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Identification of an age-related Parkinson's disease risk factor that regulates sulfur metabolism

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Abstract

Human aging is the main risk factor for Parkinson's disease (PD). To better understand age-related PD pathogenesis, we modeled PD with directly reprogrammed dopaminergic neurons (iDA) which preserve donor aging signatures. By transcriptome analysis and immunohistochemistry on postmortem tissues, we identified a sulfurtransferase, TSTD1, to be upregulated in aged and diseased individuals. TSTD1 catalyzes sulfur transfer from thiosulfate to glutathione (GSH). GSH and cysteine were significantly decreased in dopaminergic (DA) neurons with TSTD1 overexpression. Lower intracellular H₂S levels and mitochondrial membrane potential (MMP) were identified in aged, PD iDA, and TSTD1 overexpressing embryonic stem cell (ES)-derived DA neurons. TSTD1 overexpression could lead to GAPDH inhibition and energy deficiency in neurons. We hypothesize that TSTD1 upregulation in aged and PD individuals could disrupt sulfur metabolism which compromises anti-oxidant capacity and energy production in neurons; both of these mechanisms have been implicated as triggers for DA neuronal degeneration in PD.

Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease. The disease affects more than 1% of the population over the age of 60, increasing to 5% for individuals over 85 (1). Despite their importance in the development and onset of PD, genetic mutations account for only a minority of PD cases (2, 3); the majority of PD cases are classified as sporadic, without known genetic causes. While PD is a chronic progressive disorder, multiple studies have shown that clinical progression is mostly related to aging rather than to disease duration (4-6).

Dopaminergic (DA) neurons are the primary cell type that degenerates in PD (7). Like all types of neurons, DA neurons are prone to accumulation of damages and decay upon aging (8, 9). The majority of these neurons are located in the substantia nigra pars compacta (SNc). This region is rich in reactive oxidative species derived from dopamine and is relatively low in antioxidants (10, 11). The DA neurons also have high rates of oxygen metabolism due to their rapid firing nature and long projections to the dorsal striatum, nucleus accumbens and

prefrontal cortex (12). These characteristics make DA neurons prone to lifelong oxidative stress.

Sulfur-containing biomolecules, namely glutathione (GSH), cysteine, and hydrogen sulfide (H₂S), have been identified to have beneficial roles in aging and PD. A decline in concentration of these molecules in diverse systems upon aging has been reported in multiple studies. However, the reasons behind such depletion are not fully understood. GSH is the most abundant, low molecular weight thiol in animal cells (13). The molecule plays critical roles in many biological processes, including antioxidation (14), maintaining cell redox potential (15), and DNA synthesis (16). Studies with aged mice models have indicated that GSH levels decrease in the brain (17, 18). Research in humans has shown a consistent decrease in plasma GSH level in aged individuals (19, 20). In PD patients, the reduction of GSH in SNc is profound, but it is not obvious in other brain regions or in patients with other neurodegenerative diseases that also affect DA neurons (21, 22). Reduced immunostaining of GSH has been observed in DA neurons of PD patients (23). These findings suggest that GSH depletion in aging is closely linked to the development of PD.

Cysteine is a proteinogenic amino acid with a thiol sidechain. Cysteine is crucial in thiol redox homeostasis, which plays an important role in the development of neurodegenerative diseases (24). The amino acid is a precursor of GSH synthesis. The level of cysteine directly affects cellular GSH level. Nutritional supplement N-Acetyl cysteine has been shown to modulate progression of PD (25, 26) by restoring GSH levels. Cysteine is also a precursor for the production of H₂S. H₂S has been recognized as an important signaling molecule that acts on multiple hallmarks of aging (27). Decline of plasma H₂S has been shown in human subjects from 50 to 80 years of age (28). Reduced H₂S production has been found in brains of Alzheimer's and Huntington's disease patients (29, 30). Studies in rodent PD models have shown that administration of H₂S can rescue DA neurons from lesions caused by oxidative damage (31, 32).

A previous study from our lab (33) demonstrated that the neurons generated from direct reprogramming of fibroblasts (iNs) were superior to induced pluripotent stem cell (iPSC)-derived neurons as an age-equivalent, in vitro model for neuronal cell aging. The iNs generated from human donors displayed age-specific transcriptional profiles and revealed age-associated loss of nucleocytoplasmic compartmentalization. In this study, we made modifications to our current iN methodology to generate neurons of dopaminergic lineage (iDAs) from donors across a broad range of ages, with and without PD diagnosed at the time of donation. Transcriptomic analysis of iDAs revealed an enzyme in the sulfur metabolic pathway as a novel age-related PD risk factor. We further elucidated the role of this enzyme in the down-regulation of sulfur-containing compounds observed in aging and neurodegenerative diseases.

