An Optimised Method for the Proteomic Analysis of Low Volumes of Cell Culture Media and the Secretome: The Application and the Demonstration of Altered Protein Expression in iPSC-Derived Neuronal Cell Lines from Parkinson's Disease Patients.

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ABSTRACT: Traditionally, cell culture medium in iPSC-derived cell work is not the main focus of research and often considered as just "food for cells". We demonstrate that by manipulation of the media and optimised methodology, it is possible to use this solution to study the proteins that the cell secretes ("secretome"). This is particularly useful in the study of iPSC-derived neurons, which require long culture time. We demonstrate that media can be used to model diseases with optimised incubation and sampling times. The ability not to sacrifice cells allows significant cost and research benefits. In this manuscript we describe an optimised method for the analysis of the cell media from iPSC-derived neuronal lines from control and Parkinson's disease patients. We have evaluated the use of standard and supplement B27-free cell media as well as five different sample preparation techniques for proteomic analysis of the cell secretome. Mass spectral analysis of culture media allowed for the identification of > 500 proteins, in 500 µL of media which is 20-40 less volume than reported previously. Using shorter incubation times and our optimized methodology, we describe the use of this technique to study and describe potential disease mechanisms in Parkinson's disease

Keywords: Induced Pluripotent Stem Cells, Secretome, Parkinson's Disease, Proteomics, Mass Spectrometry

INTRODUCTION

Induced pluripotent stem cells (iPSC) hold great promise in understanding disease mechanisms, drug screening and regenerative medicine[1,2]. They can be generated through a reprogramming process of easily accessible human somatic cells such as fibroblasts, and can then be further differentiated into any cell type[3]. This ability is particularly interesting in the generation of cells which are not easily accessible for studies, i.e. neurons or cardiomyocytes. For example, iPSC-derived neurons are used for studying neurodegenerative disorders, such as Alzheimer's disease (AD)[4], Parkinson's disease (PD)[5,6] or other forms of dementia[7].

The majority of proteomic studies focus on the cell lysates[8–13], as it enables the in-depth analysis of the complex cellular or organellar proteome. However, this destructive approach may be problematic in the experiments requiring sampling at different time points, as it will incur growing numerous replicates and thus increasing the cost of experiments. This is of particular note as iPS-derived neurons can take up to 70-100 days before they are considered mature enough for experimental purposes. This is a significant period of time and research resources. Moreover, analysis of cell lysate does not always provide information about the secreted proteins or extracellular matrix, which is crucial in studies of the nervous system. Analysis of the cell culture media, in which cells are incubated, can complement studies of cell lysates and provide insight into the cell secretome. These liquids are intended to provide the essential nutrients to the cells and therefore support their growth and differentiation. Cells during growth not only absorb the nourishing substances, but in a two-way process they also secrete proteins and metabolites to the environment. The cell culture media is most often changed daily and therefore the analysis of cell supernatants could provide valuable information about molecules secreted by cultured cells, without increasing the experiment cost.

Thecell media can be an excellent source of information about the cell secretome andseveral reports describe proteomic analysis of this material[14–20], however only few describe the secretome of pluripotent stem cells or neural cells[16,18,21–24]. This is probably due to the technical constraints, as secreted proteins are present in the media at very low level, hampering their identification in such complex fluid, containing high levels of essential amino acids and glucose. However, perhaps the biggest hurdle in analyzing the secretome is the presence of highly abundant bovine serum albumin (BSA), transferrin, insulin or fetal calf serum, essential for the cells growth, maturation and survival. Therefore the sample preparation technique for proteomic analysis should be carefully chosen to maximize the number of detected proteins. A number

of studies have shown that analysing cell secretome in serum-free media yield higher number of identified proteins[16–19], and various protein concentration techniques were employed. These include adsorption to a carrier, extraction[17], filtration[18]or precipitation[19]. Yet all studies described above, required high volumes of cell media (1-40 mL) obtained from large-scale cultures (from flasks to 100 mm dishes). These studies led to identification of dozens to hundreds of proteins by the mean of 2D gel electrophoresis and/or mass spectrometry (MS). More sophisticated techniques, such as the combination of MS analysis and bioinformatics were used to reveal the secretome of astrocytes[21], or iterative exclusion MS approach for analysis of human embryonic stem cell secretome[23]. Here, we describe an optimized method suitable for proteomic analysis of cell media obtained from small scale cultures (35mm, 500,000-1 million cells) of iPSC-derived dopaminergic neurons, using only 500 μL of cell supernatant that produces reproducible results and which model the disease phenotype. Prepared samples were analysed using nano-liquid chromatography coupled with high resolution mass spectrometry (nanoLC-MS/MS), enabling identification of hundreds of proteins per sample.

EXPERIMENTAL PROCEDURES

Cell culture. iPSC from a familial PD patient carrying gene triplication of SNCA encoding α-synuclein and unaffected controls were maintained on Matrigel (Corning) in Essential 8 cell culture media (ThermoFisher) and passaged using collagenase IV. Details of the iPSC lines used are given in Table S-1.

Neuronal differentiation. iPSCs were differentiated into midbrain dopaminergic neurons as described previously[25]. Details are given in SI.

Microscopy. Neurons were fixed with 4% paraformaldehyde and permeabilised in 0.1% triton, 0.2M glycine and 5% donkey serum. Primary antibody dilutions were performed in PBS with 5% donkey serum; secondary antibody dilutions were performed in PBS. Hoechst was used to stain nuclei. Primary antibodies used were β III tubulin (rabbit, Covance, 1:1000), α-synuclein (mouse, BD, 1:400) and tyrosine hydroxylase (mouse, R&D Sytems, 1:400). Cells were analysed using a Leica TCS SPE confocal microscope.

