Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application

Nadia Sessarego,[1] Alessia Parodi,[1,2] Marina Podestà,[1] Federica Benvenuto,[3,4] Massimo Mogni,[5] Valentina Raviolo,[1] Mario Lituania,[5] Annalisa Kunkl,[6] Guido Ferlazzo,[7] Franca Dagna Bricarelli,[5] Antonio Uccelli,[3,4] and Francesco Frassoni[1]

[1]Centro Cellule Staminali e Terapia Cellulare, Ospedale San Martino, Genova; [2]Laboratorio di Biologia Cardiaca e Vascolare, Clinica delle Malattie Cardiovascolari, DIMI, Università di Genova; [3]Unità di Neuroimmunologia, Dipartimento di Neuroscienze Oftalmologia e Genetica and [4]Centro di Eccellenza per la Ricerca Biomedica, Università di Genova; [5]Dipartimento di Scienze Genetiche, Perinatali e Ginecologiche, Ospedale Galliera, Genova; [6]Divisione di Anatomia Patologica, Ospedale San Martino and [7]Dipartimento di Patologia Umana, Università di Messina, Italy

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Correspondence:
Francesco Frassoni, Centro Cellule Staminali e Terapia Cellulare, Ospedale San Martino, L.go R. Benzi 10, 16132 Genova Italy. E-Mail: francesco.frassoni@hsanmartino.it

The online version of this article contains a supplemental appendix.

ABSTRACT

Background
Mesenchymal stromal cells are multipotent cells considered to be of great promise for use in regenerative medicine. However, the cell dose may be a critical factor in many clinical conditions and the yield resulting from the *ex vivo* expansion of mesenchymal stromal cells derived from bone marrow may be insufficient. Thus, alternative sources of mesenchymal stromal cells need to be explored. In this study, mesenchymal stromal cells were successfully isolated from second trimester amniotic fluid and analyzed for chromosomal stability to validate their safety for potential utilization as a cell therapy product.

Design and Methods
Mesenchymal stromal cells were expanded up to the sixth passage starting from amniotic fluid using different culture conditions to optimize large-scale production.

Results
The highest number of mesenchymal stromal cells derived from amniotic fluid was reached at a low plating density; in these conditions the expansion of mesenchymal stromal cells from amniotic fluid was significantly greater than that of adult bone marrow-derived mesenchymal stromal cells. Mesenchymal stromal cells from amniotic fluid represent a relatively homogeneous population of immature cells with immunosuppressive properties and extensive proliferative potential. Despite their high proliferative capacity in culture, we did not observe any karyotypic abnormalities or transformation potential *in vitro* nor any tumorigenic effect *in vivo*.

Conclusions
Fetal mesenchymal stromal cells can be extensively expanded from amniotic fluid, showing no karyotypic abnormalities or transformation potential *in vitro* and no tumorigenic effect *in vivo*. They represent a relatively homogeneous population of immature mesenchymal stromal cells with long telomeres, immunosuppressive properties and extensive proliferative potential. Our results indicate that amniotic fluid represents a rich source of mesenchymal stromal cells suitable for banking to be used when large amounts of cells are required.

Key words: amniotic fluid, fetal stem cells, adult mesenchymal stromal cells, karyotype

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Introduction

Multipotent mesenchymal stromal cells, also known as mesenchymal stem cells (MSC)[1] were first isolated from bone marrow and initially described as precursors for fibroblasts or stromal cells.[2] More recently, their multilineage differentiation capacity has been extensively characterized. It has been shown that MSC are able to differentiate into mature cells of multiple mesenchymal tissues.[3,4] Moreover, a wider differentiating potential of MSC has been reported.[5,6]

MSC represent a rare population (approximately 0.001-0.01% nucleated cells) of adult human bone marrow cells, but they can be also identified in other adult tissues such as muscle, periosteum, adipose and other connective tissues.[7,8,9,10]

Adult MSC can be readily isolated, exploiting their adhesive property, and extensively expanded. Previous studies demonstrated that single cell-derived clones are heterogeneous and that they contain at least two subpopulations of cells: (i) small, spindle-shaped, rapidly self-renewing MSC and (ii) larger, slowly-renewing MSC.[11] It has also been observed that the subset of rapidly self-renewing MSC is able to preferentially engraft in mice; thus they appear more promising for clinical applications.[12]

