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Amniotic Fluid Cells Proliferation in Normal and Down Syndrome Subjects

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ABSTRACT
Down Syndrome/Trisomy 21 is the most common chromosomal anomaly, and it represents the most common congenital cause of infants' intellectual disability. Subjects with this syndrome are affected by degenerative processes caused by accelerated aging or unknown ethyologies. In recent years, accumulating evidence revealed increased potential of amniotic fluid-derived stem cells to be used in regenerative therapy. Our aim was to assess differences in immunophenotype, cell morphology and proliferation of amniotic fluid cells from normal and Down Syndrome pregnancies using a quantitative cytometry approach. Results revealed the emergence of a population of small sized cells in Down Syndrome derived amniotic fluid cells that are readily visible upon microscopic inspection. Hence, the fluorescence–based quantitative image cytometry determinations showed a tendency of decrease in both cell and nuclei size in trisomy, with no significant modification in nuclei circularity, as measured following actin cytoskeleton and nuclei labeling. The propensity of Ki67 positive cells was found to be increased in Down Syndrome derived cells (48.92%) as compared to normal specimens (28.68%). However, cells in S and G2/M cell cycle phases decreased from 32.91% to 4.49% in diseased cells. Further studies are devoted to understanding the molecular basis of the observed differences in the proliferation ability of Down Syndrome amniotic cells, in order to evaluate the potential therapeutic effect of amniotic fluid stem cells for tissue regeneration in subjects with trisomy and to find correlations between amniotic cells phenotype and patient prognosis.

Keywords: Down syndrome, Trisomy 21, amniotic fluid-derived stem cells, proliferation, cell cycle.

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Introduction

Down syndrome (DS), also known as Trisomy 21, ranges in incidence from 1 to 2 per 1000 live births; however, this rate underestimates the true incidence since it does not include induced and spontaneous abortions[1]. All affected subjects suffer cognitive impairment from birth and present with early onset Alzheimer's disease. However, DS phenotype shows some individual variations. Individuals with DS suffer from many congenital malformations and are at higher risk to develop transient or acute megakaryocytic leukemia[1].

Amniotic fluid (AF) obtained by amniocentesis

could serve as stem cell source to regenerate tissues in DS subjects affected by accelerated aging and degenerative diseases. The origin of amniotic fluid cells is still very much debatable[2,3]. Human AF obtained during the process of amniocentesis was shown to contain a variety of cells originating from embryonic and extraembryonic tissues[4]. Although the majority of cells present are terminally differentiated and have limited proliferative capacity [5,6], a number of studies have demonstrated the presence of a subset of cells with stemness properties [7,8]. However, amniotic fluid stem cells (AFS) are different both from pluripotent embryonic stem cells (ESC) and from multipotent adult stem cells, and may represent a new class of stem cells whose properties of plasticity exist somewhere between embryonic and adult stem cell types. Unlike ESC obtained from the inner cell mass of blastocysts, however, AFS cells do not form tumors after transplantation in mice[9]. In recent years, accumulating evidence has demonstrated the ability of AFS cells to differentiate into multiple lineages[10-15]. As a consequence, AF could represent a safe and easily available source of AFS cells to be used for therapeutic purposes, circumventing any ethical objection, given that amniocentesis is a widely accepted form of prenatal diagnostic testing[16,9,10,17]. Noteworthy, a bank of 100 000 amniotic fluid specimens could potentially supply 99 per cent of the US population with a perfect match for transplantation[18].

The types and properties of amniotic fluid cells vary with gestational age[19]. When grown in culture, AFS cells can be separated into adhering and dividing colony-forming cells, and non-adhering cells [19]. Based on their morphological and growth characteristics, amniotic fluid cells can be classified into three types: epitheloid E-type cells, amniotic fluid specific AF-type cells, and fibroblastic F-type cells[3]. AF-type and E-type both appear at the beginning of cultivation. AF-type cells persist during the cultivation process, while E-type cells soon show a significant decrease. E-type cells have been thought to derive from fetal skin and urine, AF-type cells from fetal membranes and trophoblasts, and F-type cells from fibrous connective tissue and dermal fibroblasts. AF-type cells produce estrogen, human chorionic gonadotropin, and progesterone, which suggest that these cells originate from trophoblast tissue. Also, F-type cells are considered to originate from mesenchymal tissue.

