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Original Articles

Pre-malignant transformation by senescence evasion is prevented by the PERK and ATF6alpha branches of the Unfolded Protein Response

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ABSTRACT

The incidence of carcinomas highly increases with age. However, the initial steps of the age-related molecular carcinogenic processes remain poorly characterized. We previously showed that normal human epidermal keratinocytes spontaneously and systematically escape from senescence to give rise to preneoplastic emerging cells through a process called post-senescence neoplastic emergence (PSNE). To identify molecular pathways involved in the switch from senescence to pre-transformation, we performed Connectivity Map analyses and DAVID functional annotations followed by hierarchical clustering and multidimensional scaling of the gene expression signature of PSNE cells. We identified endoplasmic reticulum stress related pathways as key regulators of PSNE. Invalidation by RNA interference of the UPR sensors PERK, ATF6α, but not IRE1α, delayed the occurrence of senescence when performed in pre-senescent cells, and increased the PSNE frequency when performed in already senescent cells. Conversely, endoplasmic reticulum stress inducers applied to already senescent cells decreased the frequency of PSNE. In conclusion, these results indicate that the activation of the UPR could protect from the early carcinogenic steps by senescence evasion. This opens new avenues to explore therapeutics that could be useful in decreasing the age-associated tumor incidence.

1. Introduction

Epidemiological data show that amongst the different types of cancer in humans, carcinomas are the most frequent and the most linked with advanced age (NCI cancer statistics). This suggests that aging may enhance carcinogenesis. Aging is accompanied by an accumulation in numerous tissues of senescent cells, which are viable but have lost their ability to divide [1]. *In vitro*, the senescent cells exhibit deep molecular and morphological changes including increase in cell size, increase in the number of lysosomes and autophagic activity, increase in the activity of the β-Galactosidase enzyme, changes in the organization of chromatin [2], polynucleation [3,4], and changes in transcriptome and proteome [5-7]. Moreover, the characterization of the senescence-associated secretory phenotype (SASP) showed high levels of pro-inflammatory cytokines, growth factors and remodeling enzymes of the extracellular matrix [8]. Senescence is characterized by a stable proliferation arrest, mainly controlled by the p53/p21^{WAF1} and p16^{INK4}/Rb pathways [9]. Because of the robustness of this cell cycle arrest, senescence was recognized as a cell-autonomous tumor suppressor mechanism [10]. However, several lines of evidence indicate that senescence would also have a tumor promoter role. This relies in part on the SASP which was shown to promote in a paracrine manner the proliferation of cancerous cells and the appearance of tumor clones [11]. However, senescence could also have cell-autonomous tumor promoter properties rather than tumor suppressor ones in certain cell types or under certain senescence inducers. We and others have shown that Normal Human Mammary Epithelial Cells (HMECs) and Normal Human Epidermal Keratinocytes (NHEKs) can spontaneously and systematically escape from the senescent state and re-enter cell cycle at a frequency of 10^{-2} to 10^{-4} to generate a progeny by an unusual asymmetric mitosis mechanism (budding). We called this process “Post-senescence neoplastic emergence” (PSNE) [12-16]. PSNE cells have undergone a slight epithelial to mesenchyme transition which is greatly enhanced when they are submitted to the SASP of senescent fibroblasts

Abbreviations: PSNE, Post-Senescence Neoplastic Emergence; UPR, Unfolded Protein Response; NHEK, Normal Human Epidermal Keratinocyte

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[14,17]. PSNE cells are mutated but do not display major karyotypic aberrations [13,14]. And more importantly, when xenografted in *nude* mice, they developed in disseminated skin lesions which were characterized as hyperplasias and small non-melanoma skin carcinomas, evidencing their tumorigenic potential [13,14]. We recently demonstrated that in contrast to most classical models of senescence such as replicative senescence of fibroblasts, senescence of NHEKs and HMECs does not involve the DNA double-strand breaks (DSBs) pathway. Instead, it involves an accumulation of oxidatively-induced DNA single-strand breaks (SSBs) which remain unrepaired because of a decrease in the expression and activity of PARP1, the enzyme that initiates the SSB repair. These unrepaired SSBs are not only the inducers of the senescent cell cycle arrest but are also the mutagenic motor of PSNE [13].

By comparing the transcriptome of proliferating, senescent and PSNE NHEKs, we established what we have called the PSNE signature. This signature comprises the genes that are up- or down-regulated in PSNE NHEKs compared to both proliferating and senescent NHEKs. It therefore might represent the transcriptome that underlies both the transformed phenotype of the PSNE cells and the switch from senescence to transformation. A first series of bioinformatics analyses of this PSNE signature confirmed that it is relevant to human epithelial cancer and characteristic of a pre-transformed state [18]. In the present study, we further explored the molecular basis of the early transformation process by senescence evasion by performing new bioinformatics analyses of the PSNE signature. We thereby identified pathways related to endoplasmic reticulum (ER) stress as potentially positively contributing to senescence and negatively impacting PSNE.

