Extracellular vesicles shed by melanoma cells contain a modified form of H1.0 linker histone and H1.0 mRNA-binding proteins

GABRIELLA SCHIERA[1], CARLO MARIA DI LIEGRO[1], VERONICA PULEO[1,2], ORIANA COLLETTA[1,2], ANNA FRICANO[1,2], PATRIZIA CANCEMI[1,3], GIANLUCA DI CARA[3] and ITALIA DI LIEGRO[2]

[1]Department of Biological Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), I-90128 Palermo; [2]Department of Experimental Biomedicine and Clinical Neurosciences (BIONEC), University of Palermo, I-90127 Palermo; [3]Center of Experimental Oncobiology (C.OB.S.), La Maddalena Hospital III Level Oncological Dept., Palermo, Italy

Received April 20, 2016; Accepted June 13, 2016

DOI: 10.3892/ijo.2016.3692

Abstract. Extracellular vesicles (EVs) are now recognized as a fundamental way for cell-to-cell horizontal transfer of properties, in both physiological and pathological conditions. Most of EV-mediated cross-talk among cells depend on the exchange of proteins, and nucleic acids, among which mRNAs, and non-coding RNAs such as different species of miRNAs. Cancer cells, in particular, use EVs to discard molecules which could be dangerous to them (for example differentiation-inducing proteins such as histone H1.0, or antitumor drugs), to transfer molecules which, after entering the surrounding cells, are able to transform their phenotype, and even to secrete factors, which allow escaping from immune surveillance. Herein we report that melanoma cells not only secrete EVs which contain a modified form of H1.0 histone, but also transport the corresponding mRNA. Given the already known role in tumorigenesis of some RNA binding proteins (RBPs), we also searched for proteins of this class in EVs. This study revealed the presence in A375 melanoma cells of at least three RBPs, with apparent MW of about 65, 45 and 38 kDa, which are able to bind H1.0 mRNA. Moreover, we purified one of these proteins, which by MALDI-TOF mass spectrometry was identified as the already known transcription factor MYEF2.

Introduction

Most eukaryotic cells are now recognized to shed extracellular vesicles (EVs) into their environment (1,2). Among these vesicles, some are known as membrane vesicles (MVs) or ectosomes and originate from the plasma membrane, through a process resembling viral budding, whereas a second class consists of smaller vesicles (exosomes), which are released after plasma membrane fusion of the so-called multivesicular bodies (MVBs), which originate in the endosomal compartment (3).

Production of EVs, initially discovered in transformed cells, has been demonstrated as a physiological mechanism involved in the horizontal transfer of several kinds of molecules in normal cells, among which different classes of cells in the nervous system (4-7). Cancer cells, however, produce and release much higher amounts of EVs (8), which can reach most biological fluids, such as blood plasma (9), breast milk (10), and saliva (11,12), where they could be used as biological markers of disease and even of the disease grade (13-15).

Concerning protein content, EVs released from cancer cells transport different classes of chaperones, tumor-specific antigens, apoptosis-inducing proteins, such as FasL and TRAIL (16,17), immune modulatory factors (18), and many other oncogenic molecules, which, once transferred into surrounding cells, can facilitate cancer development by suppressing immune responses, and stimulating tumor growth, invasion and metastasis.

Recently, we reported that oligodendroglioma cells release into EVs also the H1.0 linker histone variant (19). H1 linker histones constitute in mammals the most heterogeneous family of histones. In humans, the H1 family includes 11 members, among which 7 are expressed in somatic cells (H1.1-H1.5; H1.0 and H1X), 3 are specifically expressed in testis, and one is specifically expressed in the oocyte (H1.oo) (20-22). Moreover, among the somatic subtypes, H1.1-H1.5 are encoded by repeated genes which are transcribed during the S phase of the cell cycle, in a replication-dependent manner, to give mRNAs which are not polyadenylated. On the other hand, H1.0 is transcribed in a replication-independent way, into a polyadenylated mRNA (23), and is prevalent in differentiated cells.

The presence of H1.0 in the EVs produced by oligodendroglioma cells suggested, on one hand, that deregulation of H1.0 histone expression can be linked to tumorigenesis, and, on the other, that cancer cells can escape differentiation by discarding this protein into EVs (19). To shed more light on this hypothesis, in this study we analyzed a different kind of cancer cells: A375 melanoma cells. Here we report that these cells synthesize H1.0 histone and secrete a modified form of it into

Correspondence to: Professor Italia Di Liegro, Department of Experimental Biomedicine and Clinical Neurosciences (BIONEC), University of Palermo, I-90127 Palermo, Italy
E-mail: italia.diliegro@unipa.it

Key words: H1.0 linker histone, RNA-binding proteins, extracellular vesicles, membrane vesicles, exosomes, MYEF2

MVs. In addition, we found that EVs released from melanoma cells also contain H1.0 mRNA. Since post-transcriptional regulation of mRNA trafficking, stability and translation depends on a number of RNA-binding proteins (RBPs) (24), and since a group of H1.0 mRNA-binding proteins have been already found in the rat brain (25-28), we also looked for RBPs with this ability in melanoma cells and EVs. Here we show that H1.0-binding RBPs are indeed present in the EVs released from A375 melanoma cells, and that one of these proteins is MYEF2.

Materials and methods

Cell cultures. A375 melanoma cells (A375CL1006) were cultured in Dulbecco's modified Eagle's medium - low glucose (DMEM 5546, Sigma-Aldrich, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (F7524 Sigma-Aldrich), 2 mM glutamine (Euroclone, Milan, Italy), 0.1% MEM non essential amino acids (Sigma-Aldrich), 1 mM sodium pyruvate (Euroclone), 100,000 U penicillin, 100 mg streptomycin and 250 μg amphotericin B (Sigma-Aldrich) per liter. Cells were maintained in humidified 5% CO_2/95% air, at 37˚C. Some A375 melanoma cells were cultured in the same medium without serum.

