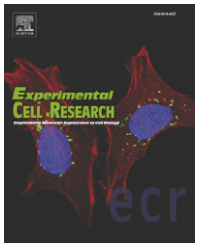
available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction

M. Guescini[a,*], *D. Guidolin*[b], *L. Vallorani*[a], *L. Casadei*[a], *A.M. Gioacchini*[a], *P. Tibollo*[a], *M. Battistelli*[c], *E. Falcieri*[c,d], *L. Battistin*[e], *L.F. Agnati*[e], *V. Stocchi*[a,*]

[a]*Department of Biomolecular Sciences, University of Urbino "Carlo Bo", 61029 Urbino, Italy*
[b]*Department of Anatomy and Physiology, University of Padova, 35100 Padova, Italy*
[c]*Department of Human, Environment and Nature Science, University of Urbino "Carlo Bo", 61029 Urbino, Italy*
[d]*Molecular Genetic Institute, CNR, Istituti Ortopedici Rizzoli, I-40136 Bologna, Italy*
[e]*IRCCS San Camillo Lido, 30100 Venezia, Italy*

ARTICLE INFORMATION

Article Chronology:
Received 8 October 2009
Revised version received 3 April 2010
Accepted 10 April 2010

Keywords:
Myoblasts
Exosomes
Mitochondrial DNA
Inter-cellular communication
Bioinformatics analysis

ABSTRACT

Micro-vesicles can be released by different cell types and operate as 'safe containers' mediating inter-cellular communication. In this work we investigated whether cultured myoblasts could release exosomes. The reported data demonstrate, for the first time, that C2C12 myoblasts release micro-vesicles as shown by the presence of two exosome markers (Tsg101 and Alix proteins). Using real-time PCR analysis it was shown that these micro-vesicles, like other cell types, carry mtDNA. Proteomic characterization of the released micro-vesicle contents showed the presence of many proteins involved in signal transduction. The bioinformatics assessment of the Disorder Index and Aggregation Index of these proteins suggested that C2C12 micro-vesicles mainly deliver the machinery for signal transduction to target cells rather than key proteins involved in hub functions in molecular networks. The presence of IGFBP-5 in the purified micro-vesicles represents an exception, since this binding protein can play a key role in the modulation of the IGF-1 signalling pathway.

In conclusion, the present findings demonstrate that skeletal muscle cells release micro-vesicles, which probably have an important role in the communication processes within skeletal muscles and between skeletal muscles and other organs. In particular, the present findings suggest possible new diagnostic approaches to skeletal muscle diseases.

Introduction

In the last ten years several groups have described the production and release of humoral factors from contracting muscle cells [1–6]. Given that skeletal muscle is the most important tissue in the human body in terms of total weight, the discovery that contracting muscle is a cytokine producing organ opened the entirely new field of the skeletal muscle as an endocrine organ. These hormone-like factors may influence metabolism in tissues and organs [7], therefore it has been suggested that cytokines and other peptides that are produced and released by muscle fibers, exerting autocrine, paracrine or endocrine effects should be classified as "myokines" (a term derived from the Greek words "muscle" and "motion").

* *Corresponding authors.* Fax: +39 0722 303401.
E-mail addresses: michele.guescini@uniurb.it (M. Guescini), vilberto.stocchi@uniurb.it (V. Stocchi).

0014-4827/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2010.04.006

Recently, it has been demonstrated that micro-vesicles can be released by many different types of cells and operate as safe containers mediating inter-cellular communication [8]. It has actually been proposed that all cell types probably release a special type of micro-vesicle, the exosome. Exosomes represent a discrete population of nanometer-sized (40–100 nm) micro-vesicles formed in the endocytic compartments called multivesicular bodies (MVBs) during endosome maturation by inward budding of their limiting membrane [9].

These micro-vesicles carry signals either in their limiting membrane and/or in their interior lumen and can be secreted from various cell types to the extracellular media both constitutively and in a regulated manner [8,10].

In view of the postulated role of skeletal muscle as an 'endocrine organ', it was important to investigate whether cultures of C2C12 cells could release exosomes as safe containers of signals for target cells. Hence, the present paper analyses micro-vesicles released by C2C12 cell cultures. They were identified by means of their specific marker proteins (Alix, Tsg101), which are involved in endosomal–lysosomal sorting [11–14]. For the first time it was also investigated whether micro-vesicles released from C2C12 cells, like those from glioblastoma cells and astrocytes [15], carry mtDNA and which type of proteins are associated with mtDNA. Furthermore, a bioinformatics analysis was carried out to gain insight into the capability of these proteins to take part in protein–protein interactions as further evidence of their possible functional role [16–18]. Finally, on the basis of these different objective approaches possible functional implications for the mtDNA and mtDNA associated proteins in the released micro-vesicles have been surmised. In particular, the hypothesis that mtDNA can reach the cytosol of target cells via the process of exosome-endocytosis and then be imported into mitochondria is introduced.

Materials and methods

Micro-vesicle isolation

C2C12 cells were cultured in DMEM containing 10% heat-inactivated FBS. FBS was previously centrifuged overnight at 4 °C and 110,000 *g* using a SW28 rotor in a Beckman ultracentrifuge, the supernatant was carefully removed with a pipette, passed through a 0.22 µm filter and then added to DMEM. Conditioned medium from 5×10^7 cells was collected after 48 h. Micro-vesicles were purified by differential centrifugation for 15 min at 1000 *g* to eliminate cell contamination. Supernatants were further centrifuged for 20 min at 12,000 *g* and subsequently for 20 min at 18,000–20,000 *g*. The resulting supernatants were filtered through a 0.22 µm filter and then micro-vesicles were pelleted by ultracentrifugation at 110,000 *g* for 70 min. The micro-vesicle pellets were washed in 13 ml PBS, pelleted again and resuspended in PBS.

