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AMNIOTIC FLUID STEM CELL EXOSOMES: THERAPEUTIC PERSPECTIVE

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AMNIOTIC FLUID STEM CELL EXOSOMES: THERAPEUTIC PERSPECTIVE

Running title: Immunomodulation by AFSC exosomes

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ABSTRACT

It is widely accepted that the therapeutic potential of stem cells can be largely mediated by paracrine factors, also included into exosomes. Thus, stem cell derived exosomes represent a major therapeutic option in regenerative medicine avoiding, if compared to stem cells graft, abnormal differentiation and tumor formation. Exosomes derived from mesenchymal stem cells (MSC) induce damaged tissue repair, and can also exert immunomodulatory effects on the differentiation, activation and function of different lymphocytes. Therefore, MSC exosomes can be considered as a potential treatment for inflammatory diseases and also an ideal candidate for allogeneic therapy due to their low immunogenicity. Amniotic fluid stem cells (AFSC) are broadly multipotent, can be expanded in culture, and can be easily cryopreserved in cellular banks. In this study, morphology, phenotype and protein content of exosomes released into amniotic fluid *in vivo* and from AFSC during *in vitro* culture (conditioned medium) were examined. We found that AFSC derived exosomes present different molecules than amniotic fluid ones, some of them involved in immunomodulation, such transforming growth factor beta and hepatic growth factors. The immunomodulatory effect of AFSC's exosomes on peripheral blood mononuclear cells stimulated with phytohemagglutinin was compared to that of the supernatant produced by such conditioned media deprived of exosomes. We present evidence that the principal effect of AFSC conditioned media (without exosomes) is the induction of apoptosis in lymphocytes, whereas exposure to AFSC-derived exosomes decreases the lymphocyte's proliferation, supporting the hypothesis that the entire secretome of stem cells differently affects immune-response.

Keywords: exosome, stem cells, immunomodulation

Abbreviations:

Amniotic fluid stem cells (AFSC) Bovine serum albumin (BSA); 1,4-diazabicyclo(2.2.2)octane (DABCO); 4',6-diamidino-2-phenylindole (DAPI); Ethylenediaminetetraacetic acid (EDTA); hepatic growth factors (HGF); immunofluorescence (IF); phosphate buffered saline (PBS); peripheral blood mononuclear cells stimulated with phytohemagglutinin (PHA-PBMC); transforming growth factor beta (TGFβ2); Tris-buffered saline (TBS); Triton-X-100 (TxTBS); western blot (WB).

For Peer Review

Introduction

Human gestational tissues have demonstrated to be a rich resource of different stem cell populations, showing properties intermediate between embryonic and adult stem cells, so much so **that** the potential to differentiate to the three germ layers has been demonstrated [1, 2]. Unlike adult stem cells, perinatal stem cells could be applied either in uterus **or** ~~of~~ postnatally, for birth defects identified during gestation, and, due to their younger age, they will have advantage of carrying fewer environmental induced mutations [3]. However, in literature are described two mechanisms playing a pivotal role in the regeneration process. One mechanism involves the differentiation of the injected stem cells towards the **specific** cyto-type of damaged tissue. This process has been described by many authors [4, 5], but the few number of exogenous cells directly and permanently integrated into the regenerated mature tissue does not completely explain the obtained regenerative result. The only partial engraftment into the recipient anatomic area, observed in literature, could be due to heterogenic level of pre-differentiation induced in the cell culture before the implant. Indeed, **even** if the expanded stem cell culture has been exposed *in vitro* to differentiation medium, may be not the entire cell population has undertaken the differentiation process. This subpopulation, however, could still maintain the low immunogenic characteristics but, more interestingly, can affect immune response and stimulate the recruitment of resident progenitor cells to regenerate damaged area. These hypotheses have been confirmed by several studies demonstrating the positive effect of the stem cell secreted molecules alone (conditioned medium) in regeneration of injured tissues where, usually, a chronic inflammation status is present [6, 7]. Basing on these assumptions, in the last decade more attention has been paid to the secretome of stem cells and, therefore, on the paracrine effect. Data published by our lab. indicate that PBMC exposed to human amniotic fluid stem cells (AFSCs) conditioned medium undergo in apoptosis at least in part for the presence of HGF secreted by AFSCs [8].

Conditioned medium includes soluble factors and extracellular vesicles. In addition to their role as biomarkers, released extracellular vesicles (EV) have been very recently included among the players contributing to MSC regenerative potential [9-11] although, to date, a comprehensive molecular definition of their cargo is missing. EV mainly include two different populations: i) microvesicles, which are micro-sized particles ranging in size from 150- up to 1000 nm, and ii) exosomes, nano-sized particles of about 40- up to 150 nm diameter. They both act as key biological effectors of paracrine signaling, being up-taken by responder cells. They include soluble, bioactive factors (proteins, lipids, etc.) and RNA (mainly regulatory microRNA – miRNA) as their complex cargo. The importance of EV in stem cells has been extensively reviewed by Han et al. [12] and in particular the role in immunomodulation of mesenchymal stem cells EV has been dissected in the review of Fierabracci et al. [2016] thus highlighting the relevance of their role in the regulation of regenerative processes. More recently, the group of Bollini [14] has extensively characterized the role of extracellular vesicles secreted by II trimester c-KIT⁺ human amniotic fluid-stem cells (AFSC-EV), but an investigation on the protein content and its effect on immune response is lacking.

