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RESEARCH ARTICLE

Transcriptional activation of genes associated with the matrisome is a common feature of senescent endothelial cells

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Abstract Cellular senescence is a stable cell cycle arrest that occurs in response to various stress stimuli and affects multiple cell types, including endothelial cells (ECs). Senescent cells accumulate with age, and their removal has been linked to reduced age-related diseases. However, some senescent cells are important for tissue homeostasis. Therefore, understanding the diversity of senescent cells in a cell-type-specific manner and their underlying molecular mechanisms is essential. Senescence impairs key ECs functions which are necessary for vascular homeostasis, leading to endothelial dysfunction and age-related vascular diseases. In order to gain insights into these mechanisms, we analyzed publicly available RNA-seq datasets to identify gene expression changes in senescent ECs induced by doxorubicin, irradiation, and replication exhaustion. While only a few genes were consistently differentially expressed across all conditions, some gene ontologies (GO) were shared. Among these, our analysis focused on validating the expression of genes associated with the matrisome, which includes genes encoding for extracellular matrix (ECM) structural components and ECM-associated proteins, in a doxorubicin-induced senescence model. Our results show that the matrisome transcriptome undergoes significant remodeling in senescent endothelial cells, regardless of the specific inducers of senescence, highlighting the importance of understanding how ECM alterations affect senescence.

Keywords Cellular senescence · Matrisome · Extracellular matrix · Endothelial cells · RNA-seq

Abbreviations

ECs	Endothelial cells
DEGs	Differentially expressed genes
ECM	Extracellular matrix
GO	Gene ontology

Introduction

Cellular senescence refers to an irreversible cell cycle arrest triggered by various stress factors such as DNA damage, oxidative stress, telomere shortening, and oncogenic signaling (Campisi and D'Adda Di Fagagna 2007). This condition can be induced either by replicative exhaustion, characterized by telomere shortening, or by external stressors such as DNA-damaging agents, including radiation or the chemotherapeutic agent doxorubicin, which leads to

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stress-induced premature senescence independent of telomere length (González-Gualda et al. 2021). Despite losing their ability to proliferate, senescent cells remain metabolically active and secrete a wide range of pro-inflammatory cytokines, chemokines, growth factors, and metalloproteases, collectively known as the senescence-associated secretory phenotype (SASP) (Coppé et al. 2008). Cellular senescence can have both positive and negative effects. On one hand, it serves as a tumor-suppressive mechanism by halting the proliferation of damaged cells, and it also plays a role in maintaining homeostasis in processes such as embryonic development and wound healing (Muñoz-Espín et al. 2013; Storer et al. 2013; Ohtani 2022). In these contexts, senescent cells are transiently present, and once their role is fulfilled, they are typically eliminated by the immune system (Prata et al. 2018). On the other hand, as organisms age, the immune system's ability to eliminate senescent cells declines, leading to their accumulation in various tissues, including endothelial tissue (Jeyapalan et al. 2007; Pantsulaia et al. 2016; Idda et al. 2020). This accumulation has been associated to the development of several age-related pathologies highlighting the dual nature of cellular senescence.

Endothelial cells (ECs), which form the inner lining of blood vessels, are more susceptible to becoming senescent than other tissues, due to their direct contact with the bloodstream (Han and Kim 2023). Because of to their unique localization, ECs are continually exposed to different circulating molecules that are carried through the blood (i.e., hormones, nutrients, glucose and lipids) as well as the changing hemodynamic forces exerted by the blood flow.

These frequent environmental changes may contribute to oxidative stress, DNA damage, and the eventual onset of endothelial senescence. One significant consequence of endothelial senescence is the disruption of vascular homeostasis, leading to endothelial dysfunction. This dysfunction is characterized by impaired nitric oxide (NO) production, increased oxidative stress, and a shift toward a pro-inflammatory and pro-thrombotic environment (Jia et al. 2019). Reduced NO bioavailability in senescent ECs results in decreased vasodilation and increased vascular stiffness. Additionally, the SASP generated by senescent endothelial cells can also contribute to vascular inflammation, tissue remodeling, and the progression of age-related vascular diseases such as atherosclerosis, hypertension, stroke, and heart failure (Han and Kim 2023). Consequently, the elimination of senescent cells through the use of compounds that selectively induce apoptosis in senescent cells termed senolytics (Lelarge et al. 2024), has shown potential to reduce the burden of senescent cells, alleviating age-related physical dysfunction (Xu et al. 2018). Research has demonstrated that long-term administration of senolytic agents can enhance vasomotor function and diminish markers of senescence in aged mice (Roos et al. 2016). Although senolytics offer promising benefits, certain senescent cells play crucial roles in maintaining organ structure and function, and their removal in mice has been linked to tissue damage and organ dysfunction (Grosse et al. 2020). Therefore, the therapeutic application of senolytics requires careful consideration of their systemic impact. In order to further refine therapeutic approaches, there is a growing need to address senescence in a cell-type-specific manner (Zhu et al. 2016).