Sucrose gradient

The 110,000 *g* pellet was resuspended with 2.5 M sucrose in 20 mM Hepes (pH 7.4) and 1 mM EDTA, and a step gradient of sucrose (2.5, 2.0, 1.75, 1.5, 1.25, 1.0, 0.75 and 0.5 M) was layered over the micro-vesicle containing 2.5 M sucrose solution. The gradient was spun at 110,000 *g* for at least 16 h using an SW28 rotor (Beckman). Fractions were collected from the top of the gradient, diluted with PBS, and spun at 150,000 *g* with a Type 90 Ti rotor (Beckman). The pelleted fractions were then used either for immunoblotting or for real-time PCR DNA quantification.

Western blotting analysis

For SDS-PAGE, samples containing 50 µg of protein were mixed with Laemmli sample buffer (1:1 ratio) and loaded onto 12% SDS-PAGE gels. Subsequently, proteins were blotted to a nitrocellulose membrane (GE Healthcare). Primary antibodies used were: Alix (1:1000 dilution, clone sc-49268 Santa Cruz) and Tsg101 (1:2000 dilution, clone 4A10 Abcam). Primary antibodies were incubated overnight at 4 °C, followed by washing and the application of secondary HRP-conjugated antibody (Pierce). Immune complexes were visualized using the Supersignal Dura reagent (Pierce).

Transmission electron microscopy (TEM)

Exosomes isolated from C2C12 culture media were adsorbed to formvar-carbon coated 200 mesh grids (Agar Scientific Ltd.) for 2 min, and briefly rinsed in filtered PBS. Exosomes on grids were immediately fixed with 2.5% glutaraldehyde for 1 min and then negatively stained with 2% (wt/vol) Na-phosphotungstate for 1 min. The observations were carried out by means of a Philips CM10 transmission electron microscope at 80 kV.

Mass spectrometry sample preparation

Micro-vesicle pellet was dissolved in 0.125 M Tris–HCl, pH 6.8, 5% β-mercaptoetanolo, 2% SDS, and 10% glycerol. Proteins were separated in vertical slab gels (16 cm × 20 cm × 1.5 mm) according to Laemmli's protocol [19] using 12.5% polyacrylamide gels run at 40 mA *per* gel under constant temperature (10 °C). Gels were stained with Brilliant Blue R 250. Method for in gel digestion was adapted from Shevchenko et al. [20]. Briefly, protein bands were rinsed with 50 mM ammonium bicarbonate/acetonitrile 1 + 1 (v/v) to destain and then dehydratated with acetonitrile. Reduction and alkylation were carried out using 10 mM DTT and 55 mM iodoacetamide, both in 25 mM ammonium bicarbonate. Proteins were digested with 150 ng trypsin (proteomics grade, Sigma) *per* spot plug at 37 °C overnight. Resulting peptides were extracted with 50% acetonitrile containing 1% formic acid.

Nanoelectrospray quadrupole time-of-flight tandem mass spectrometry (nanoESI-Q-TOF MS-MS) analysis

LC-ESI-MS/MS analysis was performed using a Q-TOF micro™ mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray nanoflow electrospray ion source and a CapLC system. The sample was analyzed using a Symmetry C18 nano column (Waters, Milford, MA, USA) as an analytical column.

For protein identification, MS/MS spectra were searched by MASCOT (Matrix science, www.matrixscience.com, U.K.) using database of NCBI nr. For unmatched peptides, however, good quality MS/MS spectra were manually sequenced using de novo sequencing process (carried out by PepSeq of the Masslynx 4.0 software, Micromass), and the obtained sequence was subsequently used in Expasy TagIdent.

Please cite this article as: M. Guescini, et al., C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction, Exp. Cell. Res. (2010), doi:10.1016/j.yexcr.2010.04.006

DNA isolation and quantification

DNA was isolated from purified micro-vesicles using the Qiamp Mini kit (Qiagen) according to the manufacturer's instructions. Thereafter, mtDNA content from purified micro-vesicles was measured by real-time PCR. Specific primers for a region of the mitochondrial D-loop were: D-loop-F: 5′-GCC CAT TAA ACT TGG GGG TA-3′ and D-loop-R: 5′-TTA TGT TGG TCA TGG GCT GA-3′. Real-time PCR amplifications were conducted using LightCycler® 480 SYBR Green I Master (Roche) according to the manufacturer's instructions, with 500 nM primers and a variable amount of DNA standard in a 20 µl final reaction volume. Thermocycling was conducted using a LightCycler® 480 (Roche) initiated by a 10 min incubation at 95 °C, followed by 40 cycles (95 °C for 5 s; 60 °C for 5 s; 72 °C for 10 s) with a single fluorescent reading taken at the end of each cycle. Each reaction was conducted in triplicate. All the runs were completed with a melt curve analysis to confirm the specificity of amplification and lack of primer dimers. *Cp* (second derivative method) values were determined by the LightCycler® 480 software version 1.2.

Bioinformatics analysis of the proteins transported by micro-vesicles

The aminoacid (AA) sequences for all the proteins included in the analysis were obtained from the ExPasy proteomic server of the Swiss Institute of Bioinformatics (http://www.expasy.org). Table 1 provides a list of the analyzed sequences together with the codes identifying them in the Swiss-prot database. Proteins were subdivided into the following six groups: trans-membrane, mitochondrial, folding, apoptosis, nuclear and signal transduction.

A first analysis was aimed to evaluate the degree of disorder of the proteins, which depends on the presence of regions lacking a clear-cut secondary structure [21]. This characteristic makes a protein very suitable for different and multiple protein–protein interactions. For each considered protein a '*disorder index*' (DI) was estimated according to Agnati et al. [16] by pooling together the results of the five predictors (all available as Web services [22–24]) reported in Table 2. Such a parameter (ranging between 0 and 1) increases as the propensity to disorder of the analyzed sequence increases.

