Insulin-like growth factor binding proteins 4 and 7 released by senescent cells promote premature senescence in mesenchymal stem cells

V Severino, N Alessio, A Farina, A Sandomenico, M Cipollaro, G Peluso, U Galderisi, and A Chambery

Cellular senescence is the permanent arrest of cell cycle, physiologically related to aging and aging-associated diseases. Senescence is also recognized as a mechanism for limiting the regenerative potential of stem cells and to protect cells from cancer development. The senescence program is realized through autocrine/paracrine pathways based on the activation of a peculiar senescence-associated secretory phenotype (SASP). We show here that conditioned media (CM) of senescent mesenchymal stem cells (MSCs) contain a set of secreted factors that are able to induce a full senescence response in young cells. To delineate a hallmark of stem cells SASP, we have characterized the factors secreted by senescent MSC identifying insulin-like growth factor binding proteins 4 and 7 (IGFBP4 and IGFBP7) as key components needed for triggering senescence in young MSC. The pro-senescent effects of IGFBP4 and IGFBP7 are reversed by single or simultaneous immunodepletion of either proteins from senescent-CM. The blocking of IGFBP4/7 also reduces apoptosis and promotes cell growth, suggesting that they may have a pleiotropic effect on MSC biology. Furthermore, the simultaneous addition of rIGFBP4/7 increased senescence and induced apoptosis in young MSC. Collectively, these results suggest the occurrence of novel-secreted factors regulating MSC cellular senescence of potential importance for regenerative medicine and cancer therapy.

Subject Category: Experimental Medicine

Senescence is the permanent cell cycle arrest that leads to the loss of cellular functions over time. Several lines of evidence correlate cellular senescence to aging, as it largely contributes to reduce tissue functions and renewal. On the other side, it is also believed to promote protective anticancer mechanisms leading to tumor growth arrest.1,2

Senescence of stem cells, such as mesenchymal stem cells (MSCs), could be very deleterious as it can greatly impair tissue homeostasis and repair. MSC are pluripotent cells that reside in postnatal organs and tissues, and can differentiate into bone, cartilage and fat. Owing to their supporting role in hematopoiesis and in the homeostatic maintenance of many organs and tissues, MSC also have a significant therapeutic potential for tissue regeneration.3–5

Cellular senescence has been for long time considered as an exclusively intracellular event unleashed by activation of a cytoplasmic signaling circuitry. Recent studies have evidenced that a pool of molecules secreted by senescent cells, for which the term ‘senescence-associated secretory phenotype’ (SASP) has been proposed, is associated and may contribute to cellular proliferative arrest through autocrine/paracrine pathways.6–8 Secreted factors can regulate the senescence response and may represent a danger signal that sensitizes normal neighboring cells to senesce, thus enhancing the ability of damaged cells to enter senescence. In advanced tumor phases, however, cancer cells can take advantage of signaling pathways induced by secreted factors for their growth.9

MSC’s beneficial influence in tissue repair has also been attributed to their paracrine activity.10 Therefore, changing the pattern of factors secreted by MSC during the senescence process may have a great impact on body’s health. Given the
paramount importance of autocrine/paracrine signaling in MSC functions, elucidating the composition of the pool of proteins secreted by MSC would enhance our understanding on how these cells affect the environment niche, and support tissue repair, immunomodulation, hematopoietic stem cell self-renew, and tumor growth. To date, studies on the MSC secretome are still in their infancy and mainly refer to the high potential of MSC secretome for the development of therapeutic strategies for the treatment of diseases.11,12

To our knowledge, a comprehensive analysis of the secretome profile of senescent MSC is lacking. In this work, the senescence-inducing properties of conditioned medium (CM) of senescent MSC have been investigated. A ‘bottom-up’ proteomics approach based on liquid chromatography-mass spectrometry (LC-MS/MS) label-free quantitative analysis has been used to identify and compare the expression levels of secreted proteins in the MSC CM at early and late cultivation phases. Our results provide a detailed characterization of the MSC secretome associated to the senescent phenotype, revealing that selected components of the insulin-like growth factor binding proteins (IGFBPs) family are strongly involved in MSC SASP. We have identified IGFBP4 and IGFBP7 as important senescence-inducing factors, as their single and/or simultaneous immunodepletion abolishes the pro-senescence effect of senescent MSC CM, reduces apoptosis and promotes cell growth. Moreover, the simultaneous addition of rIGFBP4/7 increased senescence and induced apoptosis in MSC. Predicting stem cell fate requires knowledge of how extracellular stimuli are integrated with intracellular signaling networks. Results from an in silico analysis suggest that the extracellular signal-regulated kinases (ERK 1/2) is one of the converging node of the MSC SASP. Accordingly, the induction of MSC senescence program impairs the nuclear/cytosolic localization of active ERK. This study provides an important basis for deciphering the complex extracellular protein networks implicated in MSC cellular senescence and their interplay with the corresponding cytoplasmic signaling circuitry.

Results

CM from senescent MSC triggers senescence in young cells. Senescence of stem cells is caused by a combination of intrinsic irreversible and reversible changes also influenced by circulating effectors or factors secreted by local stem cell niches.13 Therefore, we decided to investigate the effects of extrinsic signaling on MSC senescence. At first, properties of young (passage 1, P1) and senescent (passage 10, P10) MSC were evaluated. Following senescence induction, MSC showed a characteristic phenotype including larger and flattened cell morphology (Figure 1a). As expected, proliferation rate was significantly lower in P10 versus P1 cultures (Figure 1b), and this decrease was associated with an increased percentage of senescent cells (Figure 1c). No significant changes in the apoptotic rate were detected (Figure 1c), confirming the presence of a higher percentage of senescent MSC in P10 compared with P1 cultures.