In order to use this source of stem cells for use in tissue regeneration it is fundamental to characterize their proliferative capacity and differentiation abilities. To evaluate the possibility of autologous transfer of AFS cells harvested before birth for therapy of DS adults with degenerative diseases, it is necessary to validate their regenerative capacity.

In this work, we aimed to quantitatively determine the immunophenotipical and morphological changes the DS-derived AF cells are subjected to during in utero growth and to measure the proliferative potential of these cells, as compared to those from normal pregnancies. Our research is important for the characterization of AF cells phenotype in DS, for the potential use of these analyses to predict patient outcome and for the future design of tissue engineering approaches for regenerating affected organs of diseased patients.

Materials and methods

Sample collection

Human amniotic fluid samples were obtained via amniocentesis during routine prenatal genetic testing in the laboratories of Genetic Lab and Cytogenomic. The project was reviewed and approved by the ethics committee of University Ovidius Constanta. Informed consent from patients was obtained for use of amniotic fluid content in research.

Genetic molecular diagnostic

QF-PCR experiments were carried out in the laboratories of SC Genetic lab SRL. Genomic DNA extraction was performed using the QIAamp DNA Mini Kit from QIAGEN. PCR amplification was done on a Verity Thermal Cycler from Applied Biosystems using the QIAGEN Multiplex PCR Kit. Fluorescently tagged primers[20] were purchased from Applied Biosystems. The fragment analysis was carried out on an ABI PRISM 310 Genetic Analyzer. Loading

buffer, running buffer and the polymer necessary for running the analyzer were purchased from MCLAB.

Amniotic fluid cell cultivation

Amniotic fluid was transported within 24 hours to the recipient laboratory for processing. Samples were centrifuged at 110 g for 10 minutes at 4°C for separation of cells. Cell pellets were resuspended in pre-warmed AmnioMAX II media (Life Technologies) and seeded in T25 flasks (Corning). Flasks were maintained untouched for 4 days in a cell culture incubator at 37°C, in humid air containing 5% CO2 (Heraeus). Within 10 days, we obtained an average of 5-6 colonies, which were expanded for cryoconservation and further experiments. Cryostocks were generated using a 10% DMSO (SIGMA) in fetal bovine serum (FBS) (Life Technologies) freezing solution.

Immunofenotyping

Amniotic fluid derived cells were characterized for the presence of hematopoietic and mesenchymal markers at passage 2 by flow cytometry using a FACSVerse instrument (BD Biosciences). For this, 1×105 cells were labeled with phycoerytrin (PE) – conjugated antibodies against CD13, CD14, CD29, CD34, CD45 and CD90 (BD Pharmingen). Cells were washed with PBS containing 2% FBS and incubated 30 minutes with antibodies on ice. Unstained cells were used as negative control. Ten thousand cells per sample were acquired and data was analyzed using FACSuite Software (BD Biosciences). The gating strategy involved the immunophenotyping of two cell populations appearing on the forward (FSC) and side (SSC) scatter dot plot, characteristic for all amniotic fluid cells analyzed.

Cell cycle analysis

To discriminate between cells in separate phases of the cell cycle, propidium iodide (PI) staining of cells in logarithmic growth was performed. Amniotic fluid cells were detached using a 0.05% Trypsin-EDTA solution (Life Technologies) and after cell counting 200000 cells were distributed in FACS tubes. Cells were fixed and permeabilized using cold absolute ethanol and transferred to -20°C overnight. DNA was stained with 10 μg PI in 38 mM citrate buffer containing RN-ase (SIGMA) to block the labeling of intracellular RNA. Cells were stored in the dark at room temperature for 30 minutes and then resuspended in FACS buffer after centrifugation at 1500 rpm for 5 minutes. Ten thousand cells were acquired on FACSVerse (BD Biosciences) using FACSuite Software.