A second type of analysis focused on the estimation of the propensity of each protein to be involved in protein aggregation, i.e. in the formation of stable, rigid arrangements or complexes. This depends on the presence of specific motifs acting as 'hot spots' driving aggregation [25]. The public domain predictor AGGRESCAN [26] was used for the analysis and for each protein the normalised number of aggregation hot spots per 100 AA was considered as an '*aggregation index*' (AI).

Statistical analysis

The estimated DI and AI values were averaged to provide an overall mean and a 99% confidence interval. The confidence interval observed for each group was used to establish which of the analyzed proteins exhibited a significantly higher (or lower) AI and/or DI. The statistical analysis was carried out according to the parametric test for the confidence interval of the mean [27].

Table 1 – Proteomic analysis of C2C12 myoblast micro-vesicles.

UniProt A. N.	Description	Mass	Score
Plasmatic membrane associated proteins			
P11276	Fibronectin 1	272,489	1455
Q6GQT1	Alpha-2-macroglobulin	164,327	105
Q8R366	Immunoglobulin superfamily receptor PGRL	65,011	51
Q61738	Integrin alpha 7	129,428	108
P09055	Integrin beta 1	88,231	150
P41731	Cd63 antigen	25,767	56
P70296	Phosphatidylethanolamine-binding protein 1	20,830	154
Q07797	Lectin, galactoside-binding, soluble, 3 binding protein	64,491	122
Q9WV91	Prostaglandin F2 receptor negative regulator	98,708	290
O88601	Syntenin	32,263	185
Mitochondrial proteins			
P08249	Malate dehydrogenase precursor, mitochondrial	35,611	150
Q9CQN1	Heat shock protein 75, mitochondrial	80,209	137
Q9WTP6	Adenylate kinase isoenzyme 2	26,469	44
P09671	Superoxide dismutase 2	24,603	62
Protein folding			
P14602	Heat shock protein 25	23,014	111
P07901	Heat shock protein HSP 90-alpha	84,788	592
Q3UDS0	Heat shock protein 8 — HSP70	60,802	438
P63017	DnaK-type molecular chaperone hsc70 (HSP75)	70,871	681
P11499	Heat shock protein HSP 90-beta	83,325	1027
Q9CS06	Chaperonin subunit 8 (theta)	49,886	200
Apoptosis			
Q80Y09	Pdcd6ip protein — ALIX	96,312	544
Q3UGE5	Programmed cell death 6	14,384	183
Nuclear proteins			
P43277	Histone H1	22,100	290
P15864	Histone H1.2 (H1 VAR.1)	21,267	405
P62806	Hist1h4h protein	11,367	240
P70696	Histone H2B type 1-A	14,237	99
Q64475	Histone H2B type 1-B	13,952	251
P84244	H3 histone, family 3B	15,328	187
Signal transduction			
O70456	14-3-3 protein sigma	27,713	133
P62259	14-3-3 protein epsilon	29,174	694
P68254	14-3-3 protein theta	27,778	259
P63101	14-3-3 protein zeta	27,740	565
P68040	Guanine nucleotide-binding protein, beta 2	35,077	234
P62874	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta 1	37,377	162
P62880	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta 2	37,331	103
P18872	Guanine nucleotide-binding protein G(o) subunit alpha 1	40,085	66
P51150	Small GTP-binding protein Rab7	23,490	146
P62827	GTP-binding nuclear protein Ran (GTPase Ran)	24,423	129
Q07079	Insulin-like growth factor binding protein	30,372	64

Please cite this article as: M. Guescini, et al., C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction, Exp. Cell. Res. (2010), doi:10.1016/j.yexcr.2010.04.006

Table 2 – List of bioinformatics predictors used for the analysis of protein disorder propensity.

Predictor	Description	Server	References
COILS	Neural network trained on X-ray structure data to predict region devoid of regular secondary structure	http://dis.embl.de	[22]
HOT LOOPS	Neural network trained on X-ray structure data to predict highly mobile loops	http://dis.embl.de	[22]
Remark465	Neural network trained on X-ray structure data to predict regions lacking electron density in crystal structure	http://dis.embl.de	[22]
GlobPlot	Russel/Linding scale of disorder	http://globplot.embl.de	[23]
FoldIndex	Charge/hydropathy analyzed locally using a sliding window to predict regions that have a low hydrophobicity and high net charge	http://bip.weizmann.ac.il/fldbin/findex	[24]

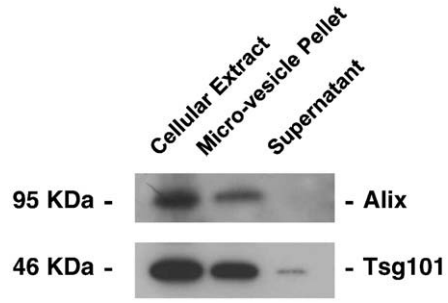


Fig. 1 – Western Blot analysis of micro-vesicles isolated from conditioned medium of C2C12 myoblasts. Micro-vesicles, supernatant and whole cellular lysate proteins from C2C12 cells were separated on SDS-PAGE and electroblotted to nitrocellulose membrane. Blots were probed with antibodies against Alix and Tsg101. Molecular mass markers are shown to the left.

Results and discussion

A topic of the utmost interest is the investigation of muscle contraction-induced humoral factors. Once released during intense exercise, these factors may trigger the exercise-induced adaptations of other organs such as the adipose tissue and the liver in order to maintain homeostasis.

About six years ago, Pedersen et al. reported that muscle-derived IL-6 fulfils the criteria of a factor released by exercise and is capable of triggering homeostatic adaptations in other peripheral organs [4,28–30]. After these pioneering contributions, it has been demonstrated that, in response to contraction, muscle can express and release other cytokines like IL-8 and IL-15 [3,31]. Recently, other myokines have been discovered leading to the new idea that skeletal muscle can act as an endocrine organ [32].

The present study focuses on the inter-cellular transferring of some functionally relevant proteins and mtDNA in C2C12 myoblast via micro-vesicles. It is now widely accepted that exosomes can operate as safe vesicular carriers for targeting messages from source cells to target cells, hence they should be considered a new mode for inter-cellular communication [14,33–35].