The heterogeneity of senescent cells and their functions complicates their detection and the design of specific senolytics to selectively eliminate harmful senescent cells. Transcriptomic and proteomic analyses have revealed that different senescence-inducing factors can lead to distinct cell-type-specific senescence molecular signatures even under the same stressors (Casella et al. 2019; Basisty et al. 2020). For instance, endothelial cells and fibroblasts show divergent responses to identical stressors, emphasizing the complexity of senescence biology (Casella et al. 2019). This underscores the importance of understanding the heterogeneity of senescent cells, their distinct functions across tissues and the unique molecular mechanisms used by different senescent cells to survive.

To gain a deeper understanding of how different senescence-inducing factors influence cellular pathways in endothelial cells, we conducted a comparative analysis of publicly available RNA-Seq datasets from human umbilical vein endothelial cells (HUVECs) subjected to senescence induced by doxorubicin, irradiation, and replicative exhaustion. We identified differentially expressed genes across all datasets and performed Gene Ontology (GO) enrichment analysis. Several common GO categories were found for both upregulated and downregulated genes. Notably, the "matrisome" and "extracellular matrix (ECM)" categories emerged as key upregulated GO terms for all

the senescence models. Based on these findings, we selected genes related to the matrisome for further validation using qPCR in a doxorubicin-induced ECs senescence model. Our results highlight the importance of assessing the role that ECM reorganization plays in the function of senescent ECs.

Methods

Data acquisition and processing

To compare gene expression in senescent endothelial cells induced under various conditions, we selected three publicly available datasets from the NCBI Gene Expression Omnibus (GEO) database (Kodama et al. 2012), shown below. The selected datasets focus on senescence induced by doxorubicin, radiation, and replication. A summary of the datasets, including control and senescent samples, is provided in Table 1.

Raw RNA reads from each dataset were processed using the Nextflow RNA-seq workflow (Ewels et al. 2020), with the human genome GRCh38 reference and annotation, applying default parameters. Gene counts were analyzed to identify differentially expressed genes (DEGs) using DESeq2 (Love et al. 2014), comparing gene expression between control and senescent samples for each condition. DEGs were defined by an absolute log2 fold change greater than 1 and an adjusted p-value ≤ 0.05. Gene ontology (GO) enrichment analysis of the DEGs was performed using Metascape (Zhou et al. 2019).

Cell culture conditions

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (cat. no. C2519A) and cultured in a humidified incubator at 37 °C with 5% CO_2. Cells were maintained in Endothelial Growth Medium-2 (EGM-2) prepared by supplementing Endothelial Basal Medium-2 (EBM-2, Lonza, cat. no. CC-3156) with the EGM-2 SingleQuots kit (Lonza, cat. no. CC-4176), which contains growth factors including hydrocortisone, human fibroblast growth factor (hFGF-B), vascular endothelial growth factor (VEGF), insulin-like growth factor (R3-IGF-1), ascorbic acid, heparin, human epidermal growth factor (hEGF), and antibiotics (GA-1000). Cells were used between passages three and eight for all experiments.

Doxorubicin-induced senescence

HUVECs were seeded in T-75 cell culture flasks at densities of 3×10^5 cells for the control group and 7×10^5 cells for the treatment group. Different seeding densities were used to account for the reduced proliferative capacity of senescent cells and to prevent overgrowth in the proliferating control cells, which were collected earlier than the senescent cells. After a 24-h attachment period, the medium was replaced with fresh medium containing either vehicle (DMSO) for the control group or 100 nM doxorubicin for the treatment group. Cells were incubated under these conditions for 24 h.

Following the incubation, control cells were collected, while doxorubicin-treated cells were washed thoroughly with PBS and cultured in fresh medium for an additional 8 days to allow the senescent phenotype to develop. Cell viability was assessed on the day of sampling using the trypan blue exclusion method. To analyze senescence-associated β-galactosidase (SA-β-gal) activity, equal numbers of cells from each condition were plated in 24-well plates. For immunofluorescence analysis, cells were plated on poly-L-lysine-coated coverslips. Additionally, cells were lysed in TRK buffer (EZNA RNA Extraction Kit, #R6834-02) for RNA extraction and in RIPA buffer

Table 1 Description of datasets used for this analysis

SRA study	Condition	Control samples	Senescent samples
SRP185629	Doxorubicin-induced senescence	SRR8564858, SRR8564859, SRR8564860	SRR8564861, SRR8564862, SRR8564863
SRP195418	Irradiation-induced senescence	SRR9016151, SRR9016152, SRR9016153	SRR9016154, SRR9016155, SRR9016156
SRP106038	Replicative exhaustion senescence	SRR5494699, SRR5494700, SRR5494701	SRR5494710, SRR5494711, SRR5494712

(Thermo Scientific, #89900) for protein collection. RNA and protein samples were stored at − 80 °C until further analysis.

