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Title: DEVELOPMENT OF A NOVEL METHOD FOR AMNIOTIC FLUID STEM CELL STORAGE

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Abstract: Background - Current procedures for collection of human Amniotic Fluid Stem Cells (hAFSCs) imply that amniotic fluid cells were cultured in flask for two weeks, than can be devoted to research purpose. However, hAFSCs could be retrieved directly from a small amount of amniotic fluid that can be obtained at the time of diagnostic amniocentesis. The aim of the study was to verify if a direct freezing of amniotic fluid cells is able to maintain and / or improve the potential of the sub-population of stem cells. Methods - We compared the potential of the hAFSCs depending on the moment in which they are frozen, cells obtained directly from amniotic fluid aspiration (D samples) and cells cultured in flask before freezing (C samples). Colony-forming-unit ability, proliferation, morphology, stemness-related marker expression, senescence, apoptosis, and differentiation potential of C and D samples were compared. Results - hAFSCs isolated from D samples expressed MSC markers until later passages, had a good proliferation rate, and exhibited differentiation capacity similar to hAFSCs of C samples. Interestingly, the direct freezing induce a higher concentration of cells positive for pluripotency stem cell markers, without teratoma formation in vivo.
Conclusions - This study suggests that minimal processing may be adequate for the banking of amniotic fluid cells, avoiding in vitro passages before the storage and exposure to high oxygen concentration affecting stem cell properties. This technique might be a reasonable approach in terms of costs and for the process of accreditation in GMP for a stem cell bank.

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Corresponding author signature, on behalf of all the authors:

Modena, April 10, 2017

Dear Editor,

We would like to thank the reviewers for the insightful and encouraging comments regarding our manuscript "DEVELOPMENT OF A NOVEL METHOD FOR AMNIOTIC FLUID STEM CELL STORAGE". We have addressed all of the critiques as indicated in the point-by-point response below. All changes have been marked using red font in the revised manuscript.

Reviewer #1:

1) After the sentence (introduction section, pag 4)" Human amniotic fluid stem cells (hAFSCs) show all these characteristics." I suggest to add the following reference "Antonucci I, Provenzano M, Rodrigues M, Pantalone A, Salini V, Ballerini P, Borlongan CV, Stuppia L. Amniotic Fluid Stem Cells: A Novel Source for Modeling of Human Genetic Diseases. Int J Mol Sci. 2016 Apr 22;17(4)"

We thank the reviewer for all the suggestions, that we applied in the text. The reference has been added after that sentence with number [1].

2) It is important to specify in the paragraph" Amniotic fluid collection " the number of amniotic fluid samples, the mean (±SD) maternal age at amniocentesis and if all women showed normal fetal karyotypes.

We added these clinical data to the M&M section as reported in this sentence: "The hAFSCs were obtained from 25 amniotic fluids collected from women (average age 36.3 ± SD 3.9) ….and no one of the fetuses showed karyotype's abnormality."

3) The crucial point of the manuscript is the applicability/suitability of a direct freezing method of the amniotic fluid. The authors used for their experiments 4 ml of amniotic fluid but unfortunately in many laboratories are processed 2-3 ml of amniotic fluid .Is possible to obtain the same results with a lower amount of amniotic fluid? Authors should discuss this aspect

The decision to collect 4 mL for the direct freezing method (D) was justified by the requirement to correctly compare with the C sample, obtained from 4 mL of fluid seeded in a T25 flask (25 cm^2). In order to process 2-3 ml of amniotic fluid maintaining a similar ratio volume/area, a petri of 35 mm (9cm^2) should be used.

This sentence has been added in "Amniotic fluid collection" section: "The fluid volume seeded in T25 of C samples by cytogenetic laboratory is of 4 mL, as well as the volume directly frozen, therefore the comparison between C and D samples is balanced. However it could be possible to store a minor volume of amniotic fluid, thawing in a smaller surface area."

Reviewer #2:

This research allows interesting evidences on maintenance of multipotency capacity of hAFSCs obtained directly from amniotic fluid aspiration, as demonstrated by results presented in the manuscript, and support in general the conclusion discussed by the Authors. However in the data presented emerging some divergences of results that merit to be clarified.

About the expression of CD44 in Figure 3A and 3B, the Authors referred the variability depending on the donor. If the sample D and C derived by the same donor, how the Authors explain the different expression of CD44 in lane 3 and 4 in WB investigation showed in Figure 3A despite the same level of actin? It would have expected a similar expression in the same sample but the image showed a downregulation of CD44 protein in a D sample (lane 3). To better support the results of flow cytometry in Figure 3B, the Figure 3A needs to be improved. It is reasonable that, if the direct storage technique of sample (sample D) proposed in the research does not change cells profile compared to sample C, it is not justified any differences in the two samples (D vs C) of the same donor.

We thank the reviewer for the suggestion, therefore we decided to show a different image of western blot for CD44 (Figure 3 mod) that better reflects the graph of cytometric analysis. The analysis by Western blot was performed for 9 couples of samples, and only for one sample the intensity of CD44 was higher in C that in D sample (as showed in Figure 3A lane 3 and 4), so in the new version of Figure 3A another set of patient has been shown. Indeed, the quantitative analysis by cytofluorimeter (and not only semi-quantitative as with WB) showed a clear (significant) trend, in particular for CD44, even with an evident error bar.

Please, the Authors will better clarify the variability depending on donor in Result and Discussion Sections.

The variability depending on the donor is an interesting point that we are trying to correlate with clinical data of the mother (smoke habit, gestosis, amniotic fluid volume, diabetic syndrome,…) and of the baby at birth (week of delivery, apgar score,…). We suppose that the intrinsic redox balance could have a role in this clue, as we demonstrated in Maraldi et al., "Nuclear Nox4 Role in Stemness Power of Human Amniotic Fluid Stem Cells" Oxidative Medicine and Cellular Longevity 2015 and Guida et al., "Inhibition of nuclearNox4 activity by plumbagin: effect on proliferative capacity in human amniotic stemcells," Oxidative Medicine and Cellular Longevity. 2013. This last reference has been added.

In fact it has been purposed that ROS homeostasis is an important modulator in stem cell power, independently on the age and the oxygen tension present during cultivation in vitro (this sentence has been added into the text). It has to be demonstrated if this redox status of AFSC (fetal cells), related to a minor stemness power, is linked to a specific anamnesis of the gestational ambient and if this observation could be related to an inclination to aging earlier in the adult life.

DEVELOPMENT OF A NOVEL METHOD FOR AMNIOTIC FLUID STEM CELL STORAGE

Running title: Collection and direct freezing of amniotic fluid cells

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ABSTRACT

Background - Current procedures for collection of human Amniotic Fluid Stem Cells (hAFSCs) imply that amniotic fluid cells were cultured in flask for two weeks, than can be devoted to research purpose. However, hAFSCs could be retrieved directly from a small amount of amniotic fluid that can be obtained at the time of diagnostic amniocentesis. The aim of the study was to verify if a direct freezing of amniotic fluid cells is able to maintain and / or improve the potential of the sub-population of stem cells. Methods - We compared the potential of the hAFSCs depending on the moment in which they are frozen, cells obtained directly from amniotic fluid aspiration (D samples) and cells cultured in flask before freezing (C samples). Colony-forming-unit ability, proliferation, morphology, stemness-related marker expression, senescence, apoptosis, and differentiation potential of C and D samples were compared. Results - hAFSCs isolated from D samples expressed MSC markers until later passages, had a good proliferation rate, and exhibited differentiation capacity similar to hAFSCs of C samples. Interestingly, the direct freezing induce a higher concentration of cells positive for pluripotency stem cell markers, without teratoma formation in vivo.