Immunofluorescence analyses. Cells were fixed in 96% ethanol and immune-stained with rat anti-integrin β1 (1:100; in-house produced), and rabbit anti-H1˚ (1:100; sc-67324 Santa Cruz, CA, USA).

The secondary antibodies used were fluorescein isothiocyanate-conjugated anti-rat-(1:100; F1763), or rhodamine-conjugated anti-rabbit-(1:200; T6778) immunoglobulins (both from Sigma, MO, USA).

Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (H1200, Vector Laboratories, Youngstown, OH, USA). Cells were observed in an Olympus BX-50 microscope (Olympus Italia S.r.l., Segrate, Italy) equipped with Vario Cam B/W camera (Nikon Instruments S.p.A., Calenzano, Italy).

Preparation of microvesicles from the A375 medium. Vesicles were prepared from A375 FBS-free conditioned medium, as follows: 24 h before collecting media, cells were washed twice in phosphate-buffered saline (PBS), pH 7.5, and then incubated with FCS-free DMEM. Conditioned media were centrifuged at 2,000 x g for 10 min and then at 4,000 x g for 15 min. The supernatant was centrifuged at 105,000 x g (Ti70 Rotor, Beckman) for 90 min at 4˚C.

To separate exosomes from MVs, before ultracentrifugation, medium was filtered with a 0.2-μm filter and centrifuged at 10,000 x g for 30 min. The pellet containing MVs was saved while the supernatant was finally centrifuged at 105,000 x g for 90 min to obtain exosomes.

Pelleted vesicles were suspended in PBS and protein concentration was determined using Qubit® Protein assay kit (Q33211, Invitrogen, OR, USA).

Vesicles analyses. The NS300 (NanoSight, London, UK) instrument is based on a conventional optical microscope and uses a laser light source to illuminate nano-scale particles. This analysis allowed measuring size and concentration of vesicles in a liquid medium based on tracking of Brownian motion.

Purification of total cell extracts. Cells were collected and homogenized in nuclei buffer (0.32 M sucrose; 50 mM sodium phosphate buffer, pH 6.5; 50 mM KCl; 0.5 mM spermine; 0.15 mM spermidine; 2 mM EDTA; 0.15 mM EGTA), containing the protease inhibitors aprotinin (2 μg/ml), antipain (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), benzamidine (1.0 mM), and phenylmethylsulfonyl fluoride (1.0 mM), all purchased from (Sigma-Aldrich). Protein concentration was determined according to Bradford (29).

Western blot analysis. Proteins (15 μg) were separated by electrophoresis on denaturing 12.5% polyacrylamide slab gels (SDS-PAGE) and transferred to PVDF membrane (IPVH00010, Immobilon P, Millipore, MA, USA), as previously described (19). Samples on the membrane were visualized by staining with Ponceau Red (Sigma-Aldrich) for 5 min. Membranes were immune-stained with rabbit polyclonal anti-H1˚ antibodies (1:500, sc-67324 Santa Cruz), mouse monoclonal anti-Hsc70 antibodies (1:1,000, sc-7298, Santa Cruz), rabbit polyclonal anti-SUMO1 (1:500, S373B, Santa Cruz). The secondary antibodies were AP-conjugated anti-mouse (1:7,500, S372B) and anti-rabbit (1:7,500, S373B) IgGs (Promega Corp., Madison, WI, USA).

Reverse transcription (RT)-PCR. Total RNA was purified from cells according to Chomczynski and Sacchi (30). RNA from vesicles was prepared using either the Pure-Link RNA microKit (12183016, Invitrogen) or the TRIzol® reagent (15596-018, Invitrogen), according to the manufacturer's instructions.

Synthesis of cDNA was performed using the Superscript II Reverse Transcriptase kit (18064-022 Invitrogen), following the manufacturer's protocol. The primer used for the reverse transcription had the following sequence: (5'→3') GGC TTT CTT GGG CGT GGC AGC C.

PCR was performed using Taq DNA polymerase (10342-020, Invitrogen), according to the manufacturer's instructions, and using the following primers: forward, (5'→3'), ATG ATC GTG GCT GCC ATC CAG GC; reverse, (5'→3'), GGC TTT CTT GGG CGT GGC AGC C.

Amplification was performed by Mastercycler Thermal Cycler 5345 (Eppendorf AG, Hamburg, Germany) using the following program: 94˚C for 30 sec, 35 cycles at 95˚C for 30 sec, 52˚C for 30 sec, 72˚C for 30 sec, and 72˚C for 5 min.

Preparation of in vitro transcripts and T1 RNase protection assay. ^{33}P-radiolabeled H1.0 RNA was prepared as previously described (25), using as a template the plasmid pMH1.0 (31), which contains the H1.0 insert (EMBL ID: X70685). In order to prepare H1.0 mRNA to be used for chromatographic experiments, in some reactions transcription was performed in the presence of biotin-21-UTP (AM8450, Ambion-Life Technologies, Paisley, UK), a UTP analog which has biotin attached to the pyrimidine ring by a 21-atom spacer arm (27).

For T1 RNase (EC 3.1.27.3; Roche, Switzerland) protection assay, radiolabeled H1.0 RNA was mixed with either total cell extracts or vesicles (15 μg), prepared as described

above, following the procedure previously described (32). RNA-protein complexes were analyzed by denaturing electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide slab gel (PAGE). At the end of the run, the gel was directly exposed to X-ray film for autoradiography (Amersham Hyperfilm™, GE Healthcare, USA). The gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich), to confirm loading of equal amounts of proteins per lane.

