HHS Public Access

Author manuscript

Exp Eye Res. Author manuscript; available in PMC 2023 July 29.

Published in final edited form as:

Exp Eye Res. 2023 January ; 226: 109351. doi:10.1016/j.exer.2022.109351.

α-Synuclein modulates fibronectin expression in the trabecular meshwork independent of TGFβ2

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Abstract

α-Synuclein (α-Syn) is implicated in Parkinson's disease (PD), a neuromotor disorder with prominent visual symptoms. The underlying cause of motor dysfunction has been studied extensively, and is attributed to the death of dopaminergic neurons mediated in part by intracellular aggregation of α-Syn. The cause of visual symptoms, however, is less clear. Neuroretinal degeneration due to the presence of aggregated α-Syn has been reported, but the evidence is controversial. Other symptoms including those arising from primary open angle glaucoma (POAG) are believed to be the side-effects of medications prescribed for PD. Here, we explored the alternative hypothesis that dysfunction of α-Syn in the anterior eye alters the interaction between the actin cytoskeleton of trabecular meshwork (TM) cells with the extracellular matrix (ECM), impairing their ability to respond to physiological changes in intraocular pressure (IOP). A similar dysfunction in neurons is responsible for impaired neuritogenesis, a characteristic feature of PD. Using cadaveric human and bovine TM tissue and primary human TM cells as models, we report two main observations: 1) α-Syn is expressed in human and bovine TM cells, and significant amounts of monomeric and oligomeric α-Syn are present in the AH, and 2) primary human TM cells and human and bovine TM tissue endocytose extracellular recombinant monomeric and oligomeric α-Syn *via* the prion protein (PrP^C), and upregulate fibronectin (FN) and α-smooth muscle actin (α-SMA), fibrogenic proteins implicated in POAG. Transforming growth factor β2 (TGFβ2), a fibrogenic cytokine implicated in ~50% cases of POAG, is also

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Author contributions

NS: conception and design of the study, interpretation of data, and manuscript preparation, AA, UP, AA: carried out experimental procedures, AK, SP, HL, PK, MK: helped with experiments, DR, EL: provided helpful input.

Declaration and competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2022.109351.

increased, and so is RhoA-associated coiled-coil-containing protein kinase 1 (ROCK-1). However, silencing of α-Syn in primary human TM cells reduces FN, α-SMA, and ROCK-1 in the *absence or presence* of over-expressed active TGFβ2, suggesting modulation of FN and ROCK-1 independent of, or upstream of TGFβ2. These observations suggest that extracellular α-Syn modulates ECM proteins in the TM independently or *via* PrP^C by activating the RhoA-ROCK pathway. These observations reveal a novel function of α-Syn in the anterior eye, and offer new therapeutic options.

Keywords

α-Synuclein; TGFβ2; Glaucoma; Trabecular meshwork; Fibronectin; α-smooth muscle actin

1. Introduction

α-Synuclein (α-Syn) is a ubiquitously expressed actin-binding protein most abundant in neuronal cells (Sousa et al., 2009; Oliveira-da-Silva & Liz, 2020). Although mainly known for its role in synaptic vesicle trafficking, α-Syn is involved in several cellular functions such as actin cytoskeleton-extracellular matrix (ECM) interaction, autophagy, mitochondrial function, and others (Auluck et al., 2010; Burré et al., 2018; Bogale et al., 2021). Aggregation of α-Syn is associated with Parkinson's disease (PD), a neurological disorder with prominent motor and visual symptoms (Armstrong, 2011; Henderson et al., 2019; Hunn et al., 2015). Some of the visual symptoms are due to aggregation of α-Syn in the outer and inner retina, where it is believed to perform diverse functions (Bodis-Wollner et al., 2013). Aggregates of α-Syn have been described in the retina of some PD cases (Leger et al., 2011; Beach et al., 2014; Bodis-Wollner et al., 2014; Miri et al., 2016; Veys et al., 2019), but are absent in others, leaving the matter unsettled (Ho et al., 2014). The association of primary open angle glaucoma (POAG) with PD, likewise, is questionable. Some reports suggest a close association, while others indicate no evidence (Barbara et al., 2017; Ekker et al., 2017; Matlach et al., 2018; Moon et al., 2018). It is believed that elevation of intraocular pressure (IOP), the hallmark of POAG, is a side-effect of anticholinergic agents and L-DOPA commonly used to alleviate motor symptoms of PD (Pescosolido et al., 2013; Reitsamer and Kiel, 2002). A comprehensive analysis of the expression pattern, functional role, and pathobiology of α-Syn in the anterior eye, in particular POAG, is lacking.

POAG is associated with chronic elevation of intraocular pressure (IOP) due to inadequate drainage of aqueous humor (AH) to the venous circulation. Normally, IOP is maintained within physiological limits by balancing the production of AH by epithelial cells of the ciliary body (CB) with drainage by the conventional and non-conventional pathways (Goel et al., 2010; Kaufman, 2020). For exit through either pathway, AH must pass through the trabecular meshwork (TM), a sheet of endothelial cells embedded in a meshwork of ECM proteins (Abu-Hassan et al., 2014; Stamer and Clark, 2017). The composition of ECM proteins changes in response to mechanical stretch and distortion produced by the changes in IOP (Ramos et al., 2009). Integrins and other ECM receptors in TM cells sense the distortion, and initiate corrective responses to adjust outflow resistance. Abnormal deposition of ECM proteins interferes with this response, predisposing to POAG (Vranka et

al., 2015). Transforming growth factor β2 (TGFβ2), a fibrogenic cytokine, is increased in the AH of ~50% of POAG cases, and predisposes to POAG by increasing the synthesis of ECM proteins (Taylor, 2012). The principal pathways implicated in POAG are the Ras homolog gene family member A (RhoA) and its downstream effector Rho-associated protein kinase (ROCK), and Suppressor of Mothers against Decapentaplegic (SMAD) that upregulate several fibrogenic proteins when activated (Prendes et al., 2013; Derynck et al., 2014; Gauthier and Liu, 2017). In fact, inhibitors of RhoA-ROCK are currently in use to alleviate the symptoms of POAG (Rao et al., 2017; Tanna and Johnson, 2018; Buffault et al., 2022).

Here, we evaluated the expression of α-Syn in the anterior eye, and whether it plays a role in POAG. Primary human TM cells and TM tissue isolated from human and bovine cadaveric eye globes were used for this study. The presence of α-Syn the AH was also examined since it is released from cells in exosomes, which are abundant in the AH and facilitate the communication between AH producing cells in the CB and draining cells in the TM (Dismuke et al., 2015; Hessvik and Llorente, 2017; Lerner et al., 2017; Liu et al., 2020). We report that α-Syn is expressed in the TM and is present in the AH, and uptake of α-Syn by TM cells occurs *via* PrP^C as reported in neuronal cells (Urrea et al., 2018; Aulić et al., 2017; Cecco and Legname, 2018; Corbett et al., 2020; Ferreira et al., 2017; Legname and Scialò, 2020; Rösener et al., 2020). Implications of our observations for POAG are discussed, keeping in mind the limitations of this study.

2. Materials and methods

2.1. Ethics statement

All experiments involving human eye globes, human AH and VH, and human primary TM cells were performed in compliance with the tenets of the Declaration of Helsinki.

2.2. Experimental design

Expression of α-Syn in primary human TM cells and human and bovine TM tissues was evaluated by immunoblotting and immunohistochemistry. The effect of intracellular and extracellular α-Syn on ECM proteins in the absence or presence of active TGFβ2 was determined by immunoblotting and immunocytochemistry.

2.3. Reagents and chemicals

Stocks of replication defective adenoviral vector expressing active TGFβ2 (AdTGFβ2) and empty vector (AdEmpty) were obtained from the University of Iowa (Shepard et al., 2010). RNAiMax was obtained from Invitrogen, USA, and was used as directed (Cat. No: 13778075). Dexamethasone (D1756) was obtained from Sigma Aldrich, USA. siRNA stocks for α-Syn (sc 29619) and scrambled sequences (sc37007) were obtained from Santa Cruz Biotechnology, USA. Monomeric human recombinant α-Syn (S-1001-2) was obtained from rPeptide, USA.

2.4. Human and bovine eye globes

Cadaveric human eye globes were obtained from Lions Gift of Sight eye bank (St. Paul, MN, USA). Donors ranged in age from 65 to 93 years (Supplementary Table 1). Seven pairs of human eyes were used for this study. After harvesting the AH, tissues were used for the isolation and culture of primary human TM cells and TM tissue. Bovine eyes were collected from a local abattoir within 15 min to 2 h of sacrifice, and processed for immunoblotting or immunocytochemistry.

