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Intercellular propagated misfolding of wild-type Cu/Zn superoxide dismutase occurs via exosome-dependent and -independent mechanisms

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Amyotrophic lateral sclerosis (ALS) is predominantly sporadic, but associated with heritable genetic mutations in 5–10% of cases, including those in Cu/Zn superoxide dismutase (SOD1). We previously showed that misfolding of SOD1 can be transmitted to endogenous human wild-type SOD1 (HuWtSOD1) in an intracellular compartment. Using NSC-34 motor neuron-like cells, we now demonstrate that misfolded mutant and HuWtSOD1 can traverse between cells via two nonexclusive mechanisms: protein aggregates released from dying cells and taken up by macropinocytosis, and exosomes secreted from living cells. Furthermore, once HuWtSOD1 propagation has been established, misfolding of HuWtSOD1 can be efficiently and repeatedly propagated between HEK293 cell cultures via conditioned media over multiple passages, and to cultured mouse primary spinal cord cells transgenically expressing HuWtSOD1, but not to cells derived from nontransgenic littermates. Conditioned media transmission of HuWtSOD1 misfolding in HEK293 cells is blocked by HuWtSOD1 siRNA knockdown, consistent with human SOD1 being a substrate for conversion, and attenuated by ultracentrifugation or incubation with SOD1 misfolding-specific antibodies, indicating a relatively massive transmission particle which possesses antibody-accessible SOD1. Finally, misfolded and protease-sensitive HuWtSOD1 comprises up to 4% of total SOD1 in spinal cords of patients with sporadic ALS (SALS). Propagation of HuWtSOD1 misfolding, and its subsequent cell-to-cell transmission, is thus a candidate process for the molecular pathogenesis of SALS, which may provide novel treatment and biomarker targets for this devastating disease.

protein misfolding | intercellular transmission | prion-like | disease-specific epitope

Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular condition that afflicts as many as 1 of 350 males and 420 females over the age of 18 (1). In ALS, degeneration of upper and lower motor neurons causes progressive muscle paralysis and spasticity, affecting mobility, speech, swallowing, and respiration (2). Half of affected individuals die within 3 y, and less than 20% survive for more than 5 y (3); 90–95% of ALS cases are sporadic (SALS) in which some apparently facilitating gene mutations, such as repeat expansions in the gene that encodes ataxin-2 (4), have been identified. The remaining 5–10% of ALS cases are familial (FALS) and predominantly associated with Mendelian-inherited mutations in the genes encoding Cu/Zn superoxide dismutase (SOD1), TAR-DNA–binding protein 43 (TDP-43), fused in sarcoma/translocated in liposarcoma (FUS/TLS), C9ORF72, and other genes (reviewed in ref. 3).

Despite the profusion of functionally diverse genes implicated in FALS and SALS, clinical and pathological similarities between all forms of ALS suggest the existence of a common pathogenic pathway that could be united by a single gene/protein (5). One of the mechanisms by which a mutant or wild-type (WT) protein can dominate pathogenesis of phenotypically diverse diseases is by propagated protein misfolding, such as that underpinning the prion diseases, which has been increasingly implicated in other neurodegenerative and systemic disorders (6, 7). A role for propagated protein misfolding in ALS is supported by the prion-like spatiotemporal progression of disease through the neuroaxis (8, 9). However, given the disparity in protein inclusion pathology between subtypes of ALS, a single unifying prion-like protein that could explain such a progression remains obscure.

Whereas it is generally accepted SOD1 is not found in large perikaryal cytoplasmic inclusions outside of SOD1 FALS cases, misfolded SOD1 has been increasingly identified in SALS and non-SOD1 FALS (5, 10, 11). Indeed, we have reported that misfolded human wild-type SOD1 (HuWtSOD1) can be detected by spinal cord immunohistochemistry (IHC) in FALS secondary to FUS mutation, and in SALS patients with cytosolic WT TDP-43 accumulation (11). Moreover, in cell models, overexpression of

Significance

Amyotrophic lateral sclerosis (ALS), an incurable motor neuron disease, is associated with mutation and misfolding of the Cu/Zn superoxide dismutase (SOD1) protein. Prior studies found that mutant misfolded SOD1 can convert wild-type (WT) SOD1 to a misfolded form inside living cells in a prion-like fashion. We now report that misfolded WT SOD1 can be transmitted from cell to cell, and that propagated protein misfolding can be perpetuated. Misfolded SOD1 transmission between cells can be mediated through release and uptake of protein aggregates or via small membrane-bounded transport vesicles called exosomes. These mechanisms may help explain why sporadic ALS, without a known genetic cause, can spread systematically from region to region in a progressive manner.

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WTTDP-43, or expression of mutant FUS or TDP-43, is associated with HuWtSOD1 misfolding (11). Collectively, these data are consistent with SOD1 being a molecular common denominator for all types of ALS. Furthermore, prion-like activity has been described for the cell-to-cell transmission of misfolding of mutant SOD1 (12), and we have reported that mutant SOD1 can confer its misfold on HuWtSOD1 (13). However, mutant SOD1 cannot explain propagation in SALS.