Chromatographic purification from A375 cell-released EVs of H1.0 RNA-binding factors. Streptavidin-conjugated paramagnetic beads (Z5481, Magnesphere, Promega) were washed three times in PBS according to the manufacturer's instructions, and then mixed with 450 μg of A375 EV proteins, in 500 μl (final volume) of binding buffer (BB: 75 mM Tris-HCl, pH 7.5; 50 mM KCl; 5 mM dithiothreitol) (27), containing proteases and phosphatases inhibitors (Sigma-Aldrich). Samples were incubated for 1 h, at 4˚C, under shaking, to allow unspecific protein binding to the particles (pre-clearing step). After centrifuging at 10,000 x g for 5 min, the pre-cleared supernatants were used for the specific binding reaction. The pre-cleared sample was divided into two aliquots, one of which was mixed with H1.0 RNA (1.2 μg) in BB, while the other one was used as an RNA-free control. Both samples were incubated for 1 h at 4˚C, after which fresh aliquots of pre-washed beads were added, and incubation was continued for 1 h, at 4˚C, under shaking. Finally, the supernatants containing unbound proteins were collected by a magnetic device (Magnesphere, Promega) and frozen. Paramagnetic beads were washed four times in BB and then resuspended in electrophoresis sample buffer, boiled and centrifuged at 10,000 x g. The supernatants, which contain bound proteins, were frozen and saved for analyses.

Silver staining. After SDS-PAGE, the gel was silver stained according to Yan *et al* (33). The region of interest was cut from the gel and analyzed by MALDI-TOF mass spectrometry.

MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometry analysis was performed using the Voyager DE-PRO (Applied Biosystems, Foster City, CA, USA) mass spectrometer as previously described (34). Briefly, silver stained band was in gel-destained with $K_3[Fe(CN)_6]$ and $Na_2S_2O_3$, reduced with dithiothreitol, S-alkylated with iodoacetamide, and subsequently digested with trypsin. The tryptic peptide extracts were desalted by μZip-TipC18 (Millipore) and loaded on the MALDI target, using the dried droplet technique and α-cyano-4-hydroxycinnamic acid as matrix. The resulting mass spectrum, was elaborated using the DataExplorer software (Applied Biosystems) and manually inspected to obtain the corresponding peak lists. Internal mass calibration was done using trypsin autolysis fragments at m/z 842.5100, 1045.5642, and 2211.1046 Da. Peptide mass fingerprinting was compared to the theoretical masses from the Swiss-Prot.

Results

A375 melanoma cells release both membrane vesicles (MVs) and exosomes. As shown in Fig. 1, A375 melanoma cells produce and release into the culture medium extracellular vesicles, at least in part from plasma membrane regions enriched in integrin β1 (Fig. 1A-C). The vesicular population is actually a mixed one, as demonstrated by NanoSight (Fig. 1D), which allowed measuring size and concentration of vesicles in the culture medium, based on tracking of Brownian motion. In addition, according to the NanoSight data (which are quantitative), the EV population is composed mainly of exosomes (compare the height of the peak at 103-131 nm, which corresponds to exosomes, with the shoulder at 270 nm, which probably corresponds to MVs). In some experiments, the medium in which melanoma cells had been cultured was filtered and centrifuged at 10,000 x g for 30 min, before ultracentrifugation, in order to pellet first only MVs. The supernatant was then centrifuged at 105,000 x g for 90 min to obtain a final pellet of exosomes. The NanoSight analysis of the separated fractions gave only single peaks (Fig. 1D, right panel, where only the analysis concerning purified exosomes is shown). The relative concentrations (expressed as μg/μl of proteins) of the two populations of vesicles obtained are reported in Fig. 1E.

EVs released from A375 melanoma cells contain both H1.0 linker histone and the corresponding mRNA. H1.0 linker histone was first discovered in non-dividing tissues (35,36), and, in general, accumulates in differentiating cells at the end of the proliferative phase. Recently, it was however found in total cell extracts and extracellular vesicles from G26/24 dividing oligodendroglioma cells (19). In this study we therefore looked for the possibility that also melanoma cells synthesize and secrete this histone via EVs. As shown in Fig. 2, A375 cells indeed produce a protein which is immune-stained by anti-H1.0 antibodies both in immunofluorescence (Fig. 2A-C) and western blot analyses (Fig. 2D). As already reported for other tumor cells, melanoma cells release EVs (both MVs and exosomes) which contain the Hsc70 chaperone (19). Interestingly, they also secrete an anti-H1.0 antibody-positive protein which, however, is larger than expected, and is specifically sorted to MVs. Since other proteins sorted to vesicles bear specific post-translational modifications, such as sumoylation (37), we looked for the presence of a SUMO moiety on this larger H1.0. As shown in Fig. 2E, anti-SUMO1 antibodies not only recognized a protein of about 38 kDa, but this band exactly co-migrates with the slow migrating protein recognized by the anti-H1.0 antibodies (Fig. 2E, asterisk).

We then analyzed both total cell lysates and the vesicular fraction for the presence of H1.0 mRNA. We used as a template for reverse transcription (RT)-PCR total RNA prepared from either whole melanoma cells or EVs (MVs plus exosomes). As shown in Fig. 3, a band of the expected size [380 base pairs (bp)] was found both in cells (Fig. 3A, A375L) and vesicles (Fig. 3B, A375v), thus indicating that, indeed, not only H1.0 protein but also the corresponding mRNA was sorted to EVs. As internal references for these RT-PCR experiments we used two positive controls: total RNA from adult rat brain (Fig. 3A, lane B), and the cDNA insert encoding H1.0 (Fig. 3C, lane cDNA); one sample which did not contain RNA was included in all the experiments as a negative control (Fig. 3B and C, lane N). Furthermore, to be sure that we were amplifying RNA and not contaminating DNA, we also performed a PCR