Senescence-associated β-galactosidase (SA-β-gal) activity assay

Senescence-associated β-galactosidase (SA-β-gal) activity was assessed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, cat. no. 9860,) following the manufacturer's protocol. After induction of senescence in the T-75 flasks, 20,000 number of cells were seeded into a 24-well plate to assess that senescence was induced in each experiment. Cells were allowed to attach to the plate 24 h and then fixed with the provided fixation solution for 10–15 min at room temperature. After fixation, cells were washed with PBS and incubated with the β-galactosidase staining solution (1 ×) containing X-gal at pH 6.0 at 37 °C in a CO_2-free incubator overnight. The next day, cells were examined under a bright-field microscope, and the percentage of cells showing blue staining (indicative of SA-β-gal activity) was calculated by counting the number of stained cells relative to the total number of cells in multiple random fields per well. Stained cells were imaged, and representative fields were captured.

Immunofluorescence

Immunodetection of proliferation and DNA damage markers was carried out as previously described (Arredondo et al. 2020). Briefly, the covers containing the cells were carefully washed three times with PBS, then permeabilized for 5 min with PBS + 0.5% Triton X-100. After washing three times with PBS, the cells were incubated for 30 min with blocking solution (PBS + 3% donkey serum) and then incubated overnight at 4 °C with primary antibodies diluted in blocking solution. On the following day, the cells were washed three times with PBS and incubated with secondary conjugated antibodies for 1 h at room temperature. The cells were washed with PBS and mounted with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA). Primary antibodies used were: rabbit anti-Ki67 (Abcam, ab16667) and rabbit anti-γH2AX (Cell Signaling, #9718). NucBlue (Life Technologies) was used as a nuclear dye. Image acquisition for analysis was performed using a ×40 objective on an Epi-Fluorescence Ti-E Microscope (Nikon). Representative images were acquired by confocal laser microscopy (Leica TCS SP8). Proliferation and DNA damage were expressed as percentages of cells positive for Ki67 or γH2AX of the total number of NucB-positive cells.

Selection of gene target for validation and RT-qPCR

To identify gene targets representing different categories of the matrisome, we accessed MatrisomeDB (Shao et al. 2023) in August 2024 and downloaded the list of all matrisome gene categories for human blood vessels. These genes included core matrisome components (collagens, ECM glycoproteins, and proteoglycans) and matrisome-associated proteins (ECM-affiliated proteins, ECM regulators, and secreted factors). We then cross-referenced the upregulated genes in doxorubicin-induced senescent cells, selecting those with the highest fold change and lowest adjusted p-value to design qPCR primers for genes representing each of the above-mentioned categories. Primers were designed using Primer-BLAST (Ye et al. 2012), specifying an amplicon length between 100 and 200 bp and ensuring that the primer pairs were separated by at least one intron in the corresponding genomic DNA.

RNA extraction and cDNA synthesis for qPCR

Total RNA was extracted using the E.Z.N.A.® RNA Kit (#R6834-02). RNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific). To remove genomic DNA contamination, RNA samples were treated with DNase I (Invitrogen, AM1907). A total of 1 µg of DNase I-treated RNA was used to synthesize complementary DNA (cDNA) with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) in a final reaction volume of 20 µL. For qPCR, all cDNA samples were diluted 1:10 in nuclease-free water. Gene expression was quantified using the Takyon™ SYBR® Green MasterMix (Eurogentec, #UF-NSMT-B0701) on an AriaMX Real-Time PCR system (Agilent). For primers used for detecting senescence markers, relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the housekeeping gene. For newly designed primers, the Pfaffl method was used, incorporating primer-specific

amplification efficiencies, determined through standard curve analysis. All statistical analyses and graph generation were performed using GraphPad Prism (v10.3.1). A complete list of qPCR primers is available in Table S1, and all primers were purchased from Integrated DNA Technologies (IDT).

Statistical analysis

All experiments were carried out in triplicate. Data points and error bars in the figures represent averages and standard errors. Data distribution was analyzed using the Shapiro–Wilk normality test. Statistical analysis was performed to identify changes between proliferating and senescent cell cultures using a Welch's t-test using Prism statistical software (GraphPad, Prism). Statistical significance was set at $p < 0.05$.

Results

Identification of differentially expressed genes in endothelial cells induced to senescence by diverse stimuli

Publicly available RNA-seq datasets from human umbilical vein endothelial cells (HUVECs) induced to senescence by doxorubicin treatment (Mongiardi et al. 2019), irradiation (Casella et al. 2019), and replication exhaustion (Sofiadis et al. 2021) were retrieved from the NCBI Gene Expression Omnibus (GEO) database for analysis (details in Table 2). Despite the different methods used to induce senescence across datasets, all the studies utilized SA-β-Galactosidase (SA-β-Gal) activity as a marker to verify senescence induction. Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al. 2014), with an absolute log2 fold change > 1 and adjusted p-value ≤ 0.05 as selection criteria. Details of data processing and bioinformatics workflows are provided in the Methods section.