MSC lack hematopoietic markers such as CD14, CD34, CD45 but express several surface proteins including SH2, SH3, CD29, CD44, CD71, CD90, CD106, and CD166.[1] They are considered to lack a specific marker although GD2, a neural ganglioside, has recently been proposed to be such a marker.[13] MSC have immunomodulatory properties because they may inhibit inflammation and immunological responses both *in vitro* and *in vivo*.[14,15,16] When administered *in vivo*, they improve the outcome of allogeneic transplantation by promoting hematopoietic engraftment[17] and also hamper graft-versus-host disease.[18,19] *In vivo* injection of MSC improved the course of chronic progressive experimental autoimmune encephalomyelitis, the mouse model of multiple sclerosis.[20,21]

Given their multipotentiality, MSC may be considered a powerful tool for tissue repair and gene therapy. Large numbers of MSC would be needed for regenerative medicine but the frequency and expansion capacity of adult MSC are limited and may decrease with age.[22] Thus, alternative sources of MSC need to be explored.

Fetal tissues, such as liver, bone marrow, blood, kidney, lung and spleen, have been found to be rich sources of MSC.[23,24] In the current study, MSC were successfully isolated from the amniotic fluid, as previously suggested.[25] Fetal cells were extensively expanded in different culture conditions to assess whether these cells can be produced on a clinical scale and, subsequently, to optimize large-scale production. MSC from the amniotic fluid (AF-MSC) were expanded and then were analyzed for chromosomal stability to validate their safety for potential utilization as a clinical grade cell therapy product.

Design and Methods

Specimen collection

Amniotic fluid samples (n=23) were collected in the second trimester (median gestational age 16 weeks, range 15-17 weeks) for prenatal diagnosis. Normal bone marrow samples (n=10) were harvested by multiple aspirations from iliac crests of healthy donors during procedures for allogeneic bone marrow transplantation. All specimens were collected after obtaining written informed consent.

Isolation and expansion of MSC from amniotic fluid and bone marrow

Specimens (2mL) were centrifuged for 10 min. at 400g and incubated in Mesencult medium (StemCell Technologies Inc., Vancouver, BC, Canada) at 37°C with 5% CO_2. After 72 hours, non-adherent cells were discarded and fresh medium added. Cells were cultured for 12-15 days, harvested with 0.05% trypsin-EDTA (Euroclone, Wetherby, West York, UK) for 5 min at 37°C and replated. Thereafter, adherent cells were subcultured until 80% confluence at weekly intervals. Mononuclear cells from bone marrow samples were isolated by centrifugation on Ficoll-Hypaque (Eurobio-Les Ulis Cedex B) as previously described.[26] Subsequently, adherent cells, which represent a MSC-enriched population, were cultured in the same way as the amniotic fluid cells.

Flow cytometry

The purity of the MSC population was determined by the lack of hematopoietic antigens CD14-FITC (BD), CD34-PE (BD), CD45-PerCP (BD), the lack of endothelial surface markers CD31-PE (BD Pharmingen, S. Diego, CA, USA), CD144 (BD), von Willebrand (BD), KDR (Sigma) and the expression of mesenchymal cell-associated antigens CD73-PE (BD Pharmingen), CD105-(Caltag) CD106-PE (Southern Biotech), CD146-FITC (BD Pharmingen), CD44-FITC (BD), CD29-PE (Coulter), and CD166-PE (BD Pharmingen) at each passage. HLA-I-APC (BD Pharmingen), HLA-II-PE (BD), CD80 (BD) and CD86 (BD) were evaluated to assess the immunogenic phenotype. 7-amino-actinomycin D (7-AAD, Sigma) was used to evaluate cell viability of the initial preparation of not fixed samples (*for details see the Online Supplementary Appendix 1).*

Functional characteristics

The ability of fetal and adult MSC to differentiate into adipogenic and osteogenic cells was determined as previously described.[3] Briefly, to assess osteogenic differentiation MSC were cultured in Osteogenic medium (Cambrex, Walkersville, MD, USA) for 2 weeks.

