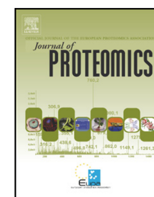
Journal of Proteomics 128 (2015) 18–29

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Proteomic analysis reveals novel common genes modulated in both replicative and stress-induced senescence

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ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 19 June 2015
Accepted 13 July 2015
Available online 20 July 2015

Keywords:
Cellular senescence
Human primary fibroblasts
2D-DIGE
Oxidative stress
Gene regulation
Chromatin immunoprecipitation

ABSTRACT

Cellular senescence causes profound changes in gene expression profile. In this study, we used a combined 2D-DIGE and nanoLC-ESI-LIT-MS/MS approach to evaluate the proteomic changes occurring both in replicative and stress-induced senescence of human IMR90 cells. Twenty protein spots were identified as shifting their quantitative representation in the same direction (over- or down-represented) in both conditions of senescence, which were associated with 25 sequence entries. Dedicated experiments demonstrated that the decreased representation of a set of these proteins is associated with the down-regulation of the corresponding mRNAs, indicating that the regulation of these genes during the senescence process occurs at a transcriptional level. We also performed functional studies by silencing nine of these genes in young cells, which demonstrated that RNA interference-mediated knockdown of LEPRE1, LIMA1/EPLIN, MAGOHA and MAGOHB induces a premature senescent phenotype in IMR90 cells. Chromatin immunoprecipitation experiments indicated that the reduced expression of these four genes is associated with changes in the histone methylation pattern of their promoters, as proved by the occurrence of increased repressive H3K27me3 along with decreased active H3K4me3 marks, respectively.

Biological significance: Cellular senescence, a stable form of cell cycle arrest, is recognized as a phenomenon related to aging and age-related pathologies as well as interfering with tumor progression. Gene expression changes are closely associated with the onset of senescence but the molecular pathways regulating this process are still poorly understood. By using proteomics coupled to functional studies, we here show that both replicative and stress-induced senescence share quantitative modification of four novel proteins, in addition to others already reported in the literature. When ectopically down-regulated, corresponding four genes induce a premature senescence in young cells. The observed parallelism concerning the down-regulation of these genes both *in vitro* and *in vivo* senescent cells may foresee a possible biomarker role of the corresponding proteins in monitoring the progression of both aging and age-related diseases. In conclusion, these results for the first time highlight a possible role of LEPRE1, LIMA1/EPLIN, MAGOHA and MAGOHB in the biology of cellular senescence/aging, thus contributing to gain a deeper knowledge of the molecular mechanisms involved in the senescence program.

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1. Introduction

Cellular senescence was first described by Hayflick and Moorhead, who reported that normal human diploid cells lose irreversibly their replicative capacity after a finite number of duplications *in vitro* [1]. This type of senescence is recognized as replicative senescence and has been essentially attributed to telomere attrition. In addition, several endogenous or exogenous stresses including reactive oxygen species (ROS), ionizing/ultraviolet irradiations, and oncogenic insults have been demonstrated to induce prematurely a senescence phenotype [2–4]. Activation of the senescence process in damaged cells represents a safety program to arrest the expansion of potential cancer cells, thus ultimately acting as a tumor suppressor mechanism [4]. According with this point, numerous studies have demonstrated the existence of

Abbreviations: Chip-qPCR, chromatin immunoprecipitation quantitative polymerase chain reaction; COL1A1, collagen alpha-1(I) chain; DDR, DNA damage response; DEM, diethylmaleate; EJC, exon junction complex; ETP, etoposide; H3K4me3, trimethylation of histone H3 at Lys4; H3K27me3, trimethylation of histone H3 at Lys27; LEPRE1, prolyl 3-hydroxylase 1; LIMA1, LIM domain and actin-binding protein 1; MAGOH, mago-nashi homolog, proliferation-associated; MMP3, matrix metallopeptidase 3; NMD, nonsense-mediated mRNA decay; PDL, population doubling level; RT-qPCR, real time quantitative polymerase chain reaction; ROS, reactive oxygen species; SA-β-gal, senescence-associated-galactosidase; SAHF, senescence-associated heterochromatin focus; SASP, senescence-associated secretory phenotype; siRNA, small interfering RNA; WS, Werner syndrome.