To test if HuWtSOD1 participates in cell-to-cell transmission of protein misfolding, we make use of previously developed mouse mAb probes for misfolded/oxidized SOD1, recognizing either full-length human mutant or WT SOD1, generated against regions that are antibody-inaccessible in natively folded SOD1 (13–15). Misfolded SOD1 mAbs used in this work are 10E11C11 and 3H1, directed against an unstructured electrostatic loop [disease-specific epitope-2 (DSE2)], and 10C12, directed against a C-terminal dimer interface peptide in which the cysteine at position 146 is substituted by a cysteic acid residue to mimic oxidation of this residue (DSE1a) (13). The use of such antibody probes have enabled us to unambiguously determine the role of misfolded mutant G127X in the induced misfolding of HuWtSOD1, which upon misfolding acquires a marked increase in sensitivity to protease digestion, consistent with global loosening of structure (13). The finding that misfolded endogenous HuWtSOD1 was observed long after transfected G127X-SOD1 was degraded suggested that HuWtSOD1, once misfolded, is capable of triggering an intracellular propagated misfolding reaction (13). We now report for the first time that misfolded HuWtSOD1 can transit cell to cell both via exosomes, and release of protein aggregates and subsequent uptake in neuronal cells. In addition, misfolded HuWtSOD1 can sustain intercellular propagated misfolding in vitro and is detectable in the spinal cord of all ALS patients tested, regardless of the genetic etiology of the disease. Collectively, these data indicate that HuWtSOD1 is competent to participate in propagated misfolding, suggesting a common pathogenic mechanism linking FALS and SALS.

Results

Cellular Import and Export of the SOD1 Transmission Particle. Previous work would suggest that the release of misfolded proteins is likely to occur either through exocytosis or cell rupture due to death (16), whereas uptake is thought to be nonspecific and mediated by fluid phase endocytosis (12). Mutant and HuWtSOD1 have been shown to be exported from NSC-34 cells via small vesicles known as exosomes (17), and a separate study shows that protein-only aggregates of mutant SOD1 are efficiently imported into mouse N2a cells by a macropinocytosis-dependent process (12). To more fully characterize possible intercellular transmission pathways of SOD1 misfolding we used the murine motor neuron-like NSC-34 cell line (18). NSC-34 cells were transfected with HuWtSOD1–GFP or mutant SOD1–GFP variants; significant cell death was observed 72 h posttransfection that was not prominent in transient GFP-alone transfection or in SOD1–GFP stably transfected models (Fig. 1*A*). SOD1–GFP aggregates are observed in transiently transfected NSC-34 cells, but not in stably transfected ones (Fig. S1). Taken together, these data demonstrate the neuron-like susceptibility of NSC-34 cells to neurotoxic species of SOD1 (19). At this time point, SOD1–GFP-positive particles, both HuWt and mutants, could be transferred to naïve cells via conditioned medium transfer and uptake of these particles could be detected in naïve NSC-34 cells (Fig. 1*B*). This uptake appeared specific as GFP by itself was not detected inside naïve cells. Given that the transmitted particle may be a complex mix of both released exosomes and protein aggregates,