Histochemical analysis of cell layers was performed evaluating alkaline phosphatase (Sigma). Adipogenic differentiation was assessed by stimulating confluent MSC with Adipogenic medium (Cambrex, Walkersville, MD, USA) for 3 weeks, then staining the cells with Oil-Red O (Bioptica, Milan, Italy).

Colony-forming unit-fibroblast (CFU-F) assay

After a median of 13 days of culture (range, 12-15) in a humidified atmosphere (37°C, 5% CO_2), the number of CFU-F was scored in terms of well-defined colonies per milliliter of amniotic fluid plated to initiate the culture. Colony growth was evaluated by staining the CFU-F with May-Grünwald-Giemsa solution.

Growth curves at different cell plating densities

The expansion of AF-MSC and of MSC from the bone marrow (BM-MSC) was assessed up to the sixth passage, seeding MSC at four different plating densities: 1) group A: 400 cells/cm^2, 2) group B: 1000 cells/cm^2, 3) group C: 2000 cells/cm^2, 4) group D: 4000 cells/cm^2. Each passage was performed at weekly intervals to avoid cell confluence during the culture. The number of population doublings was calculated at each passage as $\log_{10}(N/N_0)\times3.33$, where N represents the number of cells harvested at the end of the period of growth and N_0 is the number of cells at the previous passage.

Analysis of telomere length

The telomere length of ten samples from each source of MSC was evaluated using the Telomere PNA Kit/FITC (Dako), as described by the manufacturer. The relative telomere length value was calculated as the ratio between the telomere signal of each sample and control cells with a correction for the DNA index of G_0/G_1 cells. The above relative telomere length value indicates that the average telomere fluorescence of MSC samples was expressed as a percentage of the telomere fluorescence of the 1301 control cell line.

Hematopoietic assays

Colony-forming cell (CFC) assay

To determine the frequency of colony-forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), different volumes of amniotic fluid (from 1 to 5 mL) were plated in complete semisolid medium (StemCell Technologies Inc., Vancouver, BC, Canada) as previously described.[26] After 14 days of incubation at 37°C in a humidified 5% CO_2 atmosphere, the presence of colonies was evaluated using an inverted microscope applying standard criteria for their identification.

Long-term culture-initiating cell (LTC-IC) assay

This assay was performed by seeding a volume of amniotic fluid (from 1 to 5 mL) in dishes over a feeder layer of irradiated (1500 cGy) murine stromal cells, as previously described.[27]

Karyotype analysis

MSC derived from amniotic fluid were analyzed at the fourth and sixth passages (*for details see the Online supplementary Appendix 2*).