To determine whether extracellular factors were endowed with senescence-induction properties, proliferation rate, apoptosis and senescence were evaluated on young MSC cultured with CM from senescent cells (CM-P10). A complementary experiment was performed by culturing senescent cells with CM-P1 from young MSC (Figure 1d). Results demonstrate that CM-P10 was able to trigger senescence in young MSC as suggested by a significant reduction in their proliferation rate (Figure 1e) and confirmed by 4-methylumbelliferyl-β-D-galactopyranoside (MUG) and senescence-associated β-galactosidase (SA-β-gal) assays (Figures 1g, h–j). No modification of the apoptotic rate was detected (Figures 1i–k). The specificity of the observed senescence response was attested by the evidence that the incubation of P10 MSC cultures with CM-P1 did not induce any significant change in proliferation, senescence and apoptosis (Figures 1f–k). These results demonstrate that extrinsic factors secreted by senescent MSC are able to promote senescence phenomena in neighboring cells. Once triggered, the senescence process appears to be irreversible, or at least it cannot be reverted by secreted factors produced by young MSC.

Secretome analysis of MSC CM. To identify candidate proteins responsible for the senescence-inducing properties of CM-P10 and to delineate a hallmark of MSC senescent phenotype, a comparative secretome analysis was performed by applying a shotgun proteomics strategy. CM-P1 and CM-P10 were processed for protein precipitation by trichloroacetic acid, and LC-ESI-MS/MS analyses were carried out on peptides deriving from tryptic digestion of proteins. Using high-resolution MS and Mascot database search, 121 proteins were qualitatively identified across both conditions in the MSC secretome. Data were filtered on the basis of probability of protein identification and technical reproducibility, allowing the selection of 66 non-redundant proteins (Supplementary Information, Supplementary Tables S1 and S2). The quantitative analysis performed by spectral counting revealed that 33 proteins were common to both CM-P1 and CM-P10 with no significant differences in their expression levels (Supplementary Information, Supplementary Table S3). Among differentially expressed proteins, 7 and 13 proteins were uniquely detected in CM-P1 and CM-P10, respectively (Table 1a), whereas a set of proteins was significantly up- and downregulated in CM-P10 versus CM-P1 (Table 1b).

The identified proteins were analyzed for classical (i.e., signal peptide-driven secretion through the ER/Golgi pathway14) and non-classical (e.g., proteins released through vesicles and extracellular matrix (ECM) proteins15) secretion pathways, revealing that about 94% of the identified proteins were predicted to be secreted. Among them, 57 proteins (~93%) were endowed with the signal peptide for secretion through the ER/Golgi pathway, whereas only four additional proteins (~7%) were predicted to be released by cells through non-classical secretion mechanisms.

A bioinformatic analysis performed by using the Metacore software revealed that a significant number of differentially regulated proteins was involved in cell–matrix interactions, ECM regulation and protein-folding response (Figure 2a). In detail, the heat map distribution of these proteins show that several upregulated proteins in senescent MSC were involved in cell adhesion, ECM remodeling and connective tissue
degradation (Figure 2b). Several collagen components involved in cartilage development and integrin-mediated cell–matrix adhesion were also upregulated in senescent cells. Furthermore, proteins involved in ER-associated and cytoplasm protein-folding response were downregulated in senescent MSC (Figure 2b).

Key molecules of the IGF signaling pathway were also differentially regulated in senescent with respect to young

---

**Figure 1** CM from senescent MSC triggers senescence in young cells. (a) Induction of replicative senescence was accomplished by repeatedly passaging the cells at P10. Following senescence induction, MSC showed a characteristic phenotype including larger and flattened cell morphology with respect to young MSC (P1). (b) Cell proliferation measured by Quick Cell Proliferation Colorimetric Assay Kit II. *P < 0.05; **P < 0.01 versus P1. (c) Percentage of SA-β-gal-positive cells; **P < 0.01 versus P1. Apoptotic cells were detected using fluorescein-conjugated Annexin V staining on P1 and P10 MSC. (d) Schematic summary of the experimental workflow. (e) Cell proliferation rate evaluated on young MSC cultured with CM-P10 (P1/CM-P10); *P < 0.05; **P < 0.01 versus P1 MSC grown in control medium. (f) Cell proliferation rate evaluated on senescent MSC cultured with CM-P1 (P10/CM-P1). (g–i) MUG, SA-β-gal and Annexin V assays performed on P1/CM-P10 and on P10/CM-P1; *P < 0.05 versus MSC grown in control medium. (j) Representative microscopic fields of SA-β-gal-positive cells (blue). The mean percentage value of senescent cells (± S.D., n = 3) is reported in Figure 1h. (k) Micrographs showing merge of representative fields of P1 and P10 MSC grown under different conditions and stained with Annexin V (green) are showed. Nuclei were counterstained with Hoechst 33342 (blue). The mean percentage value of apoptotic cells (± S.D., n = 3) is indicated in Figure 1i.
MSC, including several IGFBPs, that are known to have a role in the induction of senescence and cancer. In particular, a strong upregulation of IGFBP4 and IGFBP7 was observed in senescent cells, suggesting a role for these factors in triggering senescent phenomena in MSC.