Firstly we tested the ability of C2C12 cells to release exosomes in the extracellular medium. Secreted-vesicles were characterized by Western blotting using antibodies against well-defined exosome markers such as Tsg101 and Alix [36]. As reported in Fig. 1, while the presence of these markers in total cellular and micro-vesicle extracts was clearly detected at expected size, their presence in supernatant preparations was almost undetectable under the same conditions, which means that the contamination of material derived from cell lysis was minimal.

In order to further demonstrate that C2C12 released micro-vesicles, electron microscopy experiments were performed. When examined by TEM using negative staining, the pellet from C2C12 media contained a large number of micro-vesicles of approximately 50–80 nm in diameter (Fig. 2), consistent with the reported size of exosomes [37]. In particular, these negatively stained structures appeared as rounding vesicles and most of them were aggregated in clusters of two or more. Some micro-vesicles revealed a contrast between the outer dense wall and the inner less dense region, while others seemed to contain an electron-dense diffuse material (Fig. 2). Furthermore, the panel, obtained by assembling different specimen areas, demonstrates the good quality of the purification and the absence of other cellular organelles or debris.

Hence, the present findings support the notion that C2C12 cells release exosomes in the medium.

Proteomic analysis of the released micro-vesicles led to the identification of characteristic proteins and numerous signalling proteins including guanine nucleotide-binding proteins, small GTP-binding proteins and 14-3-3 proteins. These data support the notion that myoblasts could release not only single signals, but rather sets of messages within safe containers, namely the vesicular carriers. A careful study of the biochemical characteristics and possible functions of the signals present in these micro-vesicles is a task for future investigations. A preliminary bioinformatics analysis was carried out in order to classify some of the proteins carried by micro-vesicles from a biochemical standpoint.

Overall, out of the approximately 200 proteins identified, we selected a total of 39 unique proteins for further analysis (Table 1). These included transmembrane, mitochondrial, folding, apoptosis, nuclear and signal transduction proteins frequently found in exosomes.

The results of the estimates of DI and AI are given in Table 2 and in Fig. 3. It should be noted that the confidence interval for the AI is much more limited than that of the DI. As far as the AI is concerned, it should be noted that proteins with hot spots markedly out of the confidence limits (2.66–3.22) are almost equally distributed among the different classes of analyzed proteins. On the contrary, proteins with a DI markedly out of the confidence limits (.18–.23) mainly belong to the nuclear proteins. In particular, the histone proteins showed high DI and a low number of aggregation hot spots. The possible different biochemical meanings of these two indexes are worth noting. Hence while DI gives a quantitative indication of the tendency of a protein to modify its structure in response to signals, AI

Please cite this article as: M. Guescini, et al., C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction, Exp. Cell. Res. (2010), doi:10.1016/j.yexcr.2010.04.006

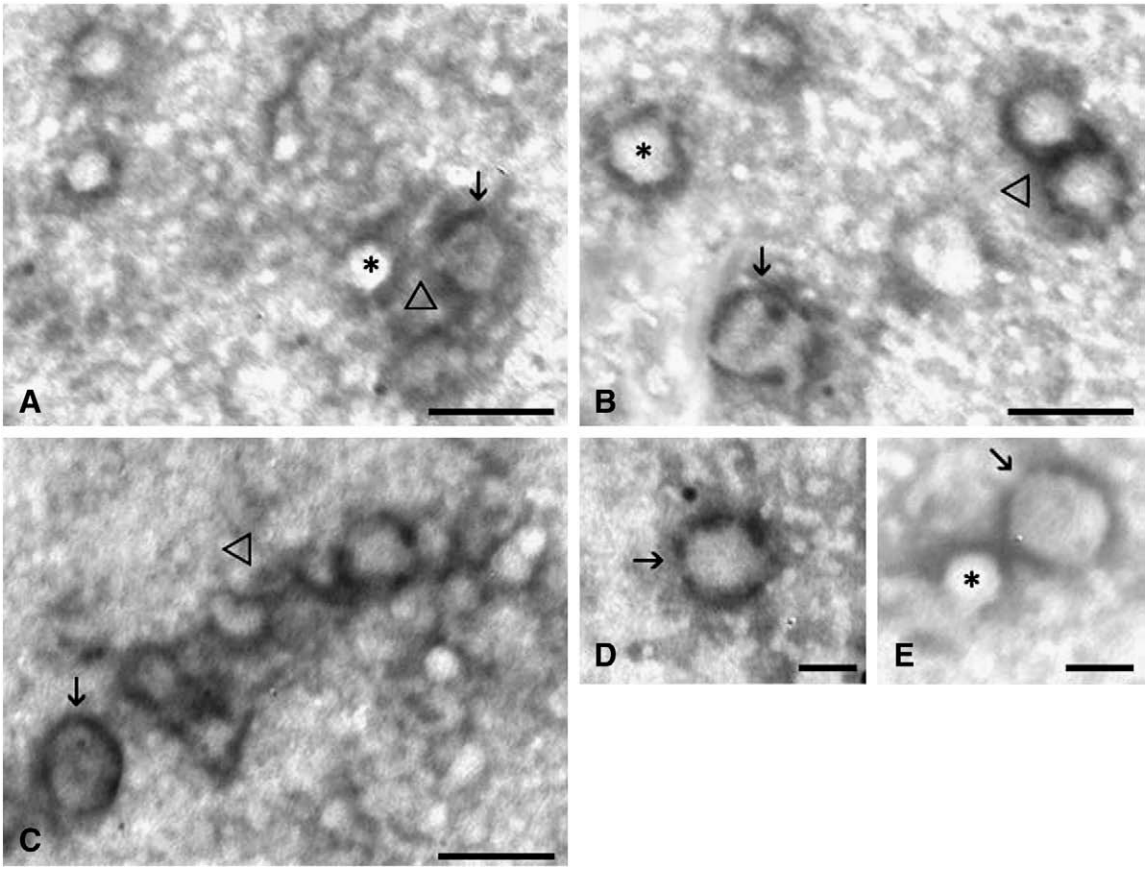


Fig. 2 – TEM images of micro-vesicles released from C2C12 cells. Micro-vesicles isolated from C2C12 culture media by 110,000 *g* centrifugation were fixed, negatively stained, and observed by TEM. The images show small vesicles of 50–80 nm in diameter. Some of these micro-vesicles apparently contain a diffuse electron-dense material (→) while others appear completely electron transparent (*). A tendency to aggregate (Δ) occasionally appears. Panels A, B and C bar = 100 nm; Panel D and E bar = 50 nm.