2.5. Isolation and culture of primary human TM cells and tissue

TM cells were isolated from human eye globes and cultured in DMEM supplemented with 1% FBS as described (Keller et al., 2018). Cultures from passage 3–7 were used for all experiments. Each culture was tested for dexamethasone-induced upregulation of myocilin before use (Ashok et al., 2019, 2020). For *in-situ* culture of TM tissue, de-identified human (Miracles In Sight, Winston-Salem, NC) and bovine corneal rims were cultured as described (Kasetti et al., 2020). In short, isolated corneal rims were divided into four quadrants, and cultured in 6-well plates in DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin (Kasetti et al., 2020). After stabilizing the culture for 3 days, 100 nM dexamethasone was added, and after an overnight culture, TM tissue was scraped and immunoblotted to check for upregulation of myocilin relative to vehicle-treated controls. Parallel cultures were treated with recombinant α-Syn or transduced with TGFβ2 for the indicated times. Harvested TM tissue was processed for immunoblotting.

2.6. Transfection of TM cells, SDS-PAGE, and immunoblotting

To silence α-Syn, primary human TM cells were plated at a density of 70%, and exposed to siRNA specific for α-syn and scrambled siRNA mixed with Lipofectamine RNAimax transfection reagent (Cat # 13778100, Thermo-Fisher) in complete medium for 48 h. Silencing of α-syn was confirmed by immunoblotting using specific antibodies (Supplementary Table 2). In-vitro transduction of TM tissue with AdTGFβ2 or AdEmpty was performed by adding AdTGFβ2 or AdEmpty to a final concentration of 7×10^7 pfu/ml in complete medium, and incubating the tissue for 48 h before evaluation. The efficiency of transfection with siRNA and transduction with TGFβ2 was confirmed by immunoblotting with specific antibodies (Supplementary Table 2). SDS-PAGE and immunoblotting were performed by conventional techniques as described in previous reports (Ashok et al., 2018; Baksi et al., 2016; Baksi and Singh, 2017).

2.7. Immunofluorescence analysis

Enucleated human and bovine eyes were fixed in 4% paraformaldehyde in PBS overnight, followed by 95% ethanol for 24 h at room temperature. Fixed eyes were embedded in paraffin, and thin sections of 4 μm were deparaffinized, rehydrated, and subjected to antigen retrieval by heating to 97 °C in the presence of 25 mM Tris-1-mM EDTA (pH8.5) for 20 min. Subsequently, sections were blocked with 1% BSA, washed with PBS, and immunostained for α-Syn and fluorophore-conjugated secondary antibody (Supplementary Table 2) as described (Ashok et al., 2018, 2019, 2020; Baksi et al., 2016). The nuclei were stained with Hoechst (# 33342, Invitrogen, USA), and the sections mounted in Fluoromount-

G (Southern Biotech, USA) and imaged. For imaging of TM cells exposed to fibrillary recombinant α-Syn, cells cultured on cover-slips were exposed to 0.5 μg/ml of α-Syn for 2 h in complete medium, washed with PBS, and fixed. The antigen-retrieval step was omitted, and the cells were immunoreacted with PrP^C-specific antibody (3F4) followed by Alexa-flour-conjugated anti-mouse secondary antibody (red). Subsequently, cells were reacted with rabbit-α-Syn antibody followed by anti-rabbit secondary antibody conjugated with FITC. The nuclei were stained with Hoechst as above, and the cells were mounted and imaged. To visualize β1-integrin, cells were exposed to fibrillary α-Syn for 2 h as above, washed with PBS, fixed, and reacted with rabbit active β1-integrin antibody and mouse α-Syn antibody followed by species-specific secondary antibodies conjugated with Alexa-fluor (red) or FITC (green). Stained cells were mounted and imaged as above. Each experiment was repeated three times using TM cells from three different donors. Representative images from 20 different fields are shown. A complete list of antibodies is provided in Supplementary Table 2.

2.8. Preparation of α-syn fibrils

Fibrils of α-Syn were prepared from 1 mg of monomeric recombinant α-Syn reconstituted in 0.5 ml of water, and incubated at 37 °C with shaking for 7 days (Narkiewicz et al., 2014). The mix was supplemented with 10X PBS, and 0.5 or 1 μg/ml of fibrillated α-Syn or vehicle was added to primary human TM cells for 2 or 24 h and processed for immunofluorescence or immunoblotting respectively.

2.9. Statistical analysis

Densitometry of images was performed with UN-SCAN-IT gels (version 6.1) software (Silk Scientific, USA) and Image J software. All data were statistically analyzed by GraphPad Prism (version 5.0) software (GraphPad Software Inc., USA), and are shown as mean ± SEM. Significant differences between control and experimental samples were determined by the Student's unpaired *t*-test or 2-way ANOVA. Differences were considered statistically significant starting at $P < .05$.

3. Results

3.1. α-Syn is detected in the trabecular meshwork and AH

Ocular tissue is known to express all three members of the Synuclein family, i.e. α, β, and γ-Synuclein (Surguchov et al., 2001). To evaluate the expression of α-Syn in particular, antibody specificity was tested by immunoblotting lysates prepared from primary human TM cells treated with scrambled sequence or siRNA unique to α-Syn (Supplementary Fig. S1). Probing with α-Syn-specific antibody revealed a single band of 14 kDa in the sample treated with scrambled (Scr) sequence, and almost complete absence in lysates where α-Syn had been silenced (Fig. 1 A, lanes 1 & 2). The membrane was re-probed for β-actin to control for protein loading.

Subsequently, the retina, CB, and TM tissue were isolated from human cadaveric eye within 24 h of death, and lysates were subjected to immunoblotting as above. Probing for α-Syn revealed a single band migrating at 14 kDa in all samples (Fig. 1 B, lanes 1–3). Evaluation

of 30 μl of non-concentrated AH and VH showed a prominent band of monomeric and oligomeric bands of α-Syn migrating at 14 kDa, ~28 kDa, and 55 kDa respectively (Fig. 1 B, lanes 4 & 5). A similar migration of oligomeric α-Syn has been reported by Lehri-Boufala et al. (2015). Re-probing for β-actin reveled more protein loading in the TM and CB sample relative to retinal lysates, indicating higher expression of α-Syn in the retina relative to the CB and TM. No reactivity for β-actin was detected in the AH and VH as expected (Fig. 1 B, lanes 1–5).

A similar analysis of freshly harvested bovine eye globes within 15 min of culling revealed monomeric α-Syn in all three tissues that increased in intensity with increasing protein loading (Fig. 1 C, lanes 1–7), confirming the antibody specificity for bovine tissue as well. The membrane was re-probed for β-actin as above (Fig. 1 C, lanes 1–7).

To evaluate the localization of α-Syn in the TM and ciliary epithelial cells, fixed sections of human and bovine anterior segment were immunostained with α-Syn antibody followed by species-specific secondary antibody conjugated to a fluorescent probe. In both human and bovine samples, immunoreaction for α-Syn was detected in TM cells (Fig. 2, panels 1 & 2, arrowhead). In the ciliary epithelium, reactivity was limited to non-pigmented epithelial (NPE) cells of the ciliary processes (Fig. 2, panels 1 & 2, arrowhead). Serial sections immunostained with isotype specific antibody and the same secondary antibody showed no reactivity, confirming the specificity of staining (Supplementary Fig. S2, panels 1–4).

Thus, α-Syn is expressed in the human and bovine retina, NPE cells, and TM cells, and soluble α-Syn is present in the AH and VH. Subsequent studies were aimed at evaluating the functional significance of α-Syn in the AH and the TM, and its role in POAG.

3.2. Extracellular α-syn upregulates fibronectin and ROCK-1 in primary human TM cells

In neurons, preformed fibrils of α-Syn induce aggregation of cell-associated α-Syn and interfere with synaptic activity (Wu et al., 2019). A similar scenario can be envisioned in the anterior eye, where oligomeric α-Syn in the AH could be taken up by TM cells. To evaluate this possibility, monomeric recombinant α-Syn was oligomerized by shaking at 37 °C for 7 days (Fig. 3 A), and 0.5 and 1 μg/ml of the mix was added to primary human TM cells in complete medium. Control cells received the same volume of buffer in complete medium. After 24 h, the cells were washed with PBS, and the lysates evaluated by immunoblotting. Probing with specific antibodies revealed a concentration-dependent increase in ROCK-1, FN, and α-SMA in cells exposed to recombinant α-Syn relative to controls (Fig. 3 B, lanes 2 & 3 vs. 1, left panel). Re-probing for TGFβ2 showed a similar increase in active TGFβ2, but no change in pro-TGFβ2 (Fig. 3 B, lanes 1–3, right panel). A concentration dependent increase in α-Syn was also noted, indicating internalization of added recombinant protein (Fig. 3 B, lanes 2 & 3 vs. 1, right panel). Quantification by densitometry after normalizing with GAPDH showed a significant increase in FN, α-SMA, ROCK-1, and active TGFβ2 by extracellular α-Syn in a concentration dependent manner relative to controls (Fig. 3 C). FN levels in the media were not assessed, which is a limitation of this study.