The central aim of this study was to examine the protein role in immunomodulation of exosomes obtained from amniotic fluid *in vivo* and from AFSC during *in vitro* culture (conditioned medium). Therefore morphology, phenotype and protein content of exosomes released into amniotic fluid and by AFSC in culture were analyzed. Then the effect of exosomes or of the other part of secretome (conditioned medium deprived of exosomes) on PBMC proliferation and cell cycle of PBMC was assessed, in order to shed a light on the different properties of AFSC secretome.

MATERIAL AND METHODS

Amniotic fluid collection

The hAFSCs were obtained, as previously reported [15], from 5 amniotic fluids collected from women between the 16th and 24st 17th week of gestation who undergo amniocentesis at Arcispedale S. Maria Nuova Hospital in Reggio Emilia. The average age of women was 34.5 (42, 38, 34, 25, 32, 36 years old). During the pre-amniocentesis interview pregnant women were informed about the purpose of the study and any risks related to. The informed consents were obtained, in accordance with the Italian law and the guidelines of the ethics committee (protocol 2015/0004362 of 02.24.2015). Informed consent, as well as all documentation relating to the invasive procedure, was signed by the pregnant woman and by a specialist before continuing the exam. All the samples were diploid. Amniotic fluids (around 2 mL for patient) were separated from the cellular part by centrifugation and then stored at – 80°C.

Amniotic fluid stem cell isolation and culture

Human amniotic fluid stem cells (hAFSCs) were isolated as previously described by De Coppi et al. 2007 [16]. Amniotic fluid cell cultures were harvested by trypsinization, and subjected to c-Kit immunoselection by MACS[®] technology (Miltenyi Biotec, Germany). hAFSCs were subcultured routinely at 1:3 dilution and not allowed to expand beyond the 70% of confluence. hAFSCs were grown in culture medium (α MEM supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin) (all from EuroClone Spa, Italy) [17].

Exosome isolation

From amniotic fluids: samples from 5 healthy donors were collected and analyzed independently. A volume of 1 ml of an amniotic fluid sample from each donor was used for exosome extraction with Total Exosome Isolation solution for other body fluids (Invitrogen, Life Technologies) performed according to manufacturer's instructions.

From conditioned medium: AFSC isolated from 5 amniotic fluid cells were grown in 75 cm² flask until subconfluence (around 1x10⁶ cells). Before exosome extraction, the cells were maintained for 4 days in 10 mL culture medium deprived of FBS in order to exclude the contamination by exosomes comprised into FBS solution. The secreted part of conditioned medium was then concentrated up to 3 mL by using Centrifugal Filter Units with 3K cutoff. Then, the concentrated CM was treated with Total Exosome Isolation solution from cell culture media (Invitrogen, Life Technologies), according to manufacturer's instructions. The surnatant (depleted of exosomes = -exo) and pellet (exosomes = +exo) were both collected and quantified by Bradford method. To obtain a sample for Western blot analysis, the pellet was re-suspended in lysis buffer. For immunomodulation experiment, exosomes were re-suspended in PBS as well for electron microscopy.

Transmission Electron microscopy

For electron microscopy, dilutions from 10 µg of exosome protein preparations were mixed with an equal volume of 4% paraformaldehyde (Sigma) in phosphate buffer. Then 5 µL of solution were deposited on 200 mesh Formvar-carbon-coated electron microscopy (EM) nickel grids and left to adsorb for 20 minutes at room temperature. Samples were fixed with 1% glutaraldehyde (Merck) in phosphate buffer, contrasted with uranyl oxalate (pH 7.0), and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose (25 cps; Sigma Aldrich) in a 1:9 ratio on ice. Grids were then removed with stainless steel loops and the excess fluid was blotted with filter paper to ensure an appropriate thickness of the methyl cellulose film [18]. After drying, grids were examined with a JEM 2010 TEM. Images were captured using a digital camera GIF energy filter + Gatan Multiscan Camera 794, and Software Digital Micrograph 3.1 su Win NT software.

Mononuclear cell separation

Human peripheral blood mononuclear cells (PBMC) were separated from peripheral blood of healthy donors by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, AXIS-SHIELD PoCAs, Oslo, Norway) at room temperature (RT) [19, 20].

The concentration of isolated PBMC was adjusted to 2×10^6 cells/mL in RPMI 1640 (EuroClone Spa, Italy) including 10% FBS. Twenty hours later, PBMC were washed, and used for experiments described below.

PBMC exposure to AFSC secretome

PBMC in the majority of experiments were activated with 5 μ g/ml phytohemagglutinin (PHA) for 24 hours prior to and during the phase of co-culture conditions.

Co-cultures with the separated parts of secretome, +exo or –exo, were obtained by adding the volume corresponding to 80 μ g of proteins to 2 mL of PBMC suspension (1×10^6 cells/mL) for 4 days.

Evaluation of cell cycle in lymphocytes by flow cytometry

Cell cycle was quantitatively analyzed using propidium iodide (PI). The staining process was performed according to the manufacturer's instructions. Data were obtained using flow cytometric analysis with FACScan within 1 hr.