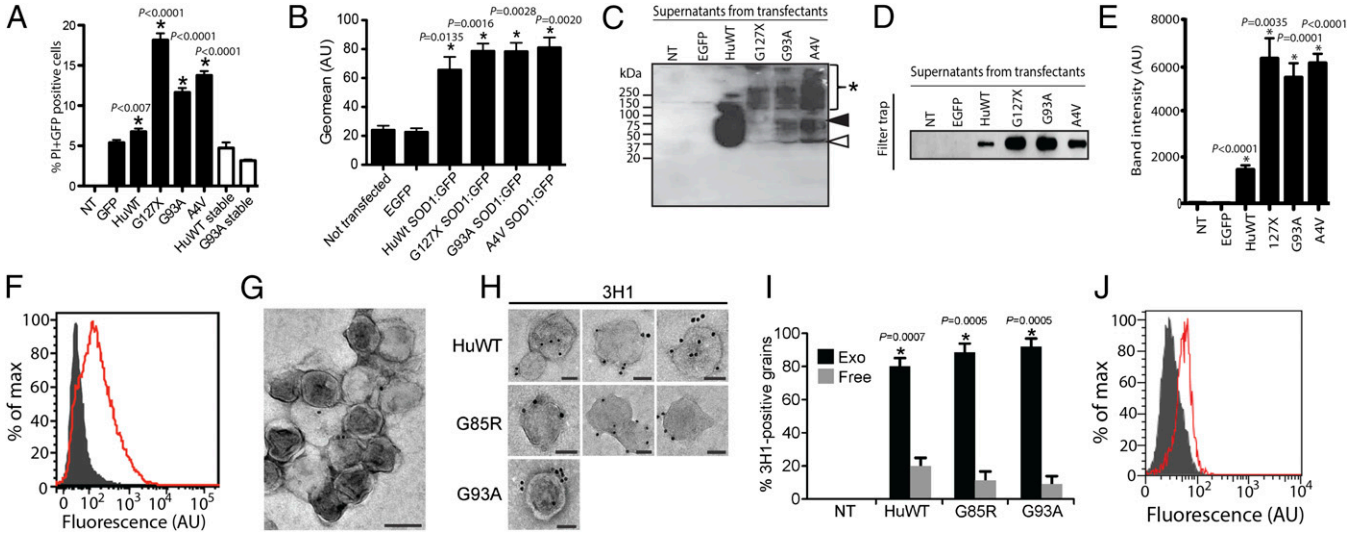

Fig. 1. Aggregated SOD1 is released from dead or dying cells and/or actively released as part of exosomes. (*A*) Cell death of GFP-positive cells after 72 h posttransfection was examined by flow cytometry. Cells stained with PI were scored as dead and these cells expressed as a proportion of total GFP-positive cells. The results are means ± SE of three independent experiments. $*P < 0.05$ compared with medium from nontransfected (NT) cells, as determined by unpaired *t* test. (*B*) Uptake of pelletable SOD1–GFP by naïve NSC-34 cells following incubation with conditioned media was measured by flow cytometry. The data shown is calculated from triplicate experiments. Error bars represent SEM. $*P < 0.05$ compared with medium from nontransfected (NT) cells, as determined by unpaired *t* test. (*C*) Detection of oligomeric SOD1 released into media 72 h posttransfection. The white arrowhead indicates SOD1–GFP monomer whereas the black arrowhead indicates SOD1–GFP dimer. The area indicated by an asterisk represents SDS-resistant oligomeric SOD1 species. Immunoblot probed with GFP pAb. (*D*) Filter-trap assay and anti-GFP immunoblot to detect SOD1–GFP aggregates in conditioned media. The filter trap was performed in triplicate and is quantified in *E*. Values obtained from the filter trap assay are the mean average of three independent experiments. Error bars represent SEM. $*P < 0.05$ compared with negative controls (NT and EGFP). (*F*) Quantitative analysis of aggregated human SOD1 internalization into NSC-34 cells using flow cytometry. Cells were either incubated with PBS (gray) or aggregated HuWtSOD1 (red line). (*G*) Visualization of exosomes secreted by NSC-34 cells by negative staining electron microscopy. (Scale bar: 100 nm.) (*H*) Immunogold labeling using 3H1 antibody shows surface-localized misfolded SOD1 on exosomes secreted by stably transfected NSC-34 cells. (Scale bar: 50 nm.) (*I*) Quantification of exosomal and proximal free misfolded SOD1 secreted by NSC-34 cells. (*J*) NSC-34 cells were treated with NSC-34–derived exosomes for 30 min and fixed. Quantitative analysis of SOD1–GFP internalization into NSC-34 cells using flow cytometry. Cells were either incubated with PBS (gray) or HuWtSOD1–GFP exosomes (red line).

the conditioned medium was analyzed by immunoblot to determine if SOD1 aggregates were released from the dying NSC-34 cells, revealing SDS-resistant oligomers released by cells transfected with SOD1–GFP variants, including HuWtSOD1 (Fig. 1*C*). Filter trap analysis confirmed aggregates larger than the 0.2-μm pore size in conditioned medium supernatants from dying NSC-34 cells transfected with all SOD1–GFP variants (Fig. 1 *D* and *E*), including HuWtSOD1–GFP. To determine if nonvesicular protein-only SOD1 aggregates can be taken up by naïve NSC-34 cells, recombinant HuWtSOD1 protein was firstly preaggregated by a seeding event initiated by mutant misfolded G93A SOD1 (Fig. S2 *A–D*). Both flow cytometry and confocal microscopy demonstrated that aggregated HuWtSOD1 is incorporated into NSC-34 cells (Fig. 1*F* and Fig. S2*E*), and that this process is mediated by macropinocytosis, as the specific inhibitor 5-(*N*-ethyl-*N*-isopropyl)-amiloride, but not the clathrin-coated pit inhibitor chlorpromazine hydrochloride, the caveolin-dependent inhibitor genestein, or lipid raft pathway inhibitor methyl-β-cyclodextrin, suppressed aggregated HuWtSOD1 uptake (Fig. S2*F*). Furthermore, uptake is SOD1-specific and aggregated forms are taken up as efficiently as nonaggregated forms, whereas the control protein, GST, was not taken up at all. (Fig. S2*G*).