Supernatant and cell sample collection. Growth media of iPSC-derived neurons was collected 3, 6 or 24 h after addition to cells (“standard” supernatant), for “B27-free” samples cells were washed with PBS and neuronal media without B27 supplement was added to cells for 24 hours, then collected and replaced with normal media. Some samples were centrifuged at 300 g for 5 min to remove cellular debris in the supernatant. Cell samples were collected by washing cells with PBS,

detaching them with accutase and centrifuging for 5 min at 300 g. Dried pellets and supernatant were stored at -20°C before being analysed.

Preparation of cell supernatants. In order to reduce biological variation, 600 µL of each cell supernatant was pooled (Table S-1). Samples of standard and B27-free media were pooled into the separate tubes. Obtained mixtures were vortexed thoroughly and 500 µL was aliquoted to the low protein binding microcentrifuge tubes. Each aliquot was subsequently prepared using different method: ultrafiltration, acetone precipitation, MeOH/CH_3Cl precipitation, TCA/Acetone precipitation or direct digestion. Detailed protocols are given in SI. For proteomic analyses of the Matrigel, 500 µL of Matrigel was spun at 13,000 rpm for 10 min to remove any precipitate. Then, proteomic digestion, solid phase extraction, peptide assay and mass spectrometry analysis were performed as described below.

Materials and reagents for proteomic analysis. All materials were of analytical and mass-spectrometry grade. DL-dithiothreitol (DTT), iodoacetamide, ASB-14, Tris base and urea were all purchased from Sigma-Aldrich. UPLC-MS grade acetonitrile (ACN), formic acid (FA) and water were obtained from Fluka, and sequencing-grade modified porcine trypsin from Promega. All buffers and solutions were prepared using ultra-pure 18 MΩ water (MilliQ) and UPLC solvents using UPLC-MS grade water.

Proteomic Digestion. Freeze-dried protein pellets were reconstituted in 20 µL of digestion buffer containing 0.1 M Tris, pH 7.8, 8 M urea and 2% of ASB-14 and were shaken at room temperature for 1 hour. Then, 1.5 µL of a 30 mg/mL solution of dithioerythritol (DTE) was added into each sample followed by shaking for 1h. Then 3 µL of 36 mg/mL iodoacetamide (IAA) solution was added and samples were shaken for 30 min at RT in the dark. 155 µL of fresh MilliQ water, followed by 10 µL of 0.1 µg/µL trypsin solution was added to the samples. Samples were vortexed and incubated for 16h at 37 ˚C in a water bath.

Solid Phase Extraction.180 µL of 0.2% Trifluoroacetid acid (TFA) was added into every sample prior to the solid phase extraction. Samples were purified using C18 Isolute 96-well plate (Biotage) according to the manufacturer's instructions. Collected peptides were freeze-dried and resuspended in 3% ACN and 0.1% TFA.

Peptide Assay. The Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay was performed to evaluate the concentration of proteins in samples, according to the manufacturer's instructions.

Online fractionation of samples.1 pmol of yeast enolase reference standard (Waters Ltd, Manchester, UK) was added to each sample and 0.4 µg of the sample per fraction was analysed using a nanoAcquity UPLC 2D-LC system coupled with

Synapt G2-Si mass spectrometer (Waters Ltd, Manchester, UK). Samples were analysed in shotgun and 4-fraction mode. In shotgun approach, peptides were separated under low pH conditions as described before[26]. In fraction approach, peptides were fractionated in 4 steps (11.3%, 15.3%, 19.3 and 50% of solvent B) under high pH conditions using XBridge BEH C18 Trap Column, 130Å, 5 µm, 180 µm x 50 mm (Waters). Solvent A was 20 mM ammonium formate in water (pH 9) and solvent B was 100% acetonitrile (ACN). Eluted peptides were separated under low pH conditions as described before[26].

Label-free UDMS^{E} mass spectrometry. Mass spectrometry analysis was performed as described previously[26]. Details are given in SI. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[27] with the dataset identifier PXD010390.

Bioinformatic analysis. Raw data were imported into Waters ProteinLynxGlobalServer version 4.1(Waters Ltd, Manchester, UK) in order to identify peptide masses corresponding to the fragmentation ion data. Mass corrections was applied based on the [glutamic acid1]-fibrinopeptide B mass delivered via an auxiliary pump. Waters Protein-Lynx Global server v 4.1 was used for protein identification and the Progenesis LC-MS software (Nonlinear dynamics) was used for differential expression analysis. The sequence of P00924 yeast enolase, P00761 porcine trypsin and P02769 BSA were manually added to a human canonical reference proteome database obtained from UniProt. Database searching parameters were set to two fragment ions matched per peptide, four fragment ions per protein and two peptides per protein. Fixed modifications of carbamidomethylation of cysteines, dynamic modifications of oxidation of methionine, 1 missed cleavage sites and maximum protein mass 800 kDa were included in protein identification parameters. 4% of false discovery rate and auto mass tolerance for ion and fragments were set. A list of proteins identified with >95% confidence were exported to open-source GeneOntology (GO) analysis tools Panther[28] and WebGestalt[29].