Conclusions - This study suggests that minimal processing may be adequate for the banking of amniotic fluid cells, avoiding in vitro passages before the storage and exposure to high oxygen concentration affecting stem cell properties. This technique might be a reasonable approach in terms of costs and for the process of accreditation in GMP for a stem cell bank.

Keywords: AFSC, direct freezing; stem cell bank

Abbreviations:

Human amniotic fluid stem cells (hAFSCs) Bovine serum albumin (BSA); carboxyfluorescein diacetate (CFDA); a colony-forming unit (CFU); cumulative population doubling (CPD); 1,4-diazabicyclo(2.2.2)octane (DABCO); 4',6-diamidino-2-phenylindole (DAPI); Ethylenediaminetetraacetic acid (EDTA); good manufacturing practice (GMP); immunofluorescence (IF); phosphate buffered saline (PBS); population doubling time (PDT); Tris-buffered saline (TBS); Triton–X-100 (TxTBS); western blot (WB).

Introduction

The current effort in regenerative medicine is the use of human stem cells that are easy to collect, high proliferating, with large plasticity and without ethical issues. Human amniotic fluid stem cells (hAFSCs) show all these characteristics [1]. Moreover, hAFSCs are becoming an important biomaterial that may be used for cell therapy and tissue engineering applications also in perinatal medicine. The harvesting protocol of hAFSCs is well established in the clinical practice as well as the selection method, based on the c-kit surface marker expression [2]. Although the donor age range has to be considered quite restricted since the sample is usually obtained between the 16th and the 20th week of pregnancy, hAFSC samples may display different stemness properties depending on the donor [3], the gestational age [4] and the number of passages in culture [5]. Usually hAFSCs samples were cultured in flask in cytogenetic laboratories for two weeks, then can be devoted to research purpose, instead of being discarded. However this long time in culture, before the step of freezing and selection, may change the properties of the stem cells sub-population.

In fact, at present, most of the cell expansion procedures are performed under atmospheric O_2 concentration (20% O_2), which is approximately 4–10 times higher than the concentration of O_2 in stem cell natural niches [6] that might cause environmental stress. Indeed, several studies have presented clear evidence regarding the negative influence of ambient O_2 concentration on stem cells, including longer population doubling time, early senescence, DNA damage [7,8], and poor engraftment following transplantation [9].

The central aim of this project is to develop a new, easy to reproduce and good manufacturing practice (GMP) -compatible method to manage amniotic fluid collection in a stem cell bank. Indeed, hAFSCs can be retrieved directly from a small amount of amniotic fluid (3–5 mL) that can be obtained at the time of diagnostic amniocentesis. Theoretically, this amniotic fluid sample can be processed and stored in an optimized banking system, so

stem cells can be isolated or expanded at any time during the prenatal and post-natal period. This procedure is designed according to the current GMP legislation since it is easy, reproducible, inexpensive, and not susceptible to technical errors [10].

Therefore in this study we verify if a direct freezing of the amniotic fluid, taken at the time of amniocentesis, is able to maintain and / or improve the potential of the sub-population of stem cells.

For this purpose, the proliferative rate during *in vitro* passages, before and after the selection process (based on c-kit antigen expression), has been analyzed by population doubling time calculation. The expression of surface mesenchymal markers for c-kit-positive cells was tested by immunofluorescence and Western blot approaches. Since hAFSCs are defined as intermediate between pluripotent and multipotent stem cells [11], the expression of pluripotency genes, such as Oct-4, Sox-2, Nanog, and SSEA-4, has been compared.

Then, hAFSCs, deriving from the two storage methods (samples C and D), were induced to differentiate towards mesenchymal, endodermal and ectodermal lineages, in order to verify if the differentiation potential is comparable.

MATERIAL AND METHODS

Amniotic fluid collection

The hAFSCs were obtained from 25 amniotic fluids collected from women (average age 36.3 ± SD 3.9) between the 16th and 21st week of gestation who undergo amniocentesis at Arcispedale S. Maria Nuova Hospital in Reggio Emilia. During the pre-amniocentesis interview pregnant women were informed about the purpose of the study and any risks related to. The informed consent 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, were signed by the pregnant woman and by a specialist before continuing the exam.

During the amniocentesis procedure 16 mL of fluid necessary for cytogenetic analysis are normally taken and put into 2 tubes of 10 mL; however, it is possible to increase the withdrawal of a few milliliters without affecting the health of the patient and fetus. Therefore, 16 mL were used for diagnostic cytogenetic routine analysis (no one of the fetuses showed karyotype's abnormality) and the additional 4 mL of amniotic fluid (D sample) were collected, encoded and treated with the GMP compatible cryoprotective medium (Cryostor CS10, STEMCELL Technologies) for freezing in liquid nitrogen.

Subsequently, also the any supernumerary (unused) flask of AF cells (C sample) cultured in the Laboratory of Genetics Arcispedale S. Maria Nuova for 2 weeks were trypsinized and treated for freezing. Frozen cells obtained from the same patient (samples C and D) were then thawed (seeded in a T25 flask) and the comparison experiments has been performed. The fluid volume seeded in T25 of C samples by cytogenetic laboratory is of 4 mL, as well as the volume directly frozen, therefore the comparison between C and D samples is balanced. However it could be possible to store a minor volume of amniotic fluid, thawing in a smaller surface area.

Adult Human Tissue isolation and cell culture

Human amniotic fluid stem cells (hAFSCs) were isolated as previously described by De Coppi et al. 2007 [2]. 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 (AmnioChrome II) including an enriched basal medium and growth supplement (all from Lonza, Italy), since it is a medium used for the culture of human amniotic fluid cells obtained from amniocentesis.

Cell proliferation and colony-forming assays

The proliferation rates of hAFSCs, from both C and D samples cryopreserved and stored for at most 1 year at -196°C, were analyzed. After reaching confluence, adherent cells, isolated as described above, were dissociated and seeded in T25 cm^2 flask at a density of $2X10^3$ cells/cm^2, cultured for 3.5 days then detached, counted and seeded again at $2X10^3$ cells/cm^2 concentration. All analyses were performed by individuals blinded to the experimental details. Cell density was expressed as the mean of cells/cm^2 ± standard deviation (SD). Cultures were performed until passage 8. The following formula was applied to all samples for each experimental group (C and D):

$PD = [\log_{10}NH - \log_{10}NS]/ \div \log_{10}2$, where NS is the cell number at seeding (2×10^3 cells/cm^2) and NH is the cell number at harvest. To calculate the cumulative number of population doublings (CPD), the PD determined for each passage was then added to the CPD of the previous passage.

The population doubling time (PDT) was calculated in the phase of exponential growth by the following formula:

$PDT= \log_{10}(2)x\Delta T/\log_{10}(NH)-\log_{10}(N1d)$

where ΔT represents the time between the cell harvesting and seeding, NH represents the harvested cell number and N1d represents the cell number at day 1.

In order to evaluate the clonogenic potential, a colony-forming unit (CFU) assay was performed. hAFSCs samples C and D from passages 3 and 4, isolated from the same patient, were plated at 80 cells/cm^2 and 160 cells/cm^2 in 12-well polystyrene tissue culture plates and stored in basal culture medium for 7 days at 37° C in a 5% CO_2 humidified atmosphere. The medium was then removed, and the cells were fixed with methanol/acetic acid 3:1 for 5 min, and stained with 0.5% crystal violet in methanol for 30 min at room temperature. A positive CFU was identified as an adherent colony containing at least 50 cells and was visualized at

10 magnification using a Nikon TE2000 inverted microscope. At the same time, a limiting dilution assay was performed, plating the cells at 250, 125, 62.5, 31, 16, 8, 4 , 2 and 1 cells/well in a 96-well plate under the CFU assay conditions described above. After 10 days, the number of CFU colonies generated was counted.