Although our transiently transfected model indicates that large aggregates of misfolded SOD1 from dead or dying cells can be efficiently taken up by neighboring cells, we and others have found that exosomal-mediated transport is relevant for misfolded protein transmission in living cells (17, 20), for which we used stably transfected NSC-34 cells that show little cell death (Fig. 1*A*). Exosomal fractions were isolated from these cells as previously described (17), with an additional high-speed (100,000 × *g*) centrifugation step included following washing to ensure microvesicles are sufficiently depleted of nonspecific material. Visualization of ultracentrifuged pellets from conditioned medium collected from cultures of stably transfected NSC-34 cells revealed microvesicles that were membrane bounded, consistent with exosomes in morphology and size of ∼100 nm (Fig. 1*G*). Exosome pellets showed enrichment of TSG101 and flotillin 1 compared with cell lysates confirming their endosomal origin (Fig. S3*A*) and the presence of prion protein (PrP) on the membrane surface by immunoelectron microscopy, another marker of exosomes (Fig. S3*B*). Immunoelectron microscopy with the 3H1 SOD1 misfolding-specific antibody revealed that ∼80–90% of grains were associated with the exterior of exosomes secreted by cells stably expressing mutant or HuWtSOD1 (Fig. 1*H*), with only a small minority labeling apparent protein aggregates not in proximity to exosomes (Fig. 1*I*). Immunoelectron microscopy of HuWtSOD1–GFP exosomes also revealed robust immunoreactivity for the C-terminal misfolded/oxidized SOD1 mAb 10C12 (Fig. S3*C*), but not the C4F6 mAb directed at more N-terminal residues 80–118 (5) (Fig. S3*D*), implying that the C-terminal third of SOD1 is preferentially exposed on the outside surface of exosomes. The configuration of misfolded SOD1 associated with the surface of exosomes was not an artifact of the GFP tag as 3H1 also labeled nontagged mutant or HuWtSOD1 exosomes from stably transfected NSC-34 cells (Fig. S3*E*). Flow cytometry and confocal microscopy of naïve NSC-34 cells exposed to exosomes secreted from cells stably expressing mutant and HuWtSOD1–GFP revealed that exosome uptake was relatively efficient (Fig. 1*J* and Fig. S3*F*).

HuWtSOD1 Misfolding Can Be Transmitted Intercellularly. Aggregation of soluble intracellular mutant SOD1 has been observed upon media exposure to the same mutants in an aggregated state (12), and we have shown that mutant SOD1 induces the misfolding of HuWtSOD1 when transfected in human, but not mouse, cell lines (13). Furthermore, we show that misfolded SOD1, both WT and mutant, may be exported from cells either associated with exosomes or as protein-only aggregates. Thus, we investigated whether cells containing mutant-induced misfolded HuWtSOD1 acquire the capacity to transmit misfolded SOD1 from cell to cell, and whether such misfolded protein could act as propagating seeds in untransfected naïve cell cultures (schematically shown in Fig. S4*A*). We selected the mesenchymal HEK293 cell line for their expression of substrate HuWtSOD1, their resistance to the toxicity of misfolded SOD1, and their efficiency of transfection (13). DSE-immunoprecipitable misfolded HuWtSOD1 was detected in lysates of HEK293 cells incubated overnight with conditioned media from HEK293 cells transiently transfected with G127X mutant SOD1, but not from lysates in which cells were incubated with media from a control empty vector transfection (Fig. 2*A* and Fig. S4*B*), reminiscent of the transfer observed in NSC-34 cells (Fig. 1*B*). Incubation of naïve HEK293 cells with media from cultures transiently transfected with GFP did not induce SOD1 misfolding (Fig. S4*C*), demonstrating that the misfolding of HuWtSOD1 in human recipient cells is specifically induced by a conditioned medium factor from SOD1-transfected HEK293 cells. Misfolded SOD1 content in recipient culture lysates was unaffected by DNase digestion of conditioned media (Fig. S4*D*), ruling out a contribution of residual transfection plasmid. Consistent with prion-like propagated misfolding of HuWtSOD1, SOD1 misfolding could be serially passaged from culture to culture via sequential media incubation (shown schematically in Fig. S4*A* and experimentally to at least five passages in Fig. 2*A*). It is notable that from the first passage onward, the DSE-immunoprecipitable misfolded SOD1 in lysates migrated as HuWtSOD1, despite being incubated with supernatants of cells transfected with the faster-migrating SOD1 mutants G127X and G85R, suggesting that misfolded HuWtSOD1 was competent to transmit its acquired conformation between cell cultures (Fig. 2*A* and Fig. S4*E*). Forced overexpression of HuWtSOD1 in the initial transfection HEK293 cultures (associated with SOD1 misfolding) (13, 21) also resulted in efficient transmission of SOD1 misfolding via media, which did not diminish with multiple serial passages (Fig. 2*A*), indicating that HuWtSOD1 was indeed fully competent to