Flow cytometer immune-assay

PBMC were stained with the following antibodies (mAbs): mouse anti-Thy-1 (CD4) PE and mouse anti-Endoglin (CD8) FITC (Miltenyi Biotec, Germany). A minimum of 10,000 cells per sample was acquired and analyzed using FACScan flow cytometer and Lysis II software (both from Becton Dickinson, San Jose, CA, USA).

Cell Proliferation-BrdU Method

Cell proliferation was measured upon completion of the 4 days incubation period with –exo or +exo as mentioned above using a CytoSelect™ BrdU Cell Proliferation ELISA Kit (Cell Biolabs, Inc) following manufacturer instructions. In brief, kit-provided BrdU labeling

solution (10 μ L) was added to each well containing 1×10^5 PBMC, pretreated or not with PHA, and the plate was incubated at 37°C for 3 hours. Thereafter, the cells were centrifuged at 300 \times g for 10 min, the labeling solution was removed, washed twice with PBS and the cells then were fixed by addition of FixDenat solution at 37° C for 30 min. The cells were then washed twice with PBS (200 μ L) and kit-provided substrate solution (100 μ L) was added to each well. After washing, primary antibody (anti-BrdU solution, 100 μ L/well) was then added and the plate was incubated at room temperature for 60 min followed by washes and secondary antibody (HRP conjugated, 100 μ L/well) for other 60 min. The plates were treated with HRP substrate, a stop solution was then added and the absorbance in each well was then measured at 450 nm in an automated plate reader (Appliskan, Thermo Scientific). Sets of blank (100 μ L culture medium alone) and control wells were included in each experiment.

Immunofluorescence and confocal microscopy

For immunofluorescence analysis, AFSC samples were processed and confocal imaging was performed by a Nikon A1 confocal laser scanning microscope, as previously described [20]. Primary antibodies were raised against the following molecules: CD9 (Invitrogen), CD63 and CD81 (Thermo Fisher, Rockford, IL, USA). Secondary antibody was anti mouse Alexa 488 (Invitrogen, Thermo Fisher, Rockford, IL, USA).

The confocal serial sections were processed with ImageJ software to obtain three-dimensional projections, as previously described. The image rendering was performed by Adobe Photoshop software.

SDS-PAGE and Coomassie brilliant blue staining

40 μ g of exosomes for each sample were separated by 10% SDS-PAGE and gels were then stained in the Coomassie brilliant blue solution (0.1% Coomassie blue in 10% acetic acid,

45% methanol) and shaken at room temperature for 1 h. The gels were destained by soaking for 2 h in destaining solution (10% acetic acid, 30% methanol) [21].

Mass Spectrometry

In gel trypsin digestion and mass spectrometry analysis were performed as discussed in Shevchenko et al. [22]. Briefly, the whole lane gel was divided in slices and then reduced in gel pieces that were treated with solution A (1:1 mixture of acetonitrile: 100 mM ammonium bicarbonate) for 30 min. The gel slices were subjected to reduction of disulfide bonds by 10 mM DTT at 56°C for 30 min. Alkylation step was then performed with 55 mM iodoacetamide for 20 min at room temperature in dark. Before trypsin digestion, the rehydration and dehydration steps were again performed with ammonium bicarbonate and solution A. Then samples were incubated at RT with acetonitrile until gel pieces become white. Trypsin digestion was performed by incubating the dry gel slices overnight in 1 µg of trypsin in ammonium bicarbonate (Trypsin Sigma) at 37°C. Following digestion, the tryptic digested fragments present in the supernatant were collected and lyophilized. Before mass spectrometry analysis, the lyophilized powder was dissolved in 0.1% TFA in 50% acetonitrile. The expressed proteins were identified using LC/MS/MS 6410B mass spectrometer. Protein identification against the peak list was performed in MASCOT version 2.1 with Swiss-Prot and cRAP database as the search engine. The search parameters for database search using Mascot were given as, taxonomy: human; enzyme used for digestion: trypsin with one missed cleavage allowed; fixed modification specified as carbamidomethylation (C), and oxidation (M) and deamidated (NQ) as variable modifications. The peptide mass tolerance was set as 40 ppm and 0.08 Da for MS/MS tolerance. The detailed list of proteins identified using MALDI is presented in Supplementary Table 1.

Bioinformatic analysis

The lists of identified proteins were subjected to PANTHER classification system, version 9.0 (<http://www.pantherdb.org/>) [23, 24], for understanding biological context of the identified proteins and their involvement in biological pathways. The list of UniProt Accession number was uploaded and mapped against reference Homo sapiens dataset to extract and summarize molecular functions, biological processes and the class of proteins. The above result was further processed in order to get the proteins of the top five categories for each of the functional domains.

Western blotting

Exosomes and whole cell lysates from PBMC were processed, as previously described [8]. Primary antibodies were raised against the following molecules: PARP, 14-3-3, HGF, clusterin α , β -actin (Santa Cruz, MA, USA), Cyclin A (Cell Signalling, MA USA), vitamin D binding protein and TGF β 2 (Novus Biologicals, Milano, Italy). Secondary antibodies, anti-mouse HRP, anti-rabbit-HRP, anti-Goat-HRP, all purchased from Pierce (Thermo Fisher, Rockford, IL, USA).