The results indicate that all three conditions—doxorubicin treatment, irradiation, and replication exhaustion—resulted in significant changes in gene expression. Specifically, doxorubicin treatment led to the upregulation of 1275 genes and the downregulation of 930 genes. Under irradiation exposure, 339 genes were upregulated while 234 were downregulated. Similarly, replication exhaustion resulted in substantial changes in gene expression, with 109 genes upregulated and 204 genes downregulated. These findings highlight the impact of different senescence-inducing stimuli on the transcriptome of endothelial cells (Table 3).

To identify the commonly regulated genes between the different senescence conditions in HUVECs, a Venn diagram analysis was performed. This approach allowed for the visualization and quantification of genes that are consistently upregulated (Fig. 1a) and downregulated (Fig. 1b) in response to doxorubicin treatment, irradiation, and replication exhaustion. The results of the Venn diagram revealed that doxorubicin treatment resulted

Table 3 Summary of DEGs for each senescence inductor

Condition	Upregulated[a]	Downregulated[b]
Doxorubicin	1275	930
Irradiation	339	234
Replication exhaustion	109	204

[a] $\log_2 FC \geq 1$, p-adj ≤ 0.05

[b] $\log_2 FC \leq -1$, p-adj ≤ 0.05

Table 2 Summary of RNA-seq datasets used for analysis

Senescence inducer	Accession	Senescence induction protocol	Senescence markers	References
Doxorubicin	GSE126426	1 h treatment with 5 µM doxorubicin, followed by 4 days in fresh medium	SA-β-Gal activity (80% beta-gal + cells)	Mongiardi et al. (2019)
Irradiation	GSE130727	Cells exposed to 4 Gy, harvested 10 days post-IR	SA-β-Gal activity and p16 mRNA level	Casella et al. (2019)
Replication	GSE98440	Cells were continuously passaged until replicative exhaustion	SA-β-Gal activity and proliferation rate	Sofiadis et al. (2021)

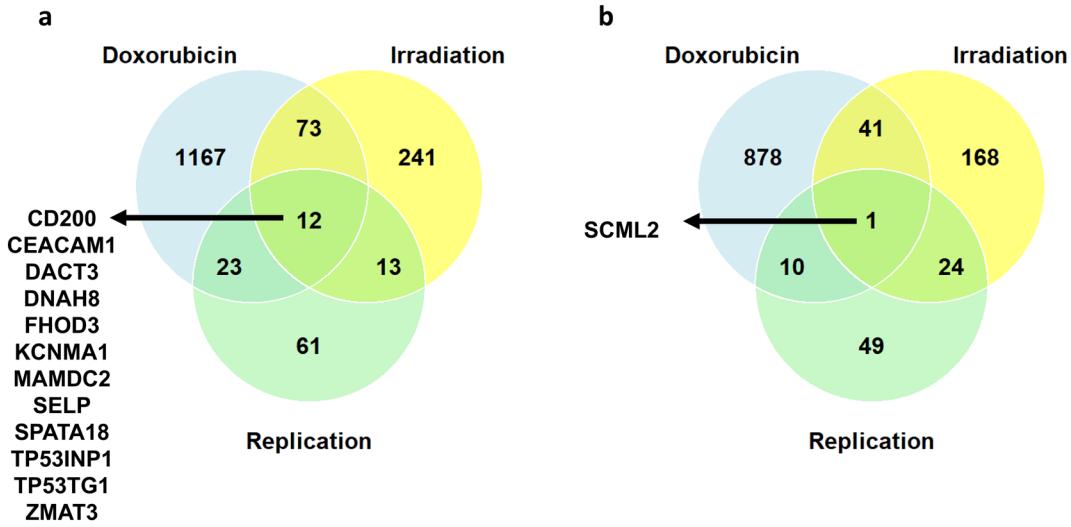


Fig. 1 Relationship of expressed genes (DEGs). The overlapping portions of the different circles represent the number of DEGs common to the different conditions for **a** upregulated and **b** downregulated genes

in 1167 uniquely upregulated genes, irradiation in 241, and replication exhaustion in 61. There were 73 genes commonly upregulated between doxorubicin and radiation, 13 between radiation and replication exhaustion, and 23 between doxorubicin and replication exhaustion. Additionally, a set of 12 genes was upregulated across all three conditions. In the distribution of downregulated genes, doxorubicin treatment led to 878 uniquely downregulated genes, while irradiation and replication exhaustion resulted in 168 and 49 unique downregulated genes, respectively. There were 41 genes commonly downregulated between doxorubicin and radiation, 24 between radiation and replication exhaustion, and 10 between doxorubicin and replication exhaustion. Only one gene was commonly downregulated across all three conditions. Common changes in gene expression included the upregulation of CD200, CEACAM1, DACT3, DNAH8, FHOD3, KCNMA1, MAMDC2, SELP, SPATA18, TP53INP1, TP53TG1, ZMAT3 (Fig. 1a) and the downregulation of SCML2 (Fig. 1b), as summarized in Table 4.