Image cytometry analysis

Morphometry and cell proliferation study was performed using imunofluorescent labeling of cells followed by microscopic automatic scanning of cover slips using TissueFAXS iPlus system (TissueGnostics). Three thousand cells per cm2 were seeded from each individual sample in expansion media in 24-well plates (Corning). After 72 hours cultivation on cover glass, cells were fixed using 4% p-formaldehyde (SIGMA) in PBS for 15 minutes at room temperature. Fixed samples were further permeabilized using a 3 minutes treatment with 0.2% Triton-X-100 (SIGMA) and blocked by an hour incubation in 0.5% BSA (Santa Cruz). Cells were labeled with AlexaFluor 488 – conjugated phalloidin (Molecular Probes), which is a yeast toxin that specifically binds actin filaments for cytoskeleton visualization. To detect proliferating cells, rabbit anti-Ki67 antibodies (NeoMarkers) were used, followed by incubation with AlexaFluor 594 donkey anti-rabbit immunoglobulins (Molecular Probes). After 30 minutes incubation with antibodies, specimens were washed with PBS and treated for 1 minute with a 1:10000 dilution of Hoechst nuclear dye (10 mg/mL stock solution). Samples were then thoroughly washed and mounted using ProLong Antifade Reagent (Invitrogen). Slides were scanned using TissueFAXS Software. Fluorescent signals quantitation was performed using TissueQuest. Values for cell areas and perimeters were generated and percent of Ki67-positive cells was determined by image cytometry analysis.

Results

Chromosomal anomalies are diagnosed following amniocentesis by testing for the presence or absence of specific genetic markers using molecular biology standard assays. To interpret a

result as normal for a particular chromosome, at least two informative markers consistent with a normal genotype are required with all other markers being uninformative. To interpret a result as abnormal for a particular chromosome, at least two informative markers consistent with an abnormal genotype are required with all other markers being uninformative.

Normal allelic pattern is determined by the marker showing two peaks of similar height/area, when the peak ratio is classified as 1:1. Abnormal allelic pattern is determined by either i) marker showing two peaks of differing height/area (peak ratio 2:1 or 1:2) or ii) marker showing three peaks of similar height/area (peak ratio 1:1:1) (Figure1, A). For example, monosomy X pattern is determined by the following conditions:

a) All X and XY markers showing homozygous allelic pattern.

b) The AMELY and SRY peaks are not detected.

c) Marker T1 showing two peaks of differing height/area and the peak ratio is classified as 2:1.

d) Marker T3 showing two peaks of differing height/area and the peak ratio is classified as 2:1.

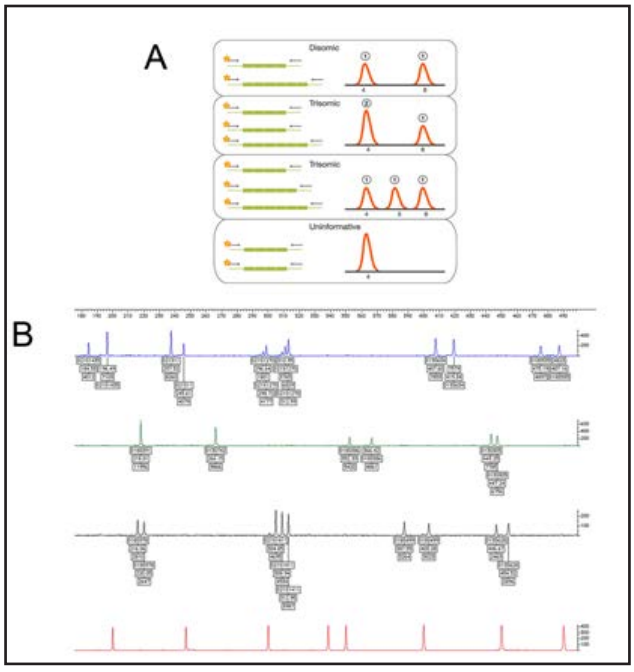

Figure 1: Principle of diagnostic of chromosomal anomalies. A. Schematic view of peaks pattern associated with chromosomal trisomy. B. Example of genetic analysis of X monosomy.