Results

Direct conversion of human skin fibroblasts into neurons expressing dopaminergic characteristics

Previous studies have demonstrated that functional DA neurons can be generated directly from human fetal fibroblasts (34, 35). These studies use forced expression of pioneering neural reprogramming factor *Ascl1*, alongside with dopaminergic lineage factors. Our lab has generated iNs with two neurodevelopmental factors, *Ngn2* and *Ascl1* (33). Without dopaminergic factors, the iNs represent a heterogeneous population of glutamatergic and GABAergic neurons. To generate human iDAs, we added a collection of dopaminergic factors to our *Ngn2*-*Ascl1*-based iN protocol (Figure 1A). Fibroblasts were lentivirally transduced to express rtTA, a 2A-peptide-linked *Ngn2* and *Ascl1*, and dopaminergic factors including *Lmx1a*, *Foxa2*, *Otx2*, *Pitx3*, *Nurr1* and *En2*. Cells were cultured in conversion media containing doxycycline (dox) to induce transgene expression (Supp Figure 1A). TGFβ/SMAD and GSK3β inhibitors were added to drive neural lineage commitment during reprogramming (Supp Figure 1A). Fibroblasts underwent morphological changes to typical neuronal morphologies during the conversion period. Four weeks post dox induction, neuronal cells stained positive for *Tuj1* and a few cells stained positive for tyrosine hydroxylase (TH) (Supp Figure 1B). To

increase the yield of iDA further and to enrich the TH-positive population, we screened for the most effective factor cocktails. Dopaminergic factors generally decrease neuron yield compared to Ngn2-Ascl1 only (Supp Figure 1C). Lmx1A appeared to be essential for generating TH-positive neurons (Supp Figure 1D). Fewer dopaminergic factors in the cocktail improved TH-positive neurons yield (Supp Figure 1D). We concluded that Ngn2, Ascl1, and Lmx1a was sufficient to generate TH-positive neurons with the highest yield (Supp Figure 1D).

In addition to transcriptional factors, hedgehog signaling activator purmorphamine, smoothened agonist (SAG), sonic hedgehog (SHH) and mid-brain patterning factor FGF-8 significantly increased the yield of iDAs (Supp Figure 1E). Since the function of purmorphamine and SAG overlaps with SHH. Eventually we used purmorphamine SAG and FGF8 in the optimized conversion medium, considering cost effectiveness. The optimized conversion medium, in addition to the Ngn2, Ascl1 and Lmx1a cocktail were used in the protocol to generate iDA (Figure 1B). With all optimizations, the reprogrammed cells were positive for pan neuron markers Tuj1, MAP2, and NeuN, dopaminergic markers TH, ALDH1a1, DDC and VMAT2 (Figure 1C-G). Around 20% of the reprogrammed cells were Tuj1-positive (Figure 1H). The TH-positive population was enriched to around 70% of Tuj1-positive cells (Figure 1I). Quantification revealed that fibroblasts from all donors yielded iDAs. Neither age nor disease status of the donor had a significant effect on the conversion efficiency (Figure 1H, I).

Functional characterization of iDAs

During week four of conversion, iDAs were gently displaced and co-cultured with either mouse astrocytes or human ES-derived neurons. In all conditions, iDAs displayed pronounced human synapsin-I promoter-driven red fluorescent protein (RFP) fluorescence (LV-hSyn-RFP) and mature neuronal morphologies after seven weeks of co-culture (Figure 2A, B).

The conditions of the co-culture had significant effects on the maturation of the iDAs. iDAs exhibited sodium and potassium currents in all coculture conditions (Figure 2D, H, L). When co-cultured with mouse astrocytes, about 15% of the neurons had several spikes in one current injection, meaning more mature neurons (Figure 2C). The iDAs exhibited very little spontaneous spiking activity (Figure 2E), and little synaptic activity (Figure 2F). When co-cultured with human ES-derived glutamatergic neurons, 100% of the iDAs had several spikes in one current injection (Figure 2G). iDAs exhibited very little spontaneous spiking activity (Figure 2I) and little synaptic activity (Figure 2J). Co-culturing with human ES-derived GABAergic neurons was the best condition, as the neurons exhibited the most mature phenotype from all conditions. About 70% of the neurons had several spikes in one current injection (Figure 2K). Neurons exhibited spontaneous spiking activity (Figure 2M), synaptic activity and even network bursts (Figure 2N). Overall, co-culturing with human ES-derived neurons produced mature iDAs, whereas in the GABAergic co-culture the synapses were most active, producing synaptic currents.