Table 4 Description of commonly downregulated and upregulated genes in all three conditions

Gene name	Description	Regulation statues
CD200	Cluster of differentiation 200	Upregulated
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	Upregulated
DACT3	Dapper antagonist of catenin 3	Upregulated
DNAH8	Dynein axonemal heavy chain 8	Upregulated
FHOD3	Formin homology 2 domain containing 3	Upregulated
KCNMA1	Potassium calcium-activated channel subfamily M alpha 1	Upregulated
MAMDC2	MAM domain containing 2	Upregulated
SELP	Selectin P	Upregulated
SPATA18	Spermatogenesis associated 18	Upregulated
TP53INP1	Tumor protein P53 inducible nuclear protein 1	Upregulated
TP53TG1	Tumor protein P53 target 1	Upregulated
ZMAT3	Zinc finger matrin-type 3	Upregulated
SCML2	Sex comb on midleg-like protein 2	Downregulated

Integration of commonly differentially expressed genes with senescence-associated databases

To better understand the significance of the DEGs in cellular senescence, we examined whether these DEGs have been previously catalogued in senescence-associated databases. This analysis aims to contextualize our findings within the established framework of known senescence markers and pathways. Specifically, we utilized the SenMayo and SeneQuest gene sets. The SenMayo gene set, developed at the Mayo Clinic, has been used to identify senescent cells across various tissues and species, including humans and mice. This set primary focuses on the expression of genes associated with the SASP (Saul et al. 2022). SeneQuest, developed by the International Cell Senescence Association (ICSA), is a comprehensive literature-based evidence database that catalogues genes associated with cellular senescence in different cell types, including HUVECs (Gorgoulis et al. 2019). Due to the limited number of differentially expressed genes common across all conditions, we chose to focus on genes that were differentially expressed in doxorubicin-treated cells and at least one additional condition for further analysis. This selection strategy was applied because we aim to validate the expression of selected target genes by RT-qPCR using a doxorubicin-induced senescence cellular model. We identified 108 upregulated and 52 downregulated genes meeting this criterion. Our analysis revealed that only one commonly upregulated gene ICAM1 (1 out of 108) and none of the commonly downregulated genes (0 out of 52) were present in the SenMayo dataset.

For SeneQuest, we conducted two separate analyses. First, we filtered genes by cell type and selected HUVECs. Using this filtering we identified 5 commonly upregulated (BTG2, CLDN1, DIO2, PTCHD4, and CDKN2B) and 7 commonly downregulated genes (CDCA5, EXO1, FEN1, HPSE, MEOX2, MYB, and SERPINB2) that were previously described in the database under this specific cell type (Table 5). To extend our analysis beyond cell type-specific findings, we performed a second analysis using the entire SeneQuest database, encompassing all available gene entries regardless of cell type specificity. We identified that a larger number of the DEGs were previously associated with cellular senescence, including 84 upregulated and 47 downregulated genes (Table 5). Furthermore, 24 upregulated (ABCA9, FAM211B, IAA1107, RP11-434C1.1, C6orf47-6, GDF7, KLHDC9, TMEM35, C8orf4, GOLGA8N-2, LOC100287896, ZNF233, CPA3, GPC5, LOC102723630, CXorf36, DDR1-4, GRAMD2, LOC90246, TNR, F2RL2, IFI27-2, PCDH15, and YAE1D1) and 5 downregulated genes (CCL23, CIDEA, GSDMC, TUBA4A, and ZNF732) have not previously been described in the SeneQuest database and are potentially novel in their association with cellular senescence.

Pathway enrichment analysis of DEGs in senescent endothelial cells shows increased expression of genes associated with extracellular matrix organization

To understand the biological roles of these DEGs, we performed a gene ontology (GO) enrichment analysis using Metascape for upregulated genes in doxorubicin-induced (Fig. 2a), irradiation-induced (Fig. 2b), and replication exhaustion senescence (Fig. 2c). Common GO categories for upregulated genes include extracellular matrix (ECM) organization and the core matrisome. The matrisome refers to a set of proteins that are structural components of the ECM and other ECM-associated proteins, including ECM regulatory proteins, which control several cellular processes such as cell survival, proliferation, differentiation (Hynes and Naba 2012). Conversely, GO analysis for downregulated genes for doxorubicin-induced (Fig. 3a), irradiation-induced (Fig. 3b), and replication exhaustion senescence (Fig. 3c) reveals several common categories. Downregulated genes

Table 5 Presence or absence of DEG in SeneQuest database

Differentially expressed genes doxorubicin and other condition	Present/absent in SeneQuest under cell type HUVEC	Present/absent in general SeneQuest
Upregulated (n = 108 genes)	5/103	84/24
Downregulated (n = 52 genes)	7/45	47/5