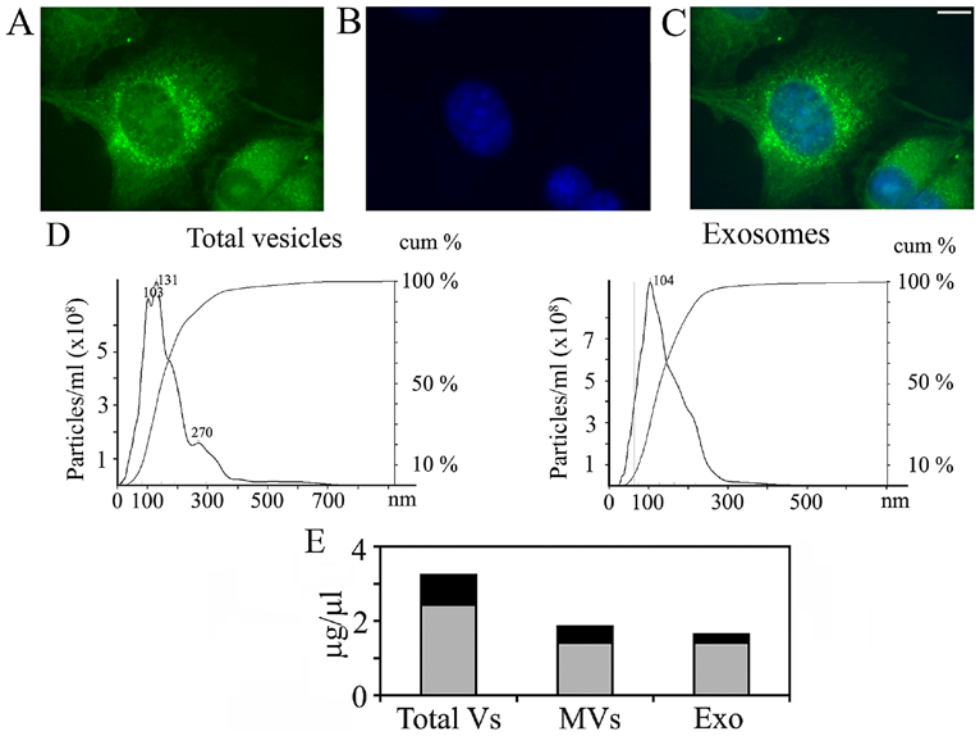

Figure 1. Analysis of extracellular vesicles produced by A375 melanoma cells. (A) A375 melanoma cells were immune-stained with anti-β1 integrin antibodies (green fluorescence). (B) Cells were also stained with DAPI (blue fluorescence). (C) Overlay of (A) and (B). Bar, 10 μm. (D and E) Nanoparticle tracking analysis (NTA) of total vesicles and exosomes from A375 melanoma cells. (D) Plot of particles showing size distribution profiles with distinct peaks at 103, 131 and 270 nm (total vesicles) and 104 nm (exosomes). (E) Grey boxes indicate average concentrations (expressed as μg/μl of proteins) of membrane vesicles (MVs) and exosomes (Exo) from at least 3 experiments; standard deviation is also indicated (black boxes).

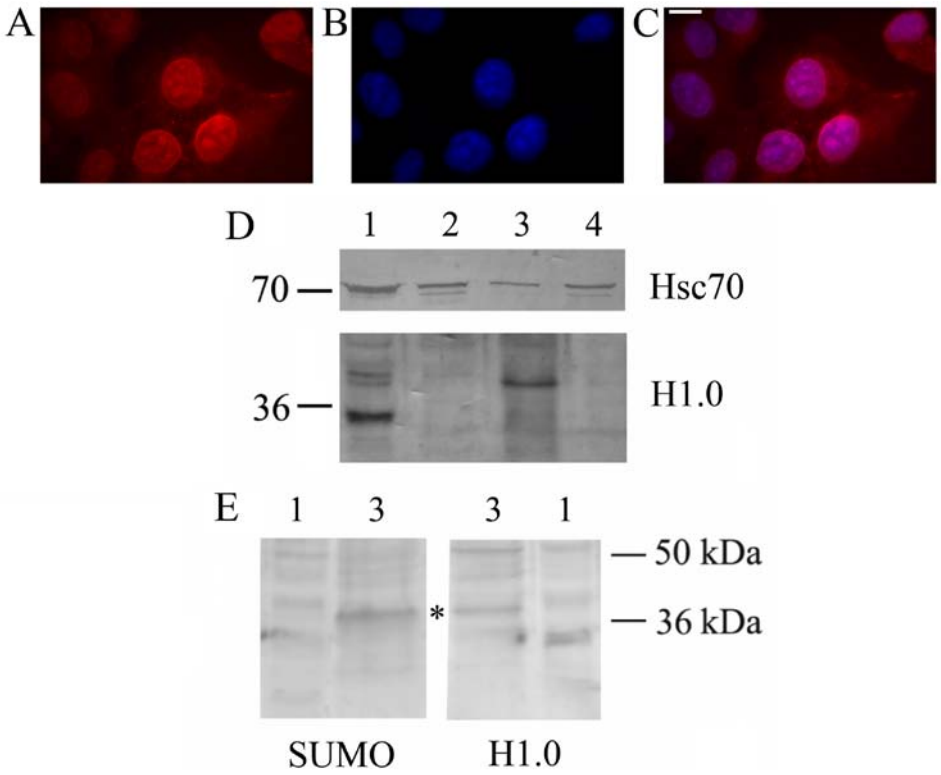

Figure 2. Expression of H1.0 histone in A375 melanoma cells. (A) A375 melanoma cells were immune-stained with anti-H1.0 antibodies (red fluorescence). (B) Cells were also stained with DAPI (blue fluorescence). (C) Overlay of (A) and (B). Bar, 10 μm. (D) Western blot analysis of total cell lysates from A375 melanoma cells (lane 1), total extracellular vesicles (lane 2), microvesicles (lane 3) and exosomes (lane 4). Proteins were immune-stained with anti-H1.0 antibodies (H1.0). The upper part of the membrane was cut out and immune-stained with anti-Hsc70 antibodies for internal reference (Hsc70). (E) Western blot analysis of total cell lysates from A375 melanoma cells and microvesicles. Twin samples of melanoma cells (lane 1) and microvesicles (lane 3) were separated by electrophoresis and blotted onto PVDF membrane. The membrane was then cut into two halves, each of which was incubated with either anti-SUMO1- or anti-H1.0-antibodies. Samples 1 and 3 are the same as those of (D). The asterisk indicates putative sumoylated H1.0.