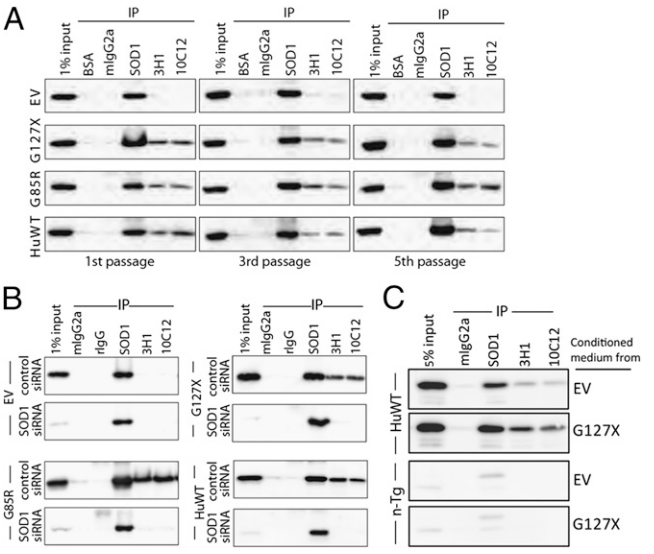

Fig. 2. Mutant SOD1-mediated HuWtSOD1 misfolding is transmissible between cells. (*A*) IP of lysates from naïve HEK cells cultured in the presence of conditioned medium from one, three, and five passages of transduction assays initiated by different SOD1 variants. mIgG2a, mouse IgG2a isotype control; rIgG, rabbit mixed IgG control; SOD1, pan-SOD1 antibody; 3H1 and 10C12, misfolded SOD1-specific antibodies. (*B*) SOD1 knockdown abolishes detectable misfolding. IP of lysates from HEK cells with knocked down SOD1 expression cultured in the presence of conditioned medium from HEK cells transfected with empty vector control (EV), SOD1-G85R, SOD1-G127X, or HuWtSOD1. (*C*) IP of lysates from naïve primary neural cell cultures derived from embryonic spinal cord of HuWtSOD1 transgenic or nontransgenic littermate (n-Tg) mice incubated in the presence of conditioned from SOD1-G127X or empty vector (EV)-transfected HEK293 cells.

support misfolding propagation in a self-sustaining reaction. To confirm the capacity of HuWtSOD1 to mediate prion-like propagated misfolding transmission between cells, we depleted endogenous HuWtSOD1 expression in HEK293 cells with siRNA oligonucleotides before exposure to conditioned media. SOD1-DSE immunoprecipitations (IPs) of lysates from HuWtSOD1-depleted cells revealed drastically reduced misfolded HuWtSOD1 compared with control incubations (Fig. 2*B* and Fig. S5*A*), arguing against the observed misfolded HuWtSOD1 resulting solely from SOD1 uptake from conditioned media, and consistent with recipient cell endogenous HuWtSOD1 being an authentic substrate for propagated protein misfolding. We also observed transmission of propagated SOD1 misfolding to primary neural cultures derived from embryonic mouse spinal cord expressing HuWtSOD1 when exogenously applied mutant misfolded SOD1 in conditioned medium is present (Fig. 2*C* and Fig. S5*B*), confirming that the process occurs in a physiologically relevant system.

Intercellular Transmission Is Mediated by Particles Displaying Misfolded SOD1. The majority of DSE-immunoprecipitable SOD1 was detectable in the ultracentrifuged pellets of conditioned media from mutant SOD1 transfected cells (Fig. 3*A*). Naïve cells were incubated with resuspended pellet or supernatant fractions of ultracentrifuged (100,000 × *g* for 1 h) conditioned media obtained from cells transfected with mutant or WT constructs of SOD1. Lysates from incubated cells revealed that ∼80% of the HuWtSOD1 misfolding activity was present in the pellet fraction (Fig. S6*A*), consistent with a relatively massive transmission particle composed of protein-only aggregates, associated with exosomes, or both. Surface plasmon resonance analysis of pellet fractions derived from conditioned media revealed 3H1-immunoreactive misfolded SOD1, even in G127X-transfected supernatants (Fig. 3*B*), signifying the extracellular presence of misfolded full-length SOD1, and consistent with exposed misfolded HuWtSOD1 being a component of the transmission particle. In the special case of G127X, for which we have generated a mutant-specific rabbit polyclonal antibody (pAb) (13), we detected limited G127X protein in G127X-transfected HEK293 supernatants (Fig. S6*B*), suggesting that on first transmission mutant SOD1 may participate in the cell-to-cell propagation of SOD1 misfolding. However, sequential dilution of mutant SOD1 in subsequent passages would militate against mutant SOD1 being an essential component of the intercellular transmission particle, and indeed in serial passages kindled by transfection by HuWtSOD1 (Fig. 2*A*), mutant SOD1 formally cannot participate. Exposure of misfolded SOD1 epitopes in the transmission particle suggests that cell-to-cell transmission might be neutralized by antibodies directed against misfolded or native HuWtSOD1. Following the blocking protocol schematically shown in Fig. S4*A*, the HuWtSOD1-converting activity of conditioned media from G127X- or G85R-transfected HEK293 cells was abolished by preincubation with SOD1-specific polyclonal rabbit IgG, but not control rabbit IgG (Fig. 4 *A* and *B*). Furthermore, HuWtSOD1 conversion in naïve HEK293 cells exposed to the conditioned medium of HEK293 cells transfected with G127X, G85R, or HuWtSOD1 was attenuated by DSE mAbs 3H1 and 10C12 expected to react with full-length misfolded/oxidized isoforms of SOD1 (Fig. 4 *A*–*C*), in keeping with misfolded HuWtSOD1 being an accessible and essential component of the cell-to-cell transmission particle.