Specifically, the selection of Trisomy 21 samples from Genetic Lab was achieved through QF-PCR testing for a set of five genetic markers located on chromosome 21: D21S11, D21S1437, D21S1409, D21S1411, D21S1435 (20). Figure 2 depicts representative capillary electrophoresis profiles for two genomic DNA samples: Normal and Trisomy 21. As shown in the figure Normal genomes present two distinct or overlapping alleles, whereas Trisomy 21 genomes are readily identifiable by the three allele pattern: either three distinct ones in a 1:1:1 ratio or two overlapping alleles along with a third one in a 2:1 or 1:2 ratio.

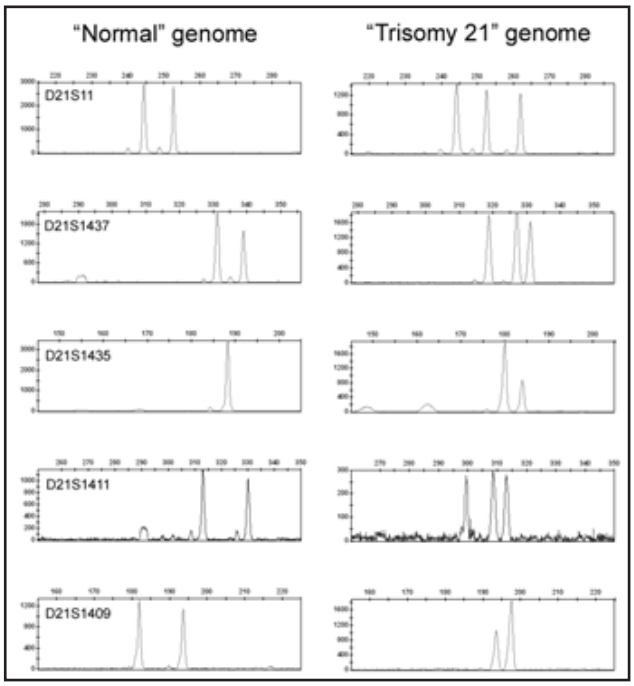

Figure 2: Representative genetic profiles of a "Normal" (left) and a "Trisomy 21" (right) sample. The five different genetic markers are indicated as labels for the capillary electrophoresis profiles represented horizontally.

During our study we have initiated cell cultures from seven samples of amniotic fluid collected from pregnancies with phenotypically normal fetuses and two from DS amniocentesis (Table I).

During flow cytometry analysis, two cell populations were constantly present in AF cells cultures, as identified on FSC/SSC dot (Figure 3A) and contour (Figure 3B) plots. The predominant

population exhibited an increased FSC, indicative of large cell size (Figure 3A,B - P2 gate).

Table I: List of amniotic fluid samples used in the present research:

Symbol used in the paper	Sample code	Disease phenotype
210	PRA15-0210	normal
213	PRA15-0213	normal
214	PRA15-0214	normal
446	PRA15-0076	normal
449	PRA15-0017	normal
450	PRA15-0063	normal
451	PRA15-0047	normal
DS	33023	DS
DS 283	PRA15-0283	DS

Second population had variable propensity between samples and decreased FSC (Figure 3A,B – P1 gate), hence small cell volume. We have analyzed the immunophenotype of each of these two cell populations using markers for hematopoietic (CD14, CD34, CD45) and mesenchymal (CD13, CD29, CD90) lineages.

Results showed that all samples were negative for hematopoietic markers and positive for CD13 and CD90 mesenchymal markers, regardless of their DS phenotype. CD29 is almost absent on all tested cell lines. CD90 had a variable expression between subjects; cells with high and low CD90 expression levels constituted two peaks with variable ratio (CD90 histograms in Figure 3 and 4).

