International Journal of
Analytical, Pharmaceutical and Biomedical Sciences

Volume: 4: Issue-3: March-2015 *ISSN:2278-0246*

www.ijapbs.com

PROTEOMICS ANALYSIS OF HUMAN ASTROCYTES AGING IN VITRO

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ABSTRACT: Since duration of ageing in humans makes it almost impossible to perform in vivo studies, one simple option is to study ageing in cell cultures. Here the astrocytes that were differentiated from mesanchymal stem cells in cell culture after 21 days were induced to ageing, and morphological variation of cells was microscopically monitored. The proteoms of young and aged cells was provided by 2DE. Among the several identified proteins, one that showed significant alteration in the density selected and was identified by MALDI mass spectrometery and MASCOT analysis. Analysis of gels base on two methods of analysis (progenesis same spot and Mass Spectrometry) detected about 1469 spots in all cells and charecterized 48 proteins. Out of 48 proteins, 30 are down regulated which might beacouse of present H-278C19 in aging process that its function is ubicuitination of some protein, ultimately degredation of them. Identified proteins in relation between neurodegeneration diseases and astrocyte aging were functionally categorized based on Gene Ontology (GO) annotation terms using the DAVID program package. Result show that the major molecular functions that annotated with PIR include acetylation while by GO include cell motion. The cellular component and molecular function showing the greatest enrichment that is response to inorganic substance are greatly enriched in our dataset. To sum up, the most protein refere to the cell cycle, cell dead and glycolysis pathway indicate that aging process are related to defect in these pathway, so aged cell try to repair themselves, neutrelize different kind of stress and regeneration their cytoskeleton.
Keywords: Proteomics, Ageing, Astrocytes, Stem Cell.

INTRODUCTION

Aging is defined a gradual loss of function associated with reduced fertility and increased mortality and morbidity (1, 2). Duration of humans aging makes it almost impossible to study in vivo. One simple option is to study ageing in cellular cultures (3). In 1961, Leonard Hayflick and Paul Moorhead discovered that normal diploid cells in cultures undergo a finite numbers of divisions before they reach a terminally non-proliferating state known as the replicative senescence (3-5). Arresting cell growth in G1 and do not enter S phase, metabolically active, and resist apoptotic death were seen in senescence cells (5). Many of cell cycle regulator such as p53 and pRb have central role in replicative senescence (6). The level of transcript of many genes is altered in senescent cells when compared to cells at early cumulative population doublings, as shown by different genomic and proteomics methods (7). Aging process and age-related changes were determined in adaptive responses including oxidative stress, mitochondrial dysfunction, telomere shortening and various genetic mechanisms (8, 9). The vertebrate central nervous system (CNS) is comprised of three predominant cell types including neuron, oligodendrocyte and astrocyte that are thought to arise from multipotent neural stem cells (NSCs) (10,11). The proper functioning of CNS include the interactive signaling between astrocytes and neurons. Astrocytes (and other glial cells, particularly microglia) directly mediate neuropathological outcomes both during CNS development and aging (10). Astrocytes make up 20 to 50% of the volume of most brain parts and correspond to a heterogenous class of cells that have many different roles (12). Astrocytes play a constitutive

role in formation of the blood-brain barrier, representing the major glycogen depots of the brain and supporting immune defense by producing various immunoactive cytokine (13).

The number and size of synapses change during aging and response to environmental stimuli (9).

Astrocyte can respond to diversity of damaging insults including physical damage, disease and chemicals, and in any of theses situation they become active and display a variety of functional changes (14-17). The common procedure for analysis of changing in the expression of many proteins in a cell is two dimensional electrophoresis techniques. The analyzed proteins can then be identified by mass spectrometry. Today this technique is used in many researches in order to identify genes that influence ageing process (18- 21) as well as in neuroscience analysis (22, 23).

So in this study will determine protein profiles of interval differentiation of stem cell to astrocyte and its aging by proteomics approach.

MATERIALS AND METHODS

Isolation of mesenchymal stem cells (MSCs)

Briefly, each aspirate was diluted 1:1 with DMEM and layered over 1:1 Ficoll (Ficoll-Paque Plus; GE Healthcare Bio-Sciences, Baie-d'Urfé, QC). After centrifugation at 900×g for 30 min, the mononuclear cell layer was removed from the interface, washed with DMEM, and resuspended in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. The cells were plated in 20 ml of medium in a 176-cm2-culture dish and incubated at 37 °C in a 5% CO2 humidified atmosphere. After 72 h, non-adherent cells were discarded and the adherent cells were thoroughly washed twice with DMEM. Thereafter, the cells were expanded as previously described (24).

Immunophenotyping of MSCs by flow cytometry

Expanded MSCs were detached from the culture flask by the use of PBS (pH 7.4) containing trypsin (0.05%) and EDTA (0.02%), washed once with DMEM and once with filtrated PBS. Cells were next suspended at a concentration of 1 × 106 cells in 50 µl PBS and incubated for 45 min at 4 °C in the dark with FITC or PE-conjugated antibodies as follows: anti-CD29-PE, anti-CD54-PE, anti-CD44-FITC, anti-CD45-FITC, anti-CD73-FITC, anti-CD166-FITC, anti-CD105-PE, anti-CD34-FITC, and anti-CD31-FITC. In parallel, cells were incubated with an irrelevant antibody (anti-Aspergillus niger glucose oxidase, Dako) as a negative isotype control to exclude non-specifically labelled cells from the calculation. Upon completion of the incubation time, cells were washed twice with PBS supplemented with 2% BSA and fixed with 1% paraformaldehyde solution in PBS. Analysis was next performed using a flow cytometer (FACsort, BD, USA). Before each test, the percentage of viability that was more than 95% was measured with trypan-blue staining and dead cells were counted with a neobar slide.

Differentiation of MSCs into Neural like cells

The adult human mesenchymal stem cells used were frozen two times and passaged a total of seven times. 2 × 105 mesenchymal stem cells were thawed and suspended in the neurosphere-inducing media in 35 mm dishes. The neurosphere media consisted of Neurobasal A media (GIBCO BRL), B27 proliferation/cell expansion supplement, 1% antibiotic/antimycotic, 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblastic growth factor (bFGF) (Sigma). The floating neurospheres that formed received additional EGF and bFGF every 3–5 days for 15 days. Ten µM of forskolin and 0.1 mM of isobutylmethylxanothine (IBMX), a phosphodiesterase inhibitor, were added to the preliminary neurosphere media to increase cyclic AMP in later experiments. An increase in intracellular cAMP has been shown to promote neuronal signaling mechanisms (25).