Statistical analysis

In vitro experiments were performed in triplicate. For quantitative comparisons, values were reported as mean \pm SD (standard deviation) based on triplicate analysis for each sample. To test the significance of observed differences among the study groups Student's t-test or One-way Anova with Bonferroni post **doc** test were applied. A P value <0.05 was considered to be statistically significant.

Results

The first purpose of this study was to characterize the exosomes pools obtained by the commercial exosome isolation kits, since we are aware that the different procedures for exosome extraction give rise to an enrichment in vesicles with similar but not equal profiles [25]. The presence of typical exosome markers, such as CD81, CD63 and CD9, after isolation process from all the samples of amniotic fluid or from AFSC in culture (CM), was verified with WB analysis. All the markers were expressed in samples obtained from the two sources (F and CM) and from different donors. In Figure 1A a representative image of WB and relative densitometric analysis of three samples are shown and a donor-dependent variability on expression level is evident mostly for CD63 and CD81, while the trend of CD9 band intensity follows that of the actin one.

TEM analysis was performed in order to measure the dimensions of isolated vesicles. Figure 1B shows representative images of one sample of exosomes isolated from F and from AFSC conditioned medium (CM). Also an image of fresh medium not exposed to cell culture, but treated with the isolation buffer following the same protocol, is reported as C. The diameter of vesicles is around 35 nm, then compatible with exosomes' characteristics.

The capability to produce exosomes by stem cells in culture was confirmed by IF analysis. Indeed, AFSC were incubated with antibodies against CD81, CD63 and CD9 and the images of Figure 1C show the presence of intracellular spots stained with exosome markers.

We have previously demonstrated that conditioned medium of AFSC induces apoptosis in PBMC [8], therefore we here tried to dissect the effect of the two components of secretome, soluble part (-exo) and vesicles (+exo), focusing on exosomes. We arranged an experimental design similar to the one previously tested, in term of days for CM collection and for exposure to PMBC, in order to compare the effect of the whole secretome with the one of -exo and +exo. Cytofluorimetric (CF) analyses demonstrated that exosome exposure affects

the percentage of cells in S and G2/M phases of PBMC, while CM depleted of exosomes (-exo) induces also apoptosis (Table I).

In particular the PBMC subpopulations CD4⁺ (T helper) significantly decreased in the presence of -exo and +exo, while CD8⁺ (T cytotoxic) cells percentage was not significantly affected (Fig. 2A), suggesting that the decline in S and G2/M phases observed with both the treatments is related to a lower number of lymphocytes T helper.

In order to better quantify the decrease in proliferation level suggested by cytofluorimetric analysis with treatments +exo or -exo, BrdU incorporation assay was performed for PBMC, activated or not with PHA, in the presence of isolated exosomes or all the other secreted molecules (-exo). BrdU test (Fig. 2B) showed that PBMC treated with PHA display a proliferation rate higher than PBMC alone, as expected, and confirmed that the incubation with +exo or -exo induces a reduction of proliferation activity of PBMC only significant when treated with PHA.

WB experiments, shown in Figure 2C, gave us a confirm of the proapoptotic effect of -exo, since the level of cleaved PARP significantly increased only in this condition, beside the decrease of proliferation rate, as shown by cyclin A profile. Indeed, densitometric analysis, shown in Figure 2D, demonstrated a reduction of expression of this member of cyclin family, which is the unique that can activate two different CDK kinases during S phase, and during the transition from G2 to M phase. However this decline is not statistically significant, while the effect of +exo treatment induced a huge decrease of cyclin A2 band intensity that can be considered statistically different from the control.

Finally, we focused on the proteomic analysis of exosome content by using mass spectrometry applied on protein samples of three biological replicates separated by monodimensional electrophoresis. Figure 3A shows the coomassie blue staining of a SDS-PAGE where exosome protein content of two samples of AFSC-CM and amniotic fluids were

loaded. Also looking at the gel, a different profile between samples from CM and from fluids can be noticed. At the same time, comparing the two samples of fluids between them, or from CM, a large similarity of protein content of exosome deriving from the same liquid source can be supposed.

Indeed, the identification, obtained from Mascot software applied after MS analysis, revealed that the two types of exosomes contain a different pattern of proteins, even if the number of molecules is similar (256 from CM vs 273 from F reported in Table I supplementary). In particular, only 60 proteins are present in both the samples. To better characterize the exosome proteome, we classified the identified proteins, differentially expressed in CM versus F, using Gene Ontology terms (<http://geneontology.org/>). The PANTHER GO analysis for biological processes (BP) indicated that the percentage of proteins into the category cellular process was slightly higher in CM samples rather than in F exosomes (from 24.4% to 28%). This bioinformatics analysis, viewed in pie charts, highlighted the presence into the class of cellular processes (part of biological process shown in the top of the figure 3B in blue navy) only in CM samples of the cell growth category, in which were included a short list of growth factors (HGF and TGF β 1 and 2), as shown in Fig. 3C, with an immunomodulatory meaning. Additionally, the analysis by Western blot also confirmed our proteomic results, as shown in Fig. 3D. The presence in the other two CM samples of these proteins is easily detectable, even if with different expression levels normalized to actin (1.5 fold difference).

