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Unveiling the effects of the Secretome of Mesenchymal Progenitors from the Umbilical Cord in Different Neuronal Cell Populations

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Abstract

It has been previously shown that the secretome of Human Umbilical Cord Perivascular Cells (HUCPVCs), known for their mesenchymal like stem cell character, is able to increase the metabolic viability and hippocampal neuronal cell densities. However, due to the different micro-environments of the distinct brain regions it is important to study if neurons isolated from different regions have similar, or opposite, reactions when in the presence HUCPVCs secretome (in the form of conditioned media-CM). In this work we: 1) studied how cortical and cerebellar neuronal primary cultures behaved when incubated with HUCPVCs CM and 2) characterized the differences between CM collected at two different conditioning time points. Primary cultures of cerebellar and cortical neurons were incubated with HUCPVCs CM (obtained 24 and 96 hours after three days of culturing). HUCPVCs CM had a higher impact on the metabolic viability and proliferation of cortical cultures, than the cerebellar ones. Regarding neuronal cell densities it was observed that with **24h** CM condition there were higher number MAP-2 positive cells, a marker for fully differentiated neurons; this was, once again, more evident in cortical cultures. In an attempt to characterize the differences between the two conditioning time points a proteomics approach was followed, based on 2D Gel analysis followed by the identification of selected spots by tandem mass spectrometry. Results revealed important differences in proteins that have been previously related with phenomena such as neuronal cell viability, proliferation and differentiation, namely 14-3-3, UCHL1, hsp70 and peroxiredoxin-6. In summary, we demonstrated differences on how neurons isolated from different brain regions react to HUCPVCs secretome and we have identified different proteins (14-3-3 and hsp70) **in** HUCPVCs CM that may explain the above referred results

1. Introduction

Mesenchymal stem/stromal cells (MSCs) have emerged in the last decade as potential tools/vehicles for regenerative medicine purposes [1,2]. These cells are characterized for: 1) their adherence to plastic in standard culture conditions; positive expression for specific markers like CD73, CD90, CD105 and negative expression for hematopoietic markers like CD34, CD45, HLA-DR, CD14 or CD11B, CD79α or CD19 and 2) *in vitro* differentiation into at least osteoblasts, adipocytes and chondroblasts [3]. In recent years it has been increasingly accepted that their regenerative effects are mainly mediated by their secretome [4-6]. The secretome, which comprises the proteins released by cells, tissues or organisms, has been shown to be crucial **to** the regulation of different cell processes [7].

Due to its low regenerative potential the Central Nervous System (CNS) has been one of the main targets of the regenerative potential of MSCs and their secretome. Initial *in vitro* studies revealed that the latter was able to promote neuronal and glial survival [8-10], neuritogenesis [8] and neural/glial differentiation [11]. These effects were then related, by different authors, with the expression of growth factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), insulin growth factor 1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), fibroblast derived growth factor 2 (FGF-2), stem cells factor (SCF) and glial derived neurotrophic factor (GDNF), as reviewed by Teixeira *et al* [12]. Similar phenomena were also reported *in vivo* [13-16]. In fact, Munoz *et al*. [13] reported that the injection of bone marrow MSCs (BM-MSCs) in the mice hippocampus **led** to an increased neuronal differentiation, mediated by neurotrophic factors. Cova *et al*. [14] and Weiss *et al*. [15] also reported that BM-MSCs and MSCs isolated from the umbilical cord

Wharton Jelly (WJ-MSCs) were able to ameliorate the condition of 6-hydroxydopamine injected Parkinsonian rats through the active secretion of growth factors. While for BM-MSCs this was attributed to the expression of epidermal growth factor (EGF), neurotrophin 3 (NT3), FGF-2, HGF and BDNF, for WJ-MSCs this was attributed to GDNF and FGF-2. Other studies have also shown that similar effects could be observed in *in vivo* models of Spinal Cord Injury (SCI) and brain ischemia [19].