Immunocytochemistry

Immunocytochemical staining was performed to examine the NF expression in MSCs at 24 h post neuron induction. The cultures were washed with D-PBS and fixed with 4% paraformaldehyde at 37°C for 10 min. The cells were then permeabilized with 1% Triton at room temperature for 10 min. Following 2 rinses with 0.5 mg/ml sodium borohydride, the cells were incubated with 5% normal goat serum and then 6% BSA, each at room temperature for 30 min, to block non-specific antibody binding. The cells were subsequently incubated with a rabbit anti-human NSE antibody at 37°C for 1 h, and a goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 at 37°C for 40 min. In other experiments, cells were incubated for 60 min at room temperature with monoclonal antibodies against neuron specific enolase (NSE; 1:20, Cymbus Biotechnology). Subsequently, slides were rinsed three times in PBS-Triton, incubated for 30 min with biotinylated antimouse IgG (1:200, Vector) and rinsed and incubated with horseradish peroxidase conjugated avidin D (1:800, Vector), followed by 10 min incubation with DAB tablet sets (SIGMA FAST). Negative controls were routinely performed for each

experiment, incubating the samples with non-immune serum and with biotinylated anti-mouse IgG. Six different experiments were performed for each marker.

Two dimensional SDS-PAGE

Briefly, young and senescent human astrocytes on 25 mm2 dishes were washed three times in PBS. 300µl lysis buffer (7M urea, 2M thiourea, 4%CHAPS, 0.2-0.3%DTT,1-2% ampholine 3-10) was added to cell culture and shaking is done at room temperature 1 h The lysate was centrifuged at 10000 g for 10 min at room temperature. The supernatant was used for protein assay according to Bradford protein assay (26) and subsequently was kept at -20C. Linear pH 3-10 Immobilized Dry Strip (17 cm) were rehydrated overnight at 20C in rehydration buffer(8.5M urea,2% CHAPS, 40mM DTT, 0.1% ampholin, 0.001% bromophenol blue). Sample (400µg) was applied during rehydration. The first dimension of 2D electrophoresis was performed on the PROTEAN IEF Cell system (Bio-Rad). Next, gels were equilibrated for 15 min in equilibration buffer I (6M urea, 2% SDS, 0.375 M Tris Hcl pH8.8, 20% glycerol, 130mM DTT). A 12% SDS-Polyacrylamide slab gel was used for second dimension gel electrophoresis. Equilibrated IPG strips were placed on the surface of second dimension gels and then sealed with 0.5% agarose in SDS electrophoresis buffer (25mM Tris base, 192mM glycine, 0.1%SDS) and were run vertically (27).

2DE gel staining:

After electrophoresis, the gels were stained with Coomassie Brilliant Blue staining (28).

Bioinformatics analysis:

2DE gels are scanned and gels are analyzed by nonlinear progenesis same spot and fliker softwares to alignment all gels together and compare the spots in one statement in gels and get the density of same spot in each of gel. Then the spots compared to data banks to detection the spots in one statement in every experiment gel and data bank references gels. Identified proteins were also functionally categorized based on Gene Ontology (GO) annotation terms using the DAVID program package.

Mass spectrometry

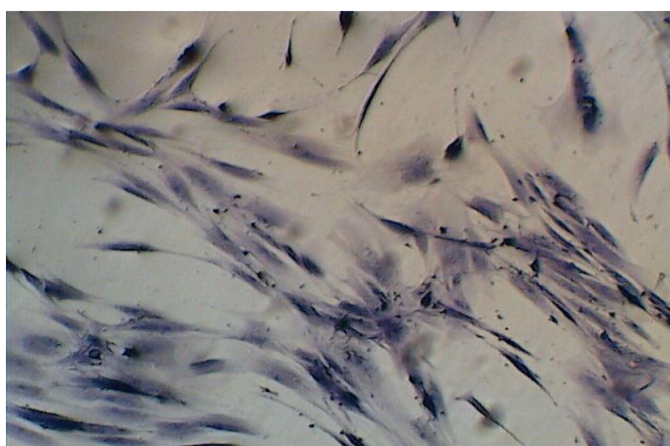
Coomassie Brilliant Blue stained protein spot containing the interested protein was destained thoroughly with 1% H2O2 (typically 1 min) and lyophilized to dryness (29). Coomassie Brilliant Blue stain removal using H2O2 was performed to enhance peptide adsorption by matrix-assisted laser desorption/ionization time-of- flight mass spectrometry (MALDI) (30). The dehydrated gel bands were hydrated with 15 µg/l (Promega, Madison, WI) of porcine trypsin in 25 mmol/L NH4HCO3, pH8.2 on ice for 45 min. Excess trypsin was removed; gel bands were covered with 25 mmol/l NH4HCO3, pH8.2 and incubated at 37°C overnight. Tryptic peptides were extracted from the gel bands with 70% acetonitrile and 0.1% trifl uoroacetic acid. Sample was desalted with C18 Zip Tips (Millipore, Bedford, MA) as per manufacturer's protocols. 0.5 µl of sample was co-crystallized with 0.5 µl of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% trifluoroacetic acid and spotted directly on a stainless steel MALDI target plate. Mass spectra were acquired using a MALDI-TOF/TOF mass spectrometer (Voyager 4700, Applied Biosystems, Foster City, CA). MALDI-TOF/TOF spectra were internally calibrated (< 20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. Spectra were submitted to Mascot (http://matrixscience.com) for peptide mass fingerprinting.

RESULTS

Figure 1A shows the human mesenchymal stem cells (MSCs), the young astrocyte cell differentiated from human mesenchymal stem cells-NSE staining (neuron specific enolase) was represented in the figure 1B. The mild aged astrosyte cells (the young astrocyte cells that are incubated two weeks in the media culture under 37oC temperature) and the aged cells (the young astrocyte cells that are incubated for 4 weeks) are shown in the figures 1C and D respectively. To study and understanding the grounds for such morphological differences like vesiculated cytosol, bigger size and more dendritic filament and the cause for ageing, 2DE in combination with mass spectrometry for protein identification were used. Following to 2DE two methods were used progenesis same spot software for image analysis, and subsequent mass spectrometry for identification of proteins which were subjected to change in expression as a result of ageing process. Gel analysis by using progenesis same spot show that 1469 spot that 940 spots have up regulated more than or equal to 2 fold that there result shown in table 1 and figure 3. Here by using progenesis same spot as depicted in table 2, it was detected that the pointed spots refer to the VIM, ACTB,ACTG, TPM1-3, TPM1-4, TPM4, TPM3, SODC, HSP27, ATP5B, GRP78, TUBB5, TTR, PDI, AACT, PRDX6, TPIS, CYPA, CFL, H-278C19.3, LGALS3, ANX2, ANX1, ALDC, PGK1, EEF1A1, GST3, APOA1, GLO1, EIF4A2, A1BG, COL6A1,AAT, AT3,ENO1, P40261, CALM2, UCH-L1,

MVP, MYL6, FGG, CRTC, CH60, PDA, GRP75, THIO, COXA, ATPD, RLA2 and PDX6 (figure 4 show 48 spots that detected by progrnrsis same spot).