Dopamine release from iDA was detected with dopamine aptamer-modified nanopipettes. These nanopipettes were developed and validated to perform live recordings from dopaminergic neurons. Upon exposure to dopamine, the target-specific aptamers underwent significant conformational rearrangements within nanoscale orifices (36) and changed the current flux through the nanopore. In the presence of dopamine, the aptamer-modified nanopipettes showed a decrease in the current response at positive bias compared to the bare medium with no neurons cultured (Figure 2O). Alternatively, for the negative control from pan neuron culture media (with no dopaminergic neurons present), minimal change in the current from the bare medium was observed. Real-time recordings where sensors were moved from the bare medium to the negative control medium (Figure 2P) vs. negative control to iDA medium (Figure 2Q) further corroborated dopamine release

from iDA neurons. The iDA samples showed a statistically significant increase in dopamine amounts vs. the negative control (Figure 2R). These measurements corroborate the electrical and transmitter characteristics of the iDA neurons. To validate the sequence specificity of the aptamer, we tested the iDA samples with sensors modified with scrambled DNA. Control sensors detected statistically lower ($p < 0.0001$, $N=3$) amounts of dopamine in the iDA samples vs. the dopamine-specific sensors (Figure 2S), further confirming the specificity of aptamer-modified nanopipette sensors.

Purification of PSA-NCAM-positive iDAs for whole transcriptome analysis

To analyze the transcriptome of iDAs using RNA-seq, neurons need to be separated from fibroblast contamination. Multiple studies have shown that reprogrammed neurons express PSA-NCAM on the cell surface (33, 37, 38) and these neurons can be purified with a fluorescence-activated cell sorting (FACS)-based protocol. PSA-NCAM-positive cells from iDA conversion were harvested with FACS sorting for RNA-seq analyses (Figure 3A, B). Fibroblasts and iDA have very different gene expression profiles, with a total of more than 9,000 genes differentially expressed (Figure 3C). Genes up-regulated in iDAs include neuronal lineage genes such as TUBB3, MAP2, NEUROD2, Synaptophysin (Figure 3D, Supp Figure 2) and dopaminergic lineage genes like SLC6A3, PITX3, SLC18A2, FOXA2 and LMX1B (Figure 3E, Supp Figure 2). Genes such as collagen, keratin, vimentin, proteins regulating cell cycle and cell division were significantly higher in fibroblasts (Supp Figure 2). We took 200 of the most significantly up-regulated genes in iDAs for STRING network analysis. Data revealed that the top three GO terms for functionally enriched biological processes were neuron fate specification, catecholamine metabolic process and neuron fate commitment (Figure 3F, G). Principal component analysis showed iDAs and iPSC DA neurons overlapped with each other and separated from fibroblasts (Figure 3H), suggesting that the transcriptome of iDAs is more similar to iPSC DA neurons than fibroblasts. These data suggest that the sorted cells carry signatures of dopaminergic neurons.

Identification of Sulfurtransferase TSTD1 as an age-related PD risk factor in iDAs but not in iPSC DAs

To identify the age-related PD risk factor, we performed RNA-seq analysis of FACS-purified iDAs ($n = 24$ samples Supp Table 1). Differential expression analysis between young (<40 years) and old (≥ 40 years) groups revealed profound, age-dependent gene expression in the iDAs, with 78 genes being significantly differentially expressed (Figure 4A). Differential expression analysis between age-matched healthy controls versus sporadic PD (sPD) and genetic PD (gPD) groups revealed disease-dependent gene expression, with 82 genes between control and sPD and 75 genes between control and gPD that were significantly differentially expressed (Figure 4B, C). GO term analysis revealed that genes involved in calcium binding and sequence-specific DNA binding were down-regulated in aged iDAs. IGF binding proteins were upregulated in gPD together with several classes of binding proteins (Supp figure 3A-D). Groups like genes up-regulated in aging and down-regulated in gPD, did not give any significant functional annotations. There was no shared functional annotation category among the aging and PD comparisons.

We looked for individual genes that were commonly up-regulated/down-regulated in all three comparisons. There was no overlapping gene in the down-regulated pool and there were two genes that were commonly up-regulated in all comparisons, namely TSTD1 and MTRNR2L1 (Figure 4D). MTRNR2L1, humanin-like protein 1, is a peptide translated from a 16S ribosomal RNA gene, MT-RNR2 or humanin. Humanin has been well documented to have beneficial effects in neurodegenerative disorders and aging related diseases (39, 40). We speculate that the up-regulation of humanin could be a self-regulatory mechanism of the cells against stress due to aging or other PD-causing mechanisms. The other candidate, TSTD1 (thiosulfate sulfurtransferase domain containing 1), is an enzyme involved in physiological sulfur metabolism (41, 42). TSTD1 is

significantly up-regulated in the old (Figure 4E), sPD and gPD donors (Figure 4F). In contrast to iDAs, TSTD1 level in iPSC-derived DA neurons did not show significant difference between PD and healthy controls (Figure 4G). We acquired postmortem tissue from SNc from donors ranging from 0-83 years of age with 11 healthy controls and three PD donors (Supp Table 2). By performing immunohistochemistry (Figure 4H), we confirmed a progressive increase in TSTD1 expression with age (Figures 4I, 4J). TSTD1 level in PD donors was significantly higher than that in healthy controls (Figure 4I, 4K). Most interestingly, TSTD1 express exclusively in TH-positive neurons (Figure 4H), which suggests that TSTD1 could be regulating cellular functions specific to dopaminergic neurons that may cause their degeneration.

