclein amino acid sequences for the human and mouse protein with the epitope marked in red.

Results

A) A clear linear monoclonal response was observed with the C-terminal consensus motif YQDYEP with a high signal-to-noise ratio. Presumably this epitope is hidden in the monomeric form of the protein as the antibody does not bind soluble monomeric alpha-synuclein. The signal against peptide LEDMPVDPDNEAYEM at 1:100 was due to a microarray artifact. B) Epitope mapping reveals the hexapeptide YQDYEP as the epitope which corresponds to amino acids 133-138 in the C-terminal.

Figure 4. MJFR14 staining patterns in the PD amygdala, substantia nigra, and striatum resemble those of the pS129 antibody



Description

A) Immunostaining of human amygdala, substantia nigra, and striatum tissue from PD patients with MJFR14 or pS129 antibodies. B) ReNcell VM cells expressing human alpha-synuclein stained with Syn205 for total alpha-synuclein and MJFR14.

Results

A) MJFR14 stains inclusions in the amygdala, substantia nigra, and striatum with good contrast. MJFR14 staining resembles that of pS129, indicating that the antibody is detecting Lewy bodies in human PD tissue. Staining was not present in tissue from control, non-PD brains. B) Staining for MJFR14 significantly overlaps with Syn205 staining. Some cells/neurites weakly stained with Syn205 do not stain with MJFR14, most likely because MJFR14 is conformation-specific for filaments.

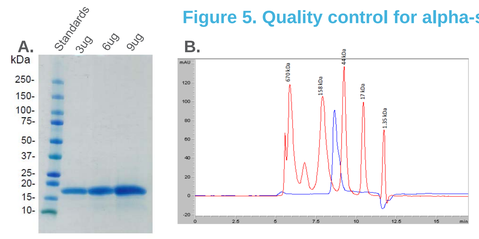
Alpha-Synuclein Protein Library

Recombinant alpha-synuclein proteins were produced and distributed by Proteos Inc. Monomers for PFFs were generated with input from Dr. Kelvin Luk. The pS129 alpha-synuclein protein was produced by CPC Scientific in close collaboration with and using the semi-synthetic strategies developed by Dr. Hilal Lashuel at EPFL. All proteins undergo quality control including gel electrophoresis and size exclusion chromatography.

Table 1. List of available alpha-synuclein proteins

Protein	Additional Information
Full-length human alpha-synuclein monomer	Monomeric form of filament protein
Full-length human alpha-synuclein filament	Used as the antigen for the MJFR14 antibody
Full-length human alpha-synuclein monomer for PFFs	Monomer for generating pre-formed fibrils (PFFs)
pS129 alpha-synuclein full-length human protein	Alpha-synuclein with phosphorylation at S129

Figure 5. Quality control for alpha-synuclein monomer protein



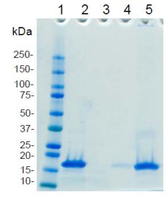
Description

A) Gel electrophoresis of 3, 6, and 9 ug alpha-synuclein monomer protein. B) Analytical SEC chromatographs with alpha-synuclein monomeric protein in blue and molecular weight standards in red.

Results

Alpha-synuclein monomers purified by heat precipitation, anion exchange, and reverse phase HPLC. A) Alpha-synuclein monomers migrate at a single band with an apparent molecular weight of 16.5 (theoretical 14.6 kDa) using gel electrophoresis. B) Sample purity is confirmed with size exclusion chromatography.

Figure 6. Quality control for alpha-synuclein filament protein



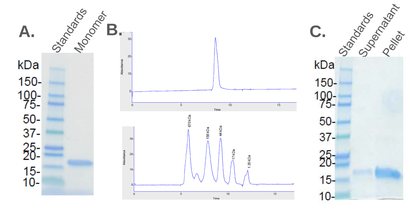
Description

Gel electrophoresis of alpha-synuclein filaments and the extracted supernatant after 14 days incubation to induce filaments and sonication or no sonication. Lane 1 contains the molecular weight standards. Lane 2 contains the filament mixture after 14 days incubation. Lane 3 contains supernatant collected after 14 days incubation. Lane 4 contains supernatant collected after 14 days incubation following sonication and clarification. Lane 5 contains the filaments pelleted after 14 days incubation following sonication and resuspension in buffer.

Results

Alpha-synuclein filaments are formed from the alpha-synuclein monomeric protein (described in Figure 5) following 14 days incubation at 37°C with gentle shaking. Gel electrophoresis reveals protein within the filamentous mixture without sonication and clarification (Lane 2) or following sonication and clarification (Lane 5). No alpha-synuclein protein is located within the supernatant fractions (Lanes 3-4).