Despite the increased knowledge on this topic, there are still a number of questions that remain to be answered. For instance, so far it was not described if neuronal cell populations isolated from different areas of the brain have the same or different **response** profiles, when exposed to MSCs secretome. This is particularly important as the CNS possesses niches with different neuroregulatory needs. Thus, different brain areas may have a different response to the secretome, a fact that can impact the range of therapeutic applications of the latter. Another important topic that should be addressed is the characterization of the secretome itself. **Although important progress has been made, it remains likely that other molecules as well as vesicles in the MSCs secretome are related with the phenomena that have been described to date**. An example of this is the work described by Lai *et al*., where the presence of exosomes in the secretome of MSCs derived from human embryonic stem cells (hESCs), was related to their cardio-protective effects [20]. **Exosomes are formed from multivesicular bodies with a bilipid membrane. They have a diameter of 40–100 nm and are known to be secreted by different cell types [20].**

Herein we have focused on determining the effects of conditioned media (CM) of Mesenchymal Progenitors isolated from the Wharton Jelly of the umbilical cord (HUCPVCs) on post-natal populations of cortical and cerebellar neurons along

withits proteomic characterization. Results revealed that the secretome of HUCPVCs increased cell viability, proliferation and neuronal cell densities in both cortical and cerebellar neuronal cultures, while exhibiting proteins with possible neuroprotective character, which had different expression profiles

2. Materials and Methods

2.1 Cell Culture

Human Umbilical Cord Perivascular Cells

HUCPVCs were isolated from umbilical cords from consenting full-term caesarean section patients. Ethical approval had been previously obtained from Hospital de S. Marcos, Braga. All human studies were conducted in accordance with the Helsinki accords. All subjects signed an informed consent document prior to their donation of tissue and participation.

They were isolated according to the procedure originally described by Sarugaser *et al*. [21]. Pieces of cord, 4–5 cm long, were dissected by first removing the epithelium of the UC section along its length to expose the underlying WJ. Each vessel, with its surrounding WJ matrix was then pulled away, after which the ends of each dissected vessel were tied together with a suture creating "loops" that were placed into a 50-ml tube containing a solution of 0.5-0.75 mg/ml collagenase (Sigma, USA) with phosphate buffered saline (PBS, Gibco, USA). After 18 hours, the loops were removed from the suspension, which was then diluted with PBS to reduce the viscosity of the suspension and centrifuged. Following the removal of the supernatant, cells were resuspended in culture media, α-MEM (Gibco) supplemented with 10%FBS (Gibco) and 1% antibiotic/antimycotic (Sigma), counted using a hemocytometer and platted in T75

flasks at a density of 4000 cells/cm^2. The culture medium was changed every 2/3 days. Upon confluence cells were trypsinized and passaged to new T75 flasks.

Primary Cultures of Cerebellar and Cortical Neurons

Cortical and Cerebellar neuronal cultures were prepared from P4 Wistar Rats [9]. Briefly, and upon dissection, brain tissue was submitted to a trypsin based enzymatic digestion followed by mechanical dissociation. Isolated cells were then plated on coverslips previously coated with Poly-D-Lysine (Sigma) at a density of 40000 cells/cm^2. Characterization of the cultures by immunocytochemistry (microtubule associated protein (MAP-2)-neurons revealed that they possessed approximately 45-50% of mature neurons.

Conditioned Medium Collection and Experiments

Conditioned media (CM) were collected from P4 HUCPVCs, as previously described [9]. For this purpose cells were plated out at a density of 4000 cells/cm^2 and allowed to grow for 3 days. Following this, culture medium was renewed and CM collected 24 and 96 hours thereafter (cell culture media was not renewed or added during this time period). Upon collection CM were frozen, being later on thawed on the day of the experiments. For CM collection Neurobasal-A medium supplemented with kanamycin (Gibco, 0.1mg/ml) was the chosen medium. Experiments with the neuronal cultures were done as follows: Upon isolation cortical and cerebellar neurons were plated out at the densities referred above and incubated from T0 with the previously collected CM (n=3/CM time point) for 7 days (with half of the volume of CM being renewed at day 4 of culture), after which cell densities, viability and proliferation were assessed (see

below). Besides kanamycin and glutamax, no further supplements were added to the HUCPVCs CM. **As the objective of these experiments was to assess if the secretome alone could induce higher levels of neuronal survival, thus without the presence of any additional factors, control cultures were kept in Neurobasal-A media supplemented with kanamycin and glutamax.**