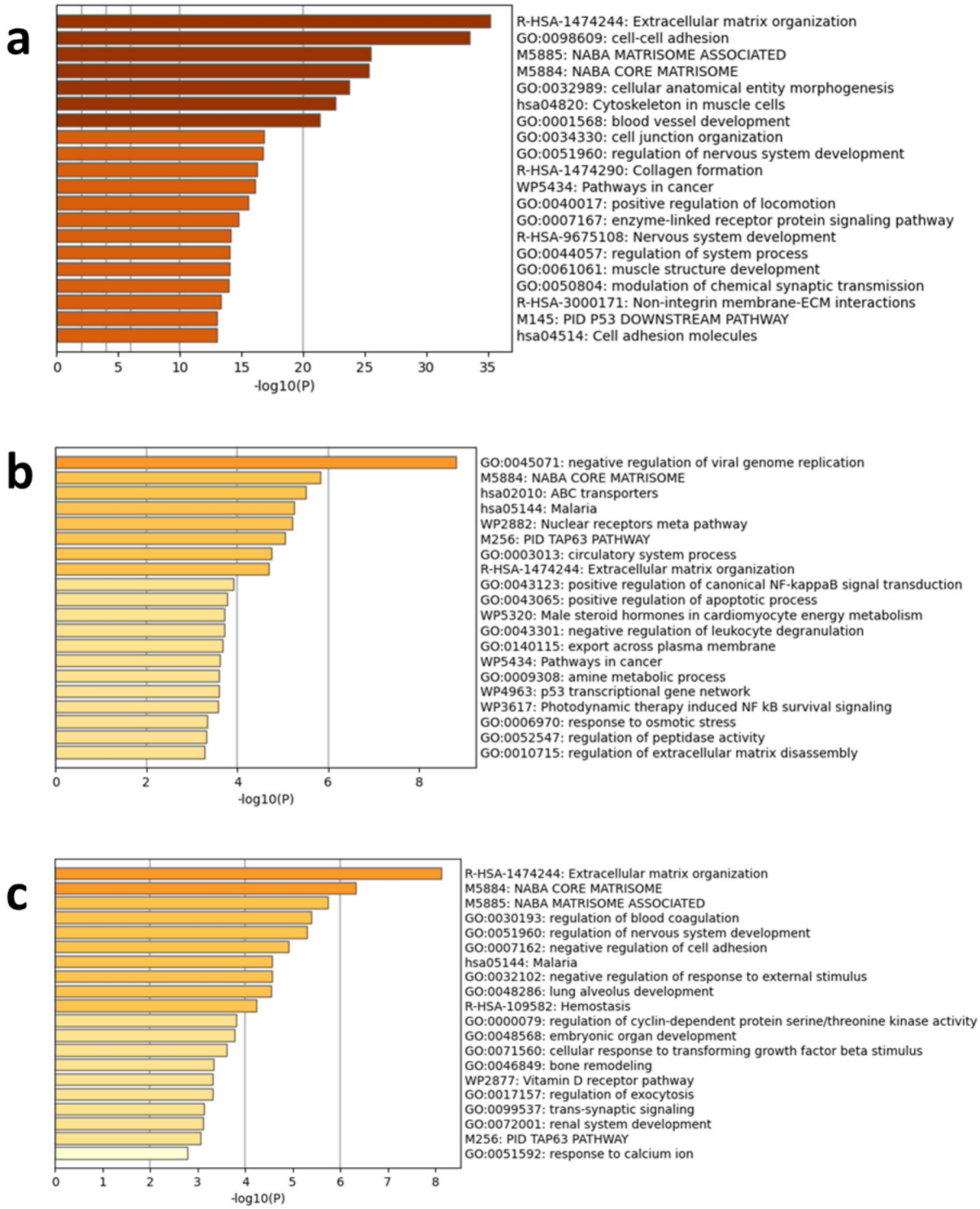


Fig. 2 Gene ontology enrichment analysis of upregulated genes in senescent HUVEC cells induced by: **a** doxorubicin; **b** irradiation; and **c** replication exhaustion