These observations indicate that extracellular α-Syn is internalized by primary human TM cells, and increases FN and α-SMA probably by activating ROCK-1. Surprisingly, levels of

active TGFβ2 were also increased, suggesting TGFβ2-mediated activation of ROCK-1 as a possibility.

3.3. TGFβ2 upregulates α-syn in primary human TM cells and bovine TM tissue

Upregulation of α-Syn by active TGFβ2 in primary human TM cells was unexpected, raising the possibility of reduced clearance due to inhibition of the autophagosomal-lysosomal pathway by TGFβ2 (Nettesheim et al., 2019).

To evaluate this possibility, primary human TM cells were transfected with adenoviral vector expressing active TGFβ2 (AdTGFβ2) or empty vector (AdEmpty) (Shepard et al., 2010). Following an incubation of 48 h, lysates were fractionated by SDS-PAGE and subjected to immunoblotting. Probing for α-Syn and the autophagosomal marker LC3-II revealed upregulation in cells over-expressing active TGFβ2 relative to vector controls (Fig. 4 A, lane 2 vs. 1). Probing for active TGFβ2 showed upregulation as expected (Fig. 4 A, lane 2 vs. 1). Quantification by densitometry after normalizing with β-actin showed significant upregulation of α-Syn and LC3-II in cells over-expressing active TGFβ2 relative to controls (Fig. 4 B).

A similar analysis was performed in *ex-vivo* cultures of bovine TM tissue isolated and cultured *in-vitro* as described (Kasetti et al., 2020). In short, corneal rims of freshly harvested bovine eye globes were cultured with TM tissue intact, and infected with AdTGFβ2 or AdEmpty as above. After 48 h, TM tissue was isolated, lysed, and clarified protein lysates were evaluated by immunoblotting. As in primary human TM cells, both α-Syn and LC3-II were increased by active TGFβ2 relative to vector controls (Fig. 4 C, lane 2 vs. 1). Quantification by densitometry after normalization with β-actin showed significant upregulation of α-Syn and LC3-II by active TGFβ2 relative to controls (Fig. 4 D).

These data indicate that active TGFβ2 increases cell-associated α-Syn partly due to inhibition of its degradation by the lysosomal pathway. Since α-Syn itself is known to inhibit autophagy (Arotcarena et al., 2019; Winslow and Rubinsztein, 2011), it is likely to accumulate in autophagolysosomes, which are known to release their content in exosomes to the extracellular milieu when overwhelmed (Hessvik and Llorente, 2017). This would create a feed-forward cycle between α-Syn and TGFβ2, predisposing to POAG.

3.4. Silencing of α-syn in TM cells disrupts TGFβ2-mediated activation of ROCK-1

To evaluate whether α-Syn activates ROCK-1 by increasing the levels of active TGFβ2, α-Syn was downregulated in primary human TM cells with siRNA for 48 h, and the cells were transfected with AdTGFβ2 or AdEmpty for additional 48 h. Control cells treated with scrambled sequence (Scr) were transfected with AdTGFβ2 or AdEmp in parallel. Subsequently, the cell lysates were fractionated on two separate gels and analyzed by immunoblotting. One membrane was probed for FN, ROCK-1, and β-actin, and the other for α-SMA and α-tubulin since the migration of β-actin overlaps with α-SMA (Fig. 5 A).

In cells treated with scrambled sequence, overexpression of active TGFβ2 induced upregulation of FN, ROCK-1, and α-SMA relative to empty vector as expected (Fig. 5 A, lane 2 vs. 1). However, to our surprise, silencing of α-Syn reduced the expression

of FN, ROCK-1, and α-SMA in the *absence* (Fig. 5 A, lane 3 vs. 1) and *presence* of active TGFβ2 (Fig. 5 A, lane 4 vs. 2). Quantification by densitometry after normalization with β-actin or α-tubulin showed significant upregulation of FN, α-SMA, and ROCK-1 by TGFβ2 as expected, and a significant decrease in all three proteins by silencing α-Syn despite over-expression of active TGFβ2 (Fig. 5 B).

To further validate the above results, α-Syn was silenced in primary human TM cells with siRNA or scrambled sequence for 48 h, and lysates were fractionated on three separate gels and evaluated by immunoblotting. Probing for FN and ROCK-1 revealed decreased expression in cells where α-Syn had been silenced relative to controls (Fig. 5 C, lane 2 *vs.* 1, left panel). Probing for α-SMA also showed decreased expression by silencing of α-Syn (Fig. 5 C, lanes 4–6 vs. 1–3, right panel). Quantification by densitometry revealed a significant decrease in FN, α-SMA, and ROCK-1 on silencing of α-Syn relative to controls (Fig. 5 D). Surprisingly, silencing of α-Syn downregulated active TGFβ2 as well (Fig. 6 A & B), though not to the same extent as FN, α-SMA, and ROCK-1.

These results indicate that α-Syn functions independently, or upstream of TGFβ2 to alter the synthesis of ECM proteins.

3.5. Extracellular α-syn is internalized by PrP^C in primary human TM cells

In neuronal cells, extracellular α-Syn is internalized by PrP^C in addition to other mechanisms, including non-specific uptake (Aulić et al., 2017; Rodriguez et al., 2018; Rösener et al., 2020). A similar scenario in the anterior eye, where α-Syn in the AH (Fig. 1 C) is internalized by PrP^C on TM cells is likely to downregulate PrP^C, resulting in upregulation of ECM proteins and POAG as described in a previous report (Ashok et al., 2019). To evaluate this possibility, primary human TM cells were cultured overnight on coverslips, and exposed to 0.5 μg/ml of recombinant α-Syn re-suspended in complete medium for 2 h. Control cells received only medium. At the indicated time, the cells were washed, fixed in paraformaldehyde, and immunostained sequentially for α-Syn and PrP^C followed by species-specific secondary antibodies conjugated with red (PrP^C) or green (α-Syn) fluorophores (Fig. 7). In control cells, reactivity for PrP^C is prominent on the plasma membrane and intracellular vesicles as expected (Fig. 7, panel 1, arrow-heads). α-Syn is mainly localized to the cytoplasm (Fig. 7, panels 2 & 3). Cells exposed to recombinant α-Syn, on the other hand, show clustering of PrP^C and co-localization with α-Syn at the plasma membrane. Co-localization in intracellular aggregates is also evident, suggesting endocytosis of the PrP^C-α-Syn complex (Fig 7, panels 4–6, arrow-heads).

Based on these observations, extracellular α-Syn is internalized by PrP^C, and is likely degraded in an intracellular compartment or externalized in exosomes.

3.6. Extracellular α-syn co-localizes with β1-integrin on primary human TM cells

Since human AH contains mostly oligomeric α-Syn (Fig. 1 C), monomeric recombinant α-Syn was oligomerized as in Fig. 3 above, and 0.5 μg/ml of this mix was added to primary human TM cells cultured overnight on coverslips. Control cells received equal volume of buffer in complete medium. After 2 h, the cells were fixed and immunostained for α-Syn and active β1-integrin followed by species-specific secondary antibody conjugated with

green (α-Syn) or red (active β1-integrin) fluorophore (Fig. 8). In control cells, reactivity for endogenous α-Syn was minimal and restricted to the cytosol (Fig. 8, panels 1–3). Reactivity for active β1-integrin was prominent on the cell surface and in intracellular compartments with minimal overlap with α-Syn (Fig. 8, panels 4–6). Incubation with recombinant α-Syn, on the other hand, revealed a punctate reaction for α-Syn and β1-integrin on the plasma membrane, and almost complete co-localization of α-Syn with β1-integrin (Fig. 8, panels 4–6, arrow-heads). Controls reacted with isotype-specific mouse and rabbit antibody and the same secondary antibodies showed no reactivity (preliminary Fig. S3, panels 1 & 2).

Thus, aggregated α-Syn induces clustering of β1-integrin on the plasma membrane of TM cells, probably by binding to PrP^C and inducing its endocytosis.