gives a quantitative indication of the ability of a protein to interact with other molecules forming stable bonds [38]. In other words, the DI gives important information of the ductility of a protein, hence of the possibility of acquiring new three-dimensional conformations, which could interact with other molecules (proteins and/or nucleic acids) playing a key role in the translational process of information (hub function). On the other hand, the AI gives important information about possible stable bonds the protein under investigation can form with interacting partners. We therefore propose the following dichotomy interpretation: a protein characterized by a high DI and low AI may operate as a recycling hub in the transductional process of information and this seems to be the case of the nuclear proteins analyzed (Fig. 3). On the other hand, a protein characterized by a low DI and a high AI probably works as a "gear" of the information flow. The identified signal transduction proteins seem to possess these characteristics (Fig. 3). With a low DI and a high AI, these proteins can stably interact with other proteins only in response to specific signals.

This evidence allows us to hypothesize that C2C12 micro-vesicles mainly deliver the machinery of transduction signals to target cells but not key proteins involved in hub functions in molecular networks. This could be a strategy selected during evolution to coordinate the cellular adaptations to environmental signals in order to obtain a uniform and prompt response by a tissue.

It should be noted that among the identified signal transduction proteins IGF-binding protein 5 (IGFBP-5) represents an exception, in fact, this protein showed a high DI and a low AI (Fig. 3). Hence, it can be surmised that this protein plays a hub role, but it probably forms only a few strong bonds with the interacting partners. In line with these results, previous studies have demonstrated that IGF-binding proteins prolong the half-life of IGFs and have been shown to either inhibit or stimulate (a switching hub?) the growth promoting effects of IGFs on cell culture [39,40].

The heat shock proteins (HSP) were abundant in C2C12 micro-vesicles. In particular, we identified HSP 25, 70, 75 and 90. Though these proteins were attractive targets for our conformational analysis, the reported values for DI and AI led us to exclude their involvement in signal decoding.

Since, we already reported the presence of mtDNA in exosomes released by glioblastoma (U87MG) and astrocyte cells [15], mtDNA presence was also investigated in purified C2C12 micro-vesicles. This analysis revealed that C2C12 micro-vesicles carry mtDNA. To confirm that mtDNA were released in association with micro-vesicles, the pelleted micro-vesicles were fractionated by sedimentation on a sucrose gradient. Real-time PCR quantification showed high mtDNA levels in Alix and Tsg101 positive fractions (Fig. 4). These results demonstrated that mtDNA could be released by an exosomal pathway in C2C12

Please cite this article as: M. Guescini, et al., C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction, Exp. Cell. Res. (2010), doi:10.1016/j.yexcr.2010.04.006