RESULTS AND DISCUSSION

Generation of iPSC-derived neurons. iPSCs derived from a patient carrying a triplication of the *SNCA* gene, encoding alpha-synuclein (referred to as patient cells), were differentiated into midbrain dopaminergic neurons alongside cells from unaffected controls. This rare mutation causes early onset and rapidly progressing PD accompanied by dementia[5,30]. To confirm the neuronal identity of iPSC-derived neurons cells were stained for neuronal markers β III tubulin (TuJ1), tyrosine hydroxlase (TH) and α-synuclein (Fig. S-1). Approximately 40-60% of cells displayed expression of these markers demonstrating that iPSCs have successfully been differentiated into dopaminergic neurons. Recently it had been shown that neurons derived from these *SNCA* triplication iPSCs display increased levels of reactive oxygen species compared to control

cells[31]. Furthermore ER-mitochondria associations are reduced in these *SNCA* triplication neurons compared to control cells[32]. Together these studies suggest that *SNCA* triplication neurons have disrupted mitochondrial function and calcium homeostasis and are subjected to increased levels of oxidative stress.

Comparison of sample preparation methods. In the first part of the study, we compared the number of identified proteins in standard and B27-free cell supernatant from cells incubated for 24h and collected without a centrifugation step. Standard cell supernatant contained commercially added proteins: BSA, catalase, human recombinant insulin, human transferrin and superoxide dismutase, while B27-free cell supernatant was deprived of these molecules. Supernatants from iPSC-derived neurons with triplication of SNCA gene locus and unaffected controls were pooled in order to reduce biological variation. This was an important step, as the biological variance would have influenced the number of identified proteins thereby affecting the results of the first part of the study. Samples were then prepared using different protein enrichment methods (Figure 2): ultrafiltration and protein precipitation using acetone, methanol/chloroform or TCA/acetone solutions and one sample was prepared without enrichment step. All samples were subsequently trypsin digested and submitted firstly to a shotgun label-free MS analysis. Moreover, the same concentration of total protein (0.4 µg of the sample per fraction) was injected on the analytical column in order to take into account differences in protein concentrations between samples. Indeed, cell numbers may vary between cell wells, which can influence the concentration of secreted proteins, it is therefore important to normalize the quantitative data.

As for standard supernatant analysis, direct digestion (without enrichment) was the most efficient preparation technique, allowing for identification of 28 proteins from 500 µL media (Figure 1 and S-2). Despite different preparation methods, BSA was the most abundant protein in all samples with sequence coverage above 85%, suggesting that its high concentration in normal cell media causes ion suppression and hampers identification of less abundant proteins secreted by cells. On the other hand, proteomic analysis of B27-free cell supernatant revealed that when removing commercially added proteins from the cell media, the number of identified proteins could be increased significantly by factor 10 or more (Figure 1). Acetone precipitation remained the most efficient sample preparation technique, enabling identification of 536 proteins (Figure 2 and 3). Protein precipitation using 10% TCA in acetone allowed for identification of 521 proteins, direct digestion- 418 proteins and ultrafiltration- 268 proteins respectively. Interestingly, BSA was also the most abundant protein detected in the B27-free samples. This can be explained by the remains of BSA present in the cell dish after washing with PBS, or absorbed, then secreted back into the media by the cells. Importantly, the observation under microscope of cells incubated for 24h in

B27-free media did not show any negative effect on the morphology of cells. After collection of B27-free cell supernatant for proteomic analysis, cell culture was successfully continued in standard cell media.

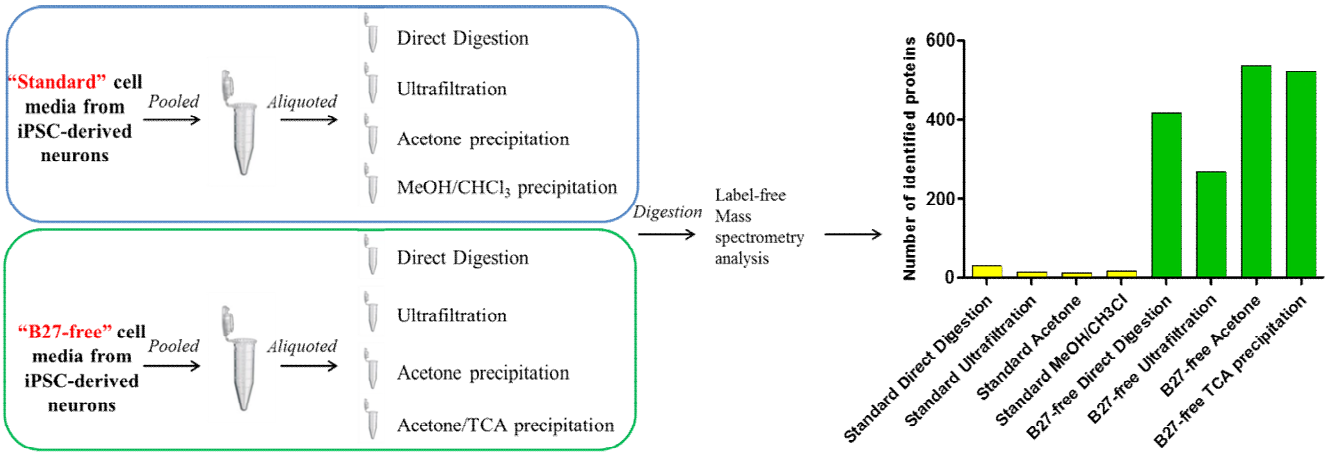

Figure 1. Different preparation techniques of iPSC-derived neurons supernatant and number of identified proteins using shotgun label free MS. Results obtained from proteomic analysis of standard cell media are shown in yellow, and from B27-free cell media in green.