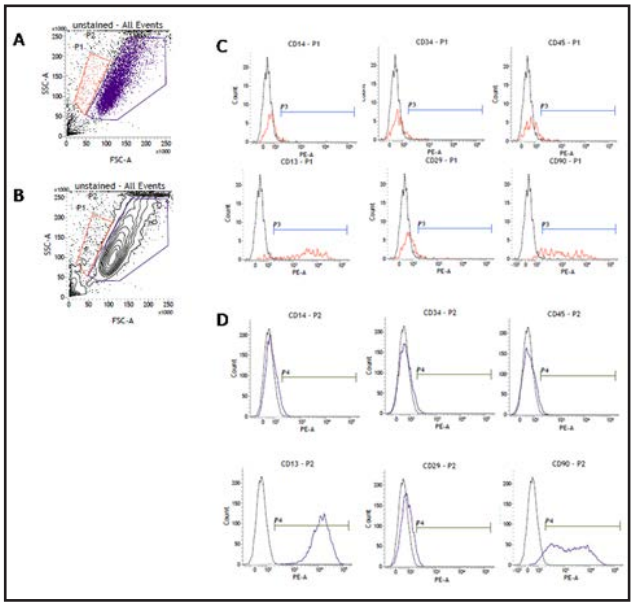

Figure 3: Immunophenotype of normal amniotic fluid cells – line 210. A. FSC/SSC dot plot of AF cells gated as low FSC (P1, red) and high FSC (P2, cyan). B. FSC/SSC contour plot of AF cells showing the limits between the two cell populations. C. Histograms showing the expression of hematopoietic and mesenchymal markers on low FSC cells. D. Histograms showing the expression of hematopoietic and mesenchymal markers on high FSC cells.

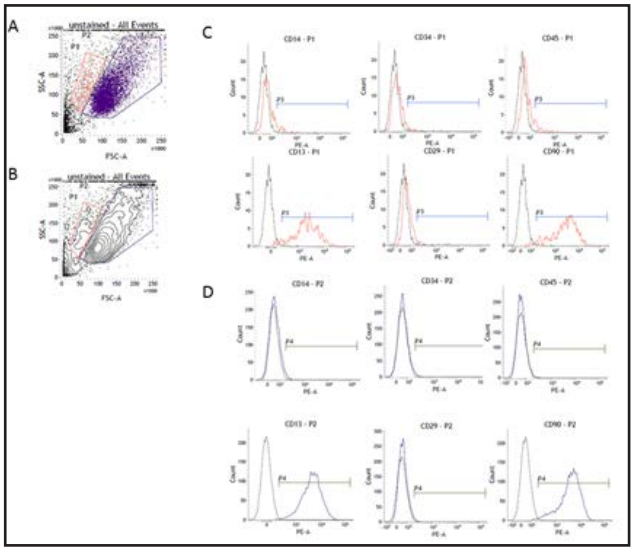

Figure 4: Immunophenotype of DS amniotic fluid cells – line DS. A. FSC/SSC dot plot of AF cells gated as low FSC (P1, red) and high FSC (P2, cyan). B. FSC/SSC contour plot of AF cells showing the limits between the two cell populations. C. Histograms showing the expression of

hematopoietic and mesenchymal markers on low FSC cells. D. Histograms showing the expression of hematopoietic and mesenchymal markers on high FSC cells.

Noteworthy, both cell subpopulations displayed similar expression patterns irrespective of their FSC value (Figure 3 and 4 – C versus D).