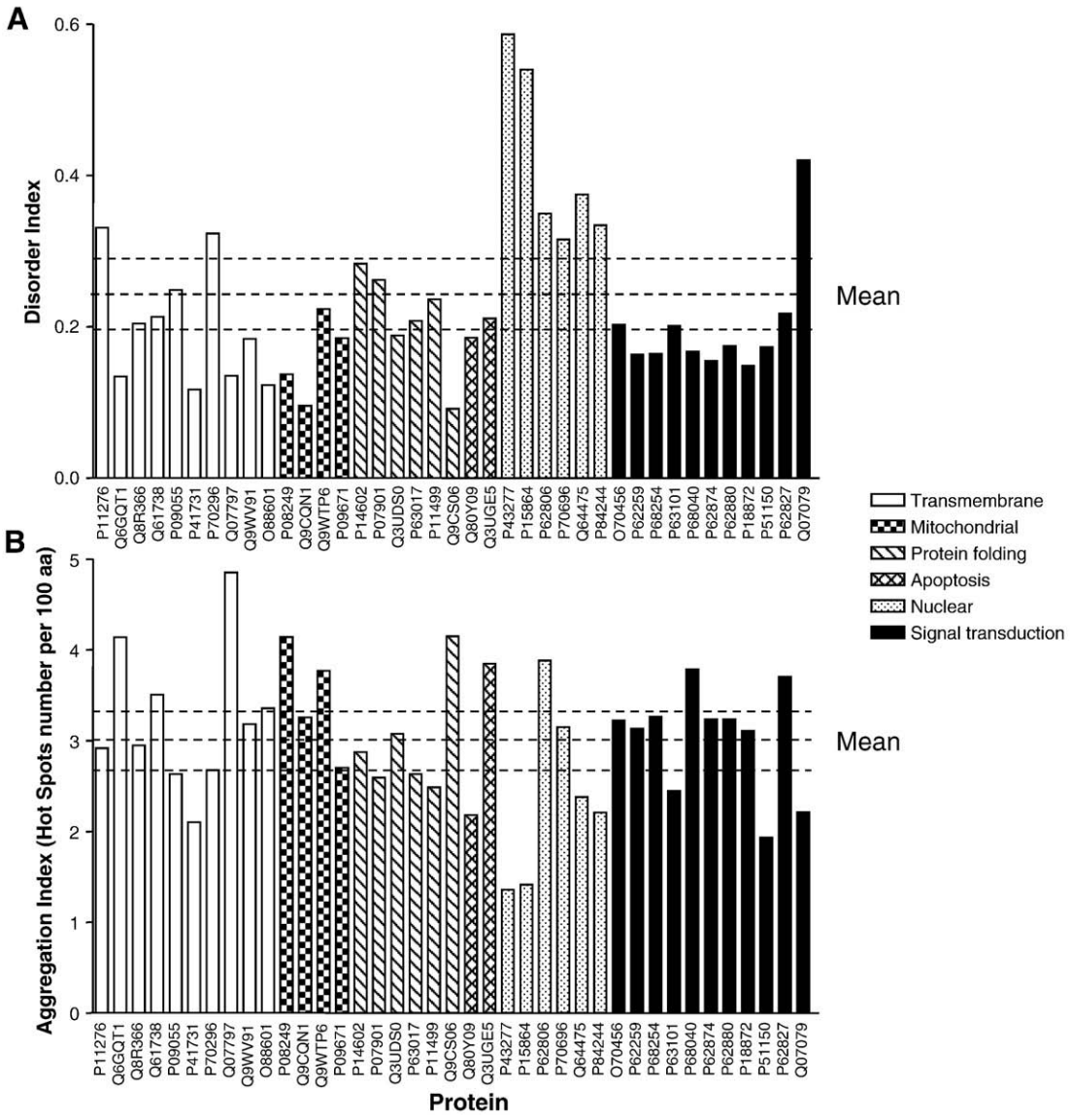


Fig. 3 – Disorder and aggregation index analyses of proteins identified by LC-MS/MS in myoblast-derived micro-vesicles. From the proteins identified by LC-MS/MS in C2C12 released micro-vesicles, we selected for disorder index (A) and aggregation index (B) analyses a total of 39 unique proteins (Table 1). These proteins were divided in the following groups: transmembrane, mitochondrial, folding, apoptosis, nuclear and signal transduction and the UniProt accession number for each protein was also reported. The broken lines show mean and the confidence interval of DI and AI, respectively.

cells. The presence of mtDNA in micro-vesicles is supported by the identification of the mitochondrial proteins: malate dehydrogenase, heat shock protein 75 kDa, adenylate kinase and superoxide dismutase 2. Furthermore, the abundance of histone proteins detected by MS/MS analysis could be related to mtDNA as previously reported by Choi et al. [41].

Recent evidence has pointed out that intracellular accumulation of mtDNA deletions causes mitochondrial dysfunction. The somatic generation and subsequent intracellular accumulation of mtDNA mutations contribute to the age-dependent loss of muscle fibers and sarcopenia [42].

The present data suggest that micro-vesicles can work as vesicular carries of mtDNA, which can therefore migrate not only from cell to neighbouring cells, but to distant target cells. This finding may be of enormous importance. It can lead us to surmise that mtDNA reaching the cytosol of the target cells can be imported into the mitochondria with the possible physiological relevance of restoring the proper mtDNA or, conversely, with pathological relevance favouring the spread of an infective-like DNA to a semi-autonomous intracellular organelle, the mitochondrion. If this is the case, another mechanism involving mtDNA, in addition to the inter-cellular migration of mitochondria via TNTs, may be at work with physiological or also pathological consequences [43–45].

Indeed, in support of this idea, it has been reported that isolated mammalian mitochondria can import DNA by a natural competence mechanism and that this DNA can act as template for both DNA and RNA synthesis [46].

Please cite this article as: M. Guescini, et al., C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction, Exp. Cell. Res. (2010), doi:10.1016/j.yexcr.2010.04.006

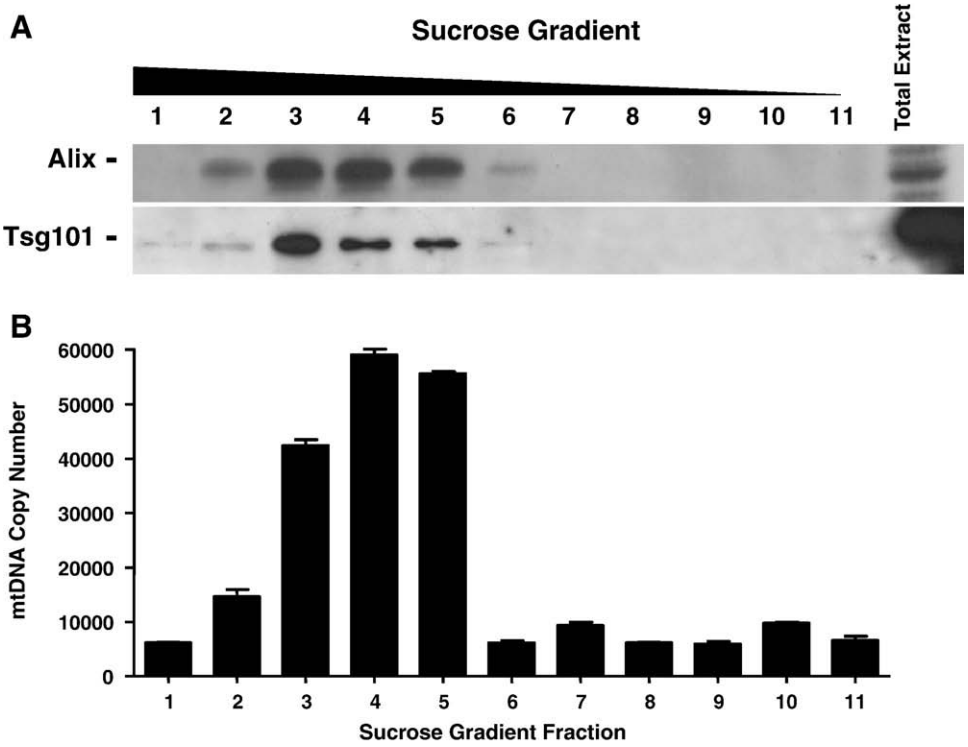


Fig. 4 – Characterization of C2C12 micro-vesicles by density gradient centrifugation. A) C2C12 myoblast micro-vesicles were harvested from conditioned medium by differential centrifugation, followed by sucrose density gradient centrifugation. Fractions containing exosomes were identified by Western blot analysis with antibodies to ALIX and Tsg101. B) Genomic DNA was isolated from the sucrose gradient fractions and mtDNA quantified using real-time PCR. These data confirmed that mtDNA could be released in association to exosomes in C2C12 myoblasts.