Evolution of the secretome in time-dependent collection. After discovery that acetone precipitation was the optimal sample preparation method for proteomic analysis, we aimed to evaluate the optimum time to sample the media during cell culture to determine both the number of proteins identified and the types of proteins identified. Any changes in cell culture media and conditions could potentially 'stress' the cells and therefore, an optimum incubation time needs evaluating to obtain a balance between the number of proteins secreted and the type of proteins secreted. i.e. those proteins secreted during normal cellular metabolism or processes and not due to stress. Therefore, we collected the B27-free cell supernatant after 3, 6 and 24h of incubation with iPSC-derived neurons. Collected samples were precipitated with acetone, trypsin digested and submitted to proteomic analysis using label-free shotgun MS. All initial screening analyses of the secretome were performed using shotgun proteomic runs. Once the optimum conditions were deduced from the shotgun analyses, a full 2D-LC experiment was performed that allowed the deeper proteomics mining experiments and hence greater proteomic coverage/protein hits.

A total of 89 proteins were identified in B27-free cell media conditioned with iPSC-derived neurons for 3h (Figure 2). The most abundant proteins detected were BSA, various types of keratins, and fibronectin. The presence of BSA and fibronectin is not surprising, as these proteins are added to the cell culture, and samples were likely contaminated with keratins during handling.

Interestingly, incubation of neurons in B27-free supernatant for 6h did not increase significantly the number of identified proteins. Ninety proteins were identified in supernatant collected after 6h of culture. Similarly as in the 3h sample, BSA, keratin, transferrin, fibronectin and collagenase IV used in cell culture were detected in the 6h sample. A total of 47 proteins were found in common for 3h and 6h samples and included the following extracellular proteins: TGFBI, versican core protein, plasminogen activator inhibitor 1, SPARC, tenascin and insulin-like growth factor binding protein 2.

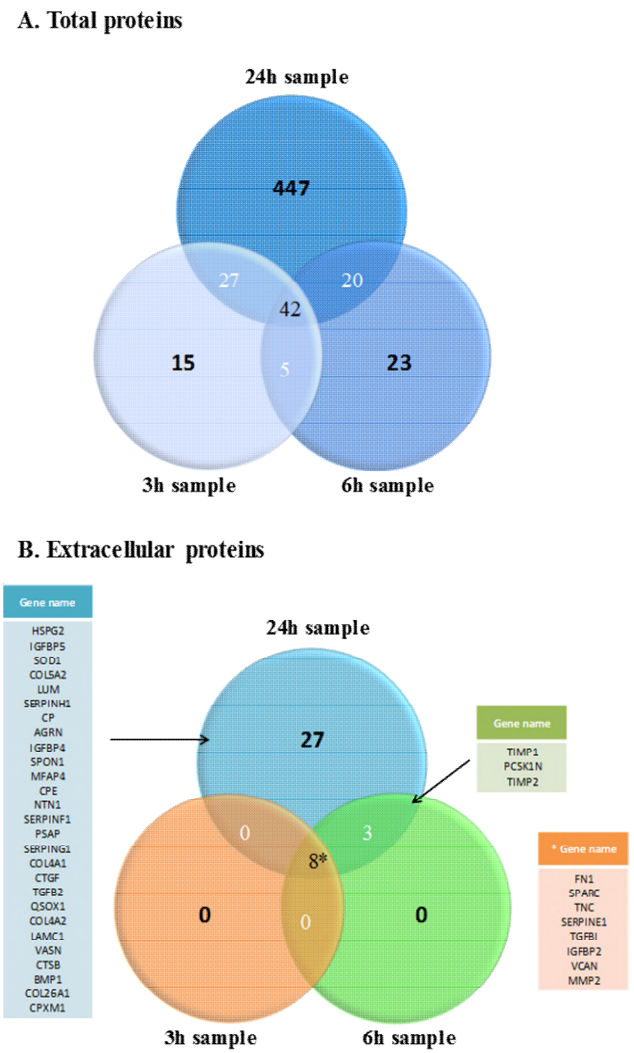

Figure 2. Venn diagram showing the number of proteins identified in B27-free cell media conditioned for 3, 6 and 24h with iPSC-derived neurons. A. Total proteins identified: 89 proteins were identified in cell media conditioned for 3h, 90 in cell media conditioned for 6h and 536 in cell media conditioned for 24h. B. Extracellular proteins identified: 8 extracellular proteins were identified in cell media conditioned for 3h, 11 in cell media conditioned for 6h and 38 in cell media conditioned for 24h.

Analysis of B27-free cell media conditioned with iPSC-derived neurons for 24h allowed for identification of 536 proteins. Of those, 42 proteins were common for supernatants derived from neurons incubated for 3, 6 and 24h (Figure 2). The 8 extracellular proteins in common included: fibronectin, collagenase IV, TGFBI, versican core protein, SPARC, plasminogen activator inhibitor 1, tenascin and insulin-like growth factor binding protein 2. Significantly more proteins were identified in the 24h incubations relative to the 3- and 6h incubations. However, the identification of increased cytoplasmic proteins identified in the 24h incubation experiments indicated, that although significantly more proteins were observed, it is probable that these could originate due to cellular stress or cell death. To clarify whether incubation of cells in B27-free media for 24 hours was toxic a cell viability assay was performed (Figure S-2). Interestingly no decrease in cell viability was observed, but a slight increase in signal was measured from wells containing cells incubated in B27-free media, which could be explained as a stress response. Therefore, although more proteins were observed during the 24 hour incubations, for a more correct and accurate determination of monitoring changes in cellular metabolism, an incubation time of 6h was decided as being optimum for monitoring the secretome. Indeed, several thousands of proteins are capable being identified after long incubations and described by other groups, but our proteomic data indicates longer incubation times induces a stress response and could influence the resulting secretome. Indeed, these responses were detected more subtlety using proteomics that conventional histopathology techniques. This is particularly important when studying the subtle pathogenesis of disease mechanisms as described in this work.