4. Discussion

We describe a novel function of α-Syn in modulating ECM proteins in the TM directly, or indirectly *via* PrP^C or other unidentified pathways. Activation of the RhoA-ROCK pathway by extracellular α-Syn, and its disruption by silencing cell-associated α-Syn in the presence or absence of over-expressed active TGFβ2 is perplexing. The first observation can be explained by the positive feedback loop between α-Syn and TGFβ2, a known stimulator of this pathway, and by downregulation of PrP^C, a known receptor for α-Syn that activates ROCK when silenced (Alleaume-Butaux et al., 2015; Kim et al., 2017, 2020). However, disruption of this pathway by silencing cell-associated α-Syn is harder to explain. It could be simply due to reduced levels of α-Syn in the extracellular milieu, or alternate signaling pathways that require further exploration.

The expression of α-Syn in the anterior segment of the human, bovine, and mouse eye (data not shown), and especially its abundant presence in the AH was unexpected. The presence of mainly oligomeric forms in the AH was another surprise. The factors that drive oligomerization of monomeric α-Syn in the AH are not clear. This is important to understand because oligomeric and aggregated α-Syn is endocytosed readily by PrP^C, as are other aggregated proteins implicated in neurodegenerative conditions (Legname and Scialò, 2020). It is possible that oligomerization of α-Syn occurs before its release from cells, within exosomes, or while in solution in the AH. This appears to be a physiological process since all human eye globes were from non-PD cases, and ranged in age from 65 to 93 years. It is unclear whether oligomerization of α-Syn in the AH increases with age or in clinical PD. Studies are ongoing to collect sufficient number of AH samples from PD cases to explore this possibility. We ruled out the possibility that α-Syn in the AH is shed from dead or dying cells lining the anterior and posterior chambers because similar levels were present in bovine eye globes harvested within 15 min of culling and stored at 4 °C for up to 1 week (data not shown).

The functional role of α-Syn has been studied extensively in neurons to understand the pathobiology of PD. An example pertinent to this study includes activation of ROCK-2, the brain isoform of ROCK, in PD (Iyer et al., 2020). This is believed to trigger various risk factors for PD, including aggregation of α-Syn, dysregulation of autophagy, and death of dopaminergic neurons. Activation of the RhoA-ROCK pathway also plays a major role

in human sporadic PD and toxin-induced animal models of PD such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and 6-OHDA (6-hydroxydopamine) (Koch et al., 2018). Collectively, there is enough evidence implicating ROCK activation in PD to consider inhibitors of this pathway as a therapeutic option (Saal et al., 2015; Tatenhorst et al., 2016; Moskal et al., 2022). However, it remains unclear whether aggregation of α-Syn *per se* plays a major role in activating ROCK-2. If this is the case, the presence of oligomeric or aggregated α-Syn in the anterior eye could have major implications where activation of ROCK-1, the isoform expressed in the eye, could upregulate ECM proteins in the TM and possibly other cell types with adverse consequences.

Based on our data, two possible scenarios by which α-Syn could upregulate FN and activate ROCK-1 can be envisioned: 1) by increasing active TGFβ2 (Medina-Ortiz et al., 2013), and 2) by inducing the endocytosis and degradation of PrP^C, which activates ROCK by inducing the clustering of β1-integrin (Schneider et al., 2021). Considering the first possibility, the mechanism by which extracellular α-Syn increases active TGFβ2, which in turn increases cell-associated and consequently extracellular α-Syn, is not very clear. One possibility is that uptake of extracellular α-Syn increases the secretion of metalloproteases and other enzymes that cleave LAP-TGFβ2 to its active form (Robertson & Rifkin et al., 2016). Once initiated, TGFβ2-mediated inhibition of the autophagosomal-lysosomal pathway (Nettesheim et al., 2019) is likely to increase cell-associated α-Syn, thus increasing its release in exosomes or by other means into the AH (Gustafsson et al., 2018). Since α-Syn itself inhibits autophagy (Arotcarena et al., 2019; Nascimento et al., 2020; Sarkar et al., 2021), a feed-forward loop between α-Syn and active TGFβ2 is likely to ensue. Silencing of α-Syn in TM cells decreases active TGFβ2, partly supporting this assumption. The fact that FN, α-SMA, and ROCK-1 are also decreased when α-Syn is silenced suggests that either secretion of cell-associated α-Syn to the extracellular milieu and subsequent endocytosis is the main event driving the synthesis of FN and possibly other ECM proteins, or α-Syn participates in a novel pathway that requires further exploration.

Regarding the second possibility, endocytosis of extracellular α-Syn is mediated by PrP^C in neurons (Urrea et al., 2018; Aulić et al., 2017; Cecco et al., 2017; Corbett et al., 2020; Ferreira et al., 2017; Legname and Scialò, 2020; Rösener et al., 2020), and in TM cells as well, as is evident from co-localization of PrP^C and α-Syn in intracellular compartments (Fig. 7), reducing the levels of PrP^C on the plasma membrane. Downregulation of PrP^C in TM cells is known to upregulate several ECM proteins, resulting in POAG (Ashok et al., 2019). Though novel in TM cells, this phenomenon is well-known in neuronal cell lines where absence of functional PrP^C on the plasma membrane induces clustering of β1-integrin and activation of ROCK-2 (Schneider et al., 2021). Since α-Syn binds amino acids 93 to 109 in the N-terminus of PrP^C (Ferreira et al., 2017; Cecco and Legname, 2018), internalization of the α-Syn-PrP^C complex is likely to decrease functional PrP^C on the plasma membrane. It is pertinent to mention here that majority of PrP^C in the human eye is physiologically cleaved near amino acid ~90, leaving the α-Syn binding site intact (Chaudhary et al., 2021). This contrasts with the human brain and ocular tissues of large animals where majority of PrP^C is cleaved at amino acids 111/112, excluding the α-Syn binding site (Chen et al., 1995). Thus, α-Syn-mediated modulation of ECM proteins *via* PrP^C, though unlikely in the

human brain and ocular tissue of most animal species, is likely to occur in the human eye, leading to increased synthesis of ECM proteins and POAG.

In conclusion, our report demonstrates an unprecedented role of intracellular and extracellular α-Syn in modulating the expression of FN and possibly other ECM proteins in the TM. Combined with the fact that α-Syn has the potential to spread to neighboring cells in a prion-like fashion (Danzer et al., 2012; Brundin and Melki, 2017), these observations have significant implications for POAG. Additional studies are necessary to understand the underlying mechanism fully.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Neil A Rana, Aaron S Wise, Shu G Chen, Luke Brandon, and Jason Walker for help experimental procedures and helpful comments on the manuscript, and Mellissa A. Pottinger at the Lions Eye Bank for cadaveric human eye globes.

Funding

This study was supported by grants NS092145, NS125228, and EY032694 to NS from the National Institutes of Health (NIH), USA.

Data availability

All pertinent data are included in the manuscript.

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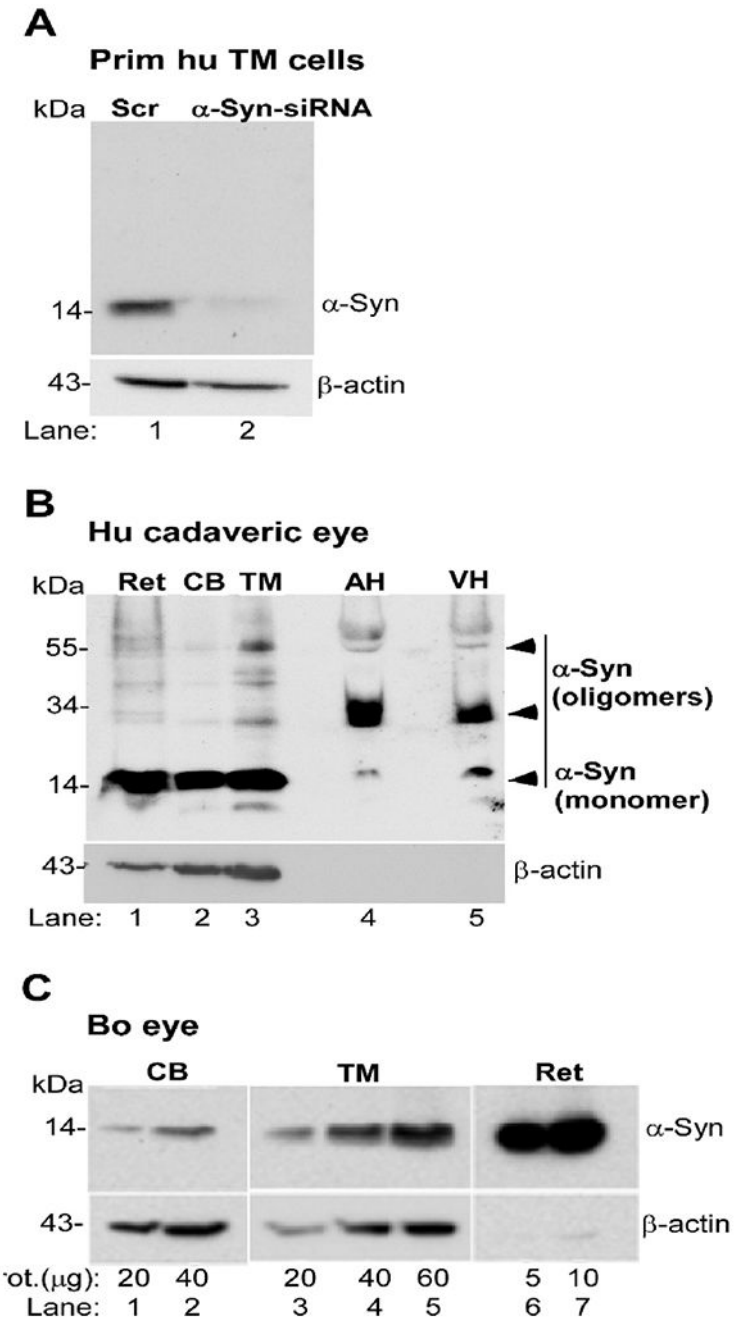