Sulfurtransferase activity of TSTD1 in neurons

TSTD1 has been shown to have rhodanese activity at alkaline pH (41, 42). The enzyme catalyzes the GSH-dependent conversion of thiosulfate to sulfite (Figure 5A). Our data showed that TSTD1 is active as a sulfurtransferase at a common intracellular pH, ranging from 7.4 to 8.0 (Figure 5B). We established human ES cell lines (H1 and H9) that host a dox-inducible TSTD1 expression cassette (Figure 5C). We generated DA neurons from these cell lines and induced TSTD1 overexpression for seven days (Figure 5C). Mass spectrometry analysis revealed a decrease in GSH in the TSTD1-overexpressing cells (TSTD1-OE) compared to the vector control cells (Figure 5D). On the other hand, there was no significant change in the oxidized form of glutathione (GSSG) (Figure 5D). The TSTD1-OE neurons had a lower GSH/GSSG ratio, suggesting the cells were experiencing higher oxidative stress (Figure 5D). In addition to the reduced GSH, the cellular levels of the precursor amino acids for GSH synthesis, cysteine, glutamate and glycine (Figure 5E), were also significantly down-regulated in TSTD1-OE neurons (Figure 5F), which indicates that precursor amino acids were mobilized to compensate TSTD1-induced GSH depletion. Despite cysteine, sulfur-containing amino acids like homocysteine, methionine and cystine, and sulfur-containing molecules such as taurine and SAM were not significantly down-regulated (Figure 5G), suggesting that TSTD1 expression affected specifically the cysteine-GSH pathway.

TSTD1 up-regulation reduces cellular hydrogen sulfide level

Metabolism of cysteine is a source for the production of hydrogen sulfide (H_2S) in cells (Supp Figure 4A). We were interested to see if TSTD1-induced cysteine leads to down-regulated cellular H_2S levels. We used a H_2S -specific detection probe, sulfidefluor-7AM, which emits green fluorescence when reacted with H_2S (Supp Figure 4E). We investigated the endogenous H_2S level in our iDA model (Figure 6A). The fluorescence signal in PSA-NCAM-labeled iDAs was recorded by flow cytometry as a representation of intracellular H_2S level. In aged (>40yrs) and PD groups, the levels of H_2S were significantly lower than in young healthy controls (Figure 6B). H_2S is metabolic pathway contributes electrons to the mitochondrial electron transportation chain (Supp Figure 4B). Therefore, we expected that reduced cellular H_2S could affect the mitochondrial membrane potential (MMP) in these cells. To measure MMPs specifically in iDA cells, we used flow cytometry analysis of PSA-NCAM-labeled iDAs with the green fluorescent cationic dye JC-1, which accumulates in red fluorescent aggregates in mitochondria with high MMPs. The data showed a significant drop in MMP in PD iDAs (Figure 6C). The observations on H_2S level and MMP in iDAs were also validated with the TSTD1-OE neurons (Figure 6D). TSTD1 overexpression induced a decrease in cellular H_2S level (Figure 6E) and MMP (Figure 6F) in hES-derived DA neurons. To validate whether the H_2S depletion was a consequence of TSTD1-induced cysteine depletion, we supplemented TSTD1-OE neurons with cysteine in the culture and observed that the TSTD1-induced decreases in both H_2S and MMP were rescued with cysteine supplementation (Figure 6G). Analysis of our RNAseq data also revealed that neither the genes involved in the H_2S production pathway (Supp Figure 4C) nor the consumption pathway were differentially expressed (Supp Figure 4D). We further

investigated the effects of knocking down endogenous TSTD1 expression in iDAs by making a dox-inducible shRNA construct for TSTD1 (TSTD1_sh). In both sPD and gPD we observed an increase in H₂S level and MMP (Figure 6H) in the TSTD1_sh group compared to scramble control, suggesting that up-regulated TSTD1 expression was a cause of down-regulation of H₂S and MMP in PD iDAs.