IGFBP4 and IGFBP7 are key factors of senescent MSC CM for triggering senescence phenomena in young MSC. To determine the role of IGFBP4 and IGFBP7 in senescence, cell proliferation, apoptosis and senescence studies were undertaken on young MSC treated with IGFBP4- and/or IGFBP7-immunodepleted CM-P10 (Figure 3a). As shown, treatment with anti-IGFBP4 and/or anti-IGFBP7 blocked the pro-senescent activity of CM-P10 even at the lowest concentration tested (Figure 3b). It is to be noted that the simultaneous treatment with both anti-IGFBP4 and anti-IGFBP7 provided an enhanced reduction of senescence induced by CM-P10 on P1 MSC (Figure 3b).

Table 1 Proteins uniquely (a) and differentially regulated (b) identified in CM-P1 and CM-P10 secretome by high-resolution LC-MS/MS

| ID | Accession | Protein name | Mr (kDa) | No. of peptides | Secretome P | Signal P |
|----|-----------|--------------|----------|----------------|-------------|----------|
| CM-P1 | P27797 | Calreticulin | 48       | 3              | 0.37        | Y        |
|      | P27658 | Collagen alpha-1(VIII) chain | 73       | 2              | 0.38        | Y        |
|      | O43854 | EGF-like repeat and discoidin I-like domain-containing protein 3 | 54       | 2              | 0.82        | Y        |
|      | P14625 | Endoplasmin | 92       | 2              | 0.50        | N        |
|      | P08238 | Heat-shock protein HSP 90-beta | 83       | 4              | 0.20        | N        |
|      | P51884 | Lumican | 38       | 8              | 0.54        | Y        |
|      | P23284 | Peptidyl-prolyl cis-trans isomerase B | 24       | 2              | 0.85        | N        |

| CM-P10 | Q9BRK5 | 45 kDa calcium-binding protein | 42       | 3             | 0.59        | Y        |
|         | P16112 | Aggrecan core protein | 250      | 6              | 0.57        | Y        |
|         | P49747 | Cartilage oligomeric matrix protein | 83       | 2             | 0.27        | Y        |
|         | P01034 | Cystatin-C | 16       | 2             | 0.94        | N        |
|         | Q4ZH4 | Fibronectin type III domain-containing protein 1 | 206      | 2             | 0.14        | Y        |
|         | P24593 | Insulin-like growth factor binding protein 5 | 31       | 2             | 0.92        | Y        |
|         | Q9Y287 | Integral membrane protein 2B | 30       | 2             | 0.84        | N        |
|         | Q09397 | Lysyl oxidase homolog 1 | 63       | 3             | 0.52        | Y        |
|         | Q09666 | Neuroblast differentiation-associated protein AHNAK | 629      | 2             | 0.24        | N        |
|         | P62937 | Peptidyl-prolyl cis-trans isomerase A | 18       | 3             | 0.34        | N        |
|         | P10124 | Serglycin | 18       | 2             | 0.72        | Y        |
|         | Q7ZG3 | Target of Nesh-SH3 | 119      | 2             | 0.21        | Y        |
|         | P24821 | Tenascin | 241      | 2             | 0.47        | N        |

| ID | Accession | Protein name | Mr (kDa) | No. of peptides | CM-P10-CM-P1 | P-value | Secretome P | Signal P |
|----|-----------|--------------|----------|----------------|--------------|---------|-------------|---------|
| Down | P21810 | Biglycan | 42       | 6             | 0.45         | 0.222   | 0.71        | Y        |
|      | P12109 | Collagen alpha-1(VI) chain | 109      | 11            | 0.58         | 0.003   | 0.23        | Y        |
|      | P12110 | Collagen alpha-2(VI) chain | 109      | 6             | 0.23         | 0.001   | 0.21        | Y        |
|      | P12111 | Collagen alpha-3(VI) chain | 344      | 10            | 0.53         | 0.006   | 0.32        | Y        |
|      | P35555 | Fibrillin-1 | 312      | 13            | 0.56         | 0.003   | 0.39        | Y        |
|      | Q15053 | Periostin | 93       | 8             | 0.33         | 0.002   | 0.27        | Y        |
| Up | Q99715 | Collagen alpha-1(XII) chain | 333      | 52            | 1.98         | 0.023   | 0.33        | Y        |
|      | P08132 | Collagen alpha-2(I) chain | 129      | 51            | 1.80         | 0.025   | 0.34        | Y        |
|      | P08572 | Collagen alpha-2(IV) chain | 168      | 9             | 2.8          | 0.034   | 0.07        | Y        |
|      | P21333 | Filamin-A | 281      | 15            | 7.75         | 1.4E-04  | 0.45        | N        |
|      | P2269 | Insulin-like growth factor binding protein 4 | 28      | 5             | 3.63         | 0.011   | 0.89        | Y        |
|      | Q16270 | Insulin-like growth factor binding protein 7 | 29      | 9             | 4.00         | 0.004   | 0.54        | Y        |
|      | P08670 | Vimentin | 54       | 29            | 2.24         | 0.013   | 0.51        | N        |

The Scaffold Software was used to improve protein identification by filtering data following the acceptance criteria of probability greater than 95.0%, as specified by the Protein-Prophet algorithm. The higher number of unique peptides for each protein identification is reported. Mr denotes relative molecular mass.

aSecretion prediction according to Secretome P 2.0 server. Proteins with NN-score ≥ 0.5 are predicted as secreted by non-classical secretory pathways.