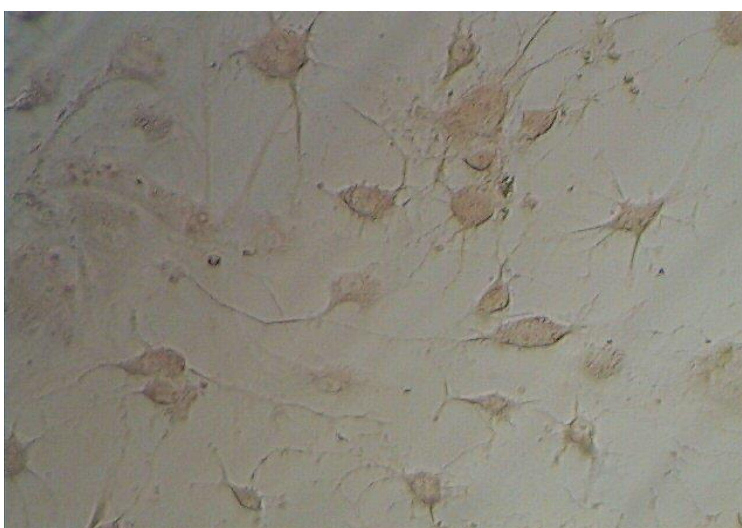
Identified proteins were functionally categorized based on Gene Ontology (GO) annotation terms using the DAVID program package. Amongst other, we found that 48 proteins were annotated with GO cellular component terms. Here 48 of the input proteins were classified. The major molecular functions that annotated with PIR include acetylation (64.6%), disease mutation(34.4%) while annotated by GO include cell motion (27.1%), response to inorganic substance(18.8%), cytosol(35.4%), response to metal ion (14.6%), anti-apoptosis(14.6%), muscle thin filament tropomyosin (6.2%), negative regulation of apoptosis(16.7%), negative regulation of programmed cell death(16.7%)negative regulation of cell death(16.7%), generation of precursor metabolites and energy(14.6%), glycolysis(8%). DAVID furthers assigns a statistical significance indicator to protein functions and classifications. The cellular component and molecular function showing the greatest enrichment are shown in Table 2. It seems that response to inorganic substance (1.8E-7) is greatly enriched in our dataset. Moreever, response to metal ion (2.6E-6), response to calcium ion (6.5E-4), cell motion (1.3E-8), structural molecule activity (1.2E-3), cytoskeleton (1.2E-3) were showing the greatest enrichment.



(a)

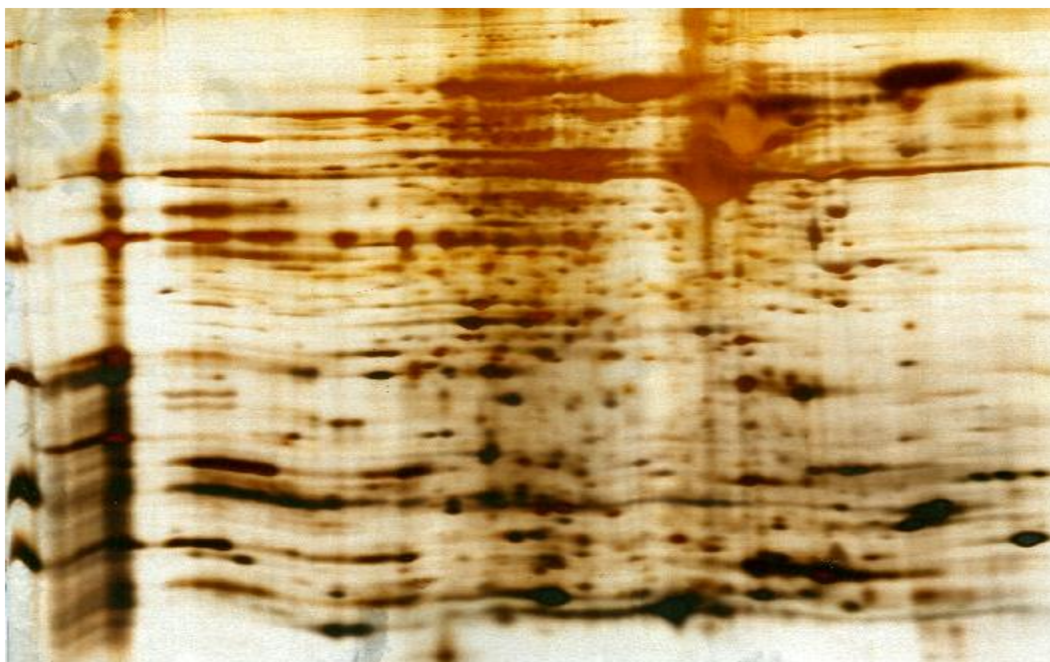
(B)

(C)