TSTD1 disrupts energy production in neurons

Glutathione persulfide (GSSH) is the product of sulfurtransferase activity of TSTD1. Studies have shown that GSSH is capable of modifying GAPDH and leading to inhibition of enzymatic activity (43, 44). Therefore, we speculated that TSTD1 up-regulation would lead to an inhibition of GAPDH activity and disrupt the downstream energy production pathway. We harvested control and TSTD1-OE DA neurons with seven days of dox induction. Neurons were treated with different concentrations of dox to induce different levels of TSTD1 expression (Figure 7A, B). There was no significant change in GAPDH level in any treatment group (Figure 7B). Commercial kits were used to determine GAPDH activity (Sigma-Aldrich) and cellular pyruvate (Cayman chemical) and ATP levels (Promega). We observed a significant drop in GAPDH activity, pyruvate levels, and ATP levels with high TSTD1 expression (Figure 7C-E). Empty-vector control neurons do not exhibit changes in GAPDH activity, or cellular pyruvate and ATP levels (Figure 7C-E), suggesting that disruption of energy production is due to TSTD1 over-expression.

Discussion

Aging has always been known as a major risk factor of PD, but the role of aging in PD development remains an unsolved puzzle. Identifying age-related risk factors has been challenging for several reasons. In vitro models like cell lines and iPSC-derived DA neurons do not accurately maintain neuronal aging signatures. Animals do not naturally develop Parkinsonian phenotypes and animal models of PD rely heavily on chemical or genetic induction. Postmortem tissue has been regarded as the gold standard to identify mechanisms in disease development by histology. However, when studying transcriptomic changes in postmortem tissues, there is a concern that DA neuron-associated risk factors might be underrepresented due to the sparse distribution of DA neurons in SNc, especially in PD patients. In a recent study, single cell RNAseq analysis of 40,000 cells in human SNc postmortem tissues only included 72 DA neurons, suggesting that previous transcriptomic analysis of bulk SNc tissue likely represents non-DA neuron cell types (45). We developed a protocol that was able to generate functional iDAs from human skin fibroblasts. These iDAs carry donor aging and disease signatures and they represent the relevant cell types that degenerate in PD.

Compared to the generation of pan neurons, reprogramming fibroblast into the dopaminergic lineage is challenging due to the lack of an efficient transcription factor cocktail and culture conditions. We were inspired by protocols generating DA neurons from human ES cells and we used compounds that drove the ventral midbrain patterning of ES cells to boost our conversion efficiency by almost 25%. In addition, we investigated co-culture conditions that support the maturation of iDA. Previous studies had co-cultured reprogrammed neurons with mouse astrocytes (31-35). We found that mouse astrocytes were not the best option, possibly due to the fact that the astrocytes were from another species that did not provide the correct growth factors for human neurons. On the other hand, neurons in the brain lives in a niche created by multiple cell types. Inter-neuron communication also provide guidance for their development and maturation. These conditions are lacking in a mouse astrocytes co-culture. ES cell-derived neurons are very robust and mature. Neurons generated this way reside with a mixture of multiple cell types emerged during the differentiation. Neurons are supported by these cells and neurons also communicate with each other. We co-cultured iDA with ES derived glutamatergic or GABAergic neurons and physiological recording data showed significant improvement in action potential firing, spontaneous firing and synaptic activity of the iDAs. Moreover, iDAs

behaved differently when co-cultured with glutamatergic and GABAergic neurons. DA neurons mainly reside in the SNc and project into the striatum, where they connect to the GABAergic medium spiny neurons. DA neurons can also project to the prefrontal cortex from ventral tegmentum and receive input from glutamatergic neurons. Both types of neurons naturally communicate with different afferents to the DA neurons which could explain why iDAs behave differently in these coculture conditions. The exact effect of different neuron subtypes on iDA physiological behavior and the possibility of using iDAs to model physiological parameters of PD could be elucidated in future studies.

In addition to observing appropriate marker expression and electrophysiological signatures, we demonstrated dopamine release from iDAs. Neurochemical recording is traditionally challenging for dopamine, due to the high degree of structural similarity with other neurochemicals such as norepinephrine and *L*-DOPA (46). State-of-the-art methodologies for dopamine detection include microdialysis sampling coupled to high-performance liquid chromatography and mass spectrometry (47), which suffers from relatively low spatiotemporal resolution. The alternative approach of fast scan cyclic voltammetry (48) encounters challenges in differentiating dopamine vs. precursors and metabolites with overlapping redox potentials. Aptamer-modified nanopipettes have been reported to overcome conventional shortcomings by providing rapid quantification of neurochemicals such as serotonin in untreated complex media with high specificity and selectivity (49, 50). The DNA aptamers endow the sensors with chemical selectivity and the confinement of sensing elements inside the ~10 nm orifice, minimizes the entry of nonspecific molecules present in complex media that cause sensor biofouling. The challenge with recording DA release from reprogrammed neurons is mostly due to the low density of iDAs in culture, in which the released DA could be easily diluted beyond detectable range. Aptamer-modified nanopipettes provided extraordinary sensitivity in detecting trace amounts of dopamine in culture medium, not only for dopamine release from iDA but also the background dopamine level in the glutamatergic neurons we used to support the iDAs. This technology could greatly facilitate the use of iDAs and other cell types in characterizing disease phenotypes regarding dopamine release.