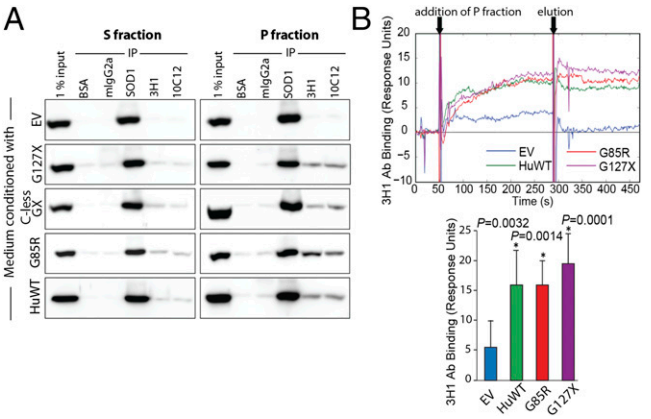

Fig. 3. Misfolded SOD1 is detectable in the culture media of cells transfected with SOD1 constructs. (*A*) Misfolded SOD1 was detected by IP in both the pellet (P) and supernatant (S) fractions of centrifugated conditioned media, but majority was detected in the P fraction. C-less GX, cysteine-less version of G127X SOD1. This was confirmed by Biacore detection of misfolded SOD1 in the P fraction by 3H1 capture (*B*). Values represent the average of seven independent experiments. An asterisk indicates significant values. Statistical significance determined by unpaired *t* test, compared with EV control (significant *P* values shown). Error bars represent SD.

Aggregates of Misfolded HuWtSOD1 Are Present in ALS Patient Spinal Cords. Using the DSE mAbs, we have previously demonstrated the presence of misfolded/oxidized SOD1 by IHC in SOD1- and FUS-FALS, and in SALS with cytoplasmic TDP43 accumulation (11). To confirm the presence of misfolded HuWtSOD1 in ALS pathology in vivo, we examined a larger series of IHC of postmortem human spinal cord sections from control, mutant-SOD1 FALS, non-SOD1 FALS (including two cases due to expansion mutations in the *C9ORF72* gene) (22, 23), and SALS patients (including one case of *C9ORF72* expansion without family history of ALS; Table S1). In all cases of ALS examined, regardless of SOD1 sequence, we detected immunoreactivity with the DSE mAbs within motor neurons ($n = 28$; Fig. 5 *A*–*F*). Similar types of structures were stained by the DSE mAbs in all ALS tissue samples, although there were appreciable differences in distribution (Table S2), with mutant SOD1 FALS showing numerous neuronal cytoplasmic inclusions that were rare in SALS and *C9ORF72*-FALS, in addition to axonal and tract immunoreactivity observed in all types of ALS. However, IHC may not accurately represent the total amount of misfolded SOD1, likely missing soluble monomers or oligomers immunoreactive with the DSE mAbs. We thus performed quantitative IP from unfixed spinal cord homogenates with DSE mAbs, normalized to total SOD1 immunoprecipitated with a pan-SOD1 pAb. IP experiments were conducted using homogenates of postmortem cervical or thoracic spinal cord from patients with mutant-SOD1 FALS and non-SOD1 SALS (including one case with *C9ORF72* expansion without family history; see 2 C in the SALS2 section of Fig. 5*G*), as well as controls (Fig. 5*G* and Table S3). Spinal cords from Alzheimer's disease (AD) patients ($n = 3$) were included as a neurological disease control, along with age- and sex-matched control spinal cord samples from normal subjects ($n = 4$). Misfolded/oxidized SOD1 was detected in an unexpectedly high concentration of ∼4% in SOD1-linked FALS and SOD1-excluded SALS, including one case of C9ORF72 mutation without family history of ALS (Fig. 5*G* and Fig. S7*A*). The amount of SOD1 immunoprecipitated by the DSE mAbs was not significantly above background in either AD or other control samples.

Similar to our previous demonstration of acquisition of protease sensitivity by induced misfolding in vitro (13), we found that that DSE-immunoreactive SOD1 species in vivo acquired profound sensitivity to protease digestion when we subjected FALS and SALS spinal cord homogenates to proteinase K (PK) digestion at a concentration of 200 μg/mL (Fig. 5*G*). PK treatment significantly decreased DSE-immunoprecipitable SOD1 by 85–90% for FALS and SALS for both DSE antibodies (Fig. S7*A*). Titrated PK digestion of control homogenates reveals that native HuWtSOD1 is highly resistant to PK digestion, even at concentrations 100-fold higher (Fig. S7*B*). Detergent-insoluble oligomers of similar intensity are observed in spinal cord homogenate from both FALS and SALS patients, in contrast to control and AD samples (Fig. S7 *C* and *D*); similar observations have been made in SOD1 mouse models of ALS (24).