The abundance of secreted or extracellular proteins detected in cell media conditioned for 3, 6 and 24h with iPSC-derived neurons followed the expected trend in increase of abundance over time, with few exceptions (Figure 3 and Table S-2). For instance, the versican core protein was detected in the same concentration in 3 and 6 h samples; tenascin was 1.5 times more concentrated in 3 h sample than in 6 h sample. Interestingly, the concentration of fibronectin increased with incubation time, suggesting that the baseline quantity of this protein added to the cell media may be augmented by the neuronal secretion. The presence of exogenous proteins added to the cell culture such as BSA or transferrin, show how challenging the proteomic analysis of cell secretome is, as many possible sources of identification bias need to be taken into account. Matrigel has

been described previously as rich source of proteins, which can potentially leach out and contribute to the cell media[33,34]. To evaluate the potential of this contributing to the proteins detected in the supernatant or secretome and to take this into account, we also analysed the Matrigel directly by label-free mass spectrometry. This analysis revealed the presence of 61 human and murine proteins (Table S-3), which were then compared with the proteins identified in the cell supernatant samples. Only 7 common proteins were identified between Matrigel and cell supernatant incubated for 3, 6 and 24h samples that could potentially, and falsely, be attributed to cellular secretion. These proteins included: Keratin type I cytoskeletal 9, transthyretin, insulin, elongation factor 1-alpha, pyruvate kinase, serotransferrin and uncharacterized protein A0A0B4J269. To ensure the accurate interpretation of our data, we decided to not take these proteins into account and were removed from any subsequent bioinformatics analyses. Nevertheless, despite cell culture artefacts present in our samples, we were able to detect many other proteins. The GO functional classification of identified proteins was performed using the open-source Panther[28] (Figure S-4) and WebGestalt[29] tools (Figures S-5 and S-6), and only the secreted proteins classified in both tools as belonging to the extracellular matrix or extracellular region were selected for further analysis (Figure 2B).

In the 3h sample, one of the proteins secreted by neurons and present in high abundance was vimentin. This cytoskeletal molecule plays role in cell adhesion and migration, and belongs to the intermediate filaments family. However, it was also reported as being secreted from astrocytes[35] and macrophages[36]. Recently, the role of vimentin in axonal growth through interaction with insulin-like growth factor 1 receptor was described[37]. Another abundant protein detected in cell supernatant was secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or BM-40. In accordance with our findings, SPARC was previously detected in the astrocyte secretome[18,35]. This extracellular protein is expressed in a variety of cells, including osteoblasts, fibroblasts, platelets and others. In nervous system, SPARC modulates the efficacy of growth factors, affecting the expression of matrix metalloproteinases and cell shape. It stimulates neural regeneration, affects synaptic plasticity and neuronal migration, and promotes neuronal survival and neurite outgrowth[38]. Other proteins identified in GO as related to extracellular matrix were: tenascin, plasminogen activator inhibitor 1, transforming growth factor-beta-induced protein (TGFBI), insulin-like growth factor binding protein 2 and versican core protein. These extracellular proteins play roles in neural plasticity, neural growth and neuron survival. Three extracellular proteins were detected only in 6h sample: tissue metalloproteinase inhibitor 1 (TIMP-1), tissue metalloproteinase inhibitor 2 (TIMP-2) and ProSAAS. These proteins were previously detected in the secretome of astrocytes[18]. TIMP-1 and TIMP-2 are multifunctional molecules with growth-promoting properties and share 40% of amino acid sequence homology[39]. In the central

nervous system, TIMP-1 is implicated in neuronal death, axonal growth and synaptic mechanism linked to learning and memory[40], and was recently described as potential biomarker candidate for AD and Lewy-body dementia (LBD)[41]. TIMP-2 is highly expressed in post-mitotic neurons, and promotes neuronal differentiation in vitro[42]. However, in one of the reports, the expression of TIMP-2 was up-regulated in response to serum-withdrawal[43], which may explain the presence of TIMP-2 in the supernatant collected from 6h culture and not from 3h culture. Detection of TIMP-1 and TIMP-2 in cell secretome is promising, as it could be applied in experiments related to neuronal function or ageing.

25 extracellular proteins were detected only in the 24h sample: agrin, basement membrane-specific heparan sulfate proteoglycan core protein, bone morphogenetic protein 1, carboxypeptidase E, cathepsin B, ceruloplasmin, collagen alpha-1(XXVI) chain, collagen alpha-1(IV) chain, collagen alpha-2(IV) chain, collagen alpha-2(V) chain, connective tissue growth factor, insulin-like growth factor-binding protein 4, insulin-like growth factor-binding protein 5, lumican, microfibril-associated glycoprotein 4, netrin-1, pigment epithelium-derived factor, plasma protease C1 inhibitor, prosaposin, probable carboxypeptidase X1, , spondin-1, transforming growth factor beta-2, sulfhydryl oxidase 1, superoxide dismutase [Cu-Zn] and vasorin. All these proteins play important roles in the central nervous system.