bSecretion prediction according to signal peptide probability of Signal P 4.0 server. Y and N indicate the presence or absence of the signal peptide for secretion.
of the apoptosis level (Figure 3g and Supplementary Information, Supplementary Figure S2). Cellular senescence implicates a stable cell growth arrest. To evaluate the effects of IGFBP4/7 soluble factors on MSC growth capability, cell proliferation rate was evaluated on young MSC cultured with CM-P10 alone or treated with anti-IGFBP4 and/or anti-IGFBP7. CM-P10 treatment with anti-IGFBP4 or anti-IGFBP7 showed a significant increase of cell proliferation rates with respect to P1 MSC cultured with untreated CM-P10 at 72 h (Figure 3h). Interestingly, the simultaneous immunodepletion of both IGFBP4 and IGFBP7 resulted in a marked increase of cell proliferation starting from 24 h, thus supporting the view that blocking of IGFBP4/7 significantly dampens the pro-senescence activity of CM-P10.

The capability of IGFBP4 and IGFBP7 of triggering senescence phenomena in MSC was further confirmed by incubating young MSC with recombinant IGFBP4 and IGFBP7 (rIGFBP4/7). Indeed, the simultaneous addition of both rIGFBP4 and rIGFBP7 increased MSC senescence and induced apoptosis at the highest concentration assayed (Figures 3i and j).

**Reversal of the DNA-damage response of young MSC induced by IGFBP4- and IGFBP7-immunodepleted CM-P10.** Extensive investigations have revealed how the DNA damage-sensing and -signaling pathways, referred to as the DNA-damage response network, are linked to cellular senescence and apoptosis. Therefore, the degree of DNA damage was investigated on young MSC cultured with IGFBP4- and IGFBP7-immunodepleted and non-immunodepleted CM-P10, by determining the level of phosphorylated histone H2A.X, which is considered a hallmark of damaged DNA nuclear foci. A marked reduction of pH2A.X-positive cells was observed when P1 MSC were cultured with CM-P10 treated with both IGFBP4 and IGFBP7 antibodies (Figures 4a and b). Moreover, we determined the percentage of 8-oxo-deoxyguanosine (8-oxo-dG), a major product of oxidative damage to DNA. In line with our results, the simultaneous blocking of IGFBP4 and IGFBP7 in CM-P10 significantly decreased the percentage of 8-oxo-dG-positive cells with damaged DNA (Figures 4c and d).

**MSC senescence program affects ERK signaling.** In response to different signals, multiple transduction pathways and targets are activated to induce cellular senescence responses. To determine the downstream pathway(s) triggered by the MSC ‘senescence-messaging secretome’ (SMS), a network map including the differentially regulated proteins identified by MS analysis was constructed in silico by using the IPA software (Ingenuity Systems Inc., Redwood City, CA, USA) (Figure 5a). As shown, a specific subset of secreted proteins was mapped on a pathway converging, at intracellular level, on ERK 1/2. Previous evidences have demonstrated that the impairment of the nuclear/cytosolic localization of active ERK affects the senescence phenotype in human fibroblasts. We therefore supposed that MSC senescence program may involve ERK signaling. To investigate this hypothesis, we analyzed the levels of total and activated ERK (pERK) in young and senescent MSC within different cellular compartments (Figure 5b). The relative distribution was computed as the ratio of the nuclear/cyttoplasmic (N/C) ERK and pERK. Although the ratio N/C for

---

**Figure 2**  SASP of senescent MSC. (a) Enriched processes networks for differentially expressed proteins identified by LC-MS/MS analysis. The probability of a random intersection between the differentially expressed proteins with functional processes was evaluated by applying the hypergeometric test by using the Metacore software. (b) Heat map view representing the functional classification of up- and downregulated proteins in CM-P10 versus CM-P1. Significant functional terms were ranked according to enrichment scores generated using the annotation clustering algorithm in Metacore software.
ERK was constant in P1 and P10, significant differences were detected with respect to pERK distribution. Indeed, the N/C ratio was strongly reduced only in P10 sample with a lower relative amount of nuclear versus cytoplasmic pERK in senescent MSC. Furthermore, together with an overall decrease of pERK levels following senescence induction,
a fourfold decrease of nuclear pERK was revealed in P10 versus P1 MSC.

**Discussion**

Senescence is a physiological phenomenon leading to permanent cell growth arrest and impairment of cellular functions.1,22 The extracellular microenvironment has a key role in regulating and determining the cellular response to senescence signaling. It is known that senescent cells secrete a unique repertoire of molecules (SASP) for which the term 'SMS' has been recently proposed.6 Although the importance of changes in SMS has long been recognized, the molecular details of senescence signaling remain elusive. Furthermore, to the best of our knowledge, the senescence-induced extracellular response of stem cells has not been yet investigated. Given the peculiar properties of stem cells, including their multilineage differentiation capability, it is of fundamental importance to understand the response of stem cells to senescence signaling, also in view of their high potential in regenerative medicine and cancer therapy.

We demonstrate that MSC SASP implements a full senescence response in young cells, suggesting the presence, in the pool of secreted molecules, of factors capable of triggering, alone and/or in combination, senescence mechanisms through autocrine/paracrine signaling.