This phenomenon could help to elucidate the mechanisms underlying age-related accumulation of high level mtDNA mutations in non-dividing tissues [47].

In conclusion, micro-vesicles seem to have a role in organ cross-talks and have now been identified in body fluids such as blood, urine, synovial fluid, breast milk and saliva [48,49]. In view of the fact that skeletal muscle represents the most important tissue in the human body in terms of total weight, it can be surmised that a large fraction of blood exosomes arise from this tissue. Hence, we are able to propose a new diagnostic approach based on a non-invasive blood sampling to obtain exosomal profiles to be used as biomarkers of skeletal muscle diseases.

REFERENCES

[1] M.H. Chan, A.L. Carey, M.J. Watt, M.A. Febbraio, Cytokine gene expression in human skeletal muscle during concentric contraction: evidence that IL-8, like IL-6, is influenced by glycogen availability, Am. J. Physiol. 287 (2004) R322–R327.

[2] R. Cuppini, S. Sartini, D. Agostini, M. Guescini, P. Ambrogini, M. Betti, L. Bertini, M. Vallasciani, V. Stocchi, Bdnf expression in rat skeletal muscle after acute or repeated exercise, Arch. Ital. Biol. 145 (2007) 99–110.

[3] T. Akerstrom, A. Steensberg, P. Keller, C. Keller, M. Penkowa, B.K. Pedersen, Exercise induces interleukin-8 expression in human skeletal muscle, J. Physiol. 563 (2005) 507–516.

[4] M.A. Febbraio, N. Hiscock, M. Sacchetti, C.P. Fischer, B.K. Pedersen, Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction, Diabetes 53 (2004) 1643–1648.

[5] Y. Izumiya, H.A. Bina, N. Ouchi, Y. Akasaki, A. Kharitonenkov, K. Walsh, FGF21 is an Akt-regulated myokine, FEBS Lett. 582 (2008) 3805–3810.

[6] N. Ouchi, Y. Oshima, K. Ohashi, A. Higuchi, C. Ikegami, Y. Izumiya, K. Walsh, Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism, J. Biol. Chem. 283 (2008) 32802–32811.

[7] B.K. Pedersen, M.A. Febbraio, Muscle as an endocrine organ: focus on muscle-derived interleukin-6, Physiol. Rev. 88 (2008) 1379–1406.

[8] E. Cocucci, G. Racchetti, J. Meldolesi, Shedding microvesicles: artefacts no more, Trends Cell Biol. 19 (2009) 43–51.

[9] G. van Niel, I. Porto-Carreiro, S. Simoes, G. Raposo, Exosomes: a common pathway for a specialized function, J. Biochem. 140 (2006) 13–21.

[10] A. Lakkaraju, E. Rodriguez-Boulan, Itinerant exosomes: emerging roles in cell and tissue polarity, Trends Cell Biol. 18 (2008) 199–209.

[11] N.R. Smalheiser, Exosomal transfer of proteins and RNAs at synapses in the nervous system, Biol. Direct 2 (2007) 35.

[12] J. Faure, G. Lachenal, M. Court, J. Hirrlinger, C. Chatellard-Causse, B. Blot, J. Grange, G. Schoehn, Y. Goldberg, V. Boyer, F. Kirchhoff, G. Raposo, J. Garin, R. Sadoul, Exosomes are released by cultured cortical neurones, Mol. Cell. Neurosci. 31 (2006) 642–648.

[13] B. Fevrier, G. Raposo, Exosomes: endosomal-derived vesicles shipping extracellular messages, Curr. Opin. Cell Biol. 16 (2004) 415–421.

[14] M. Simons, G. Raposo, Exosomes—vesicular carriers for intercellular communication, Curr. Opin. Cell Biol. 21 (2009) 575–581.

[15] M. Guescini, S. Genedani, V. Stocchi, L.F. Agnati, Astrocytes and glioblastoma cells release exosomes carrying mtDNA, J. Neural Transm. 117 (2010) 1–4.

[16] L.F. Agnati, G. Leo, S. Genedani, N. Andreoli, D. Marcellino, A. Woods, L. Piron, D. Guidolin, K. Fuxe, Structural plasticity in

Please cite this article as: M. Guescini, et al., C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction, Exp. Cell. Res. (2010), doi:10.1016/j.yexcr.2010.04.006