The ER is a network of membranous cisternae and tubules that plays major roles in calcium homeostasis, lipid synthesis, and protein synthesis, folding and post-translational modifications. ER stress is known to occur when unfolded or misfolded proteins accumulate in the ER lumen. In such situations, the Unfolded Protein Response (UPR) pathway is activated. It is initiated by three ER transmembrane sensors: PERK, ATF6α and IRE1α. All three activate specific pathways that will cooperate to globally reduce the synthesis of proteins, but specifically increase the expression of ER chaperones in order to restore ER homeostasis. They also contribute to increase autophagy and induce protein degradation through the ERAD system which involves the proteasome [19]. Numerous data strongly highlight ER stress and UPR activation as potential components of the senescent phenotype (for review see Ref. [20]). Recently, we showed that senescence of fibroblasts is associated to an ER expansion accompanied by the activation of the UPR, especially of ATF6α, and in a less extent of IRE1α. We demonstrated the key role of this UPR activation in controlling the morphological changes associated with senescence, notably the loss of the parallel organization of the fibroblasts and the loss of their fusiform shape [21]. Here, following the bioinformatics analyses, we investigated the activation and the functional involvement of the UPR in senescence of NHEKs and its impact on post-senescence emergence. Functional approaches demonstrated the involvement of the UPR, in particular of the PERK and ATF6α signaling, in the establishment of senescence in NHEKs, and, interestingly, documents for the first time that this stress response pathway protects from the first step of cancer initiation by senescence evasion.

2. Materials and methods

2.1. Reagents and cell culture

Normal human epidermal keratinocytes (NHEKs) were purchased from Clonetics (CC-2501) or Promocell. We used cells from 3 different donors of different race and age (referred as 4F0315, 2F1958, and K1MC). Cells were obtained anonymously and informed consent of each skin donor was obtained by the supplier. Cells were grown at 37 °C in an atmosphere of 5% CO2 in a KGM-Gold Bullet kit medium consisting of modified MCBD153 with 0,1 mmol/L calcium, supplemented with bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin, and epinephrin (Lonza). Such a serum-free low-calcium medium was shown to minimize keratinocyte terminal differentiation [22]. Cells were seeded at 3500 cells/cm^2 and always split at 70% confluence. The number of population doublings (PDs) was calculated at each passage by means of the following equation: PD = ln (number of collected cells/number of plated cells)/ln2. Cells were considered senescent when having reached a growth plateau during which they display increased cell size, polynucleation, accumulation of vacuoles and various damaged components, and SA-β-Gal activity, as described in Ref. [23] (Fig. S1). Emergent cells resulted from an atypical budding mitosis generating clones of PSNE cells as described in Ref. [23] (Fig. S1). Thapsigargin and ionomycin were obtained from Sigma. Lysotracker was purchased from Molecular Probes. Living cells were incubated with the Lysotracker probe directly added to the culture medium at 37 °C for 30 min as recommended by the supplier.

2.2. Calculation of PSNE frequency

After chemical treatment or siRNA transfection, senescent NHEKs were seeded into 10 cm diameter dishes at the limit density for emergence (10,000 cells per dish). Between 4 and 10 days later, cells were fixed and stained by crystal violet. The emergent clones were counted and the PSNE frequency was calculated as the ratio of the number of clones on the number of seeded senescent cells, as described in Ref. [16].

2.3. DAVID functional annotation

Genes composing the PSNE signature were annotated with gene ontology terms using DAVID (http://david.abcc.ncifcrf.gov/). The "functional annotation tool" was selected with the default parameters and additionally protein interaction data from the "BIND", "DIP", "MINT" as annotation databases. The functional groups (FGs) were determined by using the "Functional Annotation Clustering". The genes composing each FG correspond to those at the intersection between the annotation terms from the databases and genes from the PSNE signature. The obtained FGs are listed in Table S2.

2.4. Hierarchical clustering (HC) and multidimensional scaling (MDS)

Since a same gene can be shared by different FGs, similarities between all FGs were measured by the number of their shared genes (gDist). For each pair of FGs, gDist was calculated as gDist = $N(A) \cap N(B)/\min(N(A), N(B))$, where $N(A)$ denotes the number of genes in FG (A). All the genes composing a FG were used for the calculation. A similarity of 1 between two FGs indicates that the smallest FG is a subset of the largest one. The matrix of pair wise gDist values (as dissimilarities = 1-gDist) for the 25 most significant FGs from the DAVID annotation was used as input for MDS and HC. For MDS, the R function "sammon" from the R package MASS was used with default parameters. In brief, the procedure, which is a form of non-metric multidimensional scaling, places the samples (FGs) in n-dimensional space to reflect their similarity as measured by gDist. It enables to visualize similarity/dissimilarity of all samples in n-dimensional space. Here, a solution for two dimensions was sought. The coordinates of the FGs on the first two axes (dimensions) of the MDS are shown in Fig. 2A. Additionally, circles were drawn around the coordinates of FGs where the size of the circles indicates the relative size (number of genes) of the FGs. Therefore, FGs with a distance of 0 can be visualized. For HC and the heatmap in Fig. 2B, the R function "hclust" was used in combination with the "heatmap.2" function using the "average linkage" algorithm. The labels of the FGs in Fig. 2A were chosen as the most significant annotation term within each FG (see Table S2).

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