Senescence assay

In order to evaluate the presence of senescent cells in hAFSCs of samples C and D, cells at passage 7-9 were seeded in 12-well plates and processed using a senescence β-Galactosidase staining kit (Cell Signaling, MA, USA), according to the manufacturer's instructions. Six samples for each culture condition were analyzed, and the percentage of senescent cells calculated.

Apoptosis assay

hAFSCs (10^6 cells) seeded on coverslips were washed with PBS. After three washes with binding buffer (10mM HEPES, pH7.5, containing 140mM NaCl and 2.5mM $CaCl_2$), the specimen was incubated for 15min, with double staining solution (binding buffer containing Annexin V-Cy3 and CFDA), purchased from Sigma-Aldrich. At the end, the specimen was washed five times with binding buffer, mounted with 15μL of binding buffer and visualized under fluorescence microscopy.

Oncogenic activity assay

One million cells of three hAFSCs samples (C and D) at passage 4 (P4) and melanoma cells were detached and resuspended in PBS and mixed with Matrigel (Becton Dickinson) in a 1:1 ratio. The suspended cells were injected in NOD SCID mice aged 8 weeks (Charles River Laboratoires, Lecco, Italy). All procedures were carried out according to the guidelines approved by the Committee of Use and Care of Laboratory Animals of the University of Modena and Reggio Emilia.

Animal care, maintenance and procedures were conducted in accordance with the Italian Law (D.L. No. 26/2014) and European legislation (2010/63/UE).

Animals were sacrificed when the growth of a tumor mass was incompatible with the mouse well-being, usually 7 weeks, since a tumor mass was not palpable.

For histological examination, tumors or the area of injection were surgically removed and fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin. Serial section of 7 μm thickness were performed. Routine haematoxylin/eosin (H & E) staining was performed to analyze morphological details.

Staining was performed also with DAB reaction after incubation with anti-human mitochondrial protein (Millipore, CA, USA) or anti-Ki67 (Santa Cruz, CA, USA) followed by secondary antibodies (Immunopure Thermo Scie., horseradish peroxidase conjugated, USA). Histological images were obtained with a Nikon Labophot-2 optical microscope equipped with a DS-5Mc CCD color camera (Nikon Instruments, Japan).

Flow cytometer immune-assay

hAFSCs derived from either C or D at first passage were sub-cultured until reaching 80% confluence. Following trypsin dissociation, cells were re-suspended in and confirmed, using flow cytometry, to meet the minimal criteria to be defined as MSCs, according to the standards established by the International Society for Cellular Therapy [12]. Cells were stained with the following antibodies (mAbs): Rabbit anti-Thy-1 (CD90) and Rabbit anti-Endoglin (CD105) (Millipore, CA, USA); Mouse anti-SSEA4 (Cell Signaling, MA, USA); Goat anti-Integrin β1 (CD29), Rabbit anti-HCAM (CD44) (Santa Cruz CA, USA); Mouse anti-5'-Nucleotidase (CD73) (Gene Tex, CA, USA).

The expression of surface markers was analyzed by indirect staining using secondary fluorochrome Alexa 488-conjugated antibodies (Abcam, Cambridge, UK). Non-specific fluorescence was assessed by using the secondary antibody alone. A minimum of 5,000 cells

per sample was acquired and analyzed using FACScan flow cytometer and Lysis II software (both from Becton Dickinson, San Jose, CA, USA).

The same cell samples were analyzed for nuclear stem cell markers, such as Oct4 and Nanog (Cell Signalling, MA USA), after Fixation/Permeabilization process performed with the Staining Buffer Set (Miltenyi Biotec Inc, Auburn, CA, USA) optimized for nuclear staining. After exposure to the five differentiation media, nuclear differentiation markers, such as rabbit anti-Sox9 (Cell Signaling, MA, USA) and rabbit anti-PPAR (Santa Cruz, CA, USA) were analyzed by using the Staining Buffer Set (Miltenyi Biotec Inc, Auburn, CA, USA). Intracellular differentiation markers, such as rabbit anti-CK18, (Santa Cruz, CA, USA), goat anti-Aggrecan, rabbit anti-Collagen-II, mouse anti-Osteopontin, mouse anti-α-fetoprotein (Santa Cruz, CA, USA), and rabbit anti-β-Tubulin III (Millipore, CA, USA) were assessed after the FIX&PERM® Cell Fixation and Cell Permeabilization Kit (Life Technologies,MD, USA).

Cell differentiation assays

hAFSCs obtained from both C and D samples at passage 1 were tested for their ability to differentiate toward osteogenic, adipogenic, chondrogenic, hepatic and neurogenic lineages. For each differentiation experiment, at least 6 samples per condition were used. Cells were seeded at approximately 5X-10^3 cells/cm^2 on culture dishes and kept in culture medium until reaching sub-confluence. The medium was then replaced by differentiating media. Osteogenic differentiation was obtained after 3 weeks of culture in a classic osteogenic medium. Osteogenic medium was composed by culture medium supplemented with 100 µM 2P-ascorbic acid, 100nM dexamethasone, 10mM β-glycerophosphate. The medium was changed twice a week. After 3weeks of osteogenic induction, extracellular calcium deposition by Alizarin red staining, and the expression of osteogenic markers were evaluated.

Chondrogenic differentiation was obtained after 3 weeks of culture in a medium containing 5 % FBS, 100 nM dexamethasone, 10 ng/mL TGF β1 (Sigma-Aldrich, St.Louis, USA), 10 µM 2P-ascorbic acid, 1% v/v of sodium pyruvate (Invitrogen, Italy), 50 mg/mL ITS premix (BD, Franklin Lakes, NJ, USA).

Adipogenic differentiation. Cells were incubated for 3 weeks in adipogenic induction medium (culture medium supplemented with 0.5 mM isobutylmethylxanthine, 1 mM dexamethasone, 10 mM insulin, 200 mM indomethacin, and 10% FBS). Medium was changed twice a week. Lipid-rich vacuoles within the cells were evaluated by Oil red O staining and AdipoRed assay (LONZA, Basel Switzerland), following the manufacturer's instructions.

Neurogenic differentiation occurred after 3 weeks in medium containing 20 µM retinoic acid [13]. Medium was changed twice a week.

Immunofluorescence and confocal microscopy

For immunofluorescence analysis, samples were processed as previously described. Confocal imaging was performed by a Nikon A1 confocal laser scanning microscope, as previously described [14]. Primary antibodies were raised against the following molecules: CK18, AFP, CD29, CD44, Aggrecan, Osteocalcin (Santa Cruz, CA, USA); CD90, RUNX2 (Abcam, Cambridge, UK); SSEA4 Collagen-II, TRA-1-81, GFAP, Oct4, Nanog and CNPase (Cell Signaling, MA, USA); CD73 (Gene Tex, CA, USA); CD105, β-Tubulin III (Millipore, CA, 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.

Western blotting

Whole cell lysates from hAFSCs were processed, as previously described [15]. Primary antibodies were raised against the following molecules: Nanog (Cell Signalling, MA, USA), Oct4, CD44, Sox2, CD29, β-actin (Santa Cruz, CA, USA), CD73 (Gene Tex, CA, USA) and CD105 (Millipore, CA, USA).

Statistical analysis

In vitro experiments were performed in triplicate. For quantitative comparisons, values were reported as mean ± 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 was applied. A P value <0.05 was considered to be statistically significant.

Results

Samples of 25 patients have been collected, stored and then thawed, expanded and selected for c-kit expression. The samples have been analyzed for proliferation rate, expression of pluripotent and mesenchymal markers and differentiation potential.