Fig. 1. α-Syn is expressed in the anterior segment of human and bovine eye.

(A) Primary human TM cells were treated with scrambled (Scr) sequence or siRNA for α-Syn. Probing of lysates for α-Syn shows a single band migrating at 14 kDa in samples treated with scrambled sequence, and significant down-regulation in samples exposed to α-Syn siRNA (lanes 1 & 2). **(B)** Immunoblot analysis of the neuroretina, CB, and TM isolated from human cadaveric eye shows expression of monomeric α-Syn in all three samples (lanes 1–3). A similar analysis of AH and VH shows monomeric and oligomeric α-Syn (lanes 4 & 5). All membranes were re-probed for β-actin to control for loading. **(C)**

Immunoblotting of protein lysates from bovine CB, TM, and retina shows expression of α-Syn in all three tissues, with significantly higher expression in the retina (lanes 1–7).

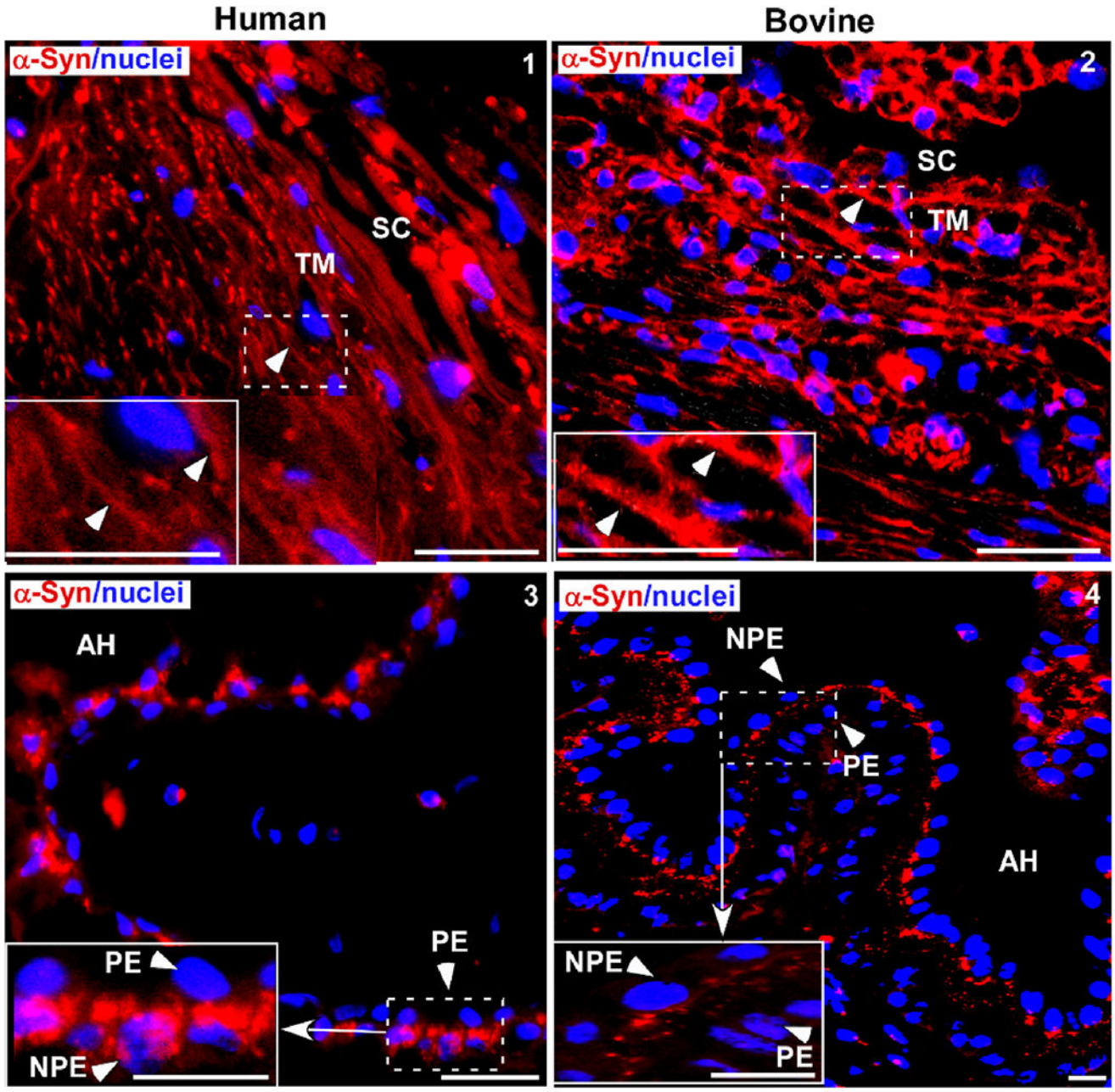

Fig. 2. Localization of α-Syn in the CB and TM:

Thin sections of fixed human and bovine anterior segment were exposed to antibody for α-Syn followed by fluorophore-conjugated secondary antibody. Strong reactivity for α-Syn is seen on the plasma membrane of human and bovine TM cells (panels 1 & 2). In the ciliary epithelium, the reactivity is limited to NPE cells (panels 3 & 4, insets). TM: trabecular meshwork, SC: Schlemm's canal, NPE: non-pigmented epithelium, PE: pigmented epithelium. Scale bar: 10 μm.