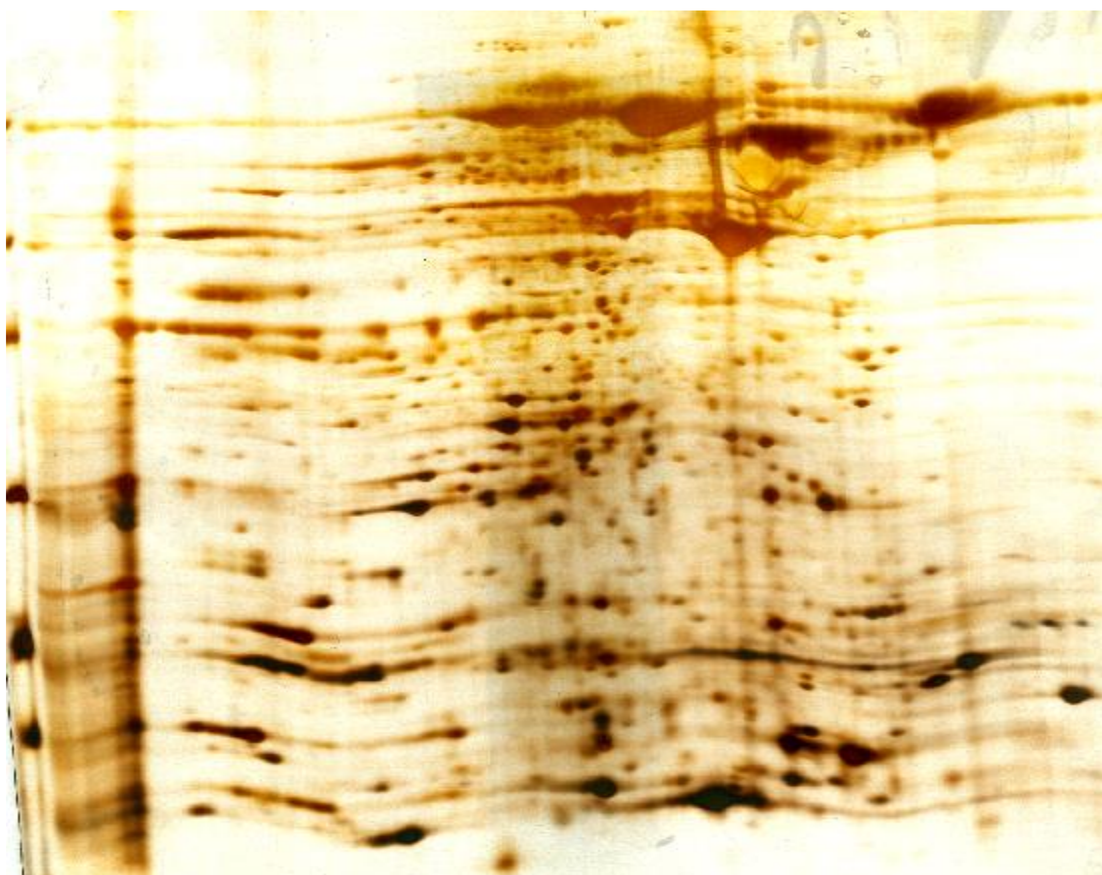


(D)

Fig 1: a) mesenchymal stem cells-NSE staining (neuron specific enolase), b) young astrocytes evolved from mesenchymal stem cells-NSE staining (neuron specific enolase), c) mild aged astrocytes, these cells are two weeks older than the young astrocytes and d) aged astrocytes, these cells are four weeks older than the young astrocytes. Morphological differences like vesiculated cytosol, bigger size and more dendritic filament respectively increase from stem cell to aged astrocyte



A



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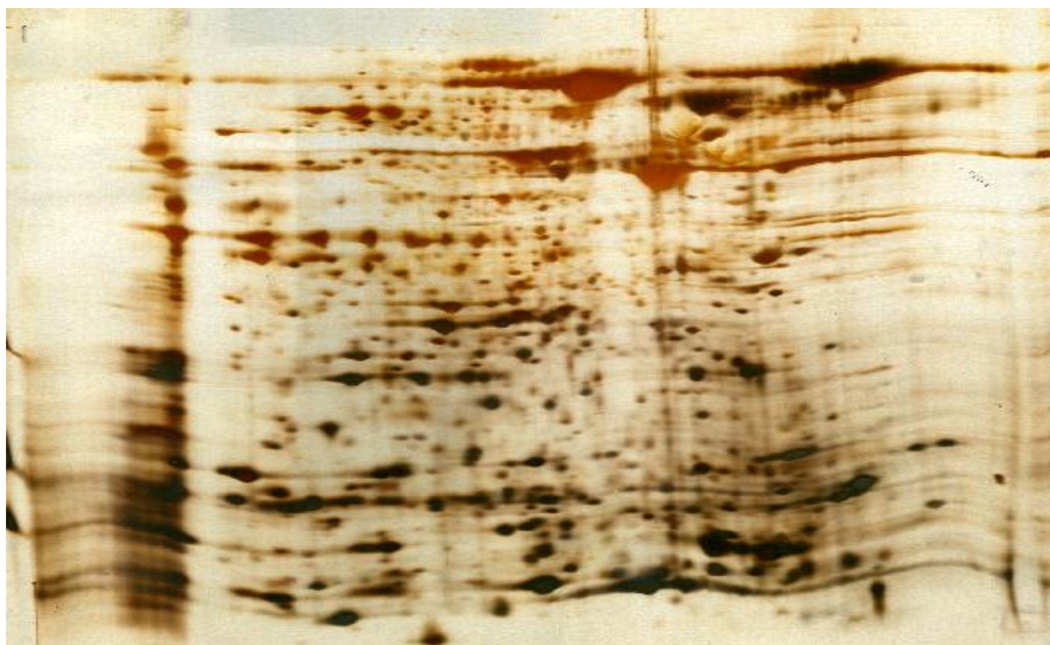
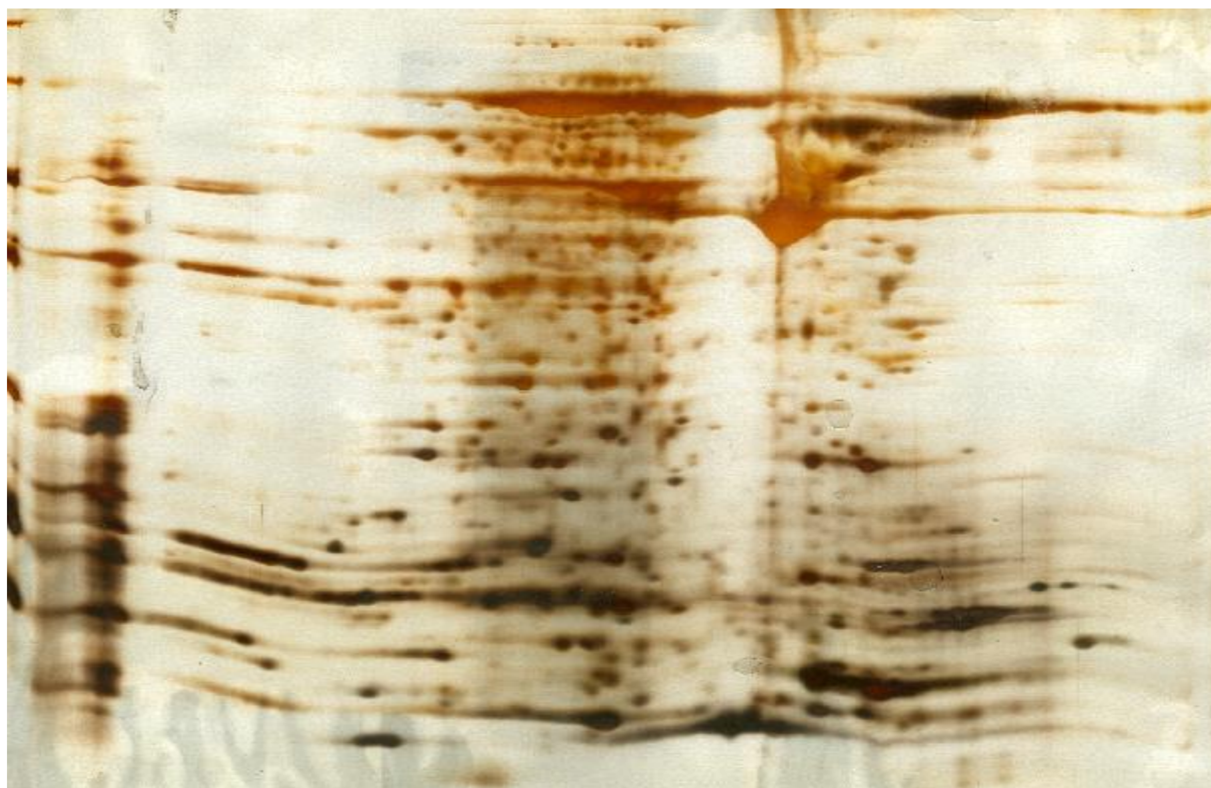