G-protein coupled receptors as demonstrated by the allosteric actions of homocysteine and computer-assisted analysis of disordered domains, Brain Res. Rev. 58 (2008) 459–474.
[17] H.J. Dyson, P.E. Wright, Intrinsically unstructured proteins and their functions, Nat. Rev. 6 (2005) 197–208.
[18] V.N. Uversky, C.J. Oldfield, A.K. Dunker, Intrinsically disordered proteins in human diseases: introducing the D2 concept, Annu. Rev. Biophys, 37 (2008) 215–246.
[19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[20] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels, Anal. Chem. 68 (1996) 850–858.
[21] A.K. Dunker, C.J. Brown, Z. Obradovic, Identification and functions of usefully disordered proteins, Adv. Protein Chem. 62 (2002) 25–49.
[22] R. Linding, R.B. Russell, V. Neduva, T.J. Gibson, GlobPlot: exploring protein sequences for globularity and disorder, Nucleic Acids Res. 31 (2003) 3701–3708.
[23] R. Linding, L.J. Jensen, F. Diella, P. Bork, T.J. Gibson, R.B. Russell, Protein disorder prediction: implications for structural proteomics, Structure 11 (2003) 1453–1459.
[24] T. Zeev-Ben-Mordehai, E.H. Rydberg, A. Solomon, L. Toker, V.J. Auld, I. Silman, S. Botti, J.L. Sussman, The intracellular domain of the *Drosophila* cholinesterase-like neural adhesion protein, gliotactin, is natively unfolded, Proteins 53 (2003) 758–767.
[25] N. Sanchez de Groot, I. Pallares, F.X. Aviles, J. Vendrell, S. Ventura, Prediction of "hot spots" of aggregation in disease-linked polypeptides, BMC Struct. Biol. 5 (2005) 18.
[26] O. Conchillo-Sole, N.S. de Groot, F.X. Aviles, J. Vendrell, X. Daura, S. Ventura, AGGRESCAN: a server for the prediction and evaluation of "hot spots" of aggregation in polypeptides, BMC Bioinformatics 8 (2007) 65.
[27] D. Colquhoun, Lectures in Biostatistics, Clarendon Press, Oxford, 1971.
[28] B.K. Pedersen, A. Steensberg, C. Fischer, C. Keller, P. Keller, P. Plomgaard, M. Febbraio, B. Saltin, Searching for the exercise factor: is IL-6 a candidate? J. Muscle Res. Cell Motil. 24 (2003) 113–119.
[29] M.A. Febbraio, B.K. Pedersen, Muscle-derived interleukin-6: mechanisms for activation and possible biological roles, FASEB J. 16 (2002) 1335–1347.
[30] B.K. Pedersen, A. Steensberg, C. Fischer, C. Keller, P. Keller, P. Plomgaard, E. Wolsk-Petersen, M. Febbraio, The metabolic role of IL-6 produced during exercise: is IL-6 an exercise factor? Proc. Nutr. Soc. 63 (2004) 263–267.
[31] A.R. Nielsen, R. Mounier, P. Plomgaard, O.H. Mortensen, M. Penkowa, T. Speerschneider, H. Pilegaard, B.K. Pedersen, Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition, J. Physiol. 584 (2007) 305–312.
[32] B.K. Pedersen, F. Edward, Adolph Distinguished Lecture: Muscle as an Endocrine Organ: IL-6 and Other Myokines, J. Appl. Physiol. 107 (2009) 1006–1014.
[33] M. Belting, A. Wittrup, Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease, J. Cell Biol. 183 (2008) 1187–1191.
[34] J. Mallegol, G. Van Niel, C. Lebreton, Y. Lepelletier, C. Candalh, C. Dugave, J.K. Heath, G. Raposo, N. Cerf-Bensussan, M. Heyman, T84-intestinal epithelial exosomes bear MHC class II/peptide complexes potentiating antigen presentation by dendritic cells, Gastroenterology 132 (2007) 1866–1876.
[35] J.S. Schorey, S. Bhatnagar, Exosome function: from tumor immunology to pathogen biology, Traffic (Copenhagen, Denmark) 9 (2008) 871–881.
[36] C. Raiborg, T.E. Rusten, H. Stenmark, Protein sorting into multivesicular endosomes, Curr. Opin. Cell Biol. 15 (2003) 446–455.
[37] C. Thery, L. Zitvogel, S. Amigorena, Exosomes: composition, biogenesis and function, Nat. Rev. Immunol. 2 (2002) 569–579.
[38] A.L. Fink, Protein aggregation: folding aggregates, inclusion bodies and amyloid, Fold. Des. 3 (1998) R9–R23.
[39] C. Duan, Specifying the cellular responses to IGF signals: roles of IGF-binding proteins, J. Endocrinol. 175 (2002) 41–54.
[40] C. Duan, Q. Xu, Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions, Gen. Comp. Endocrinol. 142 (2005) 44–52.
[41] Y.S. Choi, B.K. Ryu, H.K. Min, S.W. Lee, Y.K. Pak, Analysis of proteome bound to D-loop region of mitochondrial DNA by DNA-linked affinity chromatography and reverse-phase liquid chromatography/tandem mass spectrometry, Ann. N. Y. Acad. Sci. 1042 (2005) 88–100.
[42] A. Herbst, J.W. Pak, D. McKenzie, E. Bua, M. Bassiouni, J.M. Aiken, Accumulation of mitochondrial DNA deletion mutations in aged muscle fibers: evidence for a causal role in muscle fiber loss, J. Gerontol. 62 (2007) 235–245.
[43] H.H. Gerdes, N.V. Bukoreshtliev, J.F. Barroso, Tunneling nanotubes: a new route for the exchange of components between animal cells, FEBS Lett. 581 (2007) 2194–2201.
[44] A. Rustom, R. Saffrich, I. Markovic, P. Walther, H.H. Gerdes, Nanotubular highways for intercellular organelle transport, Science (New York, N.Y) 303 (2004) 1007–1010.
[45] L.F. Agnati, D. Guidolin, F. Baluska, G. Leo, P.W. Barlow, C. Carone, S. Genedani, A new hypothesis of pathogenesis based on the divorce between mitochondria and their host cells: possible relevances for the Alzheimer's disease., Curr Alzheimer Res. 16 (2009) 307–322.
[46] M. Koulintchenko, R.J. Temperley, P.A. Mason, A. Dietrich, R.N. Lightowlers, Natural competence of mammalian mitochondria allows the molecular investigation of mitochondrial gene expression, Hum. Mol. Genet. 15 (2006) 143–154.
[47] K. Khrapko, J. Vijg, Mitochondrial DNA mutations and aging: devils in the details? Trends Genet. 25 (2009) 91–98.
[48] R.J. Simpson, J.W. Lim, R.L. Moritz, S. Mathivanan, Exosomes: proteomic insights and diagnostic potential, Expert Rev. Proteomics 6 (2009) 267–283.
[49] S. Keller, M.P. Sanderson, A. Stoeck, P. Altevogt, Exosomes: from biogenesis and secretion to biological function, Immunol. Lett. 107 (2006) 102–108.

Please cite this article as: M. Guescini, et al., C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction, Exp. Cell. Res. (2010), doi:10.1016/j.yexcr.2010.04.006