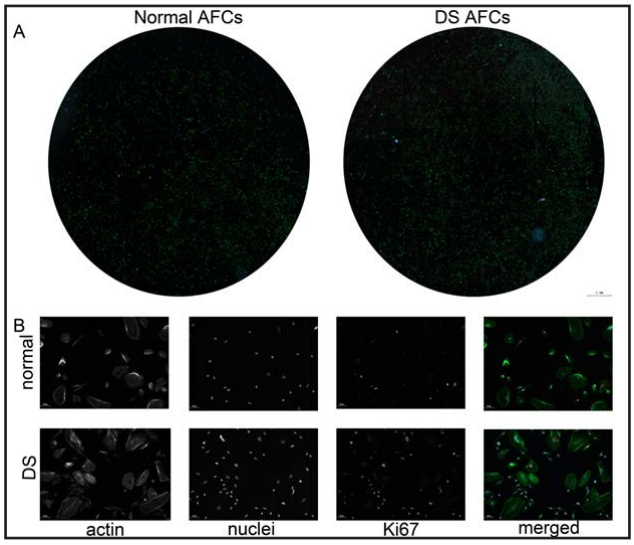

Figure 5: Image cytometry analysis of normal and DS amniotic fluid cells. A. Representative images of cover slips cultivated with normal (PRA15-0210, left) and DS (33023, right) AFCs. Whole sample view was obtained by stitching of all fields of view generated by TissueFAXS iPlus automatic scanning. B. Field of view from normal and DS AFCs scanning. Actin filaments were labeled by AlexaFluor 488-conjugated phalloidin (green), nuclei were stained by Hoechst (blue) and Ki67 nuclear antigen was stained using specific antibodies followed by AlexaFluor 594 – conjugated secondary antibodies (red). Images from each channel and merged overlays are depicted.

We next performed image analysis on seven normal and two DS derived cell lines. The entire sample area was reconstituted (Figure 5 A) and the cytoskeleton visualized together with the expression of Ki67, a nuclear marker expressed only by cycling cells, in both normal and DS cells (Figure 5B). We observed the two characteristic epithelioid and fibroblastoid cell subpopulations and the apparent increase in the propensity of small sized cells in DS samples. To obtain quantitative data, we performed image analyses aimed at measuring cell and nuclei morphometric parameters.

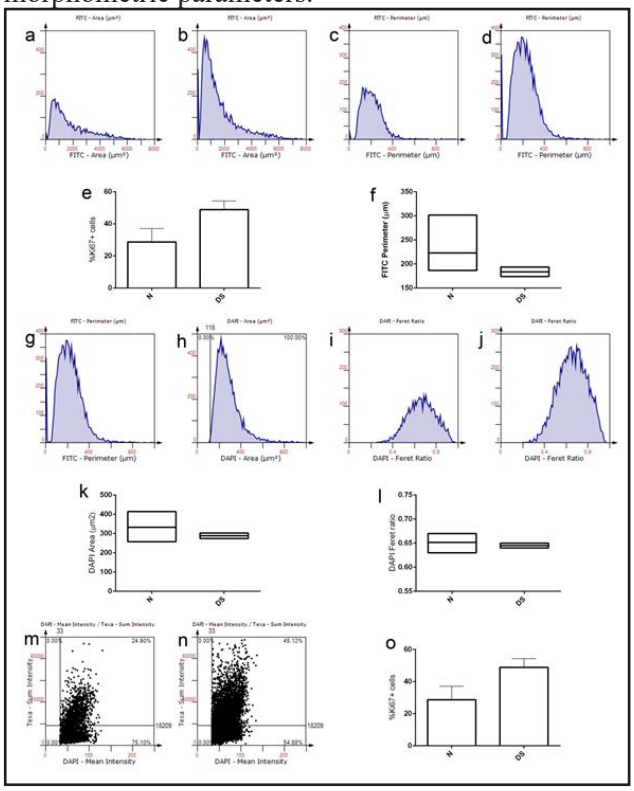

Figure 6: Quantitative analyses of cell size parameters and proliferation. Cell area values distribution in representative normal (a) and DS (b) AF cells are shown in histograms. Minimum, maximum and average values of cell areas are depicted in the subjacent graph (e) for normal (n=7) and DS (n=2) lines. Cell perimeter values distribution in representative normal (c) and DS (d) AF cells are shown in histograms. Minimum, maximum and average values of cell perimeters are depicted in the subjacent graph (f) for normal (n=7) and DS (n=2) lines. Nuclei area values distribution in representative normal (g) and DS (h) AF cells are shown in histograms. Minimum, maximum and average values of nuclei areas are depicted in the subjacent graph (k) for normal (n=7) and DS (n=2) lines. Nuclei ferret ratios distribution in representative normal (i) and DS (j) AF cells are shown in histograms. Minimum, maximum and average values of nuclei ferret ratios are depicted in the subjacent graph (l) for normal (n=7) and DS (n=2) lines. Representative scattergrams displaying % of Ki67 positive cells in normal (m) versus DS (n) AF cells are shown together with the mean percentages and standard deviations (o).