2.2 Cell Viability Assessment

Cell viability was assessed by the MTS test. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H tetrazolium) (Promega, USA) test is an assay in which the substrate – MTS – is bioreduced into a brown formazan product by NADPH or NADP produced by mitochondrial enzymes, which are active in living cells. Cell culture coverslips (n=3) were placed in culture medium containing MTS in a 5:1 ratio and incubated in a humidified atmosphere at 37ºC and 5% CO_2. After three hours of incubation 100 µl of solution from each sample were transferred to 96 well plates and the optical density was determined at 490 nm (n=3/CM time point±SD). Results are shown as a ratio between CM incubated cultures and controls (n=3/CM time point±SD)

2.3 Cell Proliferation

Cell proliferation was determined by a colorimetric assay based on 5-bromo-2'-deoxyuridine (BrdU) incorporation (Roche, Germany). Primary cortical and cerebellar neuronal cultures incubated with CM, and respective controls were incubated with BrdU on day 6. After 24 hours of incubation ELISA test was performed according to the company's instruction in the end of which O.D. was determined at 450 nm with a reference filter at 655nm. Results are shown as a ratio between CM incubated cultures and controls (n=3/CM time point±SD). **This assay will determine the profileration of**

cells other than neurons, majorly astrocytes, which are the remaining proliferative cells present in the primary cell culture systems used.

2.4 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 30 minutes, permeabilized by incubation with 0.3% Triton X-100 in PBS for 5 minutes at room temperature (for neurons and astrocytes), and washed three times in PBS. Cells were then blocked with 10% FBS/PBS followed by a 60 minute incubation with mouse anti-rat microtubule associated protein 2 (MAP-2) (Sigma, USA) to detect mature cortical and cerebellar neurons. Cells were then washed in PBS and incubated with Alexa Fluor 594 goat anti-mouse immunoglobulin G (IgG). Primary antibody was omitted to produce negative controls. Samples were then observed under a Olympus BX-61 Fluorescence Microscope (Olympus, Germany). For this purpose three cover slips *per* condition and three representative fields were chosen and analysed. Results are shown as percentage of MAP-2 positive cells over the total number of cells *per* field of observation (n=3/CM time point±SD)

2.5- Proteomic analysis of HUCPVCs CM

Protein extraction

Culture media was concentrated using 5kDa cut-off filters (Vivaspin) according to manufacturer's guidelines. The resulting solution was subject to protein precipitation using trichloroacetic acid (final concentration 20%) and acetone. The resulting pellet was resuspended in 500 μl of an isoelectric focusing (IEF) solubilization buffer [6 M urea, 1.5 M thiourea, 3% (w/v) CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid), 1.2% Destreak, 1.5% (v/v) IPG buffer and bromophenol blue].

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The suspension was sonicated and incubated at room temperature for 2 h in a rotary shaker, and then centrifuged at 20,000×g for 15 min to remove the insoluble material [22]. The total protein concentration was assessed using the 2-D Quant Kit (GE Healthcare) according to the manufacturer's guidelines, and using BSA as standard. Samples were stored at -20°C until further processing.