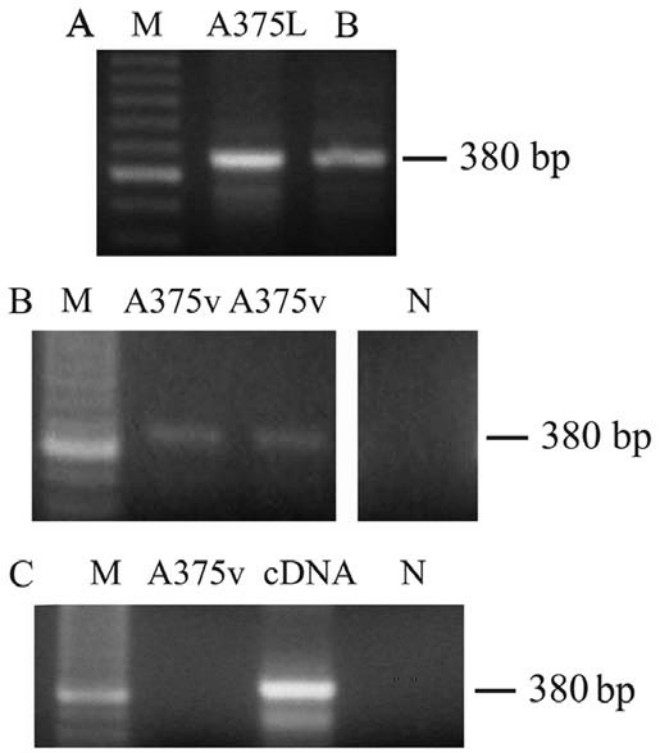

Figure 3. RT-PCR analysis of H1.0 mRNA in A375 melanoma cells and vesicles. (A) RT-PCR of RNA from total cell lysates from A375 cells (lane A375L) and adult rat brain cortices (lane B). (B) RT-PCR of RNA from extracellular vesicles released from A375 cells (lane A375v); a negative control was also included (lane N). (C) Amplification by PCR only of RNA from purified A375 vesicles (lane A375v), and from the cDNA encoding H1.0 (lane cDNA). A negative control was also included (lane N). The expected size (380 bp) of the amplified fragment is shown.

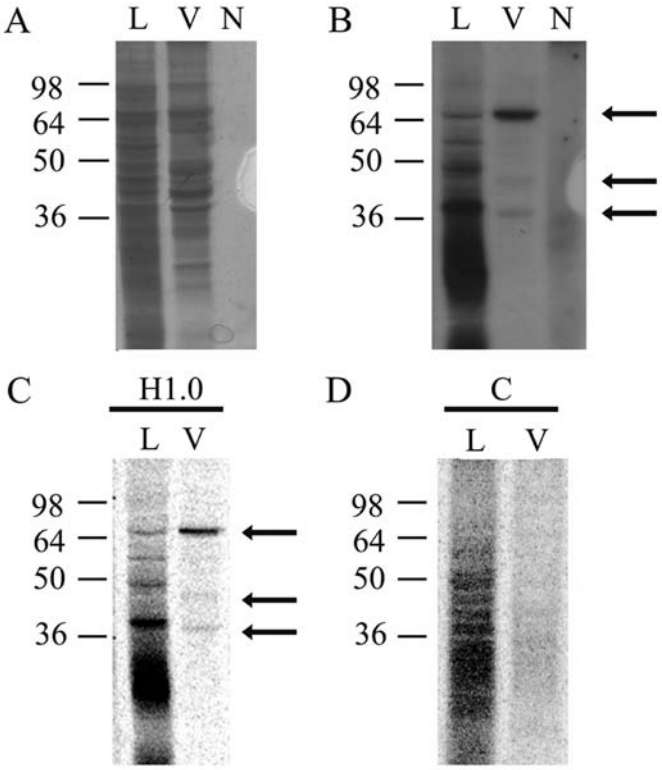

Figure 4. T1 RNase protection assay. ^{33}P-labeled H1.0 RNA was obtained by *in vitro* transcription from the T3 polymerase promoter of pMH1.0 plasmid, purified and mixed with total cell extracts (lane L) or vesicles (lane V) from A375 melanoma cells. H1.0 RNA was also treated with T1 RNase in the absence of protein (lane N), as a control. (A) Coomassie blue staining of the gel shown in (B). (B and C) Fluorography of the SDS-PAGE gels on which H1.0 RNA-protein complexes formed by proteins present in the total cell lysates (lane L) or in the extracellular vesicles (lane V) were analysed. (D) In order to ascertain the degree of specificity of RNA-protein binding, both total lysates (lane L) and vesicles (lane V) from A375 melanoma cells were also probed with another *in vitro* transcribed RNA, the one encoding CSD-C2 protein (C). This RNA was bound by several proteins when incubated with total cell lysates (lane L) but no band is visible in the vesicle fraction (lane V).

reaction not preceded by the RT step; in these PCR control experiments no band was seen (Fig. 3C, A375v).