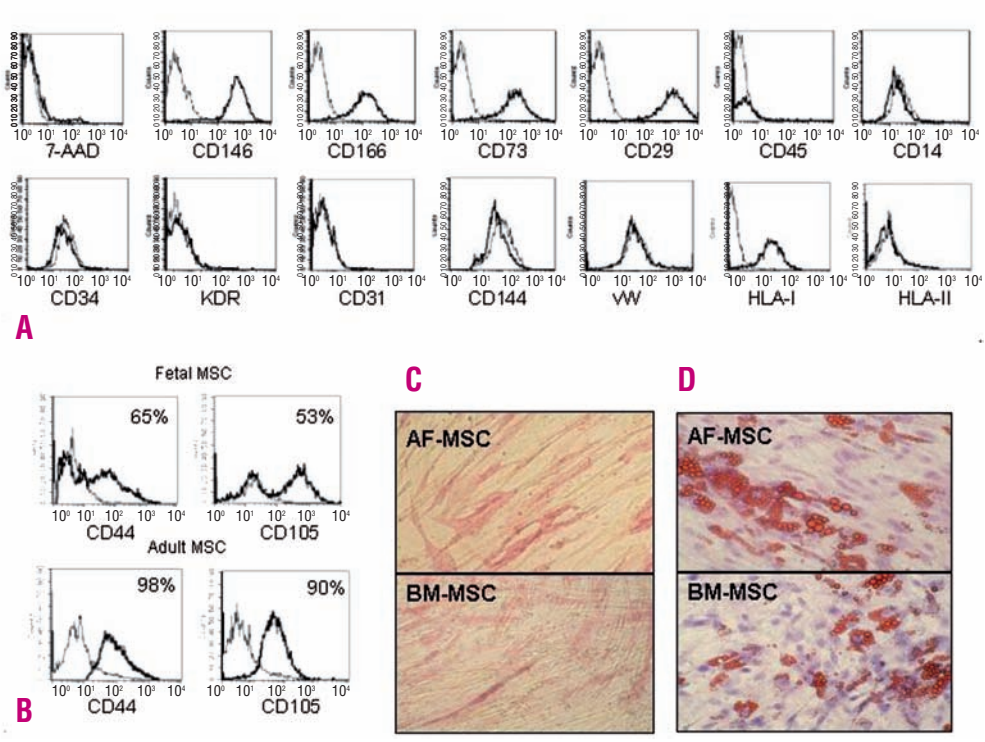

Figure 1. MSC viability, immunophenotype and differentiation capacity. A. Overlaid histograms of cells stained with labeled hematopoietic, endothelial and mesenchymal antibodies (bold line) and the respective isotype control (thin line). B. CD44 and CD105 expression is represented as the median percentage of fetal and adult MSC positively stained by the respective antibodies ($p<0.001$, t-test, for both antigens). C. Osteogenic differentiation of fetal and adult MSC. D. Adipogenic differentiation of fetal and adult MSC.

Assays for neoplastic transformation

A soft agar assay was performed to assess transformation *in vitro*. Briefly, 0.5% sea plaque agar (Biospa) was layered into plate wells as the base layer. Positive control (NB4 cell line) and test cells were resuspended in 0.3% sea plaque agar and overlayered. Following 2 weeks at 37°C in a humidified 5% CO_2 atmosphere, the presence of colonies was evaluated using an inverted microscope.

In vivo tumorigenesis was assessed with MSC expanded from different amniotic fluid samples (n=2) for 6 weeks of culture. The MSC were injected into eight NOD SCID mice. For each tested samples of amniotic fluid, two mice were infused with 2×10^6 MSC into the tail vein and two mice were injected subcutaneously with 8×10^6 MSC. Three months later the mice were sacrificed and underwent gross histological analysis. The design of the study was approved by the local Ethical Committee.

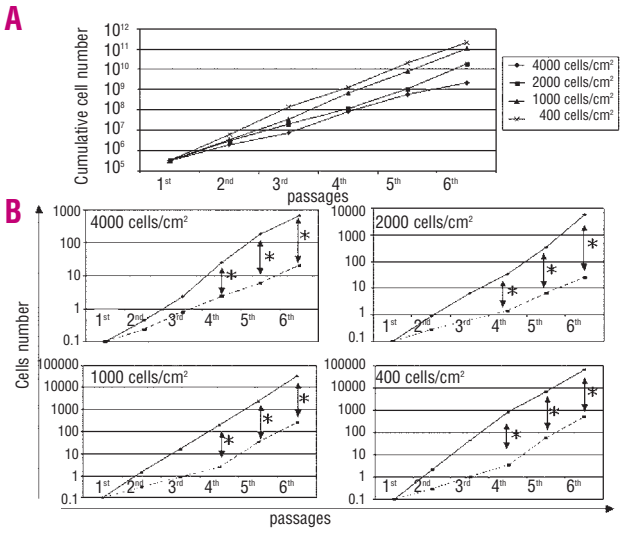

Figure 2. A. Dependence of AF-MSC isolation and expansion on the cell plating density. The MSC yield was significantly different comparing plating densities of 4000cells/cm² vs 1000cells/cm² and 4000cells/cm² vs 400cells/cm² ($p<0.05$) from the third passage. Each point represents the median value of 20 independent experiments. (B): Comparison of growth curves between AF-MSC (solid line) and BM-MSC (dashed line) starting from equal amounts of cells at the first passage. Data are median values of ten experiments. *P* values <0.05 are represented by an asterisk.

Inhibition of T-cell proliferation

To verify the immunosuppressive activity of AF-MSC, we performed standard proliferative assays on peripheral blood mononucleated cells (*for details see online supplementary Appendix 3*).