2-D Electrophoresis and image analysis

Three hundred micrograms of protein was actively rehydrated for 12 h at 50 V. IEF was performed according to the manufacturer, with slight modifications: 500 V (500 V.h step and hold (SH)), 1000 V (1000 V.h SH), 10,000 V (15,000 V.h with linear increase), and final focusing at 10,000 V during 14 h (SH), using a Protean IEF cell (BioRad, Amadora, Portugal). Strips were then equilibrated to SDS (50 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS, and trace amount of bromophenol blue) for 20 min, in the presence of 10 mg/mL DTT, followed by another 20 min step in the presence of 25 mg/mL iodoacetamide. The second dimension was performed in 10% acrylamide gels in a Protean Plus Dodeca Cell (BioRad), at 3 W/gel for 30 min, followed by 200 V for 5 h [22]. All steps were performed at 20°C. Gels were stained with silver nitrate and the images were acquired with EXQuest™ Spot Cutter (Bio-Rad).The images were imported into PDQuest™8.0 and the spots were detected and matched through the entire matchset. After automated matching, according to the parameters chosen, manual spot detection and matching was performed to confirm the results obtained using software automated functions. After matching, gel images were normalized using the "Local Regression Model" algorithm, available in PDQuest™8.0. Spots of interest were excised from stained gels with an automated picking using EXQuest™ Spot Cutter (Bio-Rad).

Protein Identification by Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS)

Gel spots were distained with 50 mM ammonium bicarbonate and 30% acetonitrile. In-gel digestion was performed overnight at room temperature with 30 μL of trypsin (10 ng/μL) in 10 mM ammonium bicarbonate. Peptides were extracted with 30%, 50%, and 98% acetonitrile in 1% formic acid, pooled, dried by rotary evaporation under vacuum, and resuspended in 2% acetonitrile and 0.1% formic acid. Protein identification was carried out on a hybrid quadrupole/linear ion-trap mass spectrometer (4000 QTrap; ABSciex) using a nanoelectrospray source and a dual gradient pump (Ultimate 3000; Dionex). The mass spectrometer was programmed for information dependent acquisition (IDA) scanning full spectra, followed by an enhanced resolution scan to determine the ion charge states, and set the appropriate collision energy for fragmentation. The IDA cycle was programmed to perform 6 MS/MS on multiple charged ions (+1 to +4) and two repeats before adding ions to the exclusion list for 60s (mass spectrometer operated by Analyst 1.5.1). Peptides were eluted into the mass spectrometer (Ultimate 3000, Dionex) with a binary gradient (250 nL/min 2% acetonitrile, 0.1% formic acid to 98% acetonitrile, 0.1% formic acid in a multiple step gradient for 50 min), using a nanoelectrospray source. Peptide identification was performed considering iodoacetamide (IAA) modification, trypsin with one miss-cleavage allowed, tolerance of 0.8 Da for both precursor and fragments, using MASCOT and Protein Pilot software (v2.0.1, ABSciex) against the Swiss-Prot or the NCBI non-redundant (nr) protein databases. Positive identifications were considered when Mascot ID protein score was above 95 % confidence and/or Protein Pilot ID was above 1.3, for confidence >95%. Protein identification based on single peptide hit had a

minimum individual score of 95% and a minimumsequence tag of 3 amino acids (4 consecutive peaks in the MS/MS spectrum) [23].

2.6- Statistical analysis

For cell culture experiments statistical evaluation was performed using one way ANOVA to assess the statistical differences between different groups. Statistical significance was defined as $p<0.05$ for a 95% confidence interval (*$p<0.05$, ** $p<0.01$, ***$p<0.001$) (n=3, mean $\pm$ SD). Regarding proteomic analysis, in order to find significant differences between the groups of samples under study, protein spots intensities were subjected to t-student test ($p < 0.05$), after data normality inspection with Kolmogorov-Smirnov test.

3. Results and Discussion

The objectives of the present study were to determine the effects of the CM of HUCPVCs on the viability, proliferation and survival of cortical and cerebellar neurons. Moreover we also intended to characterize the differences between CM of HUCPVCs collected at different time points and, identify potential molecules that could be involved in the above mentioned phenomena.