Samples deriving from the direct collection protocol (D), being composed of a small cell number, need around 7 days to start to growth in culture, since only few cell colonies begin to attach and expand during the first week. Therefore a time of 10-14 days is necessary to obtain a cell number comparable to the one of C just thawed. This data is not surprising since C samples derive from 2 weeks in culture. It is noteworthy that, both for C and D samples, only a minor cells number attach and create the cell culture, suggesting an intrinsic selection of a subpopulation.

Regarding the proliferation rate after the c-kit selection, the two samples grow in a similar manner, as shown in Fig. 1A and B. The graph of Fig. 1A shows the cumulative population doubling (CPD) curves obtained expanding representative hAFSCs samples, derived from C or D collection methods, during several passages in culture. The growth rate is similar and

the two lines overlap, since the numbers of cells at the first passage were analogous as well as the curve slopes. It is noticeable that, for a number of donors, the curve does not begin to sag still at passage 7. The second graph (Fig 1B) shows all the values of population doubling time (PDT) obtained from C and D samples at passage 2, displaying that there is not a significant difference between the average of C and D, but highlights that there is a variability among the donors, since not all the points are concentrated close to the average.

The clonogenic capacity of C and D samples are shown in Fig. 1 C,D where the first graph displays the number of colony forming unit (CFU) obtained after 10 days depending on the cell seeded density: where the initial cell concentration was 80 cells/cm^2 the number of colonies is comparable, while at 160 cells/cm^2, the D samples produce a higher CFU number. On the other hand, the second graph, showing the capacity to generate at least one colony starting from 1 or 2 seeded cells, indicates a better performance for C sample.

The viability of hAFSCs was evaluated with a combined test that highlights the presence of apoptotic cells with a probe for AnnexinV, and the level of cell metabolism, related to the viability, with CFDA probe (Fig. 1E). The intensity of green fluorescence (CFDA) is comparable between C and D samples, as well as the number of red cells (AnnexinV), while the sample incubated with staurosporin (St), inducing apoptosis, shows a decrease in green fluorescence and cells with a red ring are detectable.

The culture *in vitro* has been performed for 1 month for all the samples, and, particularly for some donor, the growth curve displayed a decrease in the proliferation rate stating from the 4th passage, as shown in Fig. 1F. Therefore the senescence grade of C and D samples has been compared, analyzing the beta-galactosidase activity. Images of Figure 1F are representative of the beta-galactosidase assay: the blue staining, indicating senescent cells, is similar for the C and D samples.

The mesenchymal and pluripotent stem cell markers analysis was performed with immunfluorescence (IF), Western blot (WB) and cytofluorimetric experiments looking at CD-44, CD-29, CD-73, CD-90, CD-105, SSEA4, TRA-1-81, Oct4, Nanog and Sox2. Figure 2A-C includes data regarding the pluripotent profile of hAFSCs derived from direct or indirect collection (D and C samples) and analyzed at second passage in culture. Interestingly, the performed quantitative and semi-quantitative analysis clearly displays a significant higher expression of Oct4, Nanog and Sox2 in D samples, rather than in C samples, suggesting the maintaining of a superior stemness capacity thanks to the direct collection protocol.

Since the high expression of embryonic markers, such as Oct4, Nanog and Sox2, could be related to teratogenic capacity, a oncogenic test *in vivo* was performed. One million of hAFSCs, seeded in hydrogel (matrigel), were subcutaneously injected in immunosuppressed mice and the formation of a palpable mass and the mice weight were monitored for 2 months. In hAFSCs treated mice the presence of a mass was not detectable and there was not a decrease in the body weight, unlike in positive control mice, injected with a melanoma cell line. Figure 2D shows that in the area of injection cells of human origin are still present, forming a loose connective -like tissue, without a high proliferative activity.

No differences between C and D have emerged regarding mesenchymal markers, except for CD44 protein that is more expressed in D samples, as demonstrated by flow cytometry (Fig. 3B). Qualitative and semi-quantitative experiments, such as IF and WB of Fig. 3A and C respectively, did not reveal any variability. On the other hand, we noticed that there is a variability depending on the donor, as already demonstrated for proliferation parameters.

We then tested the potential of differentiation into mesodermal (osteo, chondro and adipogenic), endodermal (hepatic) and ectodermal (neurogenic) lineages. For this purpose hAFSCs (C and D samples) were exposed for 3 weeks to the appropriate media of

differentiation and further the expression of protein markers of various tissues were evaluated with IF and flow cytometry, as shown in Fig. 4, 5 and Table I. For instance, cytokeratin 18 and alpha fetoprotein for hepatic differentiation, βtubulin III, GFAP and CNPase for neurogenic, osteocalcin, osteopontin and RUNX2 for osteogenic, PPAR for adipogenic and Sox9, aggrecan and collagen II for chondrogenic. Additionally, alizarin red staining has been used to reveal the mineralized matrix in osteogenic differentiation and red oil staining to demonstrate lipid drops in the adipogenic one. Immunofluorescence and histological investigation shows the capability for both the AFSC sample groups to differentiate in tissues deriving from all the three germ layers, but the analysis of quantitative data (Table I) did not reveal a clear imbalance in the differentiation capacity of C and D samples.

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 [16,17]. However, while many clinical trials currently use MSCs, the rarity of these cells - regardless of source - require vigorous ex vivo expansion prior to clinical use. Such expansion will cause replicative senescence, which limits MSC number for subsequent utilization or adversely alters MSCs functional capacity and therapeutic effects [18]. Concentration of MSCs within tissues is very low, regardless the origin, and it is not possible to isolate 50–200 million MSCs (typically used in clinical trials) from a donor for each therapy. Thus, in vitro expansion of MSCs has become an inevitable option [19].
Cellular senescence is the state of permanent inhibition of cell proliferation due to the telomeres shortening and leads to DNA damage. Oxidative stress is known to play a role in stem cell self-renewal and differentiation and several research groups have shown the relationship among oxidative stress level and both proliferative capacity and senescence of

stem cells [20]. To improve cell therapy efficacy, it is therefore necessary to modulate MSCs in order to enhance their survival upon ex vivo manipulation insults such as oxidative stress. Since counteracting cellular senescence could prolong the proliferative capacity of stem cells, strategies to overcome the stemness decline are essential [21].

Another main challenge for regenerative therapy is to develop an easy, reproducible and fast cryopreservation protocol that allows long-term storage without affecting the characteristics of the cells, thus encouraging the realization of public stem cells banks and allowing their clinical application.

This project is focused on human amniotic fluid as stem cell source, since amniotic fluid stem cells (hAFSCs) are easy to collect, highly proliferating, with large plasticity and without ethical concerns. Stem cells derived from the amniotic fluid are a new source of cells that may have therapeutic value for various diseases of pre- and post-natal life. Transplantation in uterus is used to treat congenital disorders in the fetus using cells capable of self renewal that can augment or replace the organ in the fetus [22]. The advantages of autologous transplants include a low incidence of rejection and infections and eliminate the need for external compatible donor recruitment.

Here we reported for the first time the applicability/suitability of a direct freezing method of cells of amniotic fluid, avoiding *in vitro* passages before the storage, so maintaining the GMP guidelines. This could represent a reasonable approach to store stem cells contained in amniotic fluid in a stem cell bank for clinical purpose in parallel to the storage of the same fluid amount, that may be a rich source of soluble factors or extracellular vesicles potentially interesting for clinical diagnosis or regenerative therapy [23]. This technique might be convenient both in terms of costs, with regard to the materials for the *in vitro* cultures used earlier than necessary, but also for the process of accreditation in GMP which would be required for the steps of *in vitro* expansion.