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http://dx.doi.org/10.1016/j.jprot.2015.07.010
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senescent cells *in vivo* at sites of early stages of tumorigenesis associated with mutated BRAF, NRAS and HRAS, as in lungs and naevi [4–6]. Furthermore, senescence can be induced *in vivo* through the inactivation of several tumor suppressor genes, such as PTEN, RB, NF1 and VHL [5, 7–10].

In addition to its role as a tumor suppressor, cellular senescence acts as a mechanism underlying organism aging, and as a causative factor of the onset of age-related pathologies [11–16]. Indeed, accumulation of senescent cells during the life contributes to tissue and organ deterioration *per se*; accordingly, telomere shortening-induced senescence in stem cells can prevent tissue homeostasis and regeneration. By producing a chronic senescence-associated secretory phenotype (SASP), senescent cells can also trigger paracrine senescence in neighboring cells, interfere with architecture/function of tissues, and stimulate inflammatory phenotypes paradoxically promoting a pro-oncogenic environment [16,17]. Of note, it has been recently demonstrated that the genetic elimination of senescent cells in BubR1 progeroid mice counteracts the onset of age-related pathologies in skeletal muscle, eye and fat tissues [18,19]. Finally, very recent studies have demonstrated that the cellular senescence can also represent a developmentally programmed process essential for the fine-tuning of embryogenesis [20,21], thus offering an additional layer of complexity to this phenomenon.

Senescent cells are marked by a number of distinct features that develop slowly after the initial events of irreparable DNA damage that produces an ongoing DNA damage response (DDR), necessary for both initiating and maintaining the senescent phenotype [22–24]. Canonical senescence program in normal cells is activated by two different pathways involving the tumor suppressors p53/p21 and/or p16/pRB [11, 22–24]. In some contexts, p53-independent senescence pathways have also been reported [20,21,25].

Modification of gene expression is a major determinant for the induction of the senescence program, in which are involved specific alterations of transcriptional and post-transcriptional events as well as epigenetic regulatory mechanisms [26–30]. In the latter context, selective histone modifications play a critical role. As exemplified by the formation of senescence-associated heterochromatin foci (SAHF) responsible for E2F target genes silencing [31] or by the activation of the INK4A-ARF locus that encodes p16 [32,33], the methylation status of the lysines within the H3 histone particularly contributes to the stability of the senescent phenotype. Indeed, SAHF are characterized by H3 bearing trimethylated Lys9 (H3K9me3) that acts as a repressive mark, whereas the INK4A-ARF locus displays a decreasing level of trimethylated Lys27 (H3K27me3) behaving as a repressive mark. Very recently, genome-wide studies have associated lamin B1-dependent chromatin reorganization upon senescence not only to SAHF formation but also to gene expression regulation. In fact, local increase of H3K27me3 facilitated repression of a subset of genes involved in cell proliferation [34], whereas depletion of H3K4me3 and H3K27me3 paralleled with down- and up-regulation of proliferating and SASP genes, respectively [35]. An increase of repressive marks and a decrease of active marks were also observed during oncogene-induced senescence [36].

In this study, we explored the proteomic changes occurring in cellular senescence induced either by telomere shortening or by oxidative stress of human diploid IMR90 fibroblasts with the aim to identify novel genes acting as common mediators of the senescence process. We here demonstrate that both replicative and oxidative-stress induced senescence share down-regulation of four genes, in addition to that of others already described, which are novel contributors of the senescence program. Chromatin immunoprecipitation experiments showed that *de novo* distribution at promoter level of H3K4me3 and H3K27me3 marks strongly correlates with under-expression of LEPRE1, LIMA1/EPLIN, MAGOHA and MAGOHB genes. By using fibroblasts cell lines from old humans, we finally provide preliminary evidences that declined expression of these genes is related to *in vivo* senescence.