Identification of secreted or extracellular proteins that have particular relevance to PD. Since the cell supernatant used in our study was collected from iPS-derived neurons with triplication in SNCA gene, we were particularly interested in secreted proteins which are linked to neurodegeneration, i.e. to PD. Some of the extracellular proteins detected in the cell media, were described previously as related to PD. More importantly, their role was confirmed not only through *in vitro* studies, but also two of the proteins- carboxypeptidase E and prosaposin were also described in cerebrospinal fluid (CSF) as candidate biomarkers of neurodegeneration[41]. Extracellular proteins detected in cell media and related to PD are mainly neuroprotective growth factors (i.e. netrin-1 or pigment epithelium-derived factor), and their impairment could be involved in the loss of dopaminergic neurons, which is one of the hallmarks of the disease. Another group of detected proteins are enzymes regulating oxidative stress (e.g. ceruloplasmin) and proteins implicated in degradation of α-synuclein (i.e. agrin, cathepsin B).

A range of extracellular proteins identified in our samples, have been reported previously and are associated with neurodegeneration. For instance, agrin was localized to lesions in AD, and was found to accelerate formation of amyloidogenic peptide Aβ(1-40), suggesting its role in disease pathology[44]. Importantly, it also binds to α-synuclein and influences the

formation of α-synuclein fibrils, suggesting its contribution to PD pathology[45]. Carboxypeptidase E is an enzyme affecting the function of the dopamine transporter and was recently described as a potential biomarker for AD and LBD[41]. Cathepsin B, together with cathepsin L plays an essential role in lysosomal degradation of α-synuclein, through cleavage of α-synuclein within its amyloid region[46]. Ceruloplasmin protects tissues from oxidative damage through regulation of cellular iron loading and export. In PD, this molecule has reduced enzymatic activity, impairing iron homeostasis, leading to iron accumulation and as a result to oxidative damage and neurodegeneration[47]. Netrin-1 is involved in axon guidance, and the impairment in this pathway could contribute to PD through alternating the synaptic circuits and dopamine transmission[48]. Pigment epithelium-derived factor is a neuronal growth factor which was also found to have a neuroprotective function against toxins used in *in vitro* models of PD: rotenone and 6-hydroxydopamine[49]. A chaperone protein involved in the lysosomal catabolism of glycosphingolipids, prosaposin, was also found to have protective effects on dopaminergic neurons in PD models[50]. Transforming growth factor beta-2 promotes the survival of dopaminergic neurons, which could potentially be used in the treatment of PD[51].

Deep proteomic profiling of the cell supernatant or secretome from control and PD iPSC derived neurons. In the final part of the study, we performed the deep profiling of cell supernatant secretome in order to look for the differences in protein expression between patient and control lines. For this purpose, iPSC-derived neurons with triplication of SNCA gene and unaffected controls were incubated in B27-free supernatant for 6h, centrifuged immediately after collection to remove cell debris, precipitated with acetone, trypsin digested and analyzed using 2D-nanoLC-MS/MS. A total of 311 quantifiable proteins were identified using Progenesis software. Differential analysis of triplication of SNCA and control samples have shown 25 proteins which were altered by a factor of 2-fold or more between the two groups of cell supernatant (Figure 4 and Table S-4). Similarly as for the 3, 6 and 24 h time point analyses, we compared the identified proteins with the proteins identified from the Matrigel matrix analyses. Of those, 9 proteins were common between the Matrigel matrix analyses and those proteins identified in the cell supernatant. However, only 2 proteins detected in the Matrigel: transthyretin and serpin were observed to be differentially expressed in patient and control samples. Since cell culture experiments were performed using the same stringent culture conditions, we could assume a similar level of contribution of Matrigel-derived proteins in both samples. However, we detect detected a 7.6-fold and 2-fold change in protein concentration for transthyretin and serpin, respectively. We cannot be sure whether the increase in the accumulation of these proteins were derived from Matrigel matrix and/or were cell-derived proteins. However, without performing a larger scale SILAC analyses we are unable to

prove the origin of these proteins and hence these two proteins were not taken into account during the analysis.GO pathway analysis revealed that the following pathways: inflammation mediated by chemokine and cytokine signaling, p53 and integrin signaling were enriched in the secretome of iPSC-derived neurons with SNCA mutation (Figure S-5). In line with our findings, the increase of the cytokines (such as IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ) levels were previously reported in the serum, CSF and substantia nigra brain regions of PD patients[52]. Interestingly, we have detected over 3-fold increase of the collagen type VI alpha-1 chain in triplication of SNCA samples. This protein, belonging to the inflammation pathway mediated by chemokine and cytokine and to the integrin signaling pathway, was described as having protective role in the CNS during stress. The lack of collagen VI caused increased susceptibility to oxidative stress and apoptosis of neurons[53]. On the other hand, the GO pathway analysis of proteins upregulated in the secretome of control neurons has shown their involvement in integrin, CCKR, Wnt and cadherin signaling pathways, AD-presenilin pathway and in vasopressin synthesis. Particularly interesting is the 3-fold increase in the expression of carboxypeptidase E, which plays a neuroprotective role in neurons. We hypothesize that the decrease of this molecule in patient secretome could be a sign of neuronal degeneration.