**Figure 3** SecreTed IGFBP4 and IGFBP7 induce senescence in young MSC. (a) Schematic summary of the experimental workflow for the investigation of the senescence-induction potential of IGFBP4 and IGFBP7. (b) MUG assay performed on young MSC cultured with untreated and antibody-treated CM-P10 at different concentrations (3, 9 and 20 μg/ml). Values are means of three independent experiments; *P<0.05; **P<0.01 versus P1 MSC grown with untreated CM-P10. (c and d) HP1 alpha detection performed on young MSC cultured with untreated and antibody-treated CM-P10. Micrographs showing merge of representative fields of P1 MSC grown under different conditions and stained with anti-HP1 alpha (green) to identify nuclear heterochromatin are showed. Nuclei were counterstained with Hoechst 33342 (blue); *P<0.05; **P<0.01 versus P1 MSC grown with untreated CM-P10. (e and f) H3K9me3 detection performed on young MSC cultured with untreated and antibody-treated CM-P10. Micrographs showing merge of representative fields of P1 MSC grown under different conditions and stained with anti-H3K9me3 (green) are showed. Nuclei were counterstained with Hoechst 33342 (blue); *P<0.05; **P<0.01 versus P1 MSC grown with untreated CM-P10. (g) Apoptosis assay of young MSC cultured with untreated and antibody-treated CM-P10. *P<0.05; **P<0.01 versus P1 MSC grown with untreated CM-P10. (h) Cell proliferation assay on young MSC cultured with untreated and antibody-treated CM-P10. *P<0.05; **P<0.01 versus P1 MSC grown with untreated CM-P10. (i and j) MUG and Annexin V assays performed on young MSC cultured with rIGFBP4 and/or rIGFBP7 at different concentrations (0.5, 2.5 and 10 μg/ml). Values are means of three independent experiments; *P<0.05; **P<0.01 versus P1 MSC grown in control medium.
induction of ECM protein synthesis and changes in the amount of these complexes increases with age.26 Our results show that besides the occurrence of significant changes in collagen composition, other ECM proteins (e.g., Fibrillin-1, Periostin, Lumican, Tenascin and Biglycan), involved in the maintaining of the structural and functional integrity of ECM, are differentially regulated in senescent cells. Furthermore, two components of the cartilage ECM matrix (i.e., Aggrecan and Cartilage oligomeric matrix protein, COMP) are specifically detected in senescent MSC. It has been reported that COMP interacts and is covalently cross-linked to the aggrecan core protein, and that the amount of these complexes increases with age.27 In addition to ECM components, proteins associated to unfolded protein response are downregulated in senescent MSC. This finding is in agreement with the expected attenuation of molecular chaperone function and the resulting increase of protein misfolding and aggregation occurring during aging and senescence.27 Among them, heat-shock protein 90-beta, downregulated in senescent MSC, is reported to be required for the correct assembly and function of telomerase complexes, which is an essential enzyme regulating cell life span.28

Figure 5 MSC senescence program involves the ERK 1/2 signalling. (a) Protein interaction network including a subset of differentially expressed proteins secreted by senescent MSC. The core molecules of the network were found to converge at extracellular level on IGF and TGF beta and, at intracellular level, on ERK 1/2 signaling pathways. According to IPA categorization, proteins uniquely detected or upregulated in senescent MSC are colored in orange and red, respectively; proteins uniquely detected or upregulated in young MSC are colored in blue and green, respectively. The pathway components identified by the algorithm or with no significant differences in their expression levels are reported in gray. Molecules are named according to IPA software as follows: COL12A1, Collagen alpha-1(XII); COL8A1, Collagen alpha-1(VIII); COL6A1, Collagen alpha-1(VI); COL6A3, Collagen alpha-3(VI); POSTN, Periostin; IGFBP, insulin-like growth factor binding protein; IGF, insulin-like growth factor; COL6A2, Collagen alpha-2(VI); COL1 A2, Collagen alpha-2(I); TGF beta, transforming growth factor beta; Mmp, Metalloproteinase protein family; COMP, Cartilage oligomeric matrix protein; TNC, Tenascin; ACAN, Aggrecan core protein; FBN1, Fibrillin-1; LUM, Lumican; BGN, Biglycan. (b) Western blot for ERK and pERK in N/C fractions of P1 and P10 MSC. To assess the purity of the N/C fractions, immunoblot analysis was performed with anti-GAPDH, as cytoplasmic marker, and anti-H1.2, as nuclear marker. Relative density of the bands was normalized to β-actin. (c) Schematic summary of the active ERK nucleo-cytoplasmic trafficking in the MSC response to replicative senescence induction.

Key regulators of the IGF signaling pathways are differentially expressed in senescent versus young MSC. In particular, we detect several members of the IGFBP family (i.e., IGFBP2, IGFBP3, IGFBP4, IGFBP5 and IGFBP7). Although a role in senescence has been previously reported for IGFBP3 in human endothelial cells29 and MCF-7 breast cancer cells,30 we did not detect significant expression changes for this factor and his upstream regulator plasminogen activator inhibitor 1. This result, together with the simultaneous detection of other differentially expressed IGFBPs, suggests the activation of alternative senescence-inducing pathways in MSC. In particular, IGFBP5, previously reported to trigger senescence through a p-53 dependent mechanism,31,32 is uniquely detected in senescent MSC. The strong upregulation of two additional IGFBPs (i.e., IGFBP4 and IGFBP7) in senescent MSC prompted us to investigate their involvement in triggering MSC senescence and results provide new insights on the capability of IGFBP4 and IGFBP7 in promoting senescence in MSC. We indeed find that IGFBP4 and IGFBP7 are ‘key players’ in MSC SASP, because are involved in the induction of a senescent effect that strongly influences the functions of young cells. Blocking of IGFBP4 and/or IGFBP7 in CM-P10 reduces their pro-senescent effect on P1 MSC cultures. It is noteworthy that the simultaneous inhibition of IGFBP4 and IGFBP7 suppresses apoptosis and promotes cell growth, supporting their pleiotropic effect on MSC biology. Furthermore, the simultaneous
addition of rIGFBP4/7 increased senescence and induced apoptosis in MSC, and these effects were also observed with single addition of rIGFBP7. Our findings are in agreement with recent studies, demonstrating that IGFBPs have a causal role in the induction of senescence in cancer systems.6 Reported, overexpression of IGFBP7 triggers BRAFV600E-induced senescence in human melanocytes33,34 and is considered a tumor suppressor, as it reduces breast tumor growth by induction of senescence and apoptosis pathways.35 Similarly, expression levels of IGFBP4 mRNA are increased in senescent hepatic stellate cells36 and following induction of senescence-associated genes by 5-bromodeoxyuridine in HeLa cells.37