The cohort of the donors in this study has a wide age distribution (0-74yrs) and both sPD and gPD diagnoses. We compared transcriptome signatures between young and old to determine aging dependent signatures. We then compared the PD patients with the healthy age-matched donors to determine PD-dependent signatures. We compared gPD to healthy donors separately from the sPD donors. Although it is generally believed that genetic risk factors are the main contributors towards disease development in gPD patients, aging risk factors still play a crucial role, as not all individuals carrying the mutations develop PD and those who do develop PD have an onset at earliest a few decades after birth. Comparing sPD and gPD donors with healthy donors separately helped us to screen out common gene factors between the two types of PD, resulting in the identification of two genes that were commonly up-regulated in aging, gPD and sPD.

MT-RNR2L1 is a peptide translated from humanin. Humanin has been very well documented to have neuroprotective effects in neurodegenerative disorders. Humanin specifically binds to an inactive form of Bax, inhibits its transfer to the mitochondria and suppresses initiation of apoptosis (39). Humanin also inhibit neurotoxicity induced by several familial Alzheimer's disease genes (40). Due to the strong evidence that humanin is a protective factor against neural degeneration, we speculate that the upregulation of MT-RNR2L1 in PD and aged iDAs is a defense mechanism against stress coming from disease-causing factors.

The only other common gene among the three comparisons is TSTD1. TSTD1 has been shown to catalyze the transfer of sulfur from thiosulfate to GSH, producing GSSH (41). Our data showed that TSTD1 overexpression depleted cellular GSH, which is the major antioxidant in cells. GSSH is a potent and unstable reducing agent (44). Unlike GSH, GSSH has not been shown to be utilized by enzymes involved in the antioxidation pathway. Instead, the strong spontaneous reducing ability enables GSSH to sulfhydrylate cysteine residues on proteins, alternating their activity and their corresponding molecular pathways. It is not evident that GSSH production

could compensate the loss of GSH in antioxidation. Moreover, protein sulfhydrylation resulting from GSSH could lead to undesirable biological effects, such as the inhibition of GAPDH, which is a major enzyme in the glycolysis pathway (44). Our data revealed that GAPDH activity and subsequently pyruvate and ATP levels were down-regulated upon TSTD1 overexpression. We will investigate the sulfhydrylation status of GAPDH in future studies in order to elucidate whether GSSH is responsible for inactivating GAPDH.

In addition to GSH depletion, TSTD1 overexpression leads to cysteine and H₂S downregulation. Our data showed that these changes are related as cysteine and other GSH precursor amino acids were downregulated with GSH depletion in cells. Cysteine reduction is a direct cause of H₂S down-regulation, as supplementing cysteine in culture media could rescue cellular H₂S level. These data suggested that, in addition to GSH depletion, TSTD1 up-regulation affects the metabolism of downstream sulfur-containing molecules whereas reduction of GSH, cysteine and H₂S has been well reported as a signature of aging and PD development (17-24, 27, 28). We hypothesized that TSTD1 is an age-related PD risk factor through several different mechanisms. First, TSTD1 causes depletion of GSH, which has a direct impact on the antioxidation machinery in neurons. Second, the reaction product of TSTD1, GSSH, has the potential to inhibit GAPDH and therefore to cause energy deficiency in neurons. Third, H₂S has been reported to regulate multiple aging hallmarks (27). Therefore, H₂S down-regulation caused by TSTD1 expression could have an extended range of effects on aging and PD development, which should be investigated in forthcoming studies.

In this study, we provide a methodology for generating mature functional DA neurons with direct reprogramming. The cells preserve transcriptomic features of their donors' age and disease status that are suitable to identify age-related dopaminergic risk factors. Based on transcriptome profiling and histological validation, we identified TSTD1 as a significant factor involved in PD pathogenesis. TSTD1 negatively regulates sulfur-containing molecules, which could alter aging-related cellular machinery and increase the exposure of DA neurons to oxidative stress.