The stem cell culture profile obtained from the direct collection of amniotic fluid (D samples) has been tested on cells during several passages in culture analyzing the growth rate, the capability to form colonies, the major phenotypes of premature aging, including premature loss of proliferative potential, increased number of senescence-associated–β galactosidase (SA-β-gal)–positive cells. Comparing with data obtained from the stem cell cultures isolated after expansion and cryopreservation (C samples), we did not observe relevant differences, regarding the viability, proliferation capacity and in term of number of senescent or apoptotic cells. The analysis of clonogenic activity showed divergent data. Although both the samples (C and D) were able to form a colony starting from 1 cell/well (limit dilution), demonstrating this capability related to stemness, D samples displayed a better performance only if seeded at higher concentration, suggesting that the stem cell subpopulation obtained with this methods could grow faster if seeded at this experimental density.

Linked to this observation could be the data shown in Figure 2 where D samples display a higher expression of markers of pluripotency, such as Oct4, Sox2 and Nanog. These proteins regulate self-renewal and pluripotency in human embryonic stem (hES) cells. Transplantation of hES cells into immune-deficient mice leads to the formation of differentiated tumors comprising all three germ layers, resembling spontaneous human teratomas. In order to exclude this huge side effect, we tested the potency of teratoma formation in subcutaneous space in immune-compromised mice with addition of Matrigel, and we did not found the presence of cyst after 8 weeks, but only a little loose connective tissue presenting human cells expressing low level of Ki67. Ki-67 is a nuclear protein being associated with cellular proliferation, and Ki-67 positivity confers a poor prognosis of tumor progression. These observations demonstrate that hAFSCs, obtained with the direct method, can maintain the expression of pluripotency-regulating transcription factors without inducing teratoma formation *in vivo*.

hAFSCs expressing Oct4, Nanog, Sox2 are positive also for other stem cell markers, such as SSEA4 [24], and share with BM-MSCs the expression of the mesenchymal surface markers CD29, CD44, CD73, CD90 and CD105 [25]. The quantitative analysis shows that D samples express a significant higher level of CD44, than C samples, but, even if not statistically significant, also cells positive for CD29, CD73 and CD105 are more abundant in D samples. Looking at CD44 or homing cell adhesion molecule (HCAM), it functions in cell adhesion, migration, homing, proliferation and apoptosis of stem cells and have role in stemness maintenance by involving in contact between stem cells/progenitor cells and their cellular niche [26]. The fact that the percentage of positive cells for the essential mesenchymal markers (CD105, CD73 and CD90) are less than 95%, is due to the heterogeneity of this stem cells population [24]. The width of error bar is due to the donor variability, also shown in the Western Blot analysis (Fig. 3A), that is a critical point to be investigated one by one, in order to find a correlation between the hAFSCs profile and the physio-pathological feature of the donor. We purpose that an intrinsic ROS homeostasis, depending on specific ROS sources, is an important modulator in stem cell power, independently on the age and the oxygen tension present during cultivation in vitro [3, 27].

The efficiency in differentiation towards mesenchymal lineages has been compared and histological results obtained for adipogenic and osteogenic markers, such as lipid droplets and calcium deposition respectively, suggest that D samples are more prone to be committed into cells of bone and adipose tissues. Also the quantitative assay indicates that PPAR, as a member of the nuclear receptor superfamily that promote adipogenesis, is more expressed in D samples. Runx2 plays an essential role upstream of osteoblastic differentiation, therefore a high Runx2 expression appears in early stage of osteogenic induction, but Runx2 expression reduces significantly during the differentiation into mature osteoblast, and Runx2 is undetectable during the differentiation into osteocytes [28]. Indeed IF data shows that Runx2

is still expressed into nuclei of C cells, suggesting a delay in the differentiation process, if compared to D samples. Chondrogenic differentiation seems to develop in similar manner for both the cell samples as well as for endodermal and ectodermal differentiation, when we tested the hepatogenic and neural induction, including neuronal and glial cyto-types.

In conclusion, the purpose to cryopreserve amniotic fluid cells separated from the same fluid would be more practical than direct primary isolation of stem cells, which requires further toilsome, time-consuming, and expensive processes, especially while employing current good manufacturing practice (GMP) standards for clinical use of the cells. Hence, cryopreservation of amniotic fluid cells (including the fluid) for afterwards isolation and expansion may be favorable for the banking of specimens not immediately needed and then allows to carry out cell cultures and pre-differentiation with low oxygen concentration only in case of therapy requirement. In fact cells can normally defend themselves against reactive oxygen species (ROS) damage through the use of specific enzymatic or non-enzymatic mechanisms. However, the response of stem cells to oxidative stress is not well understood but it has been related to increased rates of aneuploidy, double-stranded DNA breaks, and faster telomere shortening for MSCs cultured in ambient condition [7]. Furthermore, this study provides new knowledge for optimization of the cryopreservation process and subsequent thawing, and to increase the replicative potential of this biological material in order to obtain sufficient quantities of hAFSCs to be successfully used in clinical practice.

DECLARATION

Author Disclosure Statement: all the authors report no conflict of interest since nobody have 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

MZ, lab technician, designed and performed experimental protocols; FB, lab technician, ~~,~~ designed and performed experimental protocols; FC, PhD student, acquired experimental

data; GC, medical doctor, amniotic fluid cells processing and acquisition of clinical data; FF, biologist, amniotic fluid cells processing; VB, biologist, amniotic fluid cells processing; LB, researcher, interpretation of data; ADP, full professor, final approval of the version to be published; GBLS, full professor, revised the work critically for important intellectual content; TM, PI of the group, is responsible of the design of the work, acquisition and interpretation of data for the work and drafting the manuscript.

All authors read and approved the final manuscript.

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Table I – Cytofluorimetric analysis of differentiation marker expression

Ab	**D**	**C**
OPN	61±3	63±5
SOX9	10±4	14±2
Coll 2	19±3	16±3
PPAR	54±6	46±5
CK18	20±3	18±1
Tub III	59±5	68±6

hAFCSs from 4 samples C and D were exposed to differentiating media for 3 weeks then analyzed for differentiation markers (Osteopontin, SOX9 and Collagen 2, PPAR, Cytokeratin 18 and β-tubulinIII for osteogenic, chondrogenic, adipogenic, hepatic and neurogenic differentiation, respectively). The percentage of positive cells is shown. At least 8000 cells were evaluated for each cytofluorimetric analysis.

Legends to the figure

Figure 1. hAFSCs derived from direct (D) or indirect (C) collection protocol: comparison of the proliferation capability. A) The graph, representative of most of the samples, shows the cumulative population doubling (CPD) calculated until the 7th passage in culture. No differences in the two curves are evident. B) Population doubling time (PDT) values of all C and D samples, evaluated at the second passage in culture. The average values are not significantly different. C) The graph compares the number of colony forming units (CFU) obtained from C and D samples seeded at different density: only in the case of 160 cell/cm^2 D samples display a significant higher value (* $P \leq 0.05$). D) Percentage of well presenting at least one colony after 10 days of culture of hAFSCs (C and D samples) seeded at the density of 1 or 2 cells/well. E) Apoptotic cells in C and D samples: representative immunofluorescence (IF) images of CFDA (green), indicating the viability of the cells, Annexin V (red), showing apoptotic cells, labelling of exposed hAFSCs alone or exposed to staurosporin (St) for 4 hours, as positive control. Bar=20 µm. F) Graph showing the CPD of C and D samples of a donor that, starting from the 4th passage, displays a decrease in the growth rate. Representative images of beta-galactosidase assay performed in C and D samples. Senescent cells are blue stained. Bar=20 µm.