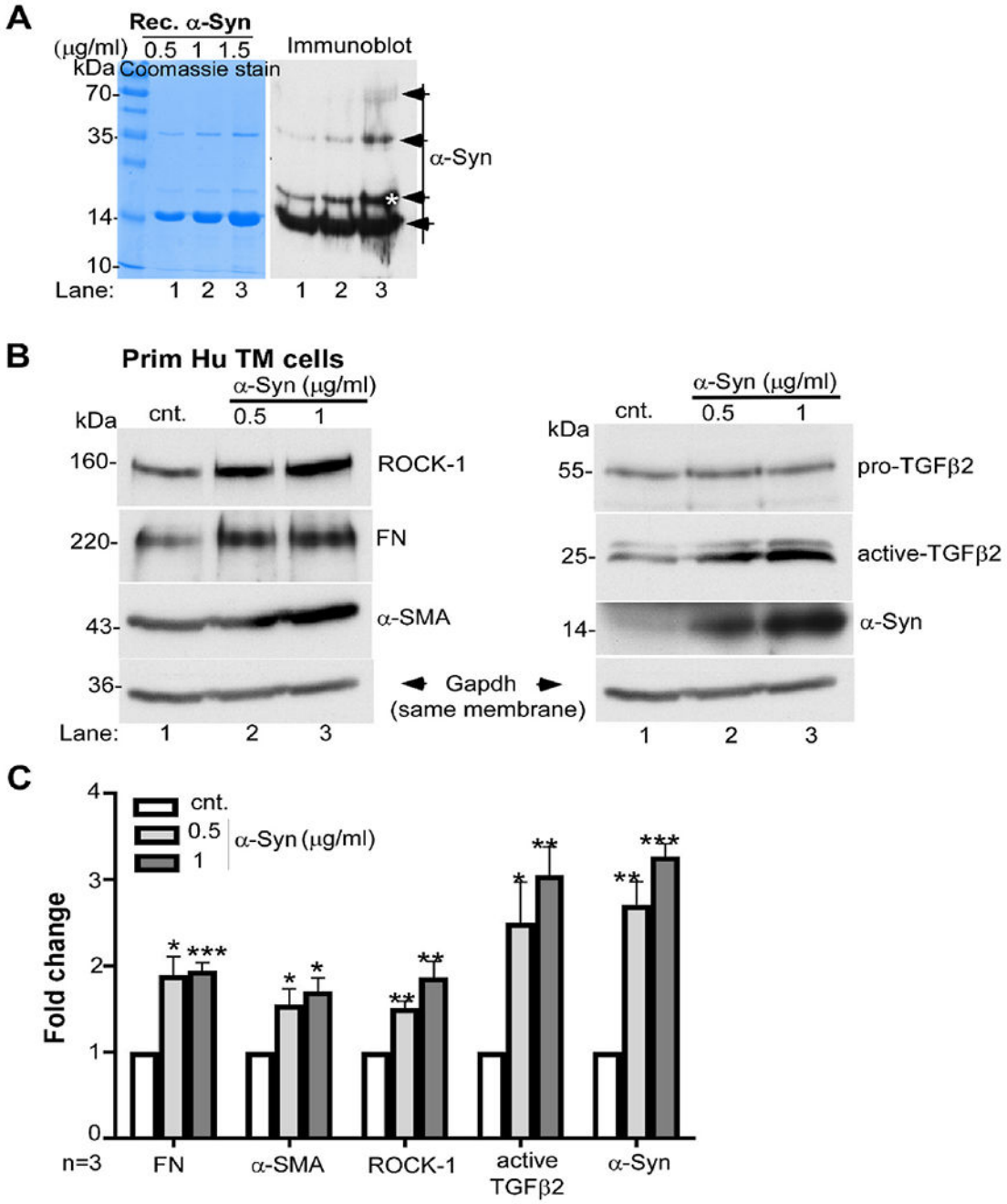

Fig. 3. Uptake of recombinant α-Syn increases FN, α-SMA, ROCK-1, and active TGFβ2 in primary human TM cells:

(A) In-gel Coomassie-blue staining of partly oligomerized recombinant α-Syn shows monomeric and oligomeric forms (left panel) that immunoreact with α-Syn antibody (right panel). **(B)** Cultures of primary human TM cells were exposed to vehicle (cnt) or 0.5 and 1 μg/ml of oligomerized α-Syn in complete medium for 24 h, and processed for immunoblotting. Probing for ROCK-1, FN, α-SMA (left panel), active TGFβ2, and α-Syn (right panel) shows a dose-dependent increase in cells exposed to extracellular α-Syn

relative to respective controls. There is no change in pro-TGFβ2 (lanes 2 & 3 *vs.* 1). Increased signal for α-Syn is probably due to internalization of the added recombinant form. Membrane was re-probed for GAPDH as a loading control (shown twice for clarity). **(B)** Densitometric analysis following normalization with GAPDH shows significant upregulation of ROCK-1, FN, and α-SMA by ~2-fold, active TGFβ2 by 2.5 and 3-fold, and α-Syn by 2.8 and 3-fold in cells exposed to α-Syn relative to controls. n represents the number of biological replicates. *p < .05, **p < .01, ***p < .001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

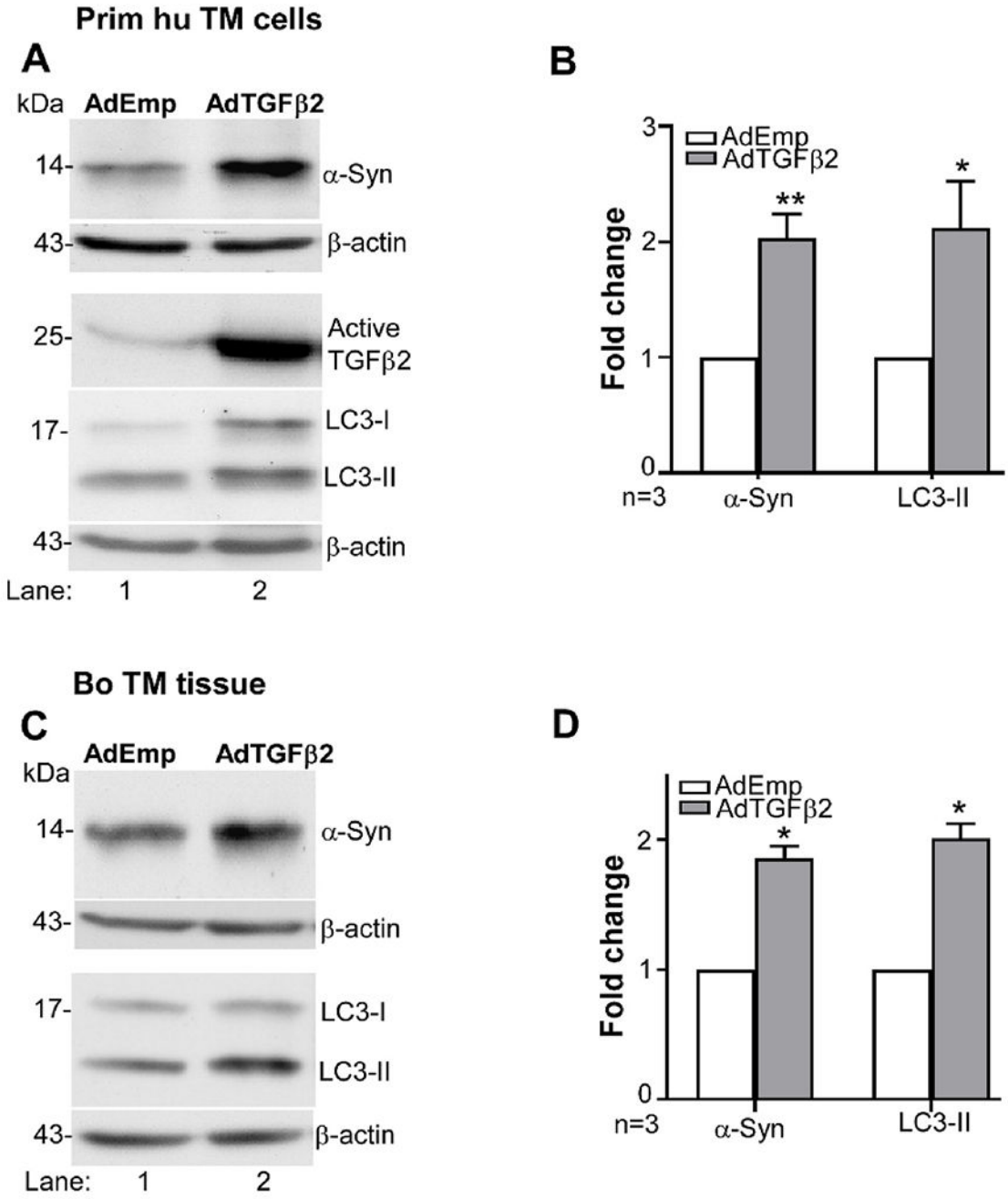

Fig. 4. TGFβ2 upregulates α-Syn in primary human TM cells and bovine TM tissue:

(A) Lysates prepared from TM cells transfected with AdEmp or AdTGFβ2 were processed for immunoblotting. Probing for α-Syn shows increased expression in cells expressing TGFβ2 relative to vector controls (lanes 1 & 2). TGFβ2 is increased as expected, and are levels of LC3-I & II (lanes 1 & 2). The membranes were reprobed for β-actin as a loading control. **(B)** Quantification by densitometiy after normalizing with β-actin shows ~ 2-fold increase in α-Syn and LC3-II in cells over-expressing TGFβ2 relative to vector controls. *p < .05, **p < .01. **(C)** A similar evaluation of bovine TM tissue shows upregulation of α-Syn

and LC3-II as in human TM cells (lanes 1 & 2). **(D)** Quantification by densitometiy after normalization with β-actin shows ~2-fold increase in α-Syn and LC3-II by TGFβ2 as in human TM cells, n represents the number of biological replicates. *p < .05.