Our results revealed that cortical and cerebellar cell populations had a distinct response profile to HUCPVCs CM (Figure 1 and 2). As it can be observed in Figure 1, the overall cell metabolic viability and proliferation of cortical cultures was positively impacted by HUCPVCs CM, namely for the CM96h ($p<0.05$ when compared to control samples). On the other hand, only the metabolic viability of cerebellar cultures was upregulated at CM24h, but not CM96h. In order to further characterize the effects of HUCPVcs CM on these cultures an immunocytochemistry for MAP-2 positive cells was performed. This analysis revealed that the incubation of HUPCVCs CM increased the number of mature neurons (MAP-2 positive cells) in both culture systems when compared to control cultures, for both CM24h and CM96 h (Figure 1 and 2, $p<0.05$). This was particularly evident in the cortical cultures, in which controls did not present MAP-2 positive cells, while the secretome incubated cultures presented values ranging from approximately 20% (CM96h) to 40% (CM24h) (Figure 1C-F). Low values for control cultures were being expected as cultures were being kept just in Neurobasal-A without any further supplementation. Finally, it was also possible to identify a decaying trend between the CM24h and CM96h incubated cultures, which was more evident for the cortical cultures. Thus, from these experiments, we conclude that cortical neurons respond more robustly to HUCPVCs CM when compared to the cerebellar ones.

In order to characterize the differences between CM24h and CM96h an exhaustive proteomics based analysis was performed. This analysis was based on a 2D gel electrophoresis followed by an analysis of selected spots by LC-MS/MS (Figure 3). For discussion purposes it should be mentioned that the analysis was focused on 2D gel spots that revealed significative differences between to the two time points of CM collection (CM24h and CM96h). The results from these analysis revealed differences on the presence of 9 proteins between CM24h and CM96h (Figure 3) which are typically know for their intracellular roles; three cytoskeletal (actin and two isoforms of vimentin) and six cytosolic proteins (hsp70, peroxiredoxin-6, UCHL1, RAD52, 14-3-3 and transgelin). These results were somehow unexpected due to the lack of known secreted factors in this list. However recent reports have increasingly shown that besides the traditional growth factors and cytokines as important players in the secretome, it now appears that most of the cells, including MSCs, secrete large amounts of micro and nano-vesicles, either constitutively or upon activation signals [24]. Although little is still known on the biogenesis and physiological role of these entities, their potential as mediators of cell interactions has been reported by different authors [25-28]. Indeed exosome or microvesicles can operate in a multitude of ways since they can be considered as complex vectors that can hold known biological molecules. These could include proteins both ubiquitous and cell specific, mRNAs, microRNA (miRNAS) and lipid molecules [24]. Regarding MSCs the importance of these vesicles has been recently reviewed and reported [19, 29, 30]. For instance Kim *et al* [30] characterized the content of MSCs derived microvesicles identifying around 730 proteins, among which mediators controlling self-renewal and differentiation. Another study evidenced that the fraction containing microvesicles/exosomes had a strong impact on the recovery of a mouse model of myochardial ischemia/reperfusion [19]. In the same

report it was suggested that the secretion of protective exosomes is a general property and function of MSCs, and is probably related with their supporting role, for instance in the bone marrow [19].

Up to now all of the identified proteins in the CM24h and CM96h have been reported to be secreted through exosomes or microvesicles by different cell types, or to be a part of MSCs proteome [31-33]. From these some may be related with neuronal survival, such as 14-3-3 proteins (upregulated in the CM24h, $p < 0.05$) which are known to play crucial roles in many biological processes including cell proliferation, response to cells damage and prevention of apoptosis, including in cells derived from the central nervous system [34]. For instance, as anti-apoptotic factors they interact with a number of apoptosis regulatory proteins such as Bad and FKHRL1 [34]. Another protein which is also upregulated in the CM24h is hsp70, which is ubiquitously expressed and displays neuroprotective effects on neuronal cells [35]. Finally the two other proteins that might be of interest for the present report are peroxiredoxin-6 and **Ubiquitin carboxy-terminal hydrolase L1 (UCHL1)**. The first has a protective role against oxidative stress, including in neurons [36]; indeed changes on its expression have been reported in neuronal death in Parkinson's disease models [36]. The second, UCHL1, is a member of a gene family whose products hydrolize small c-terminal adducts of ubiquitin to generate the ubiquitin monomer. Similarly to perodoxin-6, UCHL1 dysfunction has been reported to be involved in the pathogenesis of PD and AD [32]. With the present analysis it was possible to detect selected proteins, other than the soluble factors commonly reported for this type of studies. Moreover it was also observed that the timeline of MSCs conditioning affected the expression of the proteins. Relating the proteomics' data with the one from the cell culture, and based on what has been