Figure 2. Pluripotent capability of C and D samples of hAFSCs. A) Western Blot analysis revealed with anti-Oct4, anti-Nanog and anti-Sox2 of total lysates of hAFSCs (C and D samples) at passage 2. Actin detection was performed in order to show the amount of protein loaded in each lane. Presented data are representative of three independent experiments. The graph shows densitometric analysis of Western Blot experiments (*** $P \leq 0.0001$). B) Representative confocal images of C and D hAFSCs at passage 2 labelled with DAPI (blue)

and anti-Oct4 (green). Bar=10 µm. C) Graph showing the cytofluorimetric analysis of Nanog expression: C sample (red curve) displays 58 % of positive cells, while D sample (blue curve) 89 %. The black curve is related to the sample of cells incubated only with secondary antibody, as negative control. D) Histological examination of subcutaneous tissue derived from the area of injection of D cells in immunocompromised mice sacrificed after 8 weeks. The first image, stained with hematoxylin and eosin (H&E), shows abundant extracellular matrix with a moderate cell density. DAB reaction for revealing human cells presence, by antibody selective for human mitochondrial protein (HMit), and proliferating cells, by antibody for Ki67. Brown staining indicates positive cells. Bar=200 µm.

Figure 3. Mesenchymal profile of C and D samples of hAFSCs. A) Western Blot analysis revealed with anti-CD44, anti-CD73, anti-CD29, anti-CD105 and anti-CD90 of total lysates of three hAFSCs C and D samples at passage 2. Actin detection was performed in order to show the amount of protein loaded in each lane. B) Quantitative analysis by flow cytometry of the expression of CD44, CD73, CD29, CD105, CD90 and SSEA-4 proteins. The comparison between C and D samples shows a significant difference only for CD44 (* $P \leq 0.05$). C) Representative confocal images of three samples of C and D hAFSCs at passage 2 labelled with DAPI (blue), anti-CD29, -CD105, -CD90 (green) and anti-SSEA-4, -CD44, -CD73 (red). Bar=10 µm.

Figure 4. Differentiation capability of C and D hAFSCs samples into mesenchymal lineages. A) Osteogenic differentiation after three weeks of exposure to osteogenic medium. Evaluation of calcium deposition in the extracellular matrix through Alizarin Red staining: the intensity of red staining is related to the calcium presence typical of mineralizing tissue. Bar=100 µm. Representative confocal images of C and D hAFSCs labelled with DAPI (blue), anti-osteocalcin (OCN) in green and anti-Runx2 in red. Bar=10 µm. B) Chondrogenic differentiation. Representative confocal images of C and D hAFSCs labelled with DAPI

(blue), anti-collagen II (Coll2) in green and anti-aggrecan (Aggr) in red. Bar=10 µm. C) Adipogenic differentiation. Evaluation of intracellular lipid droplets through Red oil staining: the intensity of red staining is related to the lipid droplet presence typical of adipocytes. Bar=100 µm.

Figure 5. Differentiation capability of C and D hAFSCs samples into endodermal and ectodermal lineages. A) Hepatic differentiation. Representative confocal images of C and D hAFSCs labelled with DAPI (blue), anti-cytokeratin 18 (CK18) in green and anti-alphafetoprotein (AFP) in red. B) Neurogenic differentiation. Representative confocal images of C and D hAFSCs labelled with DAPI (blue), anti- Glial fibrillary acidic protein (GFAP) and anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in green and anti-beta tubulin III (Tub III) in red. Bar=10 µm.

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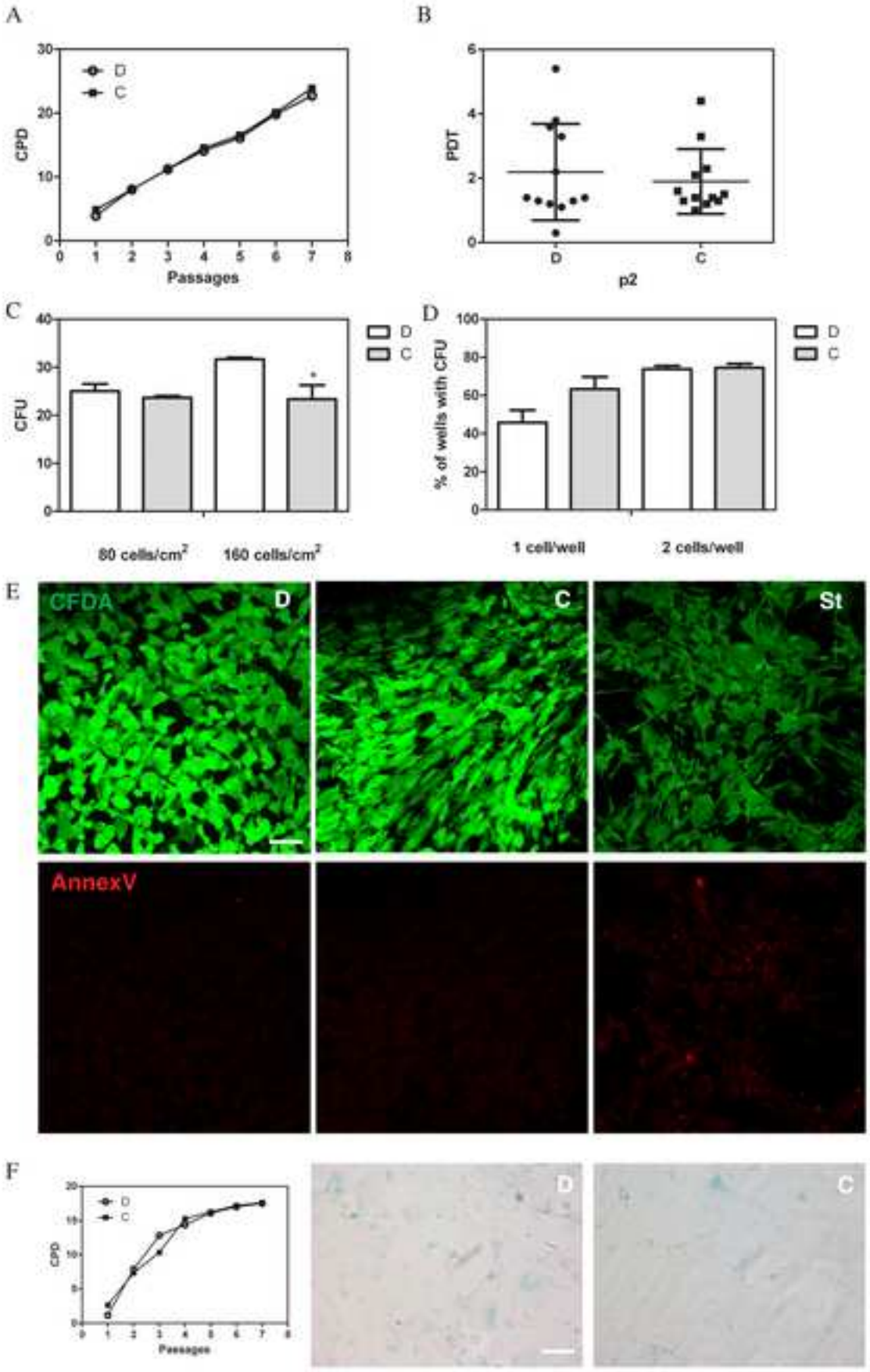