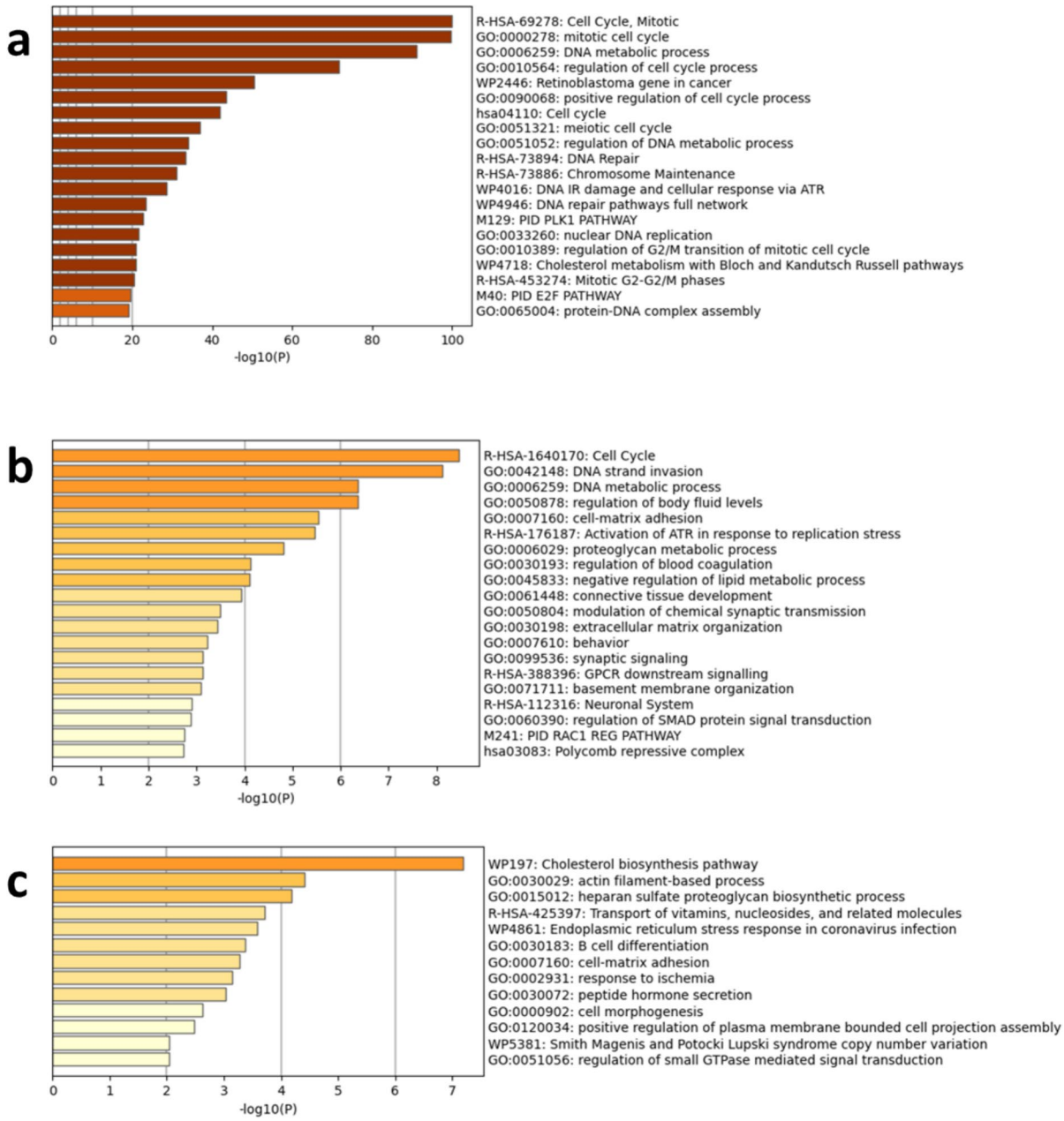


Fig. 3 Gene ontology enrichment analysis of downregulated genes in senescent HUVEC cells induced by: **a** doxorubicin; **b** irradiation; and **c** replication exhaustion

are consistently associated with cell cycle regulation, cell–matrix adhesion, and cholesterol metabolism. GO enrichment analysis allows for the identification of overrepresented functional groups in gene dataset. However, this analysis requires sufficient statistical power, which increases with the number of genes in the dataset. Due to the small number of commonly DEGs, there may not be enough statistical power to detect significant enrichment in the GO categories. As mentioned before, because we will use a doxorubicin-induced senescence model to validate the expression of selected candidate genes, we focused

on the 108 commonly upregulated and 52 commonly downregulated genes in doxorubicin and at least one other condition. Similar to the individual GO enrichment analyses, the commonly upregulated genes were enriched in categories associated with extracellular matrix organization, while downregulated genes were enriched in categories related to cell cycle regulation and cholesterol biosynthesis (Fig. 4).

Establishment of a doxorubicin-induced senescence model in HUVECs

To validate the transcriptional response of ECs to senescence, we treated HUVEC cells with 100 nM doxorubicin for 24 h, followed by an 8-day recovery period to allow for the development of the senescent phenotype. Viability assays indicated that approximately 80% of the cells remained viable at day 8 after treatment (Fig. S1). In contrast with the DMSO-treated cells, a high percentage of the doxorubicin-treated cells stained positive for senescence-associated-β-galactosidase (SA-β-gal) (Fig. 5a). However, since SA-β-gal can be upregulated in conditions such as high cell confluency or nutrient deprivation, additional senescence markers were evaluated to ensure accurate phenotypic confirmation (Severino et al. 2000).