EVs released from A375 melanoma cells also contain H1.0 mRNA-binding proteins. The presence of H1.0 RNA in EVs prompted us to also look for RNA-binding proteins (RBPs) in the vesicles. We applied a T1 RNase protection assay used by us for many years to study protein-RNA interactions (25,32). Briefly, proteins from freeze fractured vesicles were incubated with ~5.0x10^6 cpm of ^{33}P-labeled, *in vitro* transcribed H1.0 RNA, in order to allow formation of non-covalent protein-RNA complexes. The putative complexes were then treated with T1 RNase to digest all the RNA but the sequences protected by proteins. Finally, the complexes were cross-linked by UV treatment and analyzed by denaturing PAGE. As shown in Fig. 4, many H1.0 RNA-binding proteins are present in melanoma cells (Fig. 4B, lane L); in the vesicles, however, only three main bands are clearly visible, at ~65, 45 and 38 kDa, respectively (Fig. 4B, lane V). In Fig. 4A we show the stained proteins present in the same gel that was then dried and used for fluorography (Fig. 4B). In this gel a negative control was also included, which was obtained by treating H1.0 RNA with T1 nuclease and UV, but in the absence of proteins (Fig. 4B, lane N). Finally, we also probed specificity of these bands by repeating the analysis with H1.0 RNA (Fig. 4C, H1.0) and, in parallel, with a second RNA (Fig. 4C and D), encoding CSD-C2/PIPPin protein (26,38). As clearly shown in Fig. 4D, although control RNA (lane C) can be bound by several proteins in melanoma cells (Fig. 4D, lane L), no band is visible in the vesicle fraction (Fig. 4D, lane V). On the other hand, H1.0 RNA (Fig. 4C) again formed the complexes seen in Fig. 4B.

Enrichment of the H1.0 RNA-binding proteins present in EVs by affinity chromatography and analysis by MALDI-TOF mass spectrometry. Then we tried to enrich the H1.0 RNA-binding proteins evidenced in EVs by using a protocol based on affinity chromatography on biotinylated H1.0 RNA, as already described (27). The protein fractions obtained from chromatography in the presence (Fig. 5, lane 1) or in the absence (Fig. 5, lane 2) of H1.0 RNA were analyzed by SDS-PAGE and the gel was silver stained. The region indicated by the rectangle in Fig. 5 (lane 1), which clearly contains proteins not present in the negative control sample (Fig. 5, lane 2), was cut from the gel and analyzed by MALDI-TOF mass spectrometry. The highest score was found for myelin expression factor 2 (MYEF2) (Table I), a protein mainly known as a

Table I. Synopsis of information on the protein identified as MYEF 2.[a]

Protein name	Gene name	AC	Mowse score	Sequence coverage (%)	Theoretical MW (Da)-pI
Myelin expression factor 2	MYEF2	Q9P2K5	136	19	64121-8.86

[a]Protein name, gene name, AC (accession no.), theoretical MW, and pI are from Swiss-Prot database. Mowse score represent -10* Log (P), where P is the probability that the observed match is a random event.

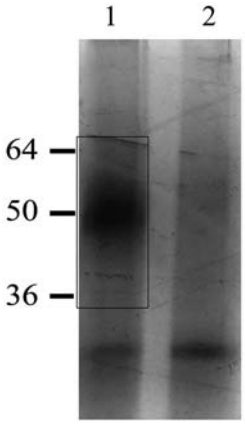

Figure 5. Silver staining of a gel (SDS-PAGE) on which RBPs obtained by affinity chromatography were separated. Affinity chromatography was performed on streptavidin-conjugated paramagnetic particles, in the presence (lane 1) or not (lane 2) of biotinylated H1.0 RNA, as described in the text.

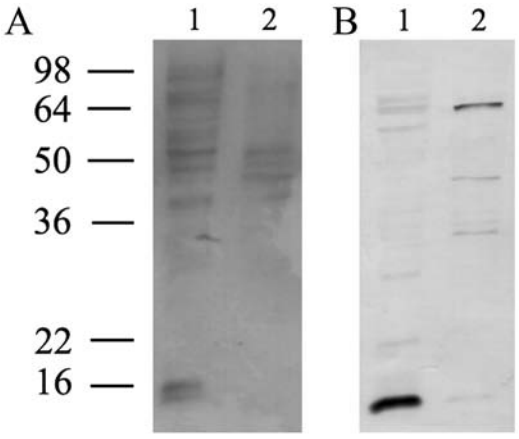

Figure 6. Western blot analysis of total cell lysates from melanoma cells (lane 1) and total extracellular vesicles (lane 2). Proteins were immune-stained with anti-MYEF2 antibodies (B). Ponceau red-staining of the same membrane as shown in (A).

DNA-binding repressor of the gene encoding the myelin basic protein (39,40).

Finally, we analyzed the proteins present in the freeze fractured vesicles from melanoma cells by western blot with anti-MYEF2 antibodies. As shown in Fig. 6, indeed, we observed three bands immune-stained by these antibodies (of ~65, 45 and 34 kDa, respectively).

Discussion

Construction of a complete multicellular organism requires a precise and complex array of events which involves cell proliferation as well as cell death, cell migration and differentiation, formation of a network of cell-to-cell and cell-to-ECM contacts, and exchange of chemical signals of many kinds. Among the ways used by cells to exchange information, production of extracellular vesicles has been recognized as a physiological process, which is highly enhanced in transformed cells. Cancer cells rely on EVs production for modifying to their own advantage activities and properties of surrounding cells. In addition, they probably use EVs to discard proteins otherwise able to counteract tumorigenesis. In both normal and cancer cells, indeed, the transcriptional potential of the cell nucleus is controlled by availability of specific transcription factors, and by the structural organization of chromatin. The structural organization of chromatin is, in turn, regulated by a series of dynamic events, involving chromatin remodelling factors (41) as well as the synthesis and incorporation of replacement histone variants (42,43), such as the linker H1.0 histone, which has been reported, long ago, to be specifically accumulated during terminal differentiation of several cell types (35,36,44).

Surprisingly, we recently found that in glial tumor cells concentration of both H1.0 mRNA and protein is high and not linked to a decrease of proliferation rate (19). Moreover, these cells secrete H1.0 by sorting it to EVs (19). In this study we therefore analysed tumor cells of different origin, A375 melanoma cells, and found that also these cells produce H1.0. In addition, like oligodendroglioma cells, they secrete it into EVs. In this case, however, the histone shows an apparent molecular mass higher than expected. Since it has been reported that some proteins, specifically sorted to EVs, are modified by sumoylation (45), the presence of a SUMO moiety in the putative H1.0 sorted to EVs was investigated. The obtained results suggest that this is indeed the case. We conclude that the still unknown mechanism responsible for the direct correlation between H1.0 expression and differentiation does not work in cancer cells. Moreover, we can hypothesize that secretion of EVs from these cells could be also involved in eliminating proteins (such as the H1.0 histone) that could be able to counteract proliferation.