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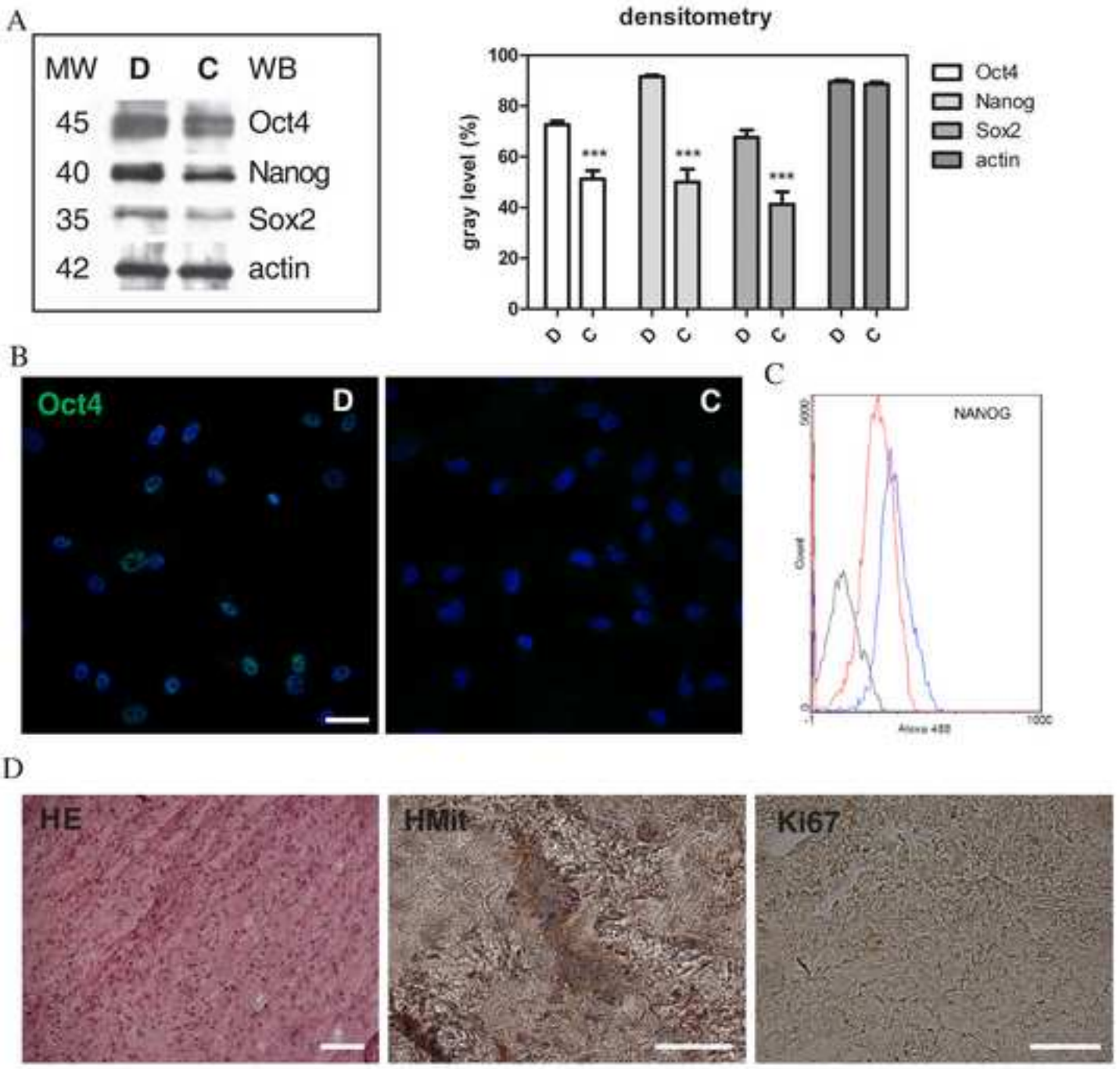

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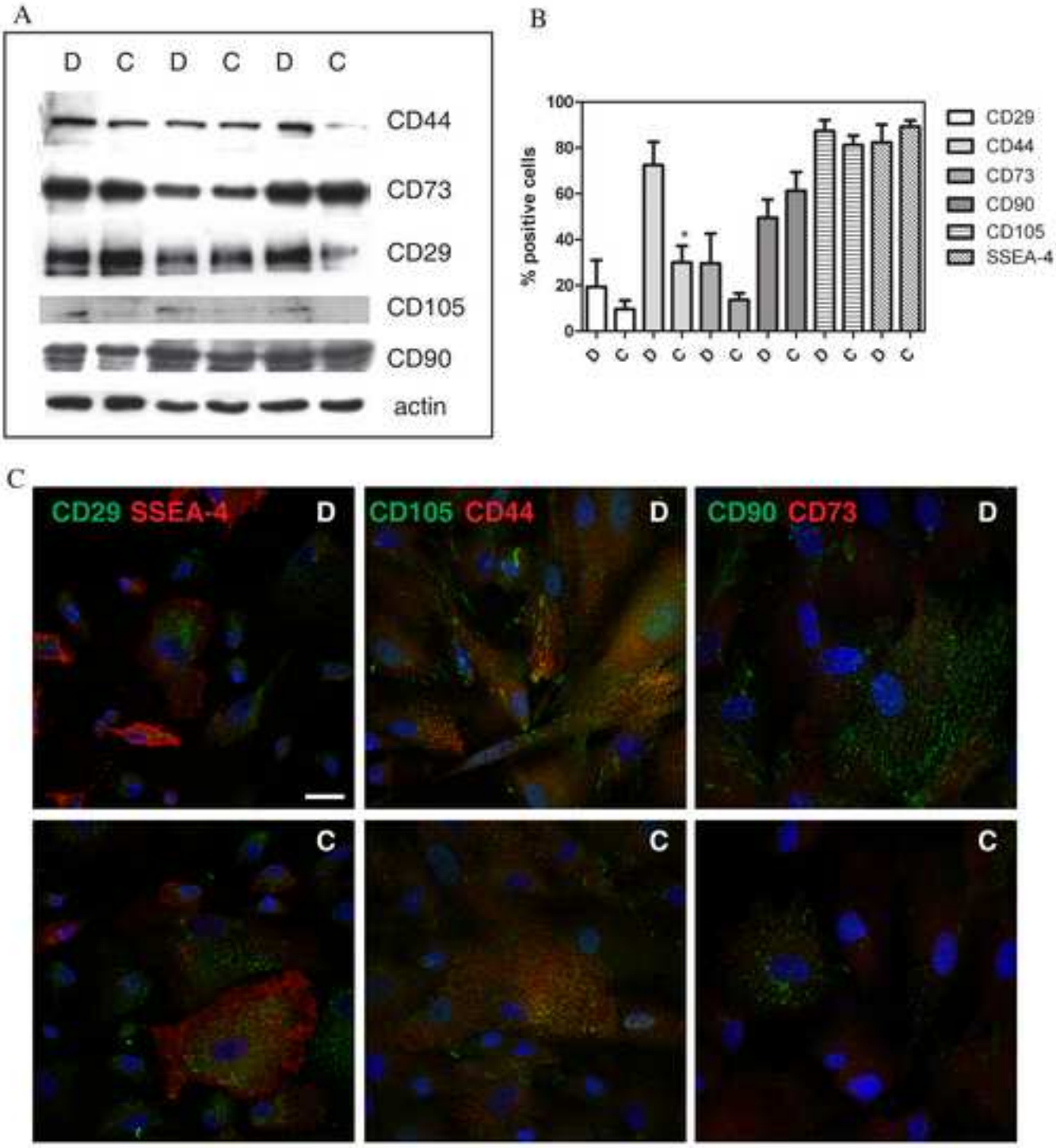