A critical aspect of senescence signaling is to unravel the connection between the SMS and the downstream effectors and pathways.6 Recent evidences support the hypothesis that SMS-associated factors may be involved in distinct but integrated signaling pathways, and that the dynamic activation of intracellular cascades is responsible for the cell decision to eventually engage a senescence program.6 However, the intracellular targets of SMS factors secreted by a given cell type have not been fully elucidated.6 By using a bioinformatic approach, a specific subset of proteins of MSC SASP was mapped on a pathway converging on ERK 1/2. Wajapeyee et al.33,38 demonstrated that IGFBP7 suppresses the phosphorylation of ERK and mediates its antiproliferative effects through a negative feedback signaling in oncogenic BRAFV600E-mediated senescence. In sharp contrast, previous evidences suggested that the expression of BRAFV600E induces the activation of ERK 1/2. These studies clearly demonstrate an involvement of the ERK signaling in the oncogene-induced senescence and point out the crucial role of ERK signaling in mediating different antiproliferative events such as apoptosis, autophagy and senescence.20 The plethora of signals that activate the ERK pathway and its numerous substrates suggest that the fine control of the spatiotemporal regulation of ERK activity is critical for determining opposite cell fates.20 Activation of cytoplasmatic ERK 1/2 through its phosphorylation leads to nuclear translocation thus allowing the interaction with specific nuclear substrates and the induction of specific programs of gene expression. The regulation of ERK nuclear-cytoplasmic trafficking modulates the transfer efficiency of the signaling carried by activated ERK to the cell.20 Accumulating evidences show that nuclear relocalization of active ERK is impaired in senescent human fibroblasts, and that this dysregulation may account for the irreversible proliferative arrest of senescent cells.20 The impaired nuclear localization of active ERK and the loss of proliferative potential have been proposed to originate from either impaired nuclear import or from ERK inactivation. This interpretation is supported by the high activity of the nuclear ERK phosphatase MKP2/DUSP4 in senescent cells.21

Our results also attest a dysregulation of active ERK localization in stem cell senescence program, as a lower amount of nuclear versus cytoplasmatic pERK is revealed in senescent MSC together with a decrease of nuclear pERK in P10 versus P1 MSC (Figure 5c). This finding strongly supports previous studies and further delineates an unexpected complexity of ERK signaling mechanisms in cellular senescence.22 Indeed, we might envisage a scenario in which the nuclear/cytosolic localization of active ERK may be related to the MSC senescent phenotype as previously demonstrated for human fibroblasts.

Although more extensive studies are needed to expand our knowledge on the mechanisms underpinning the impaired ERK nucleo-cytoplasmic trafficking in stem cells, we also believe that our results pave the way to further investigations aiming to modify, in the near future, the current in vitro MSC expansion protocols for therapeutic purposes, thereby preventing or reducing the occurrence of negative senescence-related effects, and to better understand the complex process of senescence and aging in stem cells.

Materials and Methods

MSC cultures and preparation of CM. MSC were obtained and cultured as previously described.39 Bone marrow was obtained from healthy donors after informed consent. Cells were separated on Ficoll density gradient (GE Healthcare, Milan, Italy) and the mononuclear cell fraction was collected and washed in PBS. Cells (1-2.5 × 10^5/cm²) were seeded in alpha-MEM containing 10% PBS and bFGF. After 72 h, non-adherent cells were discarded and adherent cells were further cultivated to confluence and amplified at P1. Replicative senescence was induced by repeatedly passaging the cells at P10. P1 and P10 cultures are referred to as ‘young’ and ‘senescent’ MSC, respectively. In our experimental conditions, it was verified that MSC cultures fulfilled the minimal criteria to define MSC.40 All cell culture reagents were obtained by Euroclone Life Sciences (Milan, Italy) and HyClone (Logan, UT, USA).

To harvest MSC secretome, 80% confluent P1 and P10 MSC cultures were extensively washed with PBS and transferred to a chemically defined, serum-free culture medium for an overnight incubation. Then, the CM from young (CM-P1) and senescent (CM-P10) MSC were collected and used at 50% in fresh alpha-MEM for in vitro cultivation of senescent and young MSC, respectively. Three independent culture preparations were prepared and pooled to address biological variation.