Discussion

MSCs are a highly relevant stem cell population for clinical applications due to their multilineage differentiation potential, immunomodulatory effects, and ability to home to sites of inflammation [26, 27]. Stem cells derived from amniotic fluid (AFSC) are able to suppress inflammatory responses *in vitro* and soluble factors are an essential component in the communication between lymphocytes and AFSC. Taking in account all these considerations, such as their extensive self-renewal capacity, possibility for banking and absence of tumorigenicity, AFSC can be evaluated as a superior source of stable, well characterized “off the shelf” immunomodulatory cells for a variety of immunotherapies and also have the ability to avoid allogeneic rejection [8]. It has been proved that AFS cells modulate lymphocyte proliferation in a different manner according to gestational age. Second trimester AFS cells show low expression of HLA class I molecules and HLA II absence; moreover whose features were associated with lower sensitivity to NK cell mediated lysis. Second trimester AFS cells not so efficiently inhibited T and NK cell proliferation, but could suppress B cell proliferation, which was not affected by the first and third trimester AFS cells [28].

However the employ of cell therapy is a tricky road to enter, also because of the guidelines to follow, as well as for drugs. On the other hand, the stem cell secretome has been reported to be the major player in their immunomodulating and regenerative properties [26] whereas the mechanism of hAFSCs immunoregulation by soluble factors has been only in part elucidated. Indeed, we previously demonstrated that the whole secretome of AFSC affects peripheral blood mononuclear cells [8]. Basing on these observations, we decided to deeply evaluate the immunosuppressive actions of human AFSC directed on the whole peripheral blood leukocyte population focusing on the secreted vesicles, such as exosomes.

Royo et al. [25] described that different EV enrichment methods suitable for clinical settings yield different amounts of EV protein markers. The methods were: Ultracentrifugation (CEN), Exoquick (EXQ), Invitrogen (INV), Norgen (NOR) and Lectin (LEC). INV-based methodology had high efficiency for obtaining CD26 and CD63 proteins, while EXQ method was the least efficient for most of the EV protein markers. A positive correlation between EV markers CD9 and TSG101 was found for NOR, INV and CEN methods. A significant association between CD9 and CD63 was observed for NOR and INV methods. These results suggest that there are subpopulations of EVs containing CD9 and CD63 proteins and subpopulations containing only one of the 2 markers. All these observations suggested us the choice to isolate exosomes with a procedure excluding ultracentrifugation, such as INV kit. In order to compare exosomes directly present in amniotic fluid with the ones secreted *in vitro* culture by the stem cells subpopulation isolated from amniotic fluid, we employed the kit dedicated to body fluids and to conditioned medium respectively. At first we confirmed the presence of exosome markers in both sample types obtained from 3 different patients, even if with not homogeneous expression levels, accordingly with the observed clear differences between individuals shown in several studies of EVs in human biofluids [25]. Since one of our purposes was to compare amniotic fluid exosomes with AFSC derived exosomes, we established an experimental design in part different from our previous study on the AFSC secretome and immunomodulation [8]. Thus, we decided to do not pre-activate AFSC, in order to stimulate secretion of immune related molecules, with a pre-exposure with human lymphocytes. Indeed, cells contained into amniotic fluids are usually not exposed to not-self cells. Furthermore, the idea to investigate the effect of inoculated AFSC on immune response was central in the previous study, whereas in the present one the aim is to dissect the possibility to achieve a similar result only with the secretome injection. Therefore, in order to

mimic the allogeneic cell transplant, the first study needed a pre-exposure with human lymphocytes, unlike this last.

The immunomodulatory effect of the total secretome produced by amniotic cells, divided in exosomes and the residual secretome part, was evaluated by cytofluorimetric analysis on the cell cycle of peripheral blood mononuclear cells stimulated with phytohemagglutinin (PHA-PBMC). We conventionally named +exo the exposure to isolated exosomes, and -exo the treatment with residual secretome part (depleted to exosome), but the last may even contain vesicles not extracted by the INV kit, so we cannot state that it is only soluble secretome. We present evidence **that** the secretome (+exo and -exo) of AFSC conditioned media modulates PBMC cell cycle, while the one present in amniotic fluid has not significant effect. In particular the induction of apoptosis, **which** we previously observed with the total secretome treatment, was stimulated only by AFSC -exo fraction. This effect is even stronger than in the previous study (around 30% instead of 20%) may **be** because the whole secretome, before the exosome isolation, was concentrated. Moreover, the present experimental design allowed us to investigate also modulation of other cell cycle phases, such as S and G2/M, since PBMC were treated with PHA in order to activate T-lymphocytes. Thus we noticed that AFSC -exo fraction also reduces the percentage of cells in these phases. On the other hand, the exposure to AFSC-derived exosomes decreases only, but more intensely, the lymphocyte's proliferation, as demonstrated also by BrdU experiments. Western blot analysis of apoptotic and proliferation markers confirmed these observations. Our data on proliferation do not overlap with the ones reported by Balbi et al. [14], where they stated that hAFSC-vesicles did not significantly affect hPBMC proliferation in control conditions or after stimulation. It can be explained because the ratio μg of exosomes/number of PBMC in our experimental conditions is 4 times higher and the incubation times are longer.

To understand the immunomodulatory potential of AFSC secretome, we analyzed the modifications in percentage of cells positive for markers of T-lymphocytes, such as CD4 for helper and CD8 for cytotoxic. We focused our attention on these types of T- lymphocytes since it has been recently demonstrated that hAFSC vesicles reduced the maturation of memory B cells (in PWM- stimulated PBMC), but did not influence the polarization of T reg [14]. Here we found that lymphocyte T helper seems to be the subpopulation most affected in the proliferation decrease.