To further validate the senescent phenotype, we quantified the expression of key senescence markers using qPCR (Fig. 5b). Cells treated with doxorubicin exhibited a significant upregulation SASP components, including IL6, IL8, while a reduction in LMNB1, a lamin-associated protein downregulated during senescence, was also observed. Moreover, most doxorubicin-treated cells were negative for Ki67 staining, indicating a reduction in proliferation (Fig. 5c), and presented increased DNA damage as indicated by γH2AX staining (Fig. 5d). Collectively, these data support the conclusion that doxorubicin treatment effectively induced senescence in HUVECs, as confirmed by multiple markers.

Analysis of matrisome gene expression by RT-qPCR

GO analysis of genes with increased expression in endothelial cells induced to senescence by

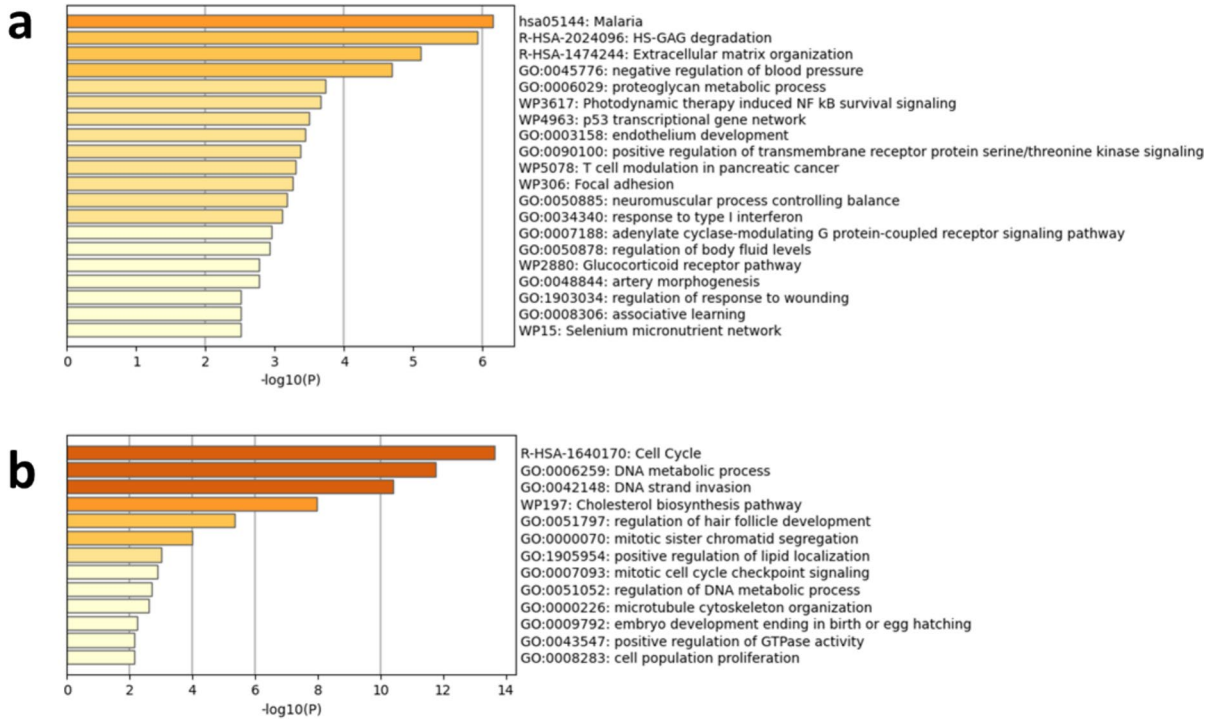


Fig. 4 Gene ontology of genes that are commonly **a** upregulated or **b** downregulated in senescence induced by doxorubicin and another condition

doxorubicin, irradiation, or replication exhaustion revealed an enrichment of matrisome-associated genes across all three conditions. Interestingly, although the specific upregulated genes differ among the stimuli, the matrisome category remained consistently enriched in all datasets. In line with these findings, we aimed to validate the expression of key genes in our doxorubicin-induced senescent endothelial cell model by RT-qPCR. To achieve this, we downloaded the matrisome-associated genes relevant to blood vessels from the MatrisomeDB database (Shao et al. 2023) and cross-referenced them with the list of upregulated genes in doxorubicin-induced senescence.

From this dataset, we selected genes associated with both the core matrisome, which includes categories such as collagens, ECM glycoproteins, and proteoglycans, as well as matrisome-associated components, comprising ECM regulators, ECM-affiliated proteins, and secreted factors involved in extracellular matrix organization. Among the upregulated genes present in the database for blood vessels, we selected at least one gene for each of these categories based on their fold change and p-adjust value in doxorubicin-induced senescence dataset. Primers were designed for 17 matrisome genes across these categories, with the goal of validating at least one gene from each category. Successful amplification was achieved for genes, associated with the categories of collagens (COL4A1 and COL25A1), proteoglycans (VCAN), ECM glycoproteins (FBN2 and LAMA4A), ECM regulators (LOXL1, MMP10, and TIMP3), Secreted factors (CX3CL1 and GDF15) and ECM affiliated proteins (GPC5).