Figure 7. Quality control for alpha-synuclein monomer protein for PFF generation



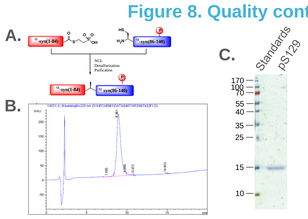
Description

A) Gel electrophoresis of alpha-synuclein monomeric protein generated for PFF production. B) Analytic SEC chromatographs for alpha-synuclein monomers (top) and molecular weight standards (bottom). C) Gel electrophoresis for alpha-synuclein PFFs generated from the monomers.

Results

Alpha-synuclein monomers purified by heat precipitation, ion exchange, and size exclusion chromatography. A) Alpha-synuclein monomers appear at the expected molecular weight of 16.5 kDa. B) Sample purity is confirmed by size exclusion chromatography. C) Monomers successfully generate alpha-synuclein pre-formed fibrils, confirmed with gel electrophoresis and Thioflavin T assay (PBS fluorescence = 28.3; monomer fluorescence = 29.0; fibril fluorescence = 735.5).

Figure 8. Quality control for alpha-synuclein protein phosphorylated at S129



Description

A) Schematic of the semi-synthetic strategy used to generate pS129 alpha-synuclein (taken from Lashuel *et al*, unpublished). B) HPLC to confirm molecular weight of the alpha-synuclein protein with phosphorylation at S129. C) Gel electrophoresis of the alpha-synuclein after phosphorylation ligation to confirm purity.

Results

A) Alpha-synuclein that is site-specifically phosphorylated at S129 was produced by native chemical ligation of a recombinant N-terminal 1-84 fragment with a C-terminal thioester and a C-terminal synthetic peptide with an N-terminal cysteine. B) HPLC confirms pS129 alpha-synuclein by size. C) The pS129 alpha-synuclein protein appears at the expected 15 kDa molecular weight after modification.

Upcoming Alpha-Synuclein Preclinical Tools

Extensive efforts are underway to continue development of alpha-synuclein molecular tools. These tools will include viral vectors, antibodies, assays, and animal models. Many of these projects were developed and supported in collaboration with the Michael J. Fox Foundation Industry Tools Consortium -- name denoted with an asterisk (*) in the table.

Table 2. List of alpha-synuclein tools currently in development

Tool Type	Name	Description	Availability
Animal Model	Alpha-synuclein KO Rat*	Rat with the alpha-synuclein gene knocked out	Early 2017
Animal Model	Alpha-synuclein A53T KI Rat*	Rat with humanized amino acids for A53T knocked into the endogenous locus	Early 2017
Antibody	Oligomeric alpha-synuclein antibody*	Conformation-specific antibody directed against the oligomeric form of alpha-synuclein	Late 2017
Assay	Alpha-synuclein aggregation assay*	Assay to measure alpha-synuclein aggregation *in vitro* (developed and provided by Charles River Discovery)	Early 2017 (Fee for Service)
Protein	Rat alpha-synuclein monomers for generating PFFs	Full-length rat alpha-synuclein monomeric protein to be used in the generation of rat PFFs	Mid 2017
Protein	Mouse alpha-synuclein monomers for generating PFFs	Full-length mouse alpha-synuclein monomeric protein for generating mouse PFFs	Mid 2017
Viral Vector	AAV1/2 3xmiR anti-SNCA (mouse)*	Viral vector encoding GFP and 3xmiR to alpha-synuclein for specific knockdown of mouse alpha-synuclein	Early 2017
Viral Vector	AAV1/2 3xmiR anti-SNCA (human)*	Viral vector encoding GFP and 3xmiR to alpha-synuclein for specific knockdown of human alpha-synuclein	Early 2017
Viral Vector	AAV2-CBA-Alpha-synuclein	Viral vector encoding human wild-type alpha-synuclein	Early 2018
Viral Vector	AAV5-CBA-Alpha-synuclein	Viral vector encoding human wild-type alpha-synuclein	Early 2018

Summary

The Michael J. Fox Foundation is invested in providing the PD research community with useful, high-quality tools to support rapid new discoveries in the PD field and encourage reliable, reproducible data. The tools described in this poster are the result of recent collaborative efforts aimed at generating molecular biology tools for alpha-synuclein-related research in particular.

More information on other alpha-synuclein tools or tools for other PD-related targets can be found in the MJFF tools catalog on our website. Information on the MJFF Industry Tools Consortium that is involved in generating many of the alpha-synuclein tools currently in development can also be found on the MJFF website. Questions regarding MJFF preclinical tools can be sent to tools@michaeljfox.org.