reported in the literature it seems that 14-3-3 protein and hsp70 have a stronger impact on neuronal cell densities. To finalize it must be said that one could also hypothesize that some of these intracellular proteins could be attributed to dying cells conditioning the media. However the high concentrations of protein found in CM, associated with very low levels of cell death of HUCPVCs, make this probability highly unlikely. Future work should be focused on the deciphering the individual role of the above referred proteins on the phenomena herein reported.

4- Conclusions

The present work demonstrates that the secretome of HUCPVCs, in the form of CM, positively impacted the metabolic viability, cell proliferation and neuronal survival/densities in cortical and cerebellar cultures, respectively. This effect was more evident in cortical cultures/neurons. A proteomic characterization of HUCPVCs CM revealed the presence of intracellular proteins, whose concentration changed according to the conditioning period that HUCPVCs were submitted, fact that could be of relevance for future therapeutic approaches.

Acknowledgments

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Figure Captions

Figure 1 - Cell metabolic viability (MTS test), Cell proliferation (BrDU assay) and cell densities, for MAP-2 positive cells, in cortical cultures after incubation with HUCPVCs CM . Results revealed that the CM of HUCPVCs was able to increase the cell metabolic viability (A) and cell proliferation (B) of cortical cultures, namely for the 96h conditioning time point, when compared to controls. Immunocytochemistry for MAP-2 revealed that HUCPVCs CM supported the maintenance of mature neurons in culture, with a stronger emphasis on CM24h (C). In control cultures it was not possible to observe MAP-2 positive cells (D). **(E) and (F) are representative examples of MAP-2 immuno-stained cultures incubated with CM24h and CM96h for 7 days, respectively** (A,B - Results shown as a ratio between CM; C- Results shown in percentage of MAP-2 positive cells; n = 3 ±SD, one way ANOVA, $p < 0.05$; A,B - # notes for statistical differences against the control, $p < 0.05$).

Figure 2 - Cell metabolic viability (MTS test), Cell proliferation (BrDU assay) and cell densities, for MAP-2 positive cells, in cerebellar cultures after incubation with HUCPVCs CM . Results revealed that the CM 24h was able to significantly increase the cell metabolic viability (A) of cortical cultures. Immunocytochemistry for MAP-2 positive neurons (C,D) revealed that both CM24h (C,E) and CM96h (C,F) supported the growth of MAP-2 positive neurons with similar percentages. (A,B - Results shown as a ratio between CM; C- Results shown in percentage of MAP-2 positive cells; n = 3±SD, one way ANOVA, $p < 0.05$; A,B - # notes for statistical differences against the control, $p < 0,05$).

Figure 3- Quantification of 9 protein spots intensities selected from 2D-electrophoresis experiment, with the respective close-up and three dimensional views. Each bar represents the mean intensity value of a specific protein spot in three different experiments ± standard deviation. To access the differences in protein spot intensities between 24h and 96h, *t*-student test was employed after data normality inspection with Kolmogorov-Smirnov test. *P<0.05, **P<0.01.