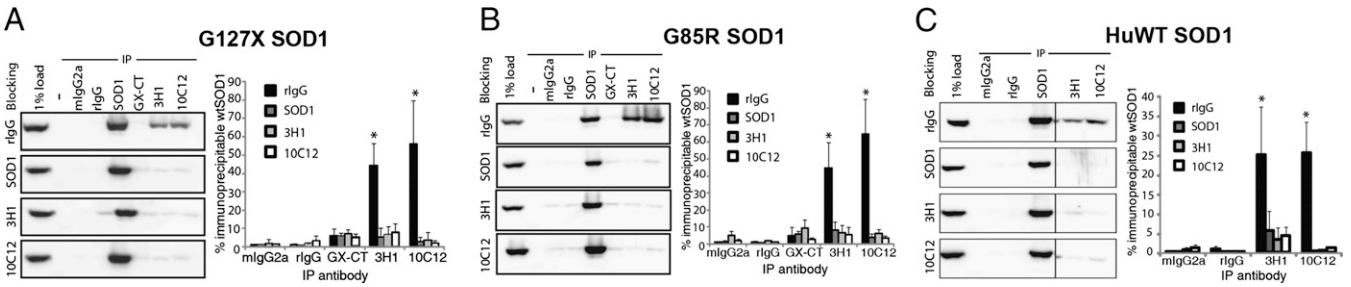

Fig. 4. SOD1 antibodies can block misfolding transmission. IP of lysates from naïve HEK cells cultured in the presence of conditioned medium from G127X-transfected HEK cells (*A*), G85R-transfected HEK cells (*B*), or HuWtSOD1-transfected HEK cells (*C*) immunologically pretreated with either rabbit mixed IgG (rIgG), pan-SOD1 pAb (SOD1), or DSE mAbs 3H1 or 10C12, all at 20 μg/mL. Corresponding quantitation is shown to the right of each immunoblot. Values represent the mean average of four independent experiments. Error bars represent SD. An asterisk indicates statistically significant blocking of wtSOD1 misfolding transmission by specific antibodies as determined by ANOVA analysis followed by Tukey (honest significant difference) multiple comparison. For G127X experiment, $P = 0.0048$; for G85R experiment, $P = 0.0100$; for HuWtSOD1 experiment, $P = 0.032$.

Discussion

Increasing evidence supports the notion that progression of pathology in neurodegenerative diseases such as AD, Parkinson disease, tauopathies, and Huntington disease is a result of propagation of misfolded or aggregated proteins (6, 7, 16). The capability of a pathogenic protein conformation to self-propagate is the central tenant of the prion hypothesis, with a necessary requirement of sporadic prion diseases being that the process of conformational conversion must be capable of being mediated by the unmutated WT PrP. Mutant SOD1 has been shown to confer its misfolded aggregated phenotype on soluble mutant SOD1 (12). Until this report, unambiguous evidence of the competence of HuWtSOD1 in propagated protein misfolding has been lacking. We and others detect SOD1 misfolding in SALS and non-SOD1 FALS, in addition to SOD1 FALS (5, 10) and here we present data that indicates that misfolded and protease-sensitive HuWtSOD1 is present in the spinal cord of SALS patients, representing ∼4% of total SOD1. Although we cannot rule out the possibility that SOD1 misfolding is a consequence of SALS pathology, taken together, these data suggest propagated misfolding of SOD1 is a strong candidate for a downstream final common pathway for all types of ALS, including its sporadic form.

We report here that HuWtSOD1 efficiently propagates from cell to cell in a HuWtSOD1 expression-dependent process by two nonexclusive mechanisms: release of protein-only aggregates and cell-derived vesicles identified as exosomes. It might be expected that aggregates of naked misfolded SOD1 might be exported from cells as they die. Fibrils of SOD1 have already been shown to be a competent seed for further SOD1 aggregation in vitro (25), and when exogenously applied can be efficiently taken up into living cells (26). In addition, aggregates of mutant SOD1 have been observed to be taken up into neuron-like cells via macropinocytosis (12). In both cases the multimeric structures of SOD1 provide competent seeds for aggregation of soluble mutant SOD1, suggesting SOD1 aggregates to be an efficient means of misfolding propagation between cells. Our data also indicates that macropinocytosis is implicated in the uptake of aggregated HuWtSOD1, also known to be involved in the propagation of mutant SOD1 aggregation (12). Although we have not identified the receptors involved in this process, our previous work on microglia would suggest a large receptor complex involving scavenger or other pattern-recognition receptors and signaling receptors may be participating (27). Heparin sulfate proteoglycans have been shown to be involved in aggregate uptake of tau, alpha-synuclein, and prion protein (28, 29), and it is interesting to speculate that this family of receptors could be involved here. However, before cell death ensues in ALS or other neurodegenerative diseases, pathology can spread from cell to cell and region to region (8), suggesting another mechanism that may be more relevant to early stages of the disease. We propose that export and uptake may be mediated by a pathological hijacking of exosome biology, which has been theorized to participate in prion propagation, and other proteopathic neurodegenerative diseases such as AD and Parkinson disease (reviewed in ref. 30). Exosomes can interact with neighboring cells by various mechanisms, including direct fusion with recipient plasma membrane, endocytosis and fusion with endosomal membrane, receptor-mediated endocytosis, and phagocytosis (31). Most recently, exosome uptake by macropinocytosis was found (32) which could lead to release of exosomal SOD1 and propagation-competent seeds delivered into the cytosol of a neighboring cell.