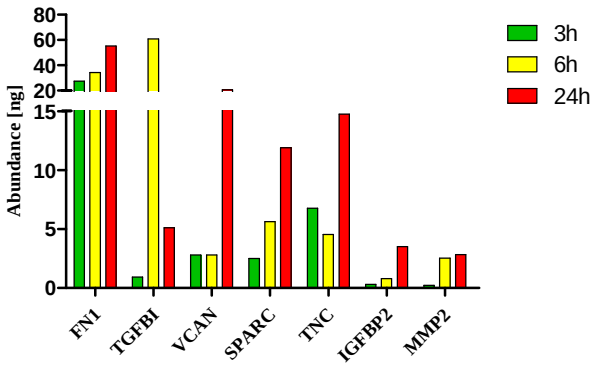

Figure 3. Changes in abundance of secreted or extracellular proteins in cell media as a function of incubation time for 3(green), 6 (yellow) and 24h (red) with iPSC-derived neurons. Proteins were quantitated using label free MS^e quantitative proteomics and using yeast enolase as internal standard.

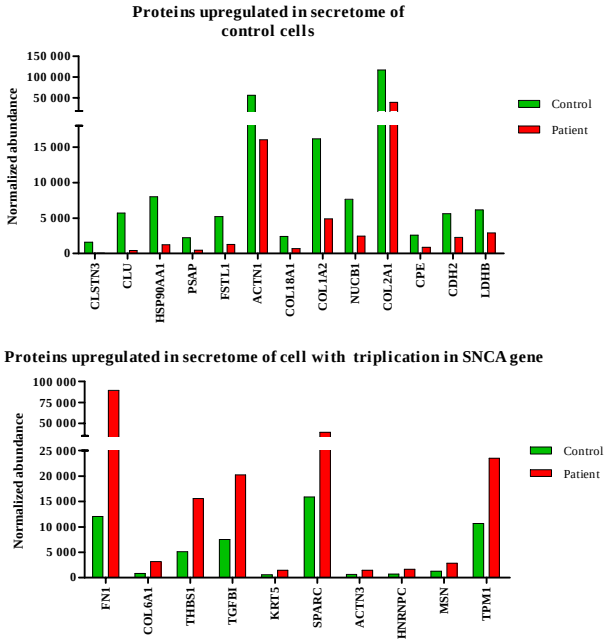

Figure 4. Proteins upregulated in the secretome of control (a) and cell line with triplication of the SNCA gene (b).

Additionally, GO analysis revealed that 5 of the 25 differentially expressed proteins can be normally found in extracellular matrix: fibronectin, transforming growth factor beta-2, SPARC, prosaposin and carboxypeptidase E. Similarly as in the samples described above, fibronectin could potentially be a contamination or cell culture artefact, but its 7-fold increase in cell media conditioned with neurons with triplication of SNCA gene suggests it can be secreted by cells. Moreover, 7 proteins were annotated in Gene Cards as related to PD: transforming growth factor beta-2, moesin, clusterin, heat shock protein HSP 90-alpha, carboxypeptidase E and L-lactate dehydrogenase B chain. Alpha-synuclein was not detected in cell supernatant; although it has been shown previously to be secreted when over-expressed in cell lines and in primary neurons and in vivo[54–56]. The reason it was not detected here may be that only a very small proportion of alpha-synuclein is secreted, either by exocytosis or in exosomes, and often only under stress conditions such as lysosomal and proteasome inhibition or microglia activation[54,57].

Interestingly, no difference in expression of amyloid precursor protein (APP) was found between supernatant conditioned with control and patient iPSC-derived neurons. However, the expression of calsyntenin-3, which stabilizes APP metabolism, was 60-times higher in control sample. Recent studies have shown that high levels of Aβ oligomer (cleavage product of APP) up-regulate the expression of calsyntenin-3, which in turn, may increase the susceptibility of neurons to degeneration and plays possible role in the AD[58].

Although clusterin, carboxypeptidase E and prosaposin were described as potential biomarkers for neurodegeneration in CSF, none of them were specific to PD[41]. In the cell supernatant, we found that these proteins were differentially expressed between control and triplication of SNCA samples. However, these findings should be further confirmed by differential analysis performed on larger cohort of the samples.

In summary, analysis of cell media can provide valuable information about the cell secretome and can complement studies of cell lysates. In the CNS, cells communicate with each other and modulate normal and pathological processes through secreted metabolites and proteins. Therefore, using these optimized methods the analysis of the extracellular media can provide insight into early changes in the proteome or metabolome of neurodegenerative disorders such as PD. This method could also be applied to develop new tools for early diagnosis of neurodegenerative disorders, for example by comparison of the secretome of iPSC-derived neurons from pre-symptomatic individuals carrying genes linked with neurodegeneration. Due to its accessibility, cell media can be used in drug screening studies to monitor therapeutic effects of agents targeting specific proteins or metabolic pathways. For example, cell media was recently studied to assess the dopamine/serotonin metabolism in SH-SY5Y cells and evaluate the involvement of mitochondrial complex I and lysosomal glucocerebrosidase (GBA1) in PD[59].

The proteomic analysis of cell secretome is used very infrequently, probably due to the composition of cell media which may hamper identification of less abundant proteins. To overcome this problem, we have used the B27-free cell media which allowed us to increase the number of identified proteins by factor 10 or more. However, even when using the B27-free supernatant, cell culture artefacts such as BSA, transferrin, fibronectin and collagenase IV could still be detected and need to be taken into account while analyzing the data of cell secretome. To date, a sophisticated methods including pulsed SILAC[60], combination of click-chemistry and pulsed SILAC[61] or dynamic SILAC[62] allowed for analysis of cell secretome with high confidence. However these methods require significant resources and are time consuming. Here we present a fast and simple preparation of cell supernatant which can be used to obtain first insights into cell secretome, prior to performing more refined quantitation techniques, such as SILAC or TMT-tagging.