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Figure Legends

Figure 1. Direct conversion of healthy and PD human fibroblasts into functional iDAs is efficient regardless of donor age and disease status

A. Conversion cocktail screening for reprogramming of human fibroblasts into DA neurons with Ngn2, Ascl1 and a collection of dopaminergic factors. B. Optimized conversion cocktail with dox-inducible Ngn2, Ascl1, Lmx1a, ventral midbrain signaling factors, and TGFβ/SMAD and GSK3β inhibitors in the medium. C-

G. Immunostaining of iDAs for (C) TujI and NeuN; (D) Vmat2 and MAP2; (E) DCC and TujI; (F) ALDH1a1 and TujI; (G) TH and TujI. The scale bars represent 50 μ m. H. Quantification of neuronal yields with TujI-positive per DAPI from 26 donors following four weeks of conversion. The bar graphs show mean $\pm$ SD. I. Quantification of DA neuronal yields with TH-positive per TujI from 26 donors following four weeks of conversion. The bar graphs show mean $\pm$ SD.

Figure 2. Electrophysiological and dopamine release recording of iDAs

A. hSyn-RFP-labelled iDAs cocultured with mouse astrocytes, human GABAergic and glutamatergic neurons. B. Electrophysiological recording of an RFP labelled iDA (electrode marked with dashed line). C-F. Physiological recording data of iDAs cocultured with mouse astrocytes. (C) Action potential firing induced by current injection; (D) Na⁺ and K⁺ current; (E) spontaneous firing (F) excitatory postsynaptic current. G-J. Physiological recording data of iDAs cocultured with human glutamatergic neurons. (G) Action potential firing induced by current injection; (H) Na⁺ and K⁺ current; (I) spontaneous firing; (J) excitatory postsynaptic current. K-N. Physiological recording data of iDAs cocultured with human GABAergic neurons. (K) Action potential firing induced by current injection; (L) Na⁺ and K⁺ current; (M) spontaneous firing (N) excitatory postsynaptic current. O. DA aptamer-modified nanopipettes detect DA in iDA medium while negligible changes from the bare medium was observed in the negative control. P, Q. Real-time recordings of sensor transitioning from (P) bare medium to the negative control medium and (Q) negative control to iDA medium. R. Quantification of DA level using specific DA sensors in iDA medium vs. negative control. S. Validation of sensor specificity – negative control sensors (scrambled DNA vs. specific aptamer) react minimally to iDA medium. Data for the DA sensors in iDA medium is reproduced in panel (R) and (S) for comparative purposes. The bar graphs show mean $\pm$ SEM (significance values: ***p < 0.0001).

Figure 3. FACSsorting of iDAs and transcriptome analysis of DA neuron lineage commitment

A. Immunostaining of iDAs for human Ncam and MAP2. The scale bar represent 100 μ m. B. Cloud plot of Ncam-positive population in FACSsorting. C. MA plot shows differential expression between fibroblast and iDA. D. Expression level of pan neuron marker TUBB3, MAP2 and NEUROD2 in iDA and fibroblast. E. Expression level of dopaminergic marker SLC6A3, VMAT2, PITX3, LMX1B, FOXA2 in iDA and fibroblast. F. STRING network analysis of 200 most significantly upregulated genes in iDA. G. GO terms for functionally enriched biological process in STRING network analysis. H. PCA analysis showing grouping of iDAs (red), fibroblasts (green) and iPSC DA neurons (blue).

Figure 4. Identification of TSTD1 as an age-related PD risk factor

A-C. MA plot shows differential gene expression between (A) young (<40yrs) and old ($\geq$ 40 years), (B) healthy vs sPD and (C) healthy vs gPD. D. Schematic Venn diagram showing the overlap of aging, sPD and gPD up-regulated genes. E. TSTD1 expression in young (<40yrs) and old ($\geq$ 40 years) iDAs. F. TSTD1 expression in healthy, sPD and gPD iDAs. G. TSTD1 expression in healthy, sPD and gPD iPSC DA neurons. H. Immunohistochemistry staining of postmortem SNc for TH and TSTD1. The scale bars represent 10 μ m. I. TSTD1 expression level quantified per TH-positive cells in healthy and PD donor of different ages. J. TSTD1 expression level in healthy young (<40yrs) and old ($\geq$ 40 years) donors. K. TSTD1 expression level in healthy and PD donors (significance values: ***p < 0.001).

Figure 5. Functional characterization of TSTD1

A. Schematics showing the enzyme activity of TSTD1. B. Enzyme activity of TSTD1 at pH7.4-8.0. C. Schematics showing generation of TSTD1-expressing DA neurons from ES cells. D. Mass spectrometry quantification of GSH, GSSG and GSH/GSSG ratio in control and TSTD1-OE DA neurons. E. Synthesis pathway of GSH from cysteine, glutamate and glycine. F. Mass spectrometry quantification of cysteine, glutamate and glycine in control and TSTD1-OE DA neurons. G. Mass spectrometry quantification of cysteine, homocysteine, methionine, SAM and taurine (significance values: *p < 0.05, **p < 0.01).