Importantly, the cell-to-cell transmission of SOD1 misfolding can be neutralized with antibodies directed against misfolded

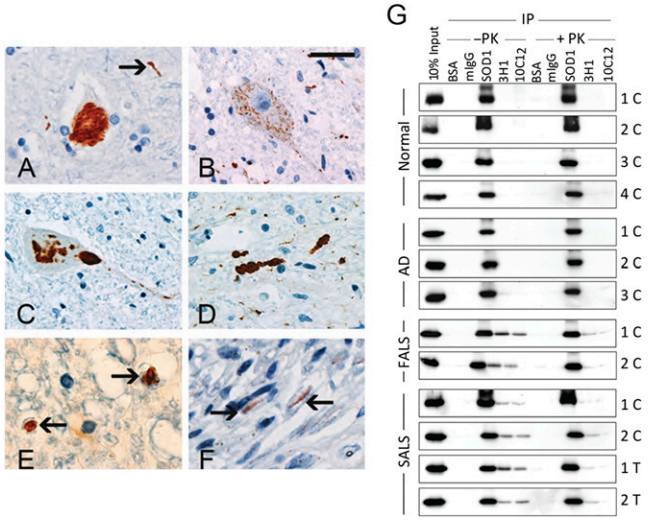

Fig. 5. Misfolded SOD1 is detectable in the spinal cords of both FALS and SALS patients. IHC using antibodies against misfolded SOD1 in cases of FALS with SOD1 mutations (*A*–*D*) and SALS (*E* and *F*). In cases with SOD1 mutations, neuronal cytoplasmic inclusions and neurites (arrow) were labeled in the primary motor cortex (*A*) and ventral gray matter of the spinal cord (*B* and *C*). The spinal gray matter (*D*) also contained numerous swollen axons. Immunopositive axons of normal caliber with a visible myelin sheath were present in the corticospinal tracts (arrows in *E*) and motor nerve roots (arrows in *F*) in sporadic cases. IHC was performed using 10E11C11 (*A*, *B*, and *D*–*F*) and 3H1 (*C*) antibodies. (Scale bars: 20 μm in *A* and *D*, 30 μm in *B* and *C*, and 12 μm in *E* and *F*.) (*G*) IP of 10% (wt/vol) spinal cord homogenate in PBS using DSE mAbs, normalized to equal concentrations of SOD1. Samples were preincubated at 37 °C for 30 min in the presence or absence of 200 μg/mL PK. All immunoblots were probed with pan-SOD1 pAb. C, cervical spinal cord; T, thoracic spinal cord.

SOD1, which is consistent with either naked aggregate transfer, or transfer via the antibody-accessible surface of exosomes. Indeed, prion or amyloid-β containing exosomes are not limited by the interior release of misfolded or aggregated protein or peptides. Most significantly, the inhibition of propagated misfolding of SOD1 by misfolding-specific antibodies in vitro suggests a unique immunotherapeutic approach to neutralizing the spread of disease in ALS patients. We note that in vitro neutralization apparently correlates with exosome exposure of misfolding-specific epitopes 3H1 and 10C12; however, an antibody directed against an epitope not exposed on the surface of exosomes, C4F6, is not effective in treatment of mouse models of ALS (33). Thus, restrictions may exist on exposure of the cognate epitopes in vivo, due to aggregation sequestration or topological constraints of misfolded SOD1 on exosome membranes. It may prove important to understand the immunological anatomy of misfolded SOD1 to generate effective immunotherapies for ALS.

Materials and Methods

Electron Microscopy. Exosomes were fixed in 2% (wt/vol) paraformaldehyde in PBS for 10 min and applied to glow-discharged 200 mesh Cu grids coated with formvar–carbon film (ProSciTech) and absorbed for 20 min. Grids were washed twice (PBS, 3 min) and blocked with 5% (wt/vol) BSA in PBS for 15 min followed by staining with 3H1 (1:200), 10C12 (1:200), or C4F6 (1:50) in PBS and 0.1% BSA for 30 min. Grids were washed six times and incubated with Aurion Protein-G gold 10 nm (ProSciTech) in PBS for 90 min. Grids were washed, postfixed with 2% (wt/vol) glutaraldehyde in PBS for 10 min, washed again, contrasted with 1.5% (wt/vol) uranyl acetate, and viewed with a 300-kV Tecnai G2 F30 electron microscope (FEI). Exosomal and proximate free 3H1-positive grains were counted from five micrographs per cell line and expressed as the percentage of total 3H1-positive grains.

Release of SOD1 Aggregates. NSC-34 cells were transfected with HuWtSOD1–GFP, G127X SOD1–GFP, G93A SOD1–GFP, or A4V SOD1–GFP. After 72 h the conditioned media was collected as in *Electron Microscopy* and 50 μg of total protein was then analyzed by filter trap assay or Western blot. The filter trap assay was performed as previously reported (34). Any trapped SOD1–GFP material was measured using an anti-GFP antibody. Conditioned media was centrifuged at 13,000 × *g* on a benchtop microfuge for 30 min and pellets resuspended in PBS. Pelleted material was then added to naïve NSC-34 cells and incubated for 60 min at 37 °C. The cells were then fixed and permeabilized and internalized SOD1–GFP was measured using flow cytometry (BD LSR II; BD Biosciences).

IP. IP of cell lysate and preparation of antibody-coupled beads were performed as previously described (13). Following IP incubation, beads were washed three times with 150 μL PBS with brief vortexing in-between washes and boiled in SDS sample buffer. Samples were loaded onto 15% acrylamide Tris·glycine gels and separated by SDS/PAGE, followed by immunoblotting. Immunoblotting, detection, and quantification were performed as previously described (13).

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