Secondly, since the extracellular proteins are present in very low concentrations in the samples, an appropriate protein enrichment method should be used for sample preparation. Here we have evaluated five different sample preparation techniques: digestion (without enrichment), ultrafiltration, and precipitation (with acetone, TCA/acetone and MeOH/$CHCl_3$). We have found that acetone precipitation of cell media conditioned with iPSC-derived neurons yielded the highest number

of identified proteins. This method was previously ruled out by other studies[17], where authors were postulating that the precipitation methods are impractical for the analysis of large volumes of media. In our studies, we have used only 500 µL of cell media, which could be easily adaptable to the classical proteomic protocols. The use of small volumes of sample is another advantage, since drug screening studies are currently moving towards high-throughput techniques, and cell cultures in large volumes being simply not cost-effective. Also it is important to note that although longer incubation times using this methodology did facilitate greater numbers of protein identifications (>2000), it was the shorter incubation times that provided more meaningful and relevant insights into the pathways affected and the disease mechanisms.

We have used our optimized protocol to perform a deep profiling of the extracellular media of iPSC-derived neurons with triplication of SNCA gene and unaffected controls. This analysis allowed for detection of 25 proteins differentially expressed between control and triplication of SNCA groups. Particularly interesting was the 60-fold decrease of calsyntenin-3 levels in triplication of SNCA sample. This transmembrane protein is usually expressed in synapses and has Ca^{2+} binding capacity. *In vitro* studies have shown that the upregulation of calsyntenin-3, caused by the Aβ oligomers may increase the vulnerability of neurons to AD[58,63]. However, the analysis of CSF from AD patients has shown that this protein was downregulated in patients carrying mutations in PSEN1 and APP[64]. To our knowledge, no studies have linked calsyntenin-3 with PD, but our preliminary results suggest that there may be a decrease of expression of this protein in patients carrying mutation in the SNCA gene. The downregulation of calsyntenin-3 could have a potential impact on calcium homeostasis in neurons, increasing their vulnerability to oxidative damage and may be a potential drug target.

CONCLUSION

We have described here an optimised protocol for the preparation of cell supernatant for proteomic analysis that enables the detection of low abundant secreted proteins. This protocol involves shorter incubation times (6 h vs 24 h) and significantly less media than previous protocols (500 µL vs. 20-40 mL). Moreover, using this methodology has been optimized to study biochemical pathways and disease mechanisms. Although, using shorter incubation times provide less protein identifications, ‘less is sometimes more’ and provides more relevant results that model disease mechanisms more closely. Using these protocols we have identified proteins described previously as being implicated in neurodegeneration. Our findings suggest that cell supernatant could potentially be used as an *in vitro* model of CSF, and because of the low level of media required be used in the screening of drugs targeting these proteins (96- or 384 well cell culture format). The proteomic data obtained from deep profiling analysis can be translated into high-throughput targeted methods to analyse cell secretomes.

This approach could be then applied to elucidate the changes in secretome for different neurological disorders and for monitoring the secretome response to therapeutic agents targeting these proteins. Our method can easily be translated to the investigation of other cell types derived from iPSC, such as cardiomyocytes, hepatocytes or renal tubular cells.

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SUPPORTING INFORMATION

Supporting Information Available: Fig. S-1. Representative confocal micrograph of iPSC-derived neurons from control and from a patient with a triplication of the SNCA gene which codes for the protein α-synuclein. Fig. S-2: Venn diagram showing proteins identified in standard cell media using different preparation techniques. Fig. S-3: Effect of B27 removal on cell

viability. Fig. S-4: Functional classification of identified proteins in cell supernatant conditioned for 3, 6 and 24h with iPSC-derived cell supernatant, using Panther software. Fig. S-5: Gene ontology analysis of B27-free supernatant conditioned for 3h with iPSC-derived neurons, using the Web-based Gene Set Analysis Toolkit (WebGestalt). Fig. S-6: Gene ontology analysis of B27-free supernatant conditioned for 6h with iPSC-derived neurons, using the Web-based Gene Set Analysis Toolkit (WebGestalt). Fig. S-7: Gene ontology analysis of pathways upregulated in the secretome of control cell line (A) and cell line with triplication in the SNCA gene (B). Fig. S-8. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant precipitated in 10% TCA in acetone prior digestion. Fig. S-9. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant precipitated in acetone prior digestion. Fig. S-10. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant ultrafiltrated prior digestion. Fig. S-11. A representative Baseline Peak Ion Chromatogram of B27-free cell supernatant digested without enrichment step. Fig. S-12. A representative Baseline Peak Ion Chromatogram of standard cell supernatant precipitated in 10% TCA in acetone prior digestion. Fig. S-13. A representative Baseline Peak Ion Chromatogram of standard cell supernatant precipitated in acetone prior digestion. Fig. S-14. A representative Baseline Peak Ion Chromatogram of standard cell supernatant ultrafiltrated prior digestion. Fig. S-15. A representative Baseline Peak Ion Chromatogram of standard cell supernatant digested without enrichment step. Table S-1: List of cell lines included in the study. Table S-2: List of identified extracellular proteins in cell media conditioned for 3, 6 and 24 h with iPSC-derived neurons. Table S-3. List of identified proteins in Matrigel. Table S-4: Differentially expressed proteins by a fold 2 or more between control and patient cell supernatant. Methodology of neuronal differentiation. Methodology of cell viability experiment. Preparation of cell supernatant. Label-free UDMSE mass spectrometry.

The Supporting Information is available free of charge on the ACS Publications website.

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