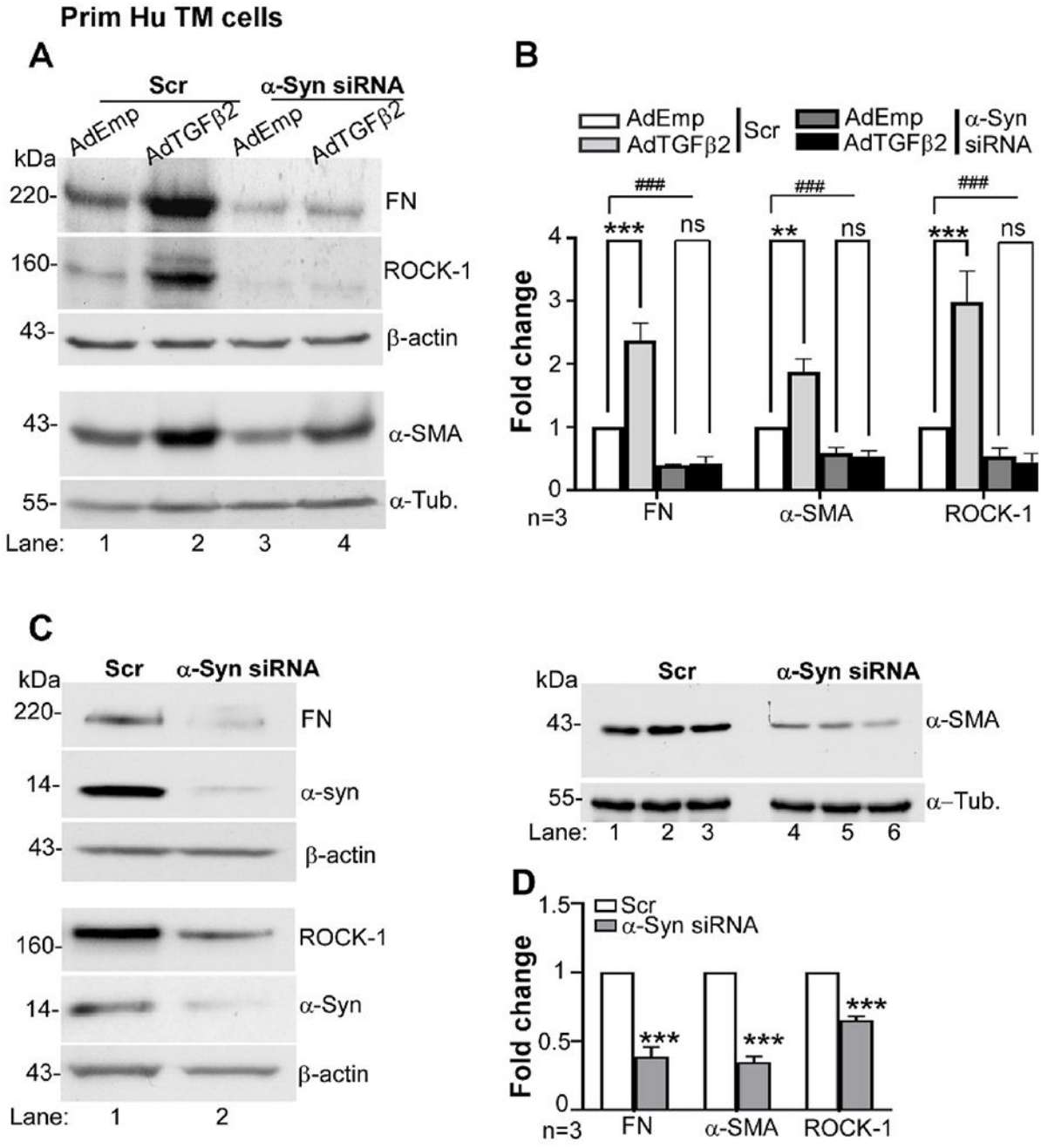

Fig. 5. Silencing of α-Syn downregulates FN, α-SMA, and ROCK-1 in primary human TM cells:

(A) Primary human TM cells were treated with scrambled sequence (Scr) or siRNA specific to α-Syn for 48 h, followed by transfection with AdEmpty or AdTGFβ2. After additional 48 h, lysates were analyzed by immunoblotting. Probing for FN, ROCK-1, and α-SMA shows increased expression in cells over-expressing TGFβ2 relative to vector controls as expected (lane 1 & 2). However, this effect is abolished by silencing of α-Syn in the absence (lane 3) or presence (lane 4) of TGFβ2 (lanes 3 & 4 *vs.* 1 & 2). **(B)** Quantification by densitometry after normalization with β-actin and α-tubulin shows significant upregu-lation

of FN, α-SMA, and ROCK-1 by 2.3 fold, 1.8 fold, and 2.8 fold respectively by TGFβ2 in the presence of endogenous α-Syn (**p < .01, ***p < .001), a significant decrease of 0.4–0.5 fold in all three proteins in cells where α-Syn had been silenced ($^{###}$ p < .001) regardless of active TGFβ2 (ns), n represents the number of biological replicates. **(C)** Lysates of primary human TM cells treated with scrambled (Scr) sequence or α-Syn specific siRNA were fractionated on three separate SDS-PAGE gels, and processed for immunoblotting. Probing of the first membrane for FN shows significant downregulation by silencing of α-Syn relative to controls (lanes 1 & 2). Probing of the second membrane for ROCK-1 also shows down-regulation by silencing of α-Syn, as is evident (lanes 1 & 2). The third membrane was probed for α-SMA, which also shows less expression in cells where α-Syn had been silenced (lanes 4–6 *vs.* 1–3). β-actin and α-tubulin were used as a loading control. **(D)** Densitometry after normalization with β-actin or α-tubulin shows significant downregulation of FN and α-SMA by ~0.7 fold, and ROCK-1 by 0.4 fold in cells where α-Syn had been silenced relative to respective controls. n represents the number of biological replicates. ***p < .001.

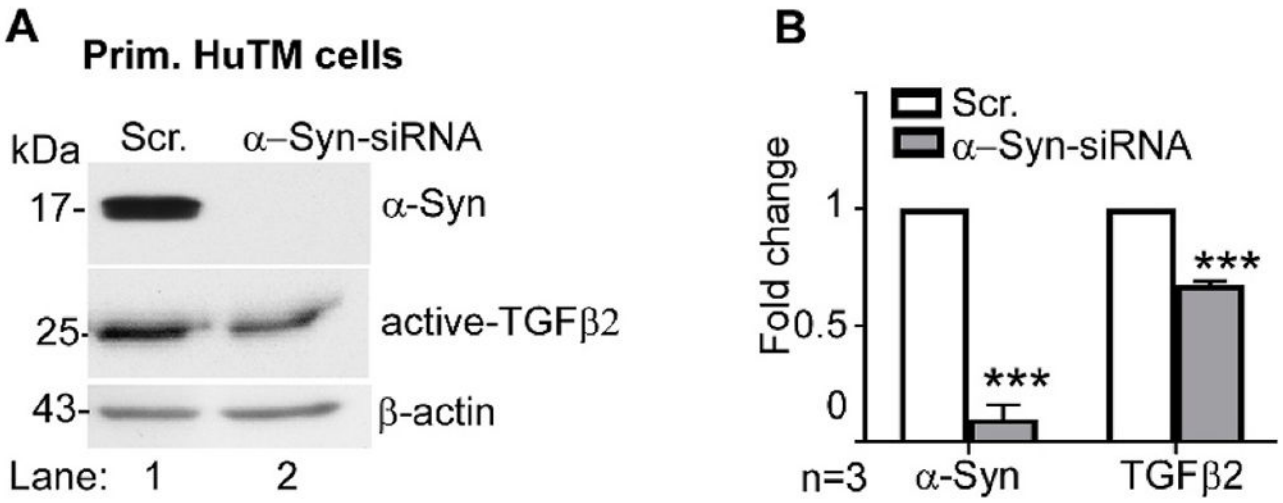

Fig. 6. Silencing of α-Syn downregulates active TGFβ2 in primary human TM cells:

(A) Lysates from primary human TM cells treated with scrambled (Scr) sequence or α-Syn specific siRNA were processed for immunoblotting. Probing for TGFβ2 shows downregulation in the absence of α-Syn relative to respective controls (lanes 1 & 2). Re probing of membrane for β-actin was performed as a loading control. **(B)** Densitometry after normalization to β-actin shows significant downregulation of active TGFβ2 by ~0.75 fold on silencing of α-Syn relative to controls. n represents the number of biological replicates. ***$p < .001$.