Figure 6. Quantification of cellular H₂S and mitochondrial membrane potential (MMP) in relation to TSTD1 expression

A. Schematic showing the generation of iDAs from human skin fibroblasts. B. Endogenous H₂S level in healthy young, healthy old, sPD and gPD iDAs. C. Endogenous MMP in healthy young, healthy old, sPD and gPD iDAs. The bar graphs in B and C show mean $\pm$ SEM. D. Schematics showing generation of TSTD1-expressing DA neurons from ES cells. E. Cellular H₂S level in control and TSTD1-OE DA neurons. F. Endogenous MMP in control and TSTD1-OE DA neurons. G. Cellular H₂S level and MMP in control, TSTD1-OE and TSTD1-OE with cysteine supplement. H. Cellular H₂S level and MMP in iDAs with TSTD1_{sh} and scramble control (significance values: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Figure 7. TSTD1 expression affects energy production in DA neurons

A. Schematics showing generation of TSTD1-expressing DA neurons from ES cells. B. Western blot of GAPDH and TSTD1 expression levels at different concentrations of dox treatment. C-E. (C) GAPDH activity, (D) pyruvate and (E) ATP level in control and TSTD1-OE DA neuron under increasing concentrations of dox treatment (significance values: *p < 0.05, ***p < 0.001).

Supp Figure 1. Optimization of iDA conversion condition

A. Schematics showing screening of conversion cocktail with dox-inducible Ngn2, Ascl1, dopaminergic factors, and TGF β /SMAD and GSK3 β inhibitors in conversion medium. B. Immunostaining of converted neuron for TujI and TH. C. Quantification of neuronal yields with TujI-positive and TH-positive per DAPI from different combinations of conversion factors. The bar graphs show mean $\pm$ SD. D. Quantification of neuronal yields with TH per TujI from different combinations of conversion factors. The bar graphs show mean $\pm$ SD. E. Quantification of neuronal yields with TH per TujI from different combinations of purmorphamine, SAG and SHH. The bar graphs show mean $\pm$ SD (significance values: *p < 0.05)

Supp Figure 2. Expression changes of DA neuron (blue) and fibroblast (red) marker genes between iDA and fibroblast after four weeks of conversion

Supp Figure 3. GO term analysis of differentially expressed genes in iDA transcriptome comparison

GO term analysis for molecular function of genes that were (A) down-regulated in (≥ 40 years) old iDAs compared to young (<40yrs), (B) up-regulated in sPD compared to healthy, (C) down-regulated in sPD compared to healthy, (D) up-regulated in gPD compared to healthy. Orange bars labelled GO terms with p-value < 0.05.

Supp Figure 4. Expression of genes in H₂S production and consumption pathway and cellular H₂S staining

A. Schematics of H₂S production pathway and enzymes involved. B. Schematics of H₂S consumption pathway and enzymes involved. C. Expression of genes involved in H₂S production pathway in young vs old and control vs PD iDAs. D. Expression of genes involved in H₂S consumption pathway in young vs old and control vs PD iDAs. E. Molecular structure of sulfidefluor-7AM and staining of cellular H₂S with sulfidefluor-7AM.

Supp Table 1. List of fibroblasts and corresponding donors' information used in this study.

Supp Table 2. List of postmortem tissues and corresponding donors' information used in this study

Supp Table 3. List of antibodies used in this study

Dilution factor are for immunofluorescent staining, if not labelled specifically for western blotting (WB).

Supp Table 4: MRM transitions in positive mode for amino acids.

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Figures

Figure 1

Direct conversion of healthy and PD human fibroblasts into functional iDAs is efficient regardless of donor age and disease status A. Conversion cocktail screening for reprogramming of human fibroblasts into DA neurons with Ngn2, Ascl1 and a collection of dopaminergic factors. B. Optimized conversion

cocktail with dox-inducible Ngn2, Ascl1, Lmx1a, ventral midbrain signaling factors, and TGF β /SMAD and GSK3 β inhibitors in the medium. C- G. Immunostaining of iDAs for (C) TuJ1 and NeuN; (D) Vmat2 and MAP2; (E) DCC and TuJ1; (F) ALDH1a1 and TuJ1; (G) TH and TuJ1. The scale bars represent 50 μ m. H. Quantification of neuronal yields with TuJ1-positive per DAPI from 26 donors following four weeks of conversion. The bar graphs show mean $\pm$ SD. I. Quantification of DA neuronal yields with TH-positive per TuJ1 from 26 donors following four weeks of conversion. The bar graphs show mean $\pm$ SD.

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- [SuppTable2.pdf](#)
- [SuppTable3.pdf](#)
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