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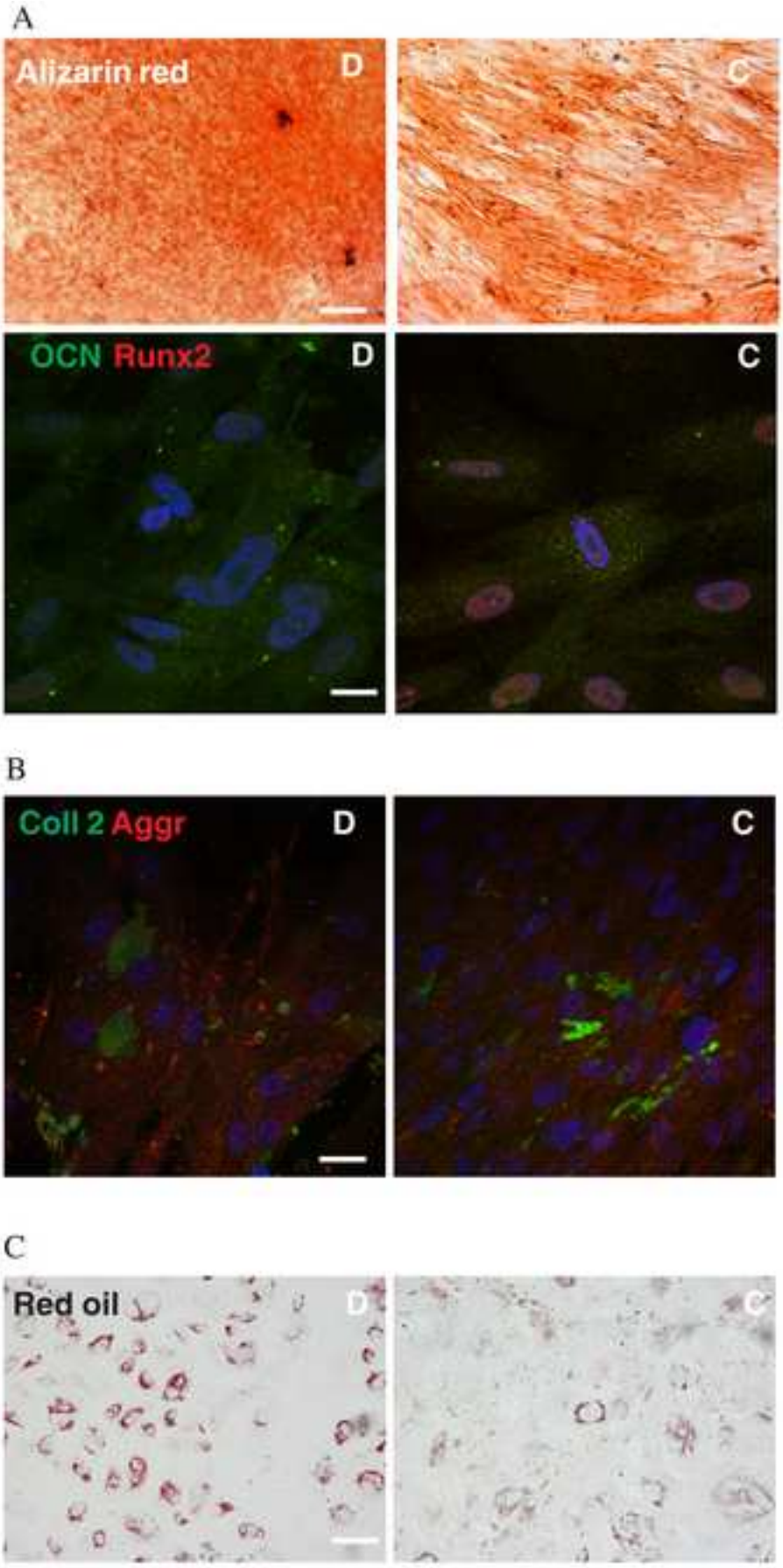

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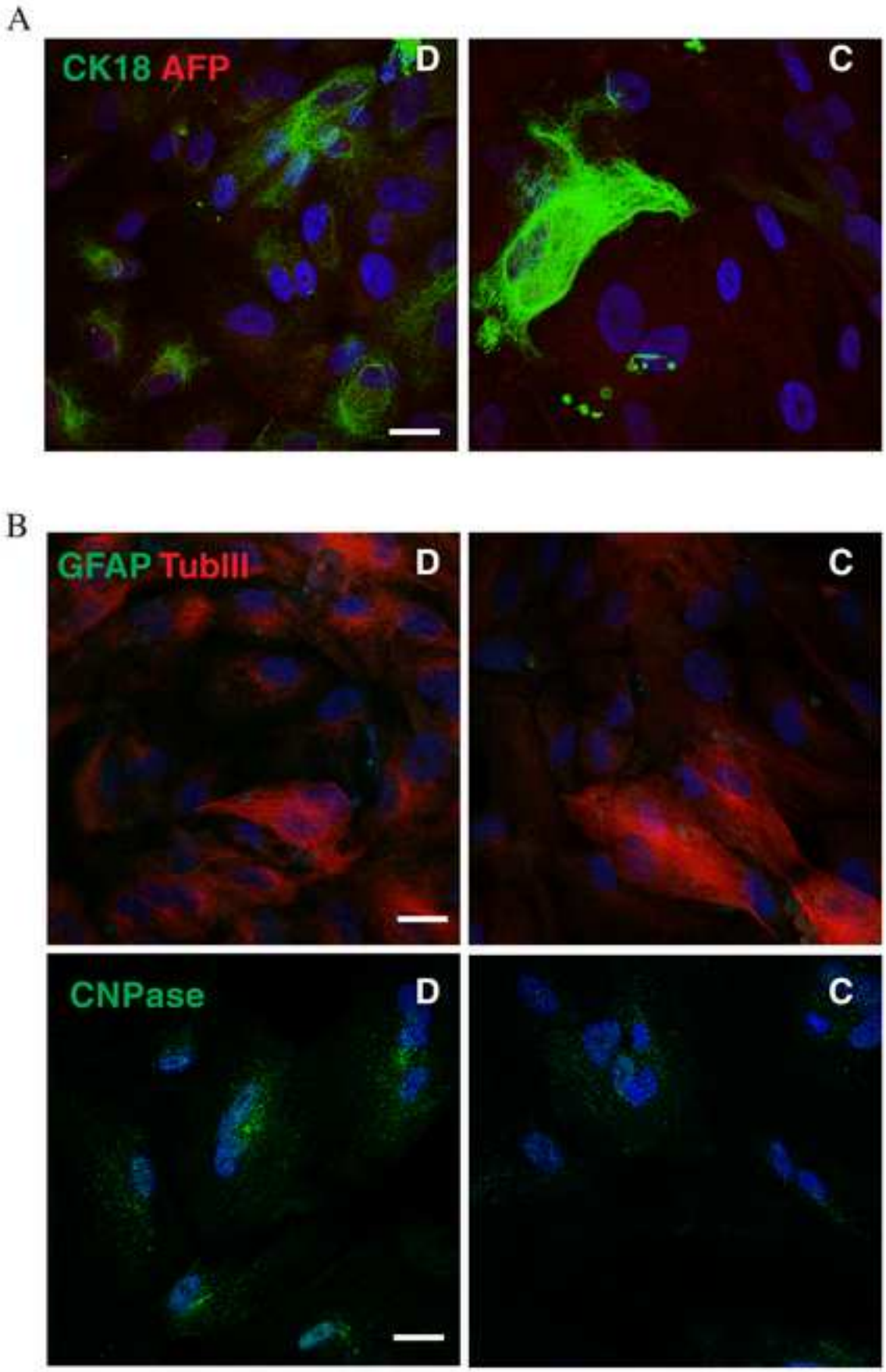

Figure 5