C



D

Fig 2: 2DE gel image of a) mesenchymal stem cells, b) young astrocytes, c) mild aged astrocytes, d) aged astrocytes

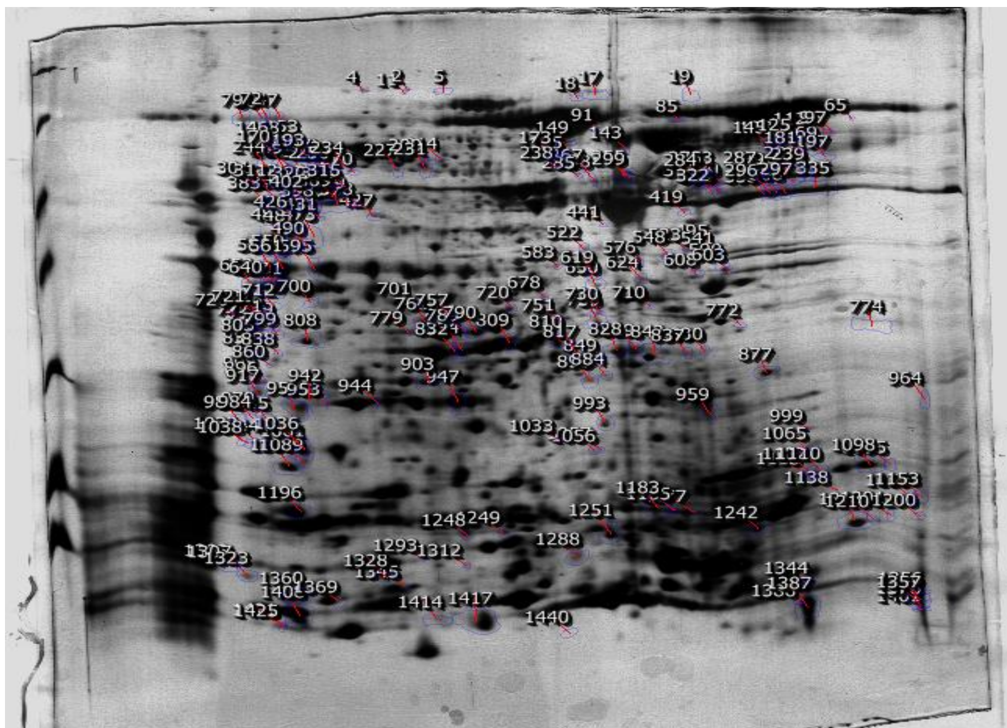


Fig 3: reference image (mild aged astrocytes) that select for analysis by progenesis same spot software

Table 1: analysis of progenesis same spot software of 2DE gels of young (condition1), mild (condition2) and late aged (condition3) of actrocyte cells and stem cell (condition4). It shows just 20 out of 1470 protein spot

#	Anova (p)	Fold	pI	MW	Average Normalised Volumes			
					Condition 1	Condition 2	Condition 3	Condition 4
1249	0	4.2	6.54	25,362	5912.725	1.05E+04	2510	3155.24
661	0	2.1	4.74	64,409	6.15E+04	1.27E+05	6.78E+04	1.16E+05
1242	0	3.1	8.58	26,000	2.94E+04	2.69E+04	9480	1.48E+04
1344	0	3.1	9	18,771	9119.406	1.07E+04	3445	4986.186
300	0	3.1	8.28	104,295	1877.642	4264.783	1362	2812.667
1159	0	5	9.85	30,229	1.32E+04	3.94E+04	7831	8640.525
352	0	3.1	8.67	99,933	1.10E+04	3.46E+04	1.55E+04	3.09E+04
624	0	2.6	7.65	66,243	9025.474	1.77E+04	6731	1.11E+04
350	0	3.2	8.75	100,282	7156.825	2.31E+04	9026	1.82E+04
637	0	2.8	4.68	65,478	1555.875	3005.935	1265	1072.342
650	0	3	7.29	65,020	1.85E+04	1.95E+04	1.21E+04	3.65E+04
1251	0	2.2	7.4	25,096	1.11E+05	8.36E+04	5.07E+04	7.63E+04
701	0	2.9	5.81	61,811	2637.093	1600.325	922	1416.288
283	0	2.7	8.27	106,913	1.81E+04	4.62E+04	1.68E+04	2.79E+04
1113	0	3.7	9.01	33,445	5352.13	4364.686	5076	1.60E+04
270	0	3.8	5.3	109,181	3888.189	3414.322	1918	7375.958

1107	0	4.4	9.06	33,922	6666.18	6469.897	8547	2.84E+04
255	0	5.4	7.54	110,228	3.18E+04	4.38E+04	8114	2.04E+04

Table 2: 2DE results of stem cell that compare to the reference Database gels by using progenesis same spot software

#of progenesis	#	Entry name	uni prot id	name
1232	1	VIM	P08670	Vimentin
610	2	ACTB	P60709	Beta-actin, Actin, cytoplasmic 1, N-terminally processed
641	3	ACTG	P63261	Gamma-actin
374	4	TPM1-3	P09493	Tropomyosin alpha-1 chain
712	5	TPM1-4	C9IZA2	Tropomyosin 1 (Alpha), isoform CRA_o
	6	TPM4	P67936	Tropomyosin alpha-4 chain
755	7	TPM3	P06753	Tropomyosin alpha-3 chain
1117	8	SODC	P00441	Superoxide dismutase [Cu-Zn]
984	9	HSP27	P04792	Heat shock protein beta-1
533	10	ATP5B	P06576	ATP synthase subunit beta, mitochondrial
226	11	GRP78	P11021	78 kDa glucose-regulated protein
426	12	TUBB5	P04350	Tubulin beta-4 chain
1178	13	TTR	P02766	Transthyretin
	14	PDI	P07237	Protein disulfide-isomerase
258	15	AACT	P01011	Alpha-1-antichymotrypsin
911	16	PRDX6	P30041	Peroxiredoxin-6
975	17	TPIS	P60174	Triosephosphate isomerase
1228	18	CYPA	P62937	Peptidyl-prolyl cis-trans isomerase A
1093	19	CFL	P23528	Cofilin-1
1094	20	H-278C19.3	O75795	UDP-glucuronosyltransferase 2B17
834	21	LGALS3	Q59FR8	LGALS3 protein variant
750	22	ANX2	P07355	Annexin A2
758	23	ANX1	P04083	Annexin A1
567	24	ALDC	P09972	Fructose-bisphosphate aldolase C
535	25	PGK1	P00558	Phosphoglycerate kinase 1
455	26	EEF1A1	P68104	Elongation factor 1-alpha 1
1096	27	GST3	O14880	Microsomal glutathione S-transferase 3
1103	28	APOA1	P02647	Apolipoprotein A-I
1090	29	GLO1	Q04760	Lactoylglutathione lyase
500	30	EIF4A2	Q14240	Eukaryotic initiation factor 4A-II
237	31	A1BG	P04217	Alpha-1B-glycoprotein
96	32	COL6A1	P12109	Collagen alpha-1(VI) chain