CM preparation for LC-MS/MS analysis. CM-P1 and CM-P10 were clarified by centrifugation, lyophilized and resuspended in 1 ml of 50 mM NH₄HCO₃. The proteins were then precipitated with 20% trichloroacetic acid for 30 min on ice and centrifuged for 15 min at 17,500 × g.41–43 Pellets washed with ethanol and acetone were air dried at room temperature, resuspended in 50 mM NH₄HCO₃, pooled and thoroughly sonicated into an ultrasonic bath for 10–15 min. Proteins were reduced with 2.5 mM dithiothreitol (final concentration) at 60°C for 30 min and carboxymethylated with 7.5 mM iodoacetamide (final concentration) at room temperature in the dark for 30 min. Enzymatic hydrolysis was performed by the addition of 10 μl of tosyl phenylalanyl chloromethyl ketone-treated trypsin solution (5 ng/μl) to the reduced and alkylated mixture. Digestion was performed by incubation at 37°C for 16 h, followed by a second addition of 10 μl of trypsin incubated at 37°C for 3 h. After digestion, samples were dried under vacuum in a SpeedVac Vacuum (Savant Instruments, Holbrook, NY, USA), resuspended in H₂O/H₂O/H₂O/formic acid (FA) 95%/5%/0.1%, and peptides were purified by using macro spin columns (Harvard Apparatus, Holliston, MA, USA). Samples were dried, resuspended in 120 μl of 0.1% FA and centrifuged at 17,500 × g for 15 min. Aliquots of the supernatant (6 μl) were analyzed by ESI LTQ-OT MS.42–43

LC-MS configuration and protein identification. ESI LTQ-OT MS was performed on a LTQ Orbitrap Velos from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters (Waters Corporation, Manchester, UK).43 Peptides were trapped on a home-made 5-μm 200 Å Magic C18 AQ (Michrom, Auburn, CA, USA) 0.1 × 20 mm pre-column and separated on a home-made 5-μm 100 Å Magic C18 AQ (Michrom) 0.75 × 150 mm column with a gravity-pulled emitter. The analytical separation was run for 85 min, using a gradient of H₂O/FA 99.9%/0.1% (solvent A) and CH₃CN/FA 99.9%/0.1% (solvent B), at a flow rate of 220 nMl/min as follows: 5% solvent B for 1 min, from 5 to 35% solvent B in 54 min and from 35 to 80% solvent B in 10 min. For MS survey scans, the Orbitrap resolution was set to 60,000 and the ion population was set to 5 × 10^5 with an m/z window from 400 to 2000. Five precursor ions were selected for collision-induced dissociation in the LTQ by setting the ion population to 7 × 10^5 isolation width of 2 m/z. The normalized collision energies were set to 35% for collision-induced dissociation.
IGFBP4 and IGFBP7 promote premature senescence

V Severino et al

Cell Death and Disease

Data processing was performed as previously described. The Mascot software (Matrix Science, London, UK; version 2.2.0) was used for database searching against the Uniprot_sprot database release 2011_09 (21 September 2011, selected for Homo sapiens, 2033 entries) assuming trypsin as digestion enzyme. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 25 p.p.m. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification.

Scaffold software (version 4.0.1, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95% probability. Protein identifications were accepted if they could be established at ≥95% probability and contained at least two identified unique peptides across replicate injection for each condition. Protein probabilities were assigned by the Protein–Prophyl algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. A false discovery rate value of 0.1% was calculated by using the Scaffold software for MS/MS validation.

Quantitative analysis. For protein quantification, for each identified protein, the number of spectral counts (the number of MS/MS spectra associated with an identified protein) was used to identify differentially expressed proteins. To this end, normalized spectral counts were calculated by dividing the spectral counts for an identified protein by the sum of the spectral counts per sample. The statistical evaluation of proteins differentially expressed between the CM-P1 and CM-P10 samples was performed by applying the Fisher’s exact test (P<0.05).

Bioinformatics analyses. Secreted and differentially expressed proteins identified in CM-P1 and CM-P10 were imported into the Ingenuity Pathways Analysis (IPA, Ingenuity Systems Inc.) or Metacore (GeneGo, St. Joseph, MI, USA) software for batch analysis and identification of the canonical pathways as previously reported. An assessment of significantly enriched processes networks for differentially expressed proteins was performed by evaluating the probability of a random intersection between the differentially expressed proteins with functional processes by applying the hypergeometric test. The heat map representing the functional classification of up- and downregulated proteins in CM-P10 versus CM-P1 remained unaltered by adding and removing functional terms to the dataset according to the MetaCore annotation algorithm by using the TIBCO Spotfire software (Somerville, MA, USA). Proteins with a predicted N-terminal signal sequence were identified by using SignalP 4.0, available at http://www.cbs.dtu.dk/services/SignalP/ (set for eukaryotes using both nuclear networks and hidden Markov models), and were considered to be secreted via a classical pathway (endoplasmic reticulum/Golgi-dependent pathway). Identified proteins were also analyzed for secretion pathways according to SecretomeP 2.0 Server (http://www.cbs.dtu.dk/services/SecretomeP) [52]. If the neural network exceeded or fell below the cut-off for each protein (NN-score), but no signal peptide was predicted, proteins were considered potentially secreted via a non-classical pathway.

Immunodepletion experiments. IGFBP4 and/or IGFBP7 were immunodepleted in CM-P10 by incubating CM and cells with a polyclonal anti-human IGFBP7 (Santa Cruz Biotechnology, Dallas, TX, USA; catalog no. sc363293) at a final concentration of 3, 9 and 20 g/ml for 30 min at 4 °C. Then, P1 and P10 MSC were cultured for 24 h at 37 °C with the CM-P10 treated with antibodies. After the incubation period, senescence was evaluated by MUG assay as described below. Cell proliferation and apoptosis were assessed by using CM-P10 immunodepleted with the highest antibody concentration (20 g/ml).