Interestingly, as demonstrated by RT-PCR, EVs released from melanoma cells also contain H1.0 mRNA. This observation prompted us to look for H1.0 mRNA-binding proteins. We used an affinity chromatography approach that allowed to search, only in the presence of a biotinylated RNA, a group of

proteins, which were further analysed by MALDI-TOF mass spectrometry. Among these proteins, the most prevalent was myelin expression factor 2 (MYEF2). This protein has been identified in undifferentiated cells, as a repressor able to bind directly to and to repress the promoter of the gene encoding the mouse myelin basic protein (39); it also contains two RNA recognition motifs (RRM) which were shown to bind DNA (40); finally, it has also been reported to form a complex with Runt-related transcription factor 1 (RUNX1), an essential transcription factor involved in generating hematopoietic stem cells (46). On the basis of these previous observations, MYEF2 expression in cancer cells is not surprising. In addition, since this protein contains RNA-recognition motifs, its binding to an mRNA is not surprising either. However, the fact that this repressor protein, mostly expressed in undifferentiated cells, binds to the mRNA encoding the differentiation-linked H1.0 histone, probably participating in its elimination from the cells via EVs, is in our opinion of great importance and sheds new light on the biochemical mechanisms involved in tumorigenesis. Moreover, H1.0 mRNA could in turn function as a MYEF2-carrier: once entered a new cell, MYEF2 could indeed also function as a transcriptional repressor, thus conditioning the expression properties of the receiving cell.

Acknowledgements

This study was supported by University of Palermo (Università degli Studi di Palermo, Palermo, Italy; ex 60%). We thank Dr Giulio Ghersi for the kind gift of anti-integrin β1 antibodies. We also thank Dr Roberto Ghiandoni for helpful advice with the NanoSight.

References

1. Iraci N, Leonardi T, Gessler F, Vega B and Pluchino S: Focus on extracellular vesicles: Physiological role and signalling properties of extracellular membrane vesicles. Int J Mol Sci 17: E171, 2016.
2. Tkach M and Théry C: Communication by extracellular vesicles: Where we are and where we need to go. Cell 164: 1226-1232, 2016.
3. Cocucci E and Meldolesi J: Ectosomes and exosomes: Shedding the confusion between extracellular vesicles. Trends Cell Biol 25: 364-372, 2015.
4. Schiera G, Proia P, Alberti C, Mineo M, Savettieri G and Di Liegro I: Neurons produce FGF2 and VEGF and secrete them at least in part by shedding extracellular vesicles. J Cell Mol Med 11: 1384-1394, 2007.
5. Proia P, Schiera G, Mineo M, Ingrassia AMR, Santoro G, Savettieri G and Di Liegro I: Astrocytes shed extracellular vesicles that contain fibroblast growth factor-2 and vascular endothelial growth factor. Int J Mol Med 21: 63-67, 2008.
6. Prada I, Furlan R, Matteoli M and Verderio C: Classical and unconventional pathways of vesicular release in microglia. Glia 61: 1003-1017, 2013.
7. Schiera G, Di Liegro CM and Di Liegro I: Extracellular membrane vesicles as vehicles for brain cell-to-cell interactions in physiological as well as pathological conditions. BioMed Res Int 2015: 152926, 2015.
8. Lorico A, Corbeil D, Pawelek JM and Alessandro R: Transmission of Information in Neoplasia by Extracellular Vesicles. BioMed Res Int 2015: 289567, 2015.
9. Arraud N, Linares R, Tan S, Gounou C, Pasquet JM, Mornet S and Brisson AR: Extracellular vesicles from blood plasma: Determination of their morphology, size, phenotype and concentration. J Thromb Haemost 12: 614-627, 2014.
10. Chen T, Xi QY, Ye RS, Cheng X, Qi QE, Wang SB, Shu G, Wang LN, Zhu XT, Jiang QY, *et al*: Exploration of microRNAs in porcine milk exosomes. BMC Genomics 15: 100, 2014.
11. Gallo A, Tandon M, Alevizos I and Illei GG: The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7: e30679, 2012.
12. Ogawa Y, Taketomi Y, Murakami M, Tsujimoto M and Yanoshita R: Small RNA transcriptomes of two types of exosomes in human whole saliva determined by next generation sequencing. Biol Pharm Bull 36: 66-75, 2013.
13. Pap E, Pállinger E and Falus A: The role of membrane vesicles in tumorigenesis. Crit Rev Oncol Hematol 79: 213-223, 2011.
14. Giusti I, D'Ascenzo S and Dolo V: Microvesicles as potential ovarian cancer biomarkers. BioMed Res Int 2013: 703048, 2013.
15. Ban LA, Shackel NA and McLennan SV: Extracellular vesicles: A new frontier in biomarker discovery for non-alcoholic fatty Liver disease. Int J Mol Sci 17: 17, 2016.
16. D'Agostino S, Salamone M, Di Liegro I and Vittorelli ML: Membrane vesicles shed by oligodendroglioma cells induce neuronal apoptosis. Int J Oncol 29: 1075-1085, 2006.
17. Lo Cicero A, Schiera G, Proia P, Saladino P, Savettieri G, Di Liegro CM and Di Liegro I: Oligodendroglioma cells shed microvesicles which contain TRAIL as well as molecular chaperones and induce cell death in astrocytes. Int J Oncol 39: 1353-1357, 2011.
18. Liu Y, Gu Y and Cao X: The exosomes in tumor immunity. OncoImmunology 4: e1027472, 2015.
19. Schiera G, Di Liegro CM, Saladino P, Pitti R, Savettieri G, Proia P and Di Liegro I: Oligodendroglioma cells synthesize the differentiation-specific linker histone H1° and release it into the extracellular environment through shed vesicles. Int J Oncol 43: 1771-1776, 2013.
20. Crane-Robinson C: Linker histones: History and current perspective. Biochim Biophys Acta 1859: 431-435, 2016.
21. Izzo A and Schneider R: The role of linker histone H1 modifications in the regulation of gene expression and chromatin dynamics. Biochim Biophys Acta 1859: 486-495, 2016.
22. Roque A, Ponte I and Suau P: Interplay between histone H1 structure and function. Biochim Biophys Acta 1859: 444-454, 2016.
23. Millán-Ariño L, Izquierdo-Bouldstridge A and Jordan A: Specificities and genomic distribution of somatic mammalian histone H1 subtypes. Biochim Biophys Acta 1859: 510-519, 2016.
24. Di Liegro CM, Schiera G and Di Liegro I: Regulation of mRNA transport, localization and translation in the nervous system of mammals (Review). Int J Mol Med 33: 747-762, 2014.
25. Scaturro M, Nastasi T, Raimondi L, Bellafiore M, Cestelli A and Di Liegro I: H1(0) RNA-binding proteins specifically expressed in the rat brain. J Biol Chem 273: 22788-22791, 1998.
26. Nastasi T, Scaturro M, Bellafiore M, Raimondi L, Beccari S, Cestelli A and Di Liegro I: PIPPin is a brain-specific protein that contains a cold-shock domain and binds specifically to H1 degrees and H3.3 mRNAs. J Biol Chem 274: 24087-24093, 1999.
27. Scaturrok M, Sala A, Cutrona G, Raimondi L, Cannino G, Fontana S, Pucci-Minafra I and Di Liegro I: Purification by affinity chromatography of H1o RNA-binding proteins from rat brain. Int J Mol Med 11: 509-513, 2003.
28. Saladino P, Di Liegro CM, Proia P, Sala A, Schiera G, Lo Cicero A and Di Liegro I: RNA-binding activity of the rat calmodulin-binding PEP-19 protein and of the long PEP-19 isoform. Int J Mol Med 29: 141-145, 2012.
29. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.
30. Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159, 1987.
31. Castiglia D, Gristina R, Scaturro M and Di Liegro I: Cloning and analysis of cDNA for rat histone H1(0). Nucleic Acids Res 21: 1674, 1993.
32. Di Liegro CM, Schiera G, Proia P, Saladino P and Di Liegro I: Identification in the rat brain of a set of nuclear proteins interacting with H1° mRNA. Neuroscience 229: 71-76, 2013.
33. Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH and Dunn MJ: A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. Electrophoresis 21: 3666-3672, 2000.
34. Cancemi P, Di Cara G, Albanese NN, Costantini F, Marabeti MR, Musso R, Riili I, Lupo C, Roz E and Pucci-Minafra I: Differential occurrence of S100A7 in breast cancer tissues: A proteomic-based investigation. Proteomics Clin Appl 6: 364-373, 2012.