Hence, we measured cell areas and perimeters based on actin filaments fluorescent staining, and

nuclei areas and ferret ratios (circularity) based on Hoechst staining. Results showed a decrease in cell and nuclei size with no significant modification of nuclear shape (Figure 6 – a-l).

In order to assess the fraction of proliferating cells cytometric measurements were performed based on Ki67 nuclear staining. Quantitative analyses scattergrams (Figure 6 – m, n) shown a significant increase of %Ki67+ cells in DS subjects derived AF cells (48.92%, SD = 5.37, n=2) as compared to normal samples (28.68%, SD = 8.44, n=7).

With a view to examine in more detail the potential cause of this increase in cell proliferation, we also performed a cell cycle analysis by flow cytometry following propidium iodide staining. Our data showed that in DS derived AF cells the proportion of cells in G0/G1 phases increased (from 61.34% to 84.89%), while the fraction of cells in S and G2/M decreased (from 20.89%+12.02% to 2.69%+1.80%), when compared with normal cells (Figure 7).

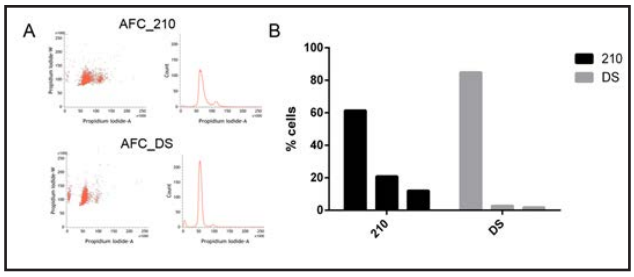

Figure 7: A. Representative flow cytometry analysis of cell cycle distribution of cells from normal (upper diagrams) and DS (lower diagrams) amniotic fluid cells. B. Graph depicting the proportion of cells in each phase of the cell cycle.

Discussion

Amniocentesis is performed as routine clinical diagnosis procedure for fetal genetic prenatal diagnosis. Therefore, amniotic fluid can be readily harvested as a source of stem cells for potential clinical application. Thus far they have been used in pre-clinical settings to treat a variety of diseases such as osteogenesis imperfecta, congenital diaphragmatic hernia, Parkinson's disease and cancer with encouraging results[21]. Finally, their usefulness for iPS (induced pluripotent stem) generation is very likely to expand their future clinical use even further [21].

Our flow and image cytometry results have confirmed the existence of two clusters of cell populations in amniotic fluid: one with low FSC and one with high FSC. The low FSC cells are probably the ones described as epithelial (E-type) AF cells, while high FSC cells are possibly those named AF-type and fibroblastoid (F-type) cells [3], based on cell size comparisons. These were also observed on scanned fluorescent microscopy specimens both in normal and DS subjects. However, in DS-derived cell lines we observed an increase in the propensity of small fibroblast-like cells. This impacted negatively on the average cell and nuclei size determined following actin and nuclei labeling. Further studies will be devoted to understand this phenotype and the functionality of this cell population. Moreover, DS-derived cells showed a significantly increased percentage of cycling cells. However, the majority of cells were found to be arrested in G1 phase, indicating a defect in cell cycle control mechanisms that merits additional investigations.

Conclusion

Our studies thrived to generate a quantitative characterization of cell immunophenotype, morphology and proliferation in normal and DS AFS cells obtained from second-trimester pregnancy during routine genetic testing. Data suggest that allogeneic transfer of AFS is advisable for treatment of DS patients' degenerative diseases. Benefits of autologous treatments are limited by the early onset of proliferation defects that could humper the regeneration of affected tissues. The understanding

of cell and molecular changes generated by genetic diseases, specifically DS, could lead to new approaches in the evaluation and therapeutic treatments of affected patients.

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