Results

Isolation and expansion of MSC from amniotic fluid

The MSC population was selected and extensively expanded from 21/23 samples of amniotic fluid. The frequency of MSC was evaluated as the median number of CFU-F per milliliter of amniotic fluid, rather than per mononuclear cells, as untouched amniotic fluid contained undetectable numbers of cells. We observed a median of 3 CFU-F/mL of amniotic fluid (range, 1-6 CFU-F/mL). Starting from a median of 3.25×10^5 cells (range, $0.1\text{-}1.4\times10^6$ cells) at the first passage, the fetal MSC were expanded for six passages, corresponding to 7 weeks of culture. These results confirm that amniotic fluid contains a low number of mesenchymal progenitors that can be effectively expanded in culture.[25]

The phenotype and functional properties of AF-MSC are similar to those of adult MSC

The viability of the cell preparations was greater than 90% in all analyzed samples. To assess the identity of the MSC population, cells expanded from amniotic fluid were characterized by flow cytometry. These cells showed bright expression of CD73, CD146, CD29, CD166 and HLA class I antigen, and variable expression of CD44 and CD105. Furthermore, the purity of fetal MSC was confirmed by the lack of expression of

Table 1b. Cumulative population doublings calculated at each single passage of the fetal MSC culture with different plating conditions.

	Second passage	*Third passage*	*Fourth passage*	*Fifth passage*	*Sixth passage*
4000 cells/cm²	2.26	4.5	7.5	8.3	10.4
2000 cells/cm²	3	5.7	8.5	11	15
1000 cells/cm²	3.8	7.2	10.9	16	20
400 cells/cm²	4.4	8.7	13	18	21.8

Table 1a. Fetal MSC yield obtained since the third passage at different plating densities.

Passage		*400 cells/cm²*	*p*	*4000 cells/cm²*
Third	Median	13.8×10^7		7.2×10^6
(n. 21)	Range	2.6×10^7 - 14×10^8	<0.05	1.2×10^6 - 5.6×10^7
Fourth	Median	1.2×10^9		79.9×10^7
(n. 21)	Range	63×10^7 - 60.85×10^9	<0.05	1.8×10^7 - 37.6×10^7
Fifth	Median	2.1×10^{10}		5.9×10^8
(n. 10)	Range	1.8×10^8 - 2.9×10^{12}	<0.05	9.5×10^7 - 50×10^8
Sixth	Median	2×10^{11}		2×10^9
(n. 10)	Range	2.1×10^{10} - 1.5×10^{14}	<0.05	1.8×10^8 – 45.9×10^9

hematopoietic and endothelial-specific surface antigens such as CD45, CD34, CD14 and CD31, CD144, von Willebrand, KDR, respectively (Figure 1A).

There was no difference between the immunophenotypes of the AF-MSC and BM-MSC, with the exception of lower expression of CD44 and CD105 on fetal MSC (Figure 1B). Both adult and fetal MSC were positive for HLA-class I and negative for HLA-class II and co-stimulatory molecules such as CD80 and CD86 (*data not shown*). Culture-expanded fetal MSC, like adult MSC, were capable of differentiating along osteogenic and adipogenic lineages in response to appropriate stimuli (Figure 1C, D).

Amniotic fluid cultures do not contain hematopoetic progenitors

We analyzed the content of hematopoietic progenitors in the samples of amniotic fluid. Neither committed (CFU-GM, BFU-E, CFU-GEMM) nor immature hematopoietic progenitor cells (LTC-IC) could be detected in any of the tested amniotic fluid samples, confirming the lack of hematopoietic progenitors.

Culture conditions affect the efficiency of fetal MSC expansion

We observed a significant correlation between the MSC yield and plating density since the third passage, as shown in Figure 2A.

After the second culture passage, there was a significant increase in the cell expansion with reducing cell density. In particular, fetal cells plated at 400 cells/cm^2 were expanded at least 1 log more than MSC plated at 4000 cells/cm^2 after 4 weeks (third passage) as well as in subsequent passages (fourth, fifth and sixth corresponding to 5, 6 and 7 weeks of culture, respectively). Comparisons of the MSC yields at each passage are reported in Table 1a. From the fifth week (fourth passage), we observed a significant difference in population doubling between fetal cells plated at 4000 cells/cm^2 and those plated at 400 cells/cm^2. In particular, MSC plated at the highest density underwent only 10.4 population doublings (median value, range 7-11), while MSC cultured at the lowest density reached up to 21.8 population doublings (median value, range 20-22; $p<0.05$) in 7 weeks (sixth passage) (Table 1b). These results suggest that, as for adult MSC,[28] low plating density of fetal MSC results in a greater cell yield.