35. Panyim S and Chalkley R: A new histone found only in mammalian tissues with little cell division. Biochem Biophys Res Commun 37: 1042-1049, 1969.
36. Zlatanova J and Doenecke D: Histone H1 zero: A major player in cell differentiation? FASEB J 8: 1260-1268, 1994.
37. Villarroya-Beltri C, Gutiérrez-Vázquez C, Sánchez-Cabo F, Pérez-Hernández D, Vázquez J, Martin-Cofreces N, Martinez-Herrera DJ, Pascual-Montano A, Mittelbrunn M and Sánchez-Madrid F: Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. Nat Commun 4: 2980, 2013.
38. Castiglia D, Scaturro M, Nastasi T, Cestelli A and Di Liegro I: PIPPin, a putative RNA-binding protein specifically expressed in the rat brain. Biochem Biophys Res Commun 218: 390-394, 1996.
39. Haas S, Steplewski A, Siracusa LD, Amini S and Khalili K: Identification of a sequence-specific single-stranded DNA binding protein that suppresses transcription of the mouse myelin basic protein gene. J Biol Chem 270: 12503-12510, 1995.
40. Muralidharan V, Tretiakova A, Steplewski A, Haas S, Amini S, Johnson E and Khalili K: Evidence for inhibition of MyEF-2 binding to MBP promoter by MEF-1/Pur alpha. J Cell Biochem 66: 524-531, 1997.
41. Zaret KS and Mango SE: Pioneer transcription factors, chromatin dynamics, and cell fate control. Curr Opin Genet Dev 37: 76-81, 2016.
42. Gaume X and Torres-Padilla ME: Regulation of reprogramming and cellular plasticity through histone exchange and histone variant incorporation. Cold Spring Harb Symp Quant Biol: Nov 18, 2015 (Epub ahead of print). pii: 027458, 2015.
43. Zink L-M and Hake SB: Histone variants: Nuclear function and disease. Curr Opin Genet Dev 37: 82-89, 2016.
44. Scaffidi P: Histone H1 alterations in cancer. Biochim Biophys Acta 1859: 533-539, 2016.
45. Kunadt M, Eckermann K, Stuendl A, Gong J, Russo B, Strauss K, Rai S, Kügler S, Falomir Lockhart L, Schwalbe M, *et al*: Extracellular vesicle sorting of α-Synuclein is regulated by sumoylation. Acta Neuropathol 129: 695-713, 2015.
46. van Riel B, Pakozdi T, Brouwer R, Monteiro R, Tuladhar K, Franke V, Bryne JC, Jorna R, Rijkers EJ, van Ijcken W, *et al*: A novel complex, RUNX1-MYEF2, represses hematopoietic genes in erythroid cells. Mol Cell Biol 32: 3814-3822, 2012.