These data support the hypothesis that the entire secretome of stem cells differently affects immune response.

In order to shed a light on the reason why exosomes from amniotic fluid stem cells are efficient in reducing immune response, unlike AF exosomes, we chose a proteomic approach, since studies with identification of the exosome protein content are limited.

Here we provide evidence that the protein pattern of the two types of exosomes is clearly different, as shown by coomassie blue staining. Analyzing the functional categories included into the cellular process, the presence, exclusive in AFSC exosomes, of the cell proliferation class, containing HGF and TGF β 1/2, attracted our attention.

In fact we previously demonstrated that the entire AFSC-CM (not deprived of exosomes) induces apoptosis of PBMC, due to the production of soluble HGF [8]. Indeed, HGF is a potent immunomodulatory factor that inhibits dendritic cell function along with differentiation of IL-10- producing T (reg) cells, a decrease in IL-17- producing T cells, and down-regulation of surface markers of T-cell activation [27]. Yeung et al. [28] found that hepatic satellite cells give an apoptotic message to T cells and at the same time inhibit antigen-specific T cells activity. TGF- β is a potent anti-inflammatory cytokine that enhances the immunomodulatory properties of MSCs. It suppresses production of proinflammatory cytokines by impact on macrophages and lymphocytes [29, 30].

HGF, as well as for TGF β in the secretome of cancer cells [31], is contained into exosomes, as demonstrated by Choi et al. [32] for adipose derived stem cells, and in a soluble form too. Comparing directly, however, the effects of exosomes and soluble growth factor, highlights the profound difference in the cell response arising. A detailed mechanistic explanation for this difference is currently lacking, and given the molecular complexity of exosomes secreted by cancer and stem cells, represents a significant challenge to define [31]. The possible co-delivery of other factors are aspects to examine. Here we found in AFSC-CM exosome samples other molecules, beside TGF β 1/2 and HGF, modulating the immune response, in particular pentraxin (see supplementary data S1), protein class that provides a regulatory pathway to control the inflammatory response to tissue injury, since induces macrophage polarization, suppresses inflammation and prevents fibrosis [33].

Taking all these observations together, we conclude that the pro-apoptotic effect in PBMC could be due to the presence of soluble HGF, ~~as previously demonstrated [8]~~, but that the exosome pool of proteins, such as HGF, TGF β and pentraxin, could finer modulate the immune response.

Interestingly, these proteins are not uniformly present in exosome deriving from different individuals. This observation can explain the minor effect of some sample, suggesting to take in account the variance in stem cells properties, typical for every single sample, that, moreover, may be unstable during several passages *in vitro* culture.

DECLARATION

Author Disclosure Statement: all the authors report no conflict of interest since nobody has commercial associations that might create a conflict of interest in connection with submitted manuscripts.

Ethics approval and consent to participate

An informed consent allowing the use of clinical data and biological samples for the specified research purpose (protocol 2015/0004362 of 02.24.2015) was signed by all infertile couples before treatment and collected by the Unit of Obstetrics & Gynecology, IRCCS - ASMN of Reggio Emilia (Italy).

Consent for publication

Not applicable since the manuscript does not contain any individual person's data in any form.

Conflict of interest

The authors report no conflict of interest.

We declare that there was not a role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

FB, lab technician, designed and performed experimental protocols; FC, PhD student, acquired experimental data; MZ, lab technician, amniotic fluid cells processing; GC, medical doctor, amniotic fluid cells processing and acquisition of clinical data; ; FF, biologist,

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3 amniotic fluid cells processing; VB, biologist, amniotic fluid cells processing; GBLS, full
4
5 professor, revised the work critically for important intellectual content; TM, PI of the group,
6
7 is responsible of the design of the work, acquisition and interpretation of data for the work
8
9 and drafting the manuscript.
10
11 All authors read and approved the final manuscript.
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Legends to the figure

Figure 1. Characterization of exosomes derived from amniotic fluid (F) and AFSC conditioned medium (CM). A) Western Blot analysis, revealed with anti-CD81, anti-CD63 and anti-CD9, of 40 µg of exosome lysates derived from 3 amniotic fluids and 3 CM of AFSC. Actin detection was performed in order to show the amount of protein loaded in each lane. The graph shows densitometric analysis of Western Blot experiments normalized to actin. Sample 2 of AFSC-CM and samples 4 and 5 of F show higher levels of CD63 and CD81 respectively. B) Representative TEM images of negative staining of exosomes obtained from amniotic fluid, F, and AFSC-CM. Background is shown in the first image (M) and was obtained loading the suspension of the pellet derived after exosome isolation process of fresh culture medium. Bar=100 nm. C) Representative confocal images of hAFSCs labelled with DAPI (blue) and anti-CD81, anti-CD63 and anti-CD9 (green). Bar=10 µm.