377	33	AAT	P01009	Alpha-1-antitrypsin
307	34	AT3	Q16650	T-box brain protein 1
259	35	ENO1	P06733	Alpha-enolase
1233	36	P40261	P40261	Nicotinamide N-methyltransferase
1050	37	CALM2	P62158	Calmodulin
895	38	UCH-L1	P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1
505	39	MVP	Q15126	Phosphomevalonate kinase
1105	40	MYL6	P60660	Myosin light polypeptide 6
283	41	FGG	P02679	Fibrinogen gamma chain
	42	CRTC	Q5MPA9	Serine/threonine-protein kinase DCLK2
488	43	CH60	P10809	60 kDa heat shock protein, mitochondrial
498	44	PDA	Q92481	Transcription factor AP-2-beta
240	45	GRP75	P38646	Stress-70 protein, mitochondrial
1229	46	THIO	P10599	Thioredoxin
1230	47	COXA	Q92954	Proteoglycan 4
1044	48	ATPD	P30049	ATP synthase subunit delta, mitochondrial
1032	49	RLA2	P05387	60S acidic ribosomal protein P2
1121	50	PDX6	O00330	Pyruvate dehydrogenase protein X component, mitochondrial

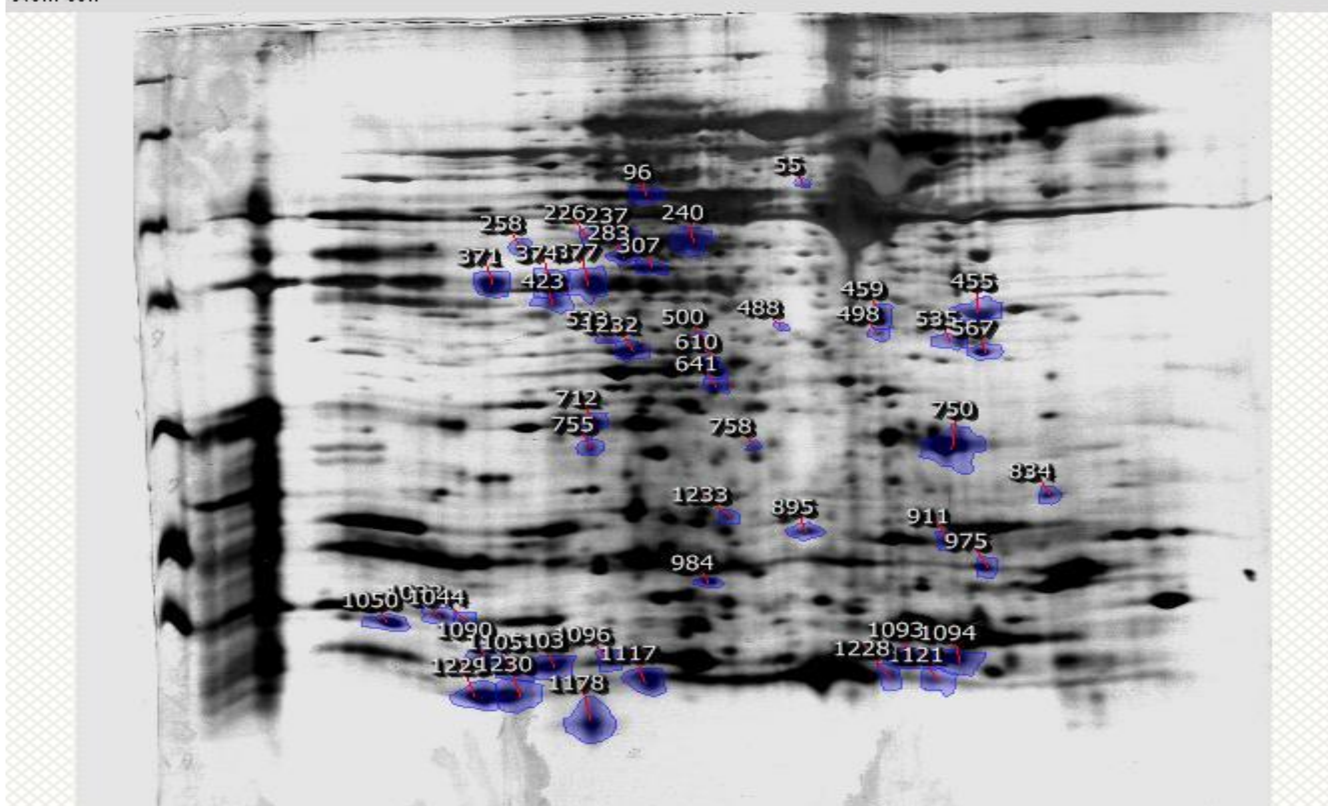


Figure4: 48 dtected protein that analysis by using of progenesis same spot software of 2DE gels of refrence image (stem cell)

Table 3: Functional Annotation Clustering by using DAVID software

Annotation Cluster 1	**Enrichment Score: 4.04**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	response to inorganic substance	9	1.80E-07	7.90E-05
GOTERM_BP_FAT	response to metal ion	7	2.60E-06	7.50E-04
GOTERM_BP_FAT	response to calcium ion	4	6.50E-04	5.40E-02
Annotation Cluster 2	Enrichment Score: 3.33	Count	P_Value	Benjamini
GOTERM_BP_FAT	cell motion	13	1.30E-08	1.10E-05
GOTERM_MF_FAT	structural molecule activity	9	1.20E-03	2.10E-01
GOTERM_CC_FAT	cytoskeleton	13	1.20E-03	1.90E-02

Table 4: Functional Related aging

	category	**term**	**Kapa**
1	GOTERM_BP_FAT	response to inorganic substance	1.00
2	GOTERM_BP_FAT	response to metal ion	0.85
3	GOTERM_BP_FAT	response to calcium ion	0.57
4	GOTERM_BP_FAT	response to wounding	0.45
5	SP_PIR_KEYWORDS	ubl conjugation	0.45
6	SP_PIR_KEYWORDS	methylated amino acid	0.45
7	GOTERM_BP_FAT	response to oxidative stress	0.45
8	GOTERM_BP_FAT	wound healing	0.45
9	GOTERM_BP_FAT	cellular response to reactive oxygen species	0.45
10	GOTERM_BP_FAT	cellular response to oxidative stress	0.45
11	GOTERM_BP_FAT	response to reactive oxygen species	0.45
12	GOTERM_BP_FAT	cellular component morphogenesis	0.40
13	SP_PIR_KEYWORDS	isopeptide bond	0.40
14	GOTERM_CC_FAT	cell cortex	0.39
15	GOTERM_BP_FAT	cellular response to stress	0.39
16	SP_PIR_KEYWORDS	methylation	0.39
17	GOTERM_BP_FAT	cytoskeleton organization	0.39
18	UP_SEQ_FEATURE	cross-link:Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin)	0.39
19	GOTERM_MF_FAT	structural constituent of cytoskeleton	0.39
20	SP_PIR_KEYWORDS	cytoskeleton	0.36