Effect of recombinant IGFBP4 and IGFBP7 on MSC senescence and apoptosis. Recombinant human IGFBP4 and/or IGFBP7 (rIGFBP4 and rIGFBP7; PeproTech, London, UK; catalog nos. 350-566 and 350-08, respectively) were added to young MSC (P1) cultures at a final concentration of 0.5, 2.5 and 10 g/μl. After a 36-h incubation period, senescence and apoptosis were evaluated by MUG and annexin V assays, respectively, as described below.

Cell proliferation and annexin V assays. Cell proliferation was evaluated by quick cell proliferation assay kit II (Biovision, Milpitas, CA, USA). One thousand cells were seeded in 96-well culture plates. At 1, 2 and 5 days post plating, cells were collected and counted. The ratio of the total number of cells at day 'n' to the number of cells at day 'n-1' was regarded as the cell proliferation rate. Apoptotic cells were detected using fluorescein-conjugated annexin V (Roche, Milan, Italy) following the manufacturer’s instructions.

Senescence assays. The percentage of senescent cells was calculated in situ by the number of blue β-galactosidase-positive cells out of at least 500 cells in different microscope fields as already reported. For the quantitative SA-β-gal assay, 4-MUG was used as a substrate of β-galactosidase. 4-MUG does not fluoresce until cleaved by the enzyme to generate the fluorophore 4-methylumbelliferylone. Assay was carried out on lysates obtained from cells that were grown in 96-wells plates as reported. The production of the fluorophore was monitored at an emission/excitation wavelength of 365/460 nm.

Cell fractionation. Cell fractionation was performed essentially as previously described. P1 and P10 MSC were lysed in 10 mM Tris-Cl pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5 mM spermidine and 0.5% Nonidet P40. After an incubation of 5 min at 4 °C, nuclear and cytosolic fractions were obtained after centrifugation for 5 min at 500 × g. The nuclei were washed three times in PBS, resuspended in the lysis buffer and further centrifuged at 17,000 × g.

Immunochemistry. Mouse monoclonal anti-8-oxo-dG (clone 2E2; Trevigen, Gaithersburg, MD, USA), rabbit monoclonal anti-phospho histone H2A.X (pH2A.X), rabbit polyclonal anti-H3K9me3, Abcam, Cambridge, UK) antibodies were used for the detection of 8-oxo-quinone, pH2A.X, H1 alpha and H3K9me3, respectively, according to the manufacturer’s protocol. Nuclei were counterstained with Hoechst 33342 and cells were observed through a fluorescence microscope (Leica, Milan, Italy). The percentage of 8-oxo-dG-, H1 alpha-, pH2A.X- and H3K9me3-positive cells was calculated by counting at least 500 cells in different microscope fields.

Western blot analysis. For western blot analysis, proteins from P1 and P10 MSC nuclear and cytosolic fractions (15 μg) were resolved by SDS-PAGE on a 4–15% Mini-Protein TGX precast gel (Bio-Rad, Milan, Italy) under reducing conditions and transferred onto nitrocellulose membrane (Bio-Rad) with a Trans-Blot Turbo electrobott apparatus (Bio-Rad). For the assessment of the purity of the nuclear and cytoplasmatic fractions, monoclonal anti-GAPDH (1:2000, Sigma, Milan, Italy; catalog no. G8795) and polyclonal anti-H1.2 (1:1000, Abcam; catalog no. ab17677) were used as cytoplasmic and nuclear markers, respectively. For the detection of ERK 1/2, anti-ERK 1 was used at a dilution of 1:2000 (Santa Cruz Biotechnology; catalog no. sc-93). Active (pERK) was detected by using an anti-phospho ERK 1/2 diluted 1:1000 (Cell Signaling; catalog no. 9101 L). Immunoreactive protein bands were visualized by the enhanced chemiluminescence (ECL) Kit Western Blotting Detection System (GE Healthcare) according to the manufacturer’s instructions. Western blot results were normalized by stripping and reprobing the membranes with anti-β-actin monoclonal antibody (Sigma-Aldrich, Milan, Italy; catalog no. A2228) used for blot normalization. Densitometry analysis was performed with ImageJ software (http://rsb.info.nih.gov/ij/)

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. This work was partially supported by Ministry of Foreign Affairs Republic of Italy (Senescence of stem cells and Rett syndrome), by European Atomic Community FP-7-Fission 2012 RISK-IR) to UG and by Progetto PON - ‘Ricerca e Competitività 2007–2013’. PON01_00802 entitled: ‘Sviluppo di molecole capaci di modulare vie metaboliche intracellulari rodox-sensitive per la prevenzione e la cura di patologie inflittive, tumorali, neurodegenerative e loro delivery mediante piattaforme nano tecnologiche’.

1. Campisi J, d’Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 2007; 8: 729–740.
2. d’Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer 2008; 8: 513–520.
3. Beyer Nardi N, da Silva Meirelles L. Mesenchymal stem cells: isolation, in vitro expansion and characterization. Handb Exp Pharmacol 2006; 174: 249–282.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al. Multilineage potential of adult human mesenchimal stem cell. Science 1999; 284: 143–147.
IGFBP4 and IGFBP7 promote premature senescence
V Severino et al

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)