Figure 2. Immunomodulation potential of AFSC secretome. A) PBMC, activated with PHA, were treated for 4 days with 80 µg of isolated exosomes (+exo) or of CM deprived of exosomes (-exo). The graph shows the cytofluorimetric analysis of the percentage of PBMC positive for CD4 (grey columns) and CD8 (white columns) surface markers. CD4⁺ cells significantly decrease by 16 % with both the treatments (* P≤0.05). Presented data are the average of three independent experiments. B) PBMC, activated or not with PHA, were treated for 4 days with 80 µg of isolated exosomes (+exo) or of CM deprived of exosomes (-exo). The graph shows the BrdU assay performed three times in quintuplicate. Values were normalized setting the average of control without PHA as 1. Only for PHA group, proliferation declines significantly decrease with both the treatments, but with +exo it gets halved (***) P≤0.0001; ** P≤0.01). C) Western Blot analysis revealed with anti-PARP (total

and cleaved forms were recognized) and anti-Cyclin 2A total lysates of PBMC treated as described above. Actin detection was performed in order to show the amount of protein loaded in each lane. Presented data are representative of three independent experiments. D) The graph shows densitometric analysis normalized to actin (control samples were set as 1) of Western Blot experiments (*** $P \leq 0.0001$ ** $P \leq 0.01$).

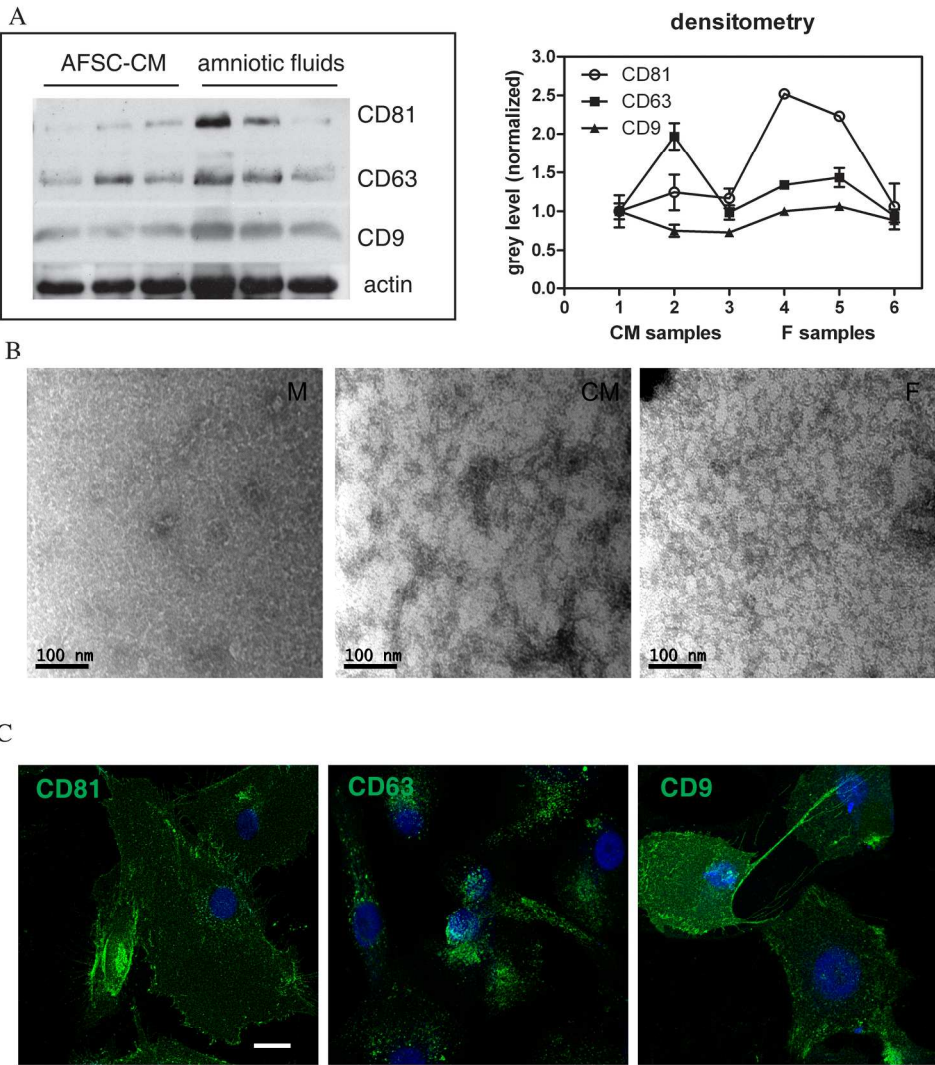
Figure 3. Proteomic profile of exosomes derived from amniotic fluid (F) and AFSC conditioned medium (CM). A) SDS-PAGE 10% stained with Coomassie blue of 2 samples of F and 2 of CM. B) Panther GO-Slim classification of differentially expressed proteins in CM and F according to their biological process (upper) and the subset of cellular process (lower). Cellular process pie charts display classification of differentially expressed proteins divided into 8 subsets for CM sample and only 5 for F. Molecular functions regulated by the differentially expressed proteins as classified by Gene Ontology. C) Panther gene list of the proteins included in cell proliferation subset of CM samples. D) Western Blot analysis, revealed with anti-HGF, anti-TGF β 2 of 40 μ g of exosome lysates derived from 2 CM of AFSC. Actin detection was performed in order to show the amount of protein loaded in each lane. Results of densitometric analysis are into the text.