DISCUSSION

Astrocytes are a major cell type in the CNS that cooperate with neurons and other glial cells in the development and maintenance of CNS functions (30). Astrocytes play different roles at different stages of the lifespan. While the role of astrocytes during ageing is not as well understood, the correct migration of neurons and growth cones

provide by glial cells in early development(31-34). As it is shown in figure 1, the more age, the more different shape, so there are morphological differences in aged astrocyte than young one like vesiculated cytosol, bigger size and more dendritic filament. . Immature astrocytes possess a polygonal shape, have no processes, and continue to proliferate, whereas mature astrocytes have a stellate cell morphology, increased glial fibrillary acidic protein (GFAP) expression, and proliferate slowly (30).This variation refers to the numerous differences in the expression of genes in the compared cells due to the aging possess. One of the best ways for detection of the gene expression is proteomics. 2DE analysis determined 1469, in all cells are determined and their expressions were calculated. 48 spots in table 2, 3 and 4 determined the protein charectrictis and function. The mass results confirm that the progenesis same spot finding is real and the considered proteins are Prx-cis, aldolase C, lectin and VDAC. The expression of detected proteins was different in the lifespan of astrocyte. Apolipoprotein (apo) was gradually increased in ageing of astrocyte. It belongs to the lipocalin superfamily member that transports small hydrophobic molecules (35, 36). In the CNS apo D expressed in neurons, oligodendrocytes, astrocytes, and perivascular cells (37). Previous studies have been observed the increments of its expression associat with aging as well as in the elderly brain and with some types of neuropathology (36) that involved in repair and regeneration after neurodegeneration (38). Beta-actin is one of six different actin isoforms which have been identified in humans (39). In this study beta actin just was seen in aged astrocyte. Previous study demonstrated that beta-actin mRNA levels are differentially regulated during development of astrocytes in primary culture (40). The next protein is transthyretin (TTR), a second major cerebrospinal fluid protein that transports thyroxine from the bloodstream to the brain. It was gradually increased in ageing of astrocyte (41). Alpha 1-antichymotrypsin (AACT), a serine protease inhibitor, was respectively increased and decreased in aging of astrocyte. It was found in normal human brain in astrocytes, in various human neuropathological states (42). Aldolase C, a glycolytic enzyme, was gradually decreased in aging of astrocyte (43, 44). Cellular life span can modulate by glycolytic enzyme. Replicative senescence followed early passage declines glycolytic activity and inhibition of glycolytic enzymes can induce senescence (44).

Out of 48 detected proteins, about 30 proteins down regulate, since in this profile detected ubiquitin carboxyl-terminal hydrolase isozyme L1 that it is ubiquitinated proteins. This enzyme is a thiol protease with ATP-independent ubiquitin ligase activity. Down regulation of some protein can because of present this protein that guide the protein to degradation.

Identified proteins were functionally categorized based on Gene Ontology (GO) annotation terms using the DAVID program package (14, 15). Amongst other, we found that 48 proteins were annotated with GO cellular component terms. As shown in table 6, The major molecular functions that annotated with PIR include acetylation (64.6%), disease mutation(34.4%) while annotated by GO include cell motion (27.1%), response to inorganic substance(18.8%), cytosol(35.4%), response to metal ion (14.6%), anti-apoptosis(14.6%),muscle thin filament tropomyosin (6.2%), negative regulation of apoptosis(16.7%), negative regulation of programmed cell death(16.7%)negative regulation of cell death(16.7%), generation of precursor metabolites and energy(14.6%), glycolysis(8%). The cellular component and molecular function showing the greatest enrichment are shown in Table 5. It seems that response to inorganic substance (1.8E-7) is greatly enriched in our dataset. Further, response to metal ion (2.6E-6), response to calcium ion (6.5E-4), cell motion (1.3E-8), structural molecule activity (1.2E-3), and cytoskeleton (1.2E-3) were showing the greatest enrichment. The most function related to the aging can categorize as detected in table7 that conclude response to inorganic substance, response to metal ion , response to calcium ion, response to wounding, ubl conjugation , methylated amino acid, response to oxidative stress , wound healing, cellular response to reactive oxygen species, cellular response to oxidative stress, response to reactive oxygen species, cellular component morphogenesis, isopeptide bond, cell cortex , cellular response to stress, methylation , cytoskeleton organization, cross-link:Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin), structural constituent of cytoskeleton , cytoskeleton. Our finding indicates that most of protein that are detected involve in neurodegeneration diseases like Alzheimer, Parkinson and Huntington. They are the most diseases that associated to aging. In the other hand neurodegeneration diseases and aging of astrosyte have improve in same pathways. Beside of the role of different proteins in the various repair pathways it plays the important role on the prevention of astrocyte aging. We can conclude that use of signature proteins in aging of astrocyte can help to find target biomarker to prevent or delay of astrocyte aging.

ACKNOWLEDGMENT

The financial support from proteomics research center of shahid beheshti university medical science is gratefully acknowledged.