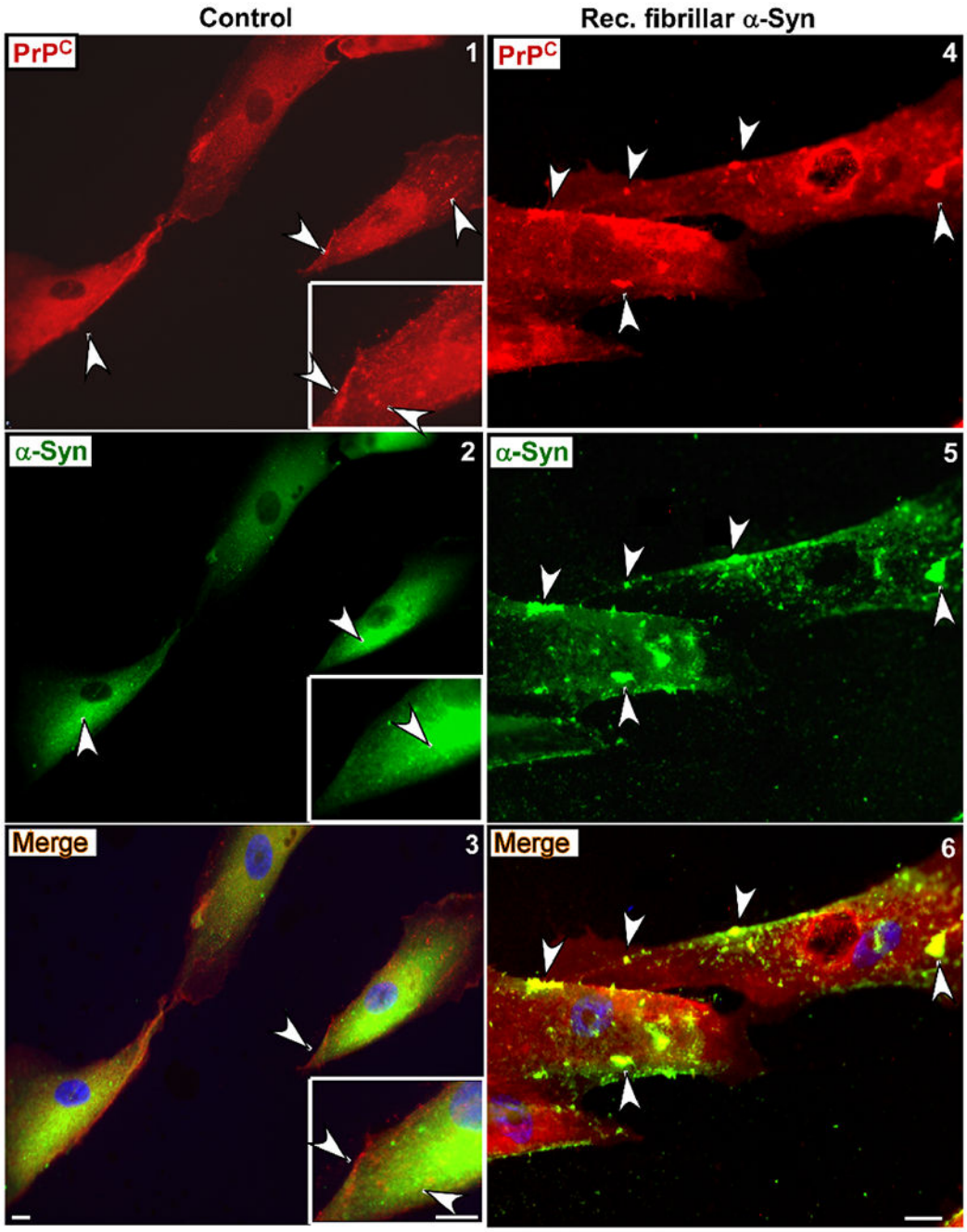

Fig. 7. Recombinant oligomerized α-Syn is internalized by PrP^C in primary human TM cells:
(A) Primary human TM cells were exposed to medium (controls) or 0.5 μg/ml of recombinant α-Syn in complete medium for 2 h. Immunoreaction of control cells for PrP^C shows localization on the cell surface and in intracellular vesicles as expected (panel 1). Reactivity for α-Syn is localized to the cytoplasm (panel 2). There is no co-localization of α-Syn with PrP^C (panel 3). In cells exposed to recombinant α-Syn, PrP^C shows clustering and co-localization with α-Syn on the plasma membrane, and of PrP^C-α-Syn aggregates (panels 4–6). Scale bar: 10 μm.

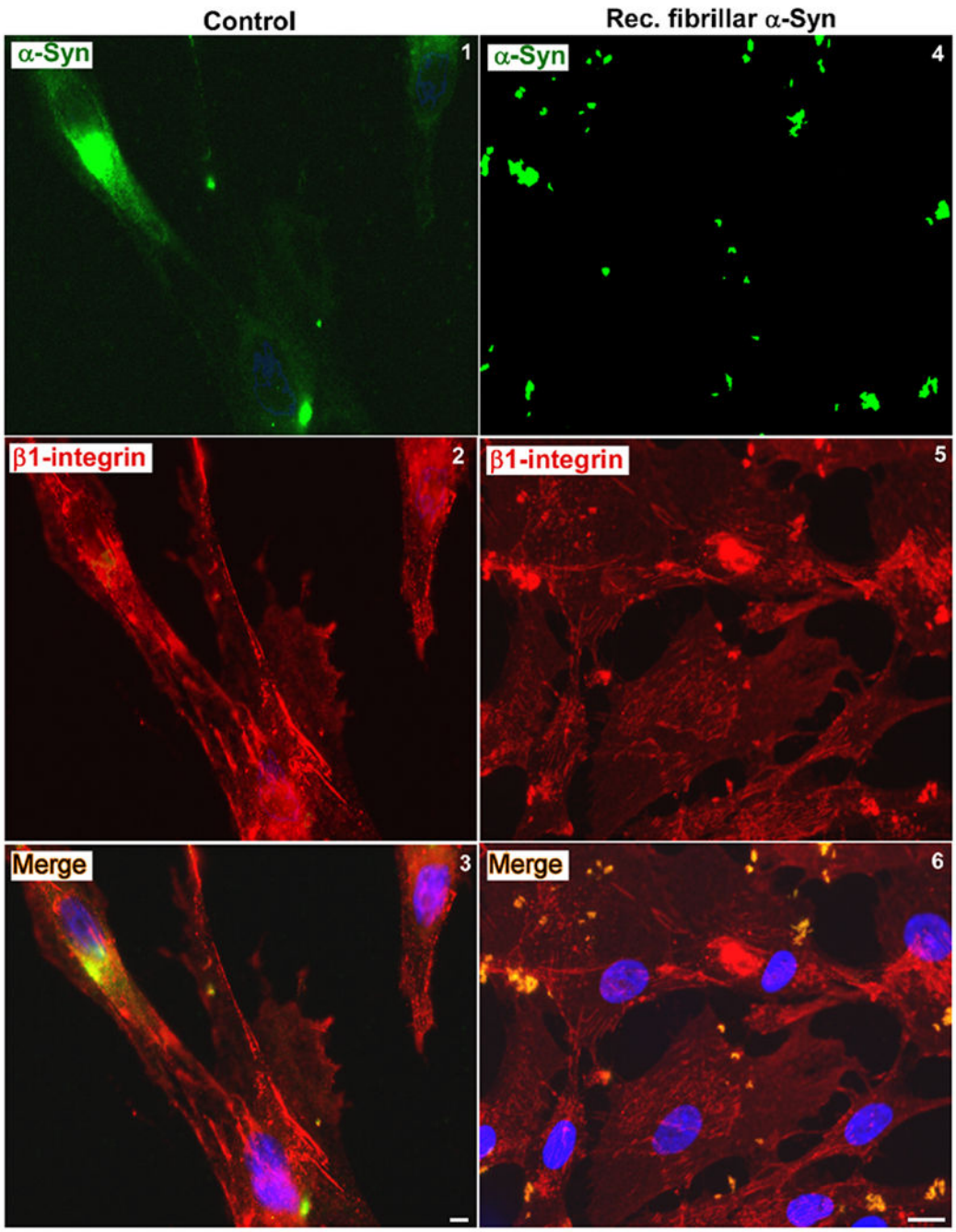

Fig. 8. Recombinant α-Syn binds to β1-integrin on primary human TM cells:
Primary human TM cells were exposed to medium (controls) or 0.5 μg/ml of recombinant α-Syn for 2 h. Immunoreaction for α-Syn shows rare cytosolic reaction in control cells (panel 1). Immunoreaction for β1-integrin is localized to the cytosol (panel 2). Minimal co-localization of α-Syn with β1-integrin is noted (panel 3). In cells exposed to α-Syn, on the other hand, prominent punctate reactivity for α-Syn (panel 4) that colocalizes with β1-integrin is prominent (panels 5 & 6). Scale bar 10 μm.