CORTICAL CULTURES
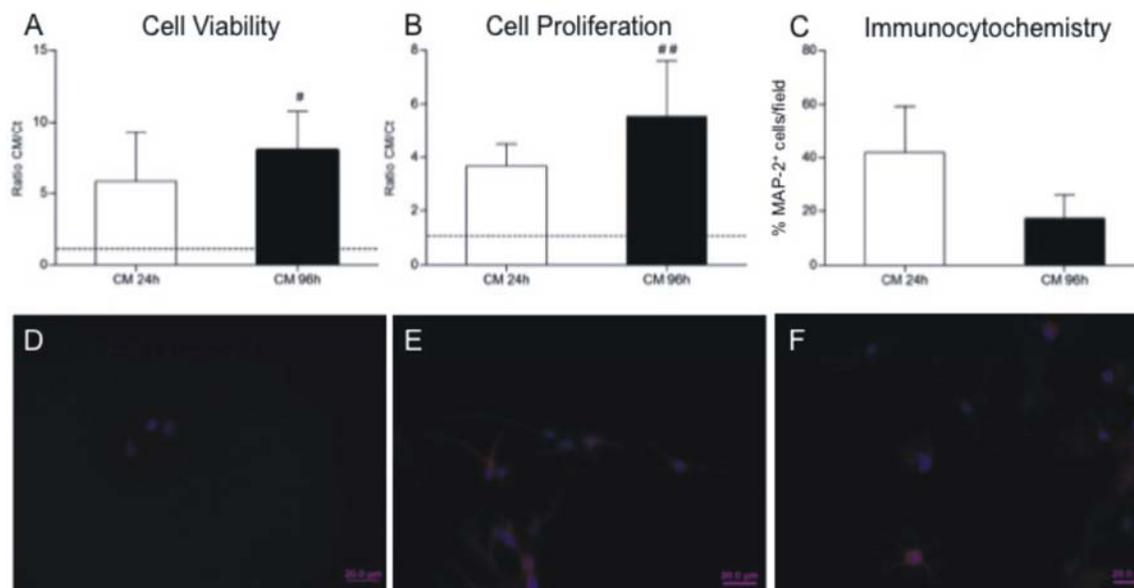
CEREBELLAR CULTURES
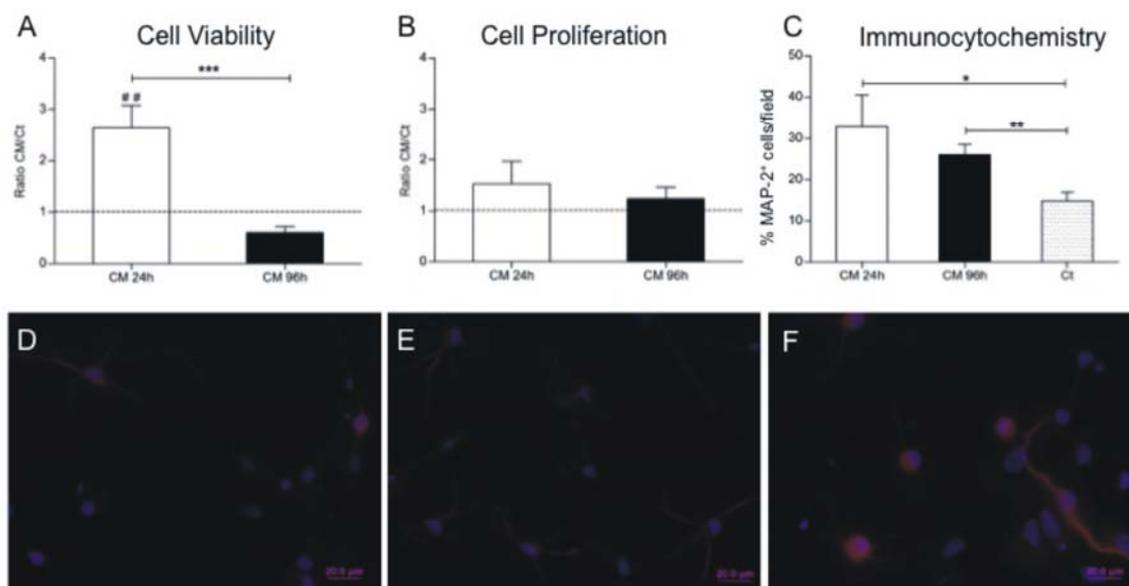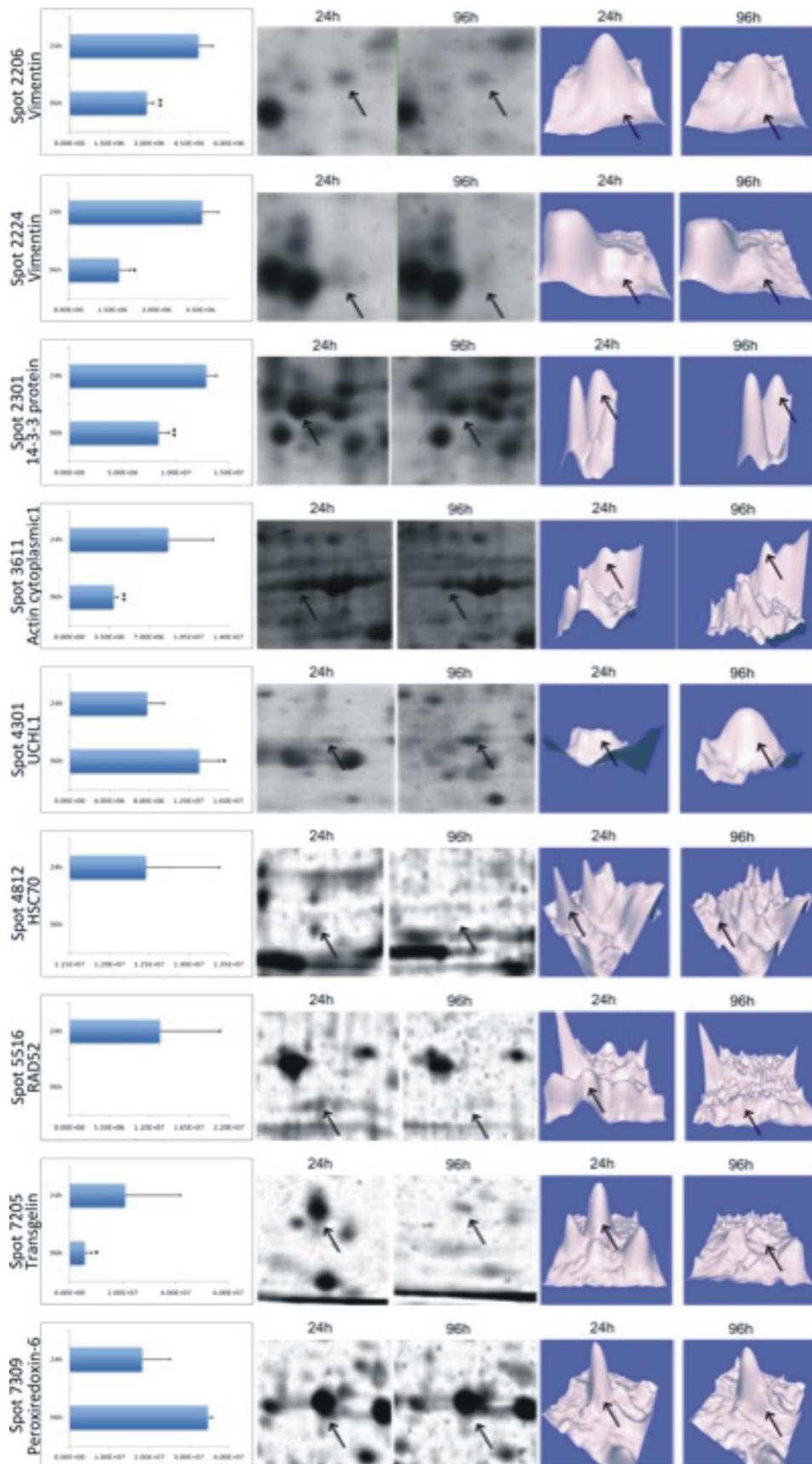
Highlights

- The secretome of human umbilical cord perivascular cells (HUCPVCs) increases the survival of cortical and cerebellar neurons
- Cortical neurons have a more robust HUCPVCs Secretome
- Proteomic analysis revealed the presence hsp70, peroxiredoxin-6, UCHL1, RAD52, 14-3-3 and transgelin in HUCPVCs secretome
- The secretome of HUCPVCs, in the form of conditioned media, changes according to its time-points of collection