REFERENCES

[1] Allan J.McLEAN, David G. LE COUTEUR, Aging biology and geriatric clinical pharmacology, Pharmacol Rev, 2004, 56, 163-184.
[2] Brian T. Weinert, Poala S. Timiras, Physiology of aging, J Appl Physiol, 2003, 1706- 1716.
[3] Joao Perdo de Magalhaes, From cells to ageing: a review of models and mecha-nisms of cellular senescence and their impact on human ageing, Experimental Cell research, 2004,300,1.
[4] Hyun-Seok Kim et al, immortalization of human embryonic fibroblasts by overex-pression of c-myc and simian virus 40 large T antigen ,EXPERIMENTAL AND MOLECULLAR MEDICINE, 2001,33,293.
[5] Goberdhan P.DIMRI et al, A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc.Natl.Acad.Sci.USA, 1995,92,9363.
[6] Koji Itahana et al, Regulation of senescence by p53, Eur.J.Biochem.2001,268,2784.
[7] Jean-Francois Dierick et al, Identification of 30 protein species involved in replicative senescence and stress-induced premature senescence, FEBS Letters, 2002,531,499.
[8] Allan J.McLEAN, David G. LE COUTEUR, Aging biology and geriatric clinical pharmacology, Pharmacol Rev, 2004, 56, 163-184.
[9] Mark P. MATISON, SIC L. CHAN, Wenzhen Duan, Modification of brain and neurodegenerative disorders by genes, diet, and behavior, Physiol Rev, 2002, 82, 637-672.
[10] Michael Aschner et al, Astrocyte modulation of neurotoxic injury, Brain Pathol, 2002, 12,475.
[11] Robert M. Bachoo, molecular diversity of astrocytes with implications for neurolog-ical disorders, PNAS,2004,101.
[12] Raymonde Porchet et al, Analysis of glial acidic fibrillary protein in the human entorhinal cortex during aging and in Alzheimer,s disease, Proteomics, 2003, 3, 1476-1485.
[13] Andrea Goncalves Trentin, Thyroid hormone and astrocyte morphogenesis, Jour-nal of Endocrinology,2006.
[14] Hongjun Song, Charles F. Stevens , Fred H. Gage, Astroglia induce neurogenesis from adult neural stem cells, Nature, 2002, 417,39.
[15] Zsuzsanna Kornyei et al, Humoral and contact interactions in astroglia/stem cell co-cultures in the course of glia-induced neurogenesis, GLIA, 2005, 49,430-444.
[16] Karen S. Christopherson et al, Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis, Cell, 2005, 120, 421-433.
[17] Jiansong Sheng et al, Bystin as a novel marker for reactive astrocytes in the adult rat brain following injury, European Journal of Neuroscience, 2004, 20, 873-884.
[18] Silvia Benvenuti et al, Differential Proteome Analysis of Replicative Senescence in Rat Embryo Fibroblasts, Molecular & Cellular Proteomics 1.4, 2002,1,280-292.
[19] Bok- Hee Choi and Jong-Suk Kim, Age-related decline in expression of calnexin, Experimental and Molecular Medicine, 2004, 36, 499-503.
[20] Hiroki Kamino et al, Searching for genes involved in arteriosclerosis: Proteomic analysis of cultured human umbilical vein endothelial cells undergoing replicative senescence, Cell Structure and Function,2003, 28, 495-503.
[21] Jean-Francois Dierick et al, Identification of 30 protein species involved in replicative senescence and stress-induced premature senescence, FEBS Letters, 2002,531,499.
[22] Gert Lubec, Kurt Krapfenbauer, Michael Fountoulakis, Proteomics in brain re-search: potentials and limitations, Progress in Neurobiology,2003, 611, 1-19.
[23] Richard S. Morrison et al, Proteomic Analysis in the Neurosciences, Molecular & Cellular Proteomics, 2002, 1, 553-560.
[24] D.C. Colter, R. Class, C.M. DiGirolamo and D.J. Prockop, Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow, Proc Natl Acad Sci (USA) 97 (2000), pp. 3213–3218.

[25] Sherri S. Schultz and Paul A. Lucas, Human Stem Cell isolated from adult muscle differentiate into neural phenotypes, Journal of Neuroscience Methods, 2006, 152, 144-155.

[26] Brad ford, MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the princil of protein. Analytical Biochemistry 1976;72: 248-254

[27] Gi Zhou, Hongmei Li, Dianne Decamp, et al, 2d differential in-gel electrophoresis for the identification of esophageal scans cell cancer-specific protein markers, Mo-lecular and Cellular Proteomics, 2002, 1,2: 117-123.

[28] Sasse J, Gallagher SR., Staining proteins in gels. Curr Protoc Immunol. 2004;Chapter 8:Unit 8.9.

[29] Martin R. Larsen, Stuart j. Cordwell, Peter Roepstroff, Graphite powder as an alternative or reversed-phase material for desalting and concentration of peptide mixtures prior to matrix-assisted laser desorption/ionization-mass spectrometry, Proteomics, 2002, 2, 1277-1287.

[30] Sumner LW, Wolf-Sumner B, White SP, Asirvatham VS. Silver stain removal using H2O2 or enhanced peptide mass mapping by matrix-assisted laser desorp-tion/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spec-trom 2002; 16: 160-168.

[31] Endo T. Glycans and glycan-binding proteins in brain: galectin-1-induced expres-sion of neurotrophic factors in astrocytes. Curr Drug Targets. 2005 Jun;6(4):427-

[32] Glia-neuron intercommunications and synaptic plasticity. Prog Neurobiol. 1996 Jun;49(3):185-214

[33] Kandel, E. R., Schwartz, J. H. & Jessell, T. M. Principles of Neural Science (McGraw-Hill, New York, 2000).

[34] Nicholls, J. G., Martin, A. R. & Wallace, B. G. From Neuron To Brain: A Cellular and Molecular Approach to the Function of the Nervous System 3rd edn. 149–152 (Sinauer, Sunderland, Massachusetts, 1992).

[35] Haydon, P. G. Glia: listening and talking to the synapse. Nat. Rev. Neurosci. 2, 185–193 (2001).

[36] Rassart E, Bedirian A, Do Carmo S, Guinard O, Sirois J, Terrisse L, Milne R (2000) Apolipoprotein D. Biochim Biophys Acta 1482:185–198

[37] Eva del Valle, Ana Navarro, Aurora Astudillo, and Jorge ToliviaApolipoprotein D Expression in Human Brain Reactive Astrocytes. The Journal of Histochemistry & Cytochemistry 51(10): 1285–1290, 2003

[38] Boyles JK, Notterpeck LM, Wardell MR, Patel SC (1990) Rat iden-tification, charac-terization and tissue distribution of apolipopro-tein D in rat. J Lipid Res 31:2057–2065

[39] Ghiso J, Matsubara E, Koudinov A, Choi–Leuba G, Kraftsik R (1994) Visual cortex in Alzheimer's disease: occurrence of neu-ronal death and glial proliferation, and correlation with patho-logical hallmarks. Neurobiol Aging 15:29–43

[40] Gunning P, Weinberger R, Jeffrey P(1997). "Actin and tropomyosin isoforms in morphogenesis.". Anat. Embryol. 195 (4): 311–5.

[41] da Cunha A, Aloyo VJ, Vitković L . Developmental regulation of GAP-43, gluta-mine synthetase and beta-actin mRNA in rat cortical astrocytes. Brain Res Dev Brain Res. 1991 Dec 17;64(1-2):212-5.

[42] Schwarzman AL, Goldgaber DInteraction of transthyretin with amyloid beta-protein: binding and inhibition of amyloid formation. Ciba Found Symp. 1996;199:146-60; discussion 160-4.

[43] Abraham CR, Shirahama T, Potter H. Alpha 1-antichymotrypsin is associated solely with amyloid deposits containing the beta-protein. Amyloid and cell locali-zation of alpha 1-antichymotrypsin. Neurobiol Aging. 1990 Mar-Apr;11(2):123-9.

[44] Henriette Skala et al, Upstream Elements Involved in vivo in activation of the brain-specific rat aldolase C gene, The Journal of Biological Chemistry, 1998, 273, 31806-31814.

[45] Rafael Canete-Soler, Aldolases A and C are ribonucleolytic components of a neu-ronal complex that regulates the stability of the light-neurofilament mRNA, The Journal of Neuroscience, 2005, 25(17), 4353-4364.