2. Materials and methods

2.1. Cell cultures and reagents

Normal human primary fibroblasts IMR90 were obtained from Coriell Institutes for Medical Research (CCR; New Jersey, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Groningen, The Netherlands) supplemented with 10% v/v fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). WI38 at passage 11 (p11) or 15 (p15) were kindly provided by Fabrizio d'Adda di Fagagna (Istituto di Oncologia Molecolare, Fondazione Italiana per la Ricerca sul Cancro, Milan, Italy) and cultured in modified Eagle's medium supplemented with 10% FBS, 10 mM non-essential amino acids, and 1 mM sodium pyruvate (Invitrogen) until passage 23 (p23). The population doubling level (PDL) was calculated by using the formula $PDL = \log(n_h/n_i)/\log2$, where n_i is the initial number of cells and n_h is the final number of cells at each passage. To induce premature senescence, IMR90 at PDL34 or WI38 cells at PDL30 were treated with 100 μM diethylmaleate (DEM) (Sigma-Aldrich) for ten days or challenged with 50 μM etoposide (Sigma-Aldrich) for 24 h, and then subcultured for another 10 days [37,38].

2.2. Two-dimensional differential in-gel electrophoresis (2D-DIGE)

For 2D-DIGE analysis, total proteins from three independent biological replicates of IMR90 at PDL34, IMR90 at PDL58, or IMR90-DEM treated cells, were extracted with UTC solution (7 M urea, 2 M thiourea, and 4% w/v 3-[(3-cholamidopropyl)dimethylammonium]-1-propane sulfonate). Lysates were precipitated in 8:1 v/v acetone/methanol for 16 h, at −20 °C, and centrifugated at 16,000 ×*g* for 30 min, at 4 °C. Protein pellets were dissolved in UTC solution and samples (30 μg in a final volume of 10 μL) were labeled with the appropriate CyDye (240 pmol of Cy2, Cy3, or Cy5) according to the DIGE minimal labeling protocol (GE Healthcare, Milan, Italy) by implementation of a dye-swapping strategy [39]. An equal mixture of all three samples of each biological replicate was labeled with Cy2 as internal standard during gel electrophoresis to allow gel-to-gel matching and in-gel analyses of relative protein spot intensities. Three sample mixtures, made of appropriate Cy3- and Cy5-labeled pairs and a Cy2-labeled control, were supplemented with UTC solution containing 130 mM DTT, 2% immobilized pH gradient (IPG) buffer (pH 3–10 NL), and 2.8% DeStreak reagent (GE Healthcare, Little Chalfont, UK), and used for passive hydration of immobilized pH gradient (IPG) strips (24 cm) to run the isoelectric focusing on IPGphor II apparatus (GE Healthcare). After the first dimension, the strips were equilibrated and transferred into 12% polyacrylamide gels to perform the second dimension. SDS-PAGE was performed on a DALT II electrophoresis unit (GE Healthcare) at 1 W/gel for 16 h. Gels were scanned on a Typhoon 9400 variable mode imager (GE Healthcare), with the indicated excitation/emission wavelengths for Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm). 2D-DIGE images were acquired in the Image Quant software (GE Healthcare) and analyzed by using the DeCyder 6.0 software (GE Healthcare). A DeCyder differential in-gel-analysis module was used for spot detection and pairwise comparison of each sample (Cy3 and Cy5) to the Cy2 mixed standard present in each gel. The DeCyder biological variation analysis module was then used to simultaneously match all of the protein-spot maps from the gels, and to calculate average abundance ratios and *p* values across the triplicate sets of samples (Student's *t*-test). Only protein spots with a value of $p \le 0.005$ were investigated further.

For preparative protein separations, 600 μg of unlabeled total proteins from pooled IMR90 at PDL34, IMR90 at PDL58 and IMR90-DEM extracts were used for passive hydration of 24 cm strips for the first gel dimension (pH 3–10 NL IPG strips, GE Healthcare). The first and second-dimension runs were carried out as described above. After 2-DE, gels were fixed and stained with SyproRuby fluorescent stain (Invitrogen-Life Technologies Italia, Monza, Italy). Finally, spots were

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