Table I – Cytofluorimetric analysis of secretome effects on PBMC cell cycle

	Apoptosis	G0/G1	S phase	G2/M
CTRL	8.7 ± 0.9	80.0 ± 1.2	5.7 ± 0.2	5.1 ± 0.3
-EXO CM	31.2 ± 1.3 ***	65.1 ± 2.0 ***	0.9 ± 0.1 ***	1.6 ± 0.2 ***
+EXO CM	9.6 ± 0.5	86.7 ± 1.7 *	0.8 ± 0.2 ***	1.2 ± 0.3 ***
-EXO F	9.2 ± 0.6	78.0 ± 1.0	4.1 ± 0.8	4.8 ± 0.7
+EXO F	8.8 ± 0.8	81.0 ± 1.2	3.5 ± 1.3	4.3 ± 0.9

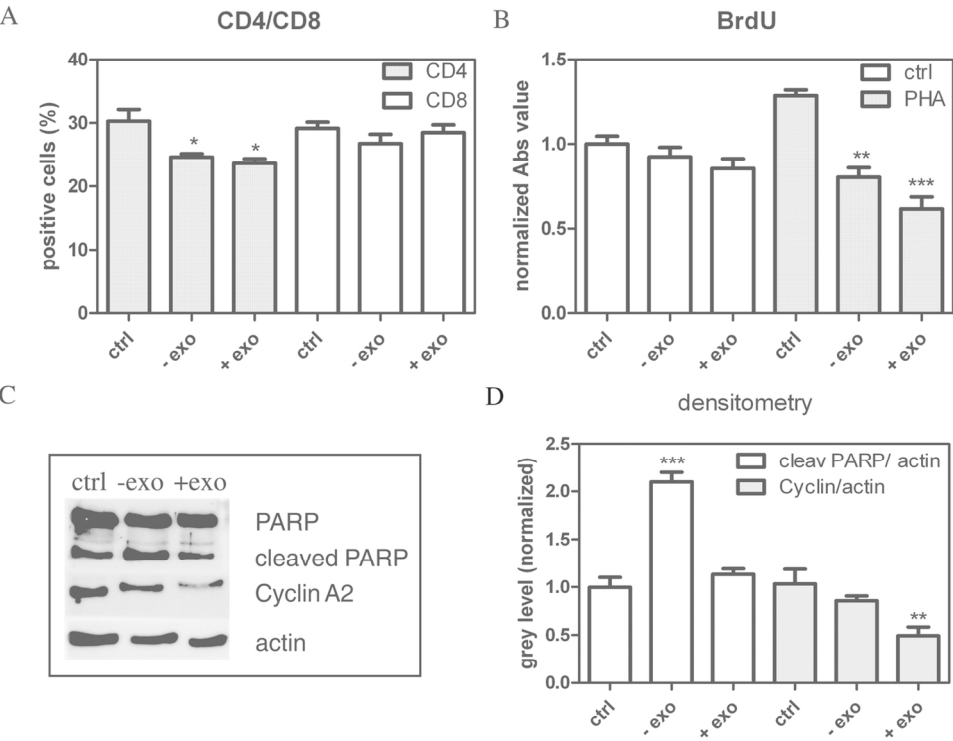
PBMC, treated with PHA, were exposed for 4 days to secretome of AFSC-CM or of amniotic fluid (F): 80 µg of exosomes (+exo) or a comparable quantity of CM depleted of exosomes (-exo). PBMC were then analyzed for PI fluorescence and the percentage of positive cells is shown and the values represent the means of three different experiments ±SD, *P<0.05; ***P<0.0001 vs control; At least 10000 cells were evaluated for each cytofluorimetric analysis.

Figure 1

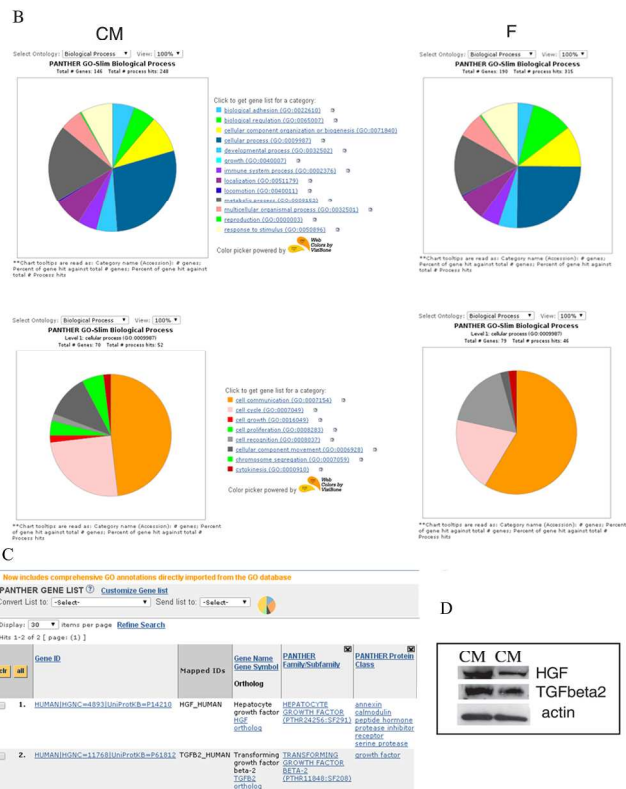


199x248mm (300 x 300 DPI)

Figure 2



127x102mm (300 x 300 DPI)



150x285mm (300 x 300 DPI)