The expansion potential of AF-MSC is greater than that of adult MSC

Equal numbers of fetal and adult MSC at first passage were plated to calculate the expansion capacity of both populations. Growth curves were constructed using ten samples of BM-MSC and ten samples of AF-MSC.

We observed that fetal MSC are endowed with a significantly greater expansion potential than are adult BM-MSC ($p<0.05$ at the fourth, fifth and sixth passages), as shown in Figure 2B. Plating density had a great impact on the growth potential of both fetal and adult MSC.

AF-MSC contain small cells with high proliferative capacity

When MSC were seeded at 400 cells/cm^2, phenotypic evaluation revealed a main subpopulation (70-90% of total cells) of relatively small cells (median size, 27 μm), which were CD106 negative and showed low side scatter. When cells were plated at a higher concentration (4000 cells/cm^2), a large cell subpopulation (70-85% of total cells) appeared (median size, 37 μm), showing high forward side scatter, high side scatter and bright CD106 expression (Figure 3). These two sub-fractions were maintained constantly up to the sixth passage. At later passages, cells showing high side scatter and forward side scatter became predominant also in the population plated at a low density. Adult MSC showed a predominant subpopulation of large cells when plated at any of the tested densities (55-70% and 80-95% of total cells at 400 cells/cm^2 and 4000 cells/cm^2, respectively). As for the fetal MSC subpopulations, the large cells had bright expression of CD106, while the small cells were CD106 negative. MSC proliferation was assessed at different time points, comparing fetal and adult MSC growth at low (400 cells/cm^2) and high plating density (4000 cells/cm^2). At 24 hours after CFSE staining, AF-MSC showed a daughter cell peak at both plating densities, whereas BM-MSC displayed only the parental peak (*Online Supplementary Figure 1*). After 5 days, fetal MSC showed virtually nine generations, whereas adult MSC

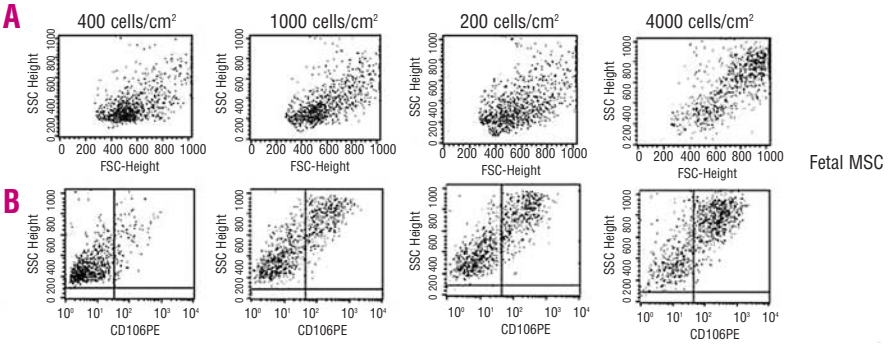

Figure 3. Subpopulations of AF-MSC. Flow cytometry analysis was performed at the same time point to assess: size and complexity (A), and CD106 expression (B) of fetal MSC cultured at different cell densities. Data are from one representative experiment of ten independent experiments.

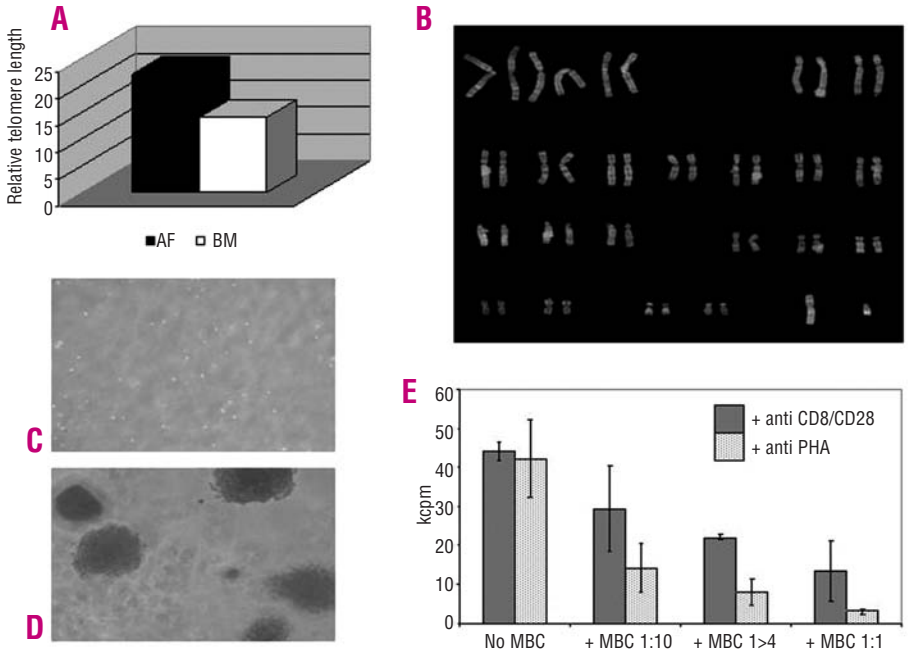

Figure 4. (A) Telomere length of fetal and adult MSC was evaluated in cells expanded for three passages. Results are representative of ten independent experiments. (B) Karyotype of fetal MSC revealed chromosomal stability. (C) Soft agar assay showed that AF-MSC are unable to form colonies. (D) Colony growth occurred in the NB4 cell line used as a positive control. (E) AF–MSC inhibit T-cell proliferation in a dose-dependent manner. PHA, phytohemagglutinin

displayed no more than six generations. After 10 days, the fetal MSC parental peak was no larger detectable for either culture densities, whereas the parental peak of the adult MSC was still present. Furthermore, at the last time point, we observed that MSC cultured at 400 cells/cm^2 showed enhanced proliferation compared to cells cultured at 4000 cells/cm^2.

AF-MSC have longer telomeres than adult MSC

MSC expanded from amniotic fluid (n=10) and from bone marrow (n=10) were analyzed for their telomere length, expressed as the relative telomere length (Figure 4A). We found a significant difference between the relative telomere length of AF-MSC (median value 22%; range, 16-67%) and BM-MSC (median value 14%; range, 5-22%; p=0.007, calculated by Student's t-test).

These results are in line with the higher proliferative capacity of AF-MSC compared to BM-MSC.

Cultured AF-MSC have a stable karyotype and do not display tumorigenic potential

The karyotype of fetal MSC was analyzed at different culture passages (fourth and sixth) to evaluate the occurrence of spontaneous chromosomal alterations during expansion (Figure 4B). All samples remained genetically stable, even after 7 weeks of culture in both density conditions. Using the soft agar assay we did not observe any colony growth from fetal MSC. These cells were not able to grow in an anchorage-independent manner, suggesting that they did not undergo any transformation even after extensive expansion (Figure 4C, D).

Moreover, when 2×10^6 fetal MSC, expanded for 6 weeks in culture, were administered either intravenously or subcutaneously to eight NOD-SCID mice, no tumor formation was observed during 3 months of follow-up (*data not shown*).

AF–MSC inhibit T–cell proliferation

Finally, we investigated the effects of AF-MSC on T-cell proliferation following stimulation of peripheral blood mononuclear cells from healthy donors with either anti-CD3/anti-CD28 or phytohemagglutinin. Given their high proliferative kinetics, we utilized irradiated AF-MSC.

A statistically significat inhibition of T-cell proliferation was observed at peripheral blood mononuclear cell/AF-MSC ratios of 1:1 (p=0.002) and 1:4 (p=0.0001) and a decrease at low AF-MSC concentration (p=0.07) following dose-dependent kinetics. A comparable inhibition was observed for peripheral blood mononuclear cell stimulated with phytohemagglutinin (Figure 4E).

Discussion

In this study we demonstrated that it is possible to isolate and extensively expand fetal MSC from AF and that these cells are suitable for therapeutic purposes.

The optimal MSC dose for clinical applications is currently unknown, but it is likely that large amounts of MSC would be needed for both immunomodulation and regenerative medicine. In fact, a very high dose of MSC was used in preclinical studies.[20] In the mouse, an efficacious MSC dose is in the order of magnitude of a million cells, which is equivalent to a billion cells for treating human patients. In the perspective of clinical applications, the yield of MSC from bone marrow or other adult

tissues may be insufficient. Potentially important therapies could, therefore, be discarded prematurely because of non-appropriately designed clinical trials.

In humans, the most appropriate dose is difficult to identify even in settings in which MSC have been proven to be effective, such as graft-versus-host disease;[29] this is because of the limited availability of MSC expanded from adult bone marrow. In the search for a more abundant source of MSC, this study shows that amniotic fluid is a better source of MSC than bone marrow and, may, therefore offer a way to overcome the limitation of MSC dose.

Despite the extremely low frequency of MSC in the starting cell population, we successfully expanded MSC up to the fourth passage from 2 mL of 21/23 (91%) consecutive samples of amniotic fluid and up to the sixth passage of 10/23 samples (43%).

Cell density plays an important role in expansion of both BM-MSC and AF-MSC.[30] The proliferation pattern showed that at a low cell plating density, several generations of MSC were rapidly produced.

Interestingly, fetal MSC plated at low density (400 cells/cm^2) consisted mainly (70% of total cells) of small cells characterized by a high proliferative potential. This finding differs from previous observations,[31] in which a similar MSC subpopulation (named RS) derived from adult bone marrow rapidly disappeared from the culture, turning into large flattened cells. In contrast, small adherent cells derived from the amniotic fluid are maintained for a long time, although their number declined in the late passages.

Since it seems that MSC have a hierarchical organization with small cells being an immature progenitor capable of self-renewal[32] we may conclude that amniotic fluid represents an enriched source of immature MSC, that the persistence of these small cells implies self-renewing capacity division during the culture period and that such immature cells enable the production of a large MSC output.

Although AF-MSC proliferate extensively in culture, no karyotypic abnormalities or transformation potential in soft agar assay were observed in the samples tested at different passages. This is remarkable since we observed four karyotype abnormalities out of 23 clinical grade MSC expansions performed from adult bone marrow (*Sessarego N., unpublished data*). Moreover, in our hands that fetal MSC were non tumorigenic even after prolonged expansion.

We observed that AF-MSC had an immunosuppressive on induced T-cell proliferation *in vitro*, which is similar to effect that reported for adult bone marrow-derived MSC.[33] Thus, AF-MSC may be utilized in immune-mediated disorders as well as in the treatment of graft-versus-host disease.[34]

Recently, amniotic stem cell lines were described as a c-Kit positive population isolated by immunoselection.[35] Such fetal pluripotent lines reached 250 population doublings exceeding the *Hayflick limit* typical of post-embryonic stem cells (about 50 population doublings); in fact such cells avoided senescence and maintained a stable telomere length. Although this report is scientifically very interesting, it seems to describe a cell line that is still far from being usable in clinical practice. Moreover, it is not clear the frequency at which such cells can be isolated with respect to the number of analyzed samples. In contrast, the AF-MSC described here have functional characteristics comparable to those of adult bone marrow-derived MSC, which have already been successfully utilized in clinical practice.[17-19] AF-MSC have longer telomeres than adult MSC throughout the period of *ex vivo* expansion, which probably explains their higher proliferative capacity, without giving rise to an *immortalized* proliferation behavior. It is reassuring that there was complete chromosomal stability and no evidence of transformation during the *in vitro* expansion; furtheremore, there was no undesired differentiation into other cell types after infusion into mice.

These properties make amniotic fluid a rich source of MSC suitable for banking and universal application when large amounts of cells or repeated infusions are required.

Authorship and Disclosures

NS, AP, MM, VR, FB, AK performed the experimental research and participated in the collection and analysis of data; NS, AP, ML, GF, FDB, AU participated in the interpretation of data; FDB, ML provided the samples; FDB, FB, ML, AU, GF were involved in critically revising the manuscript; NS wrote the draft paper; FF, MP had the original idea, designed the study, and revised the draft article; NS, AP created Figure 1; NS created Figure 2 and Table 1; AP created Figure 3 and supplementary Figure 1; NS, AP, FB created Figure 4. The authors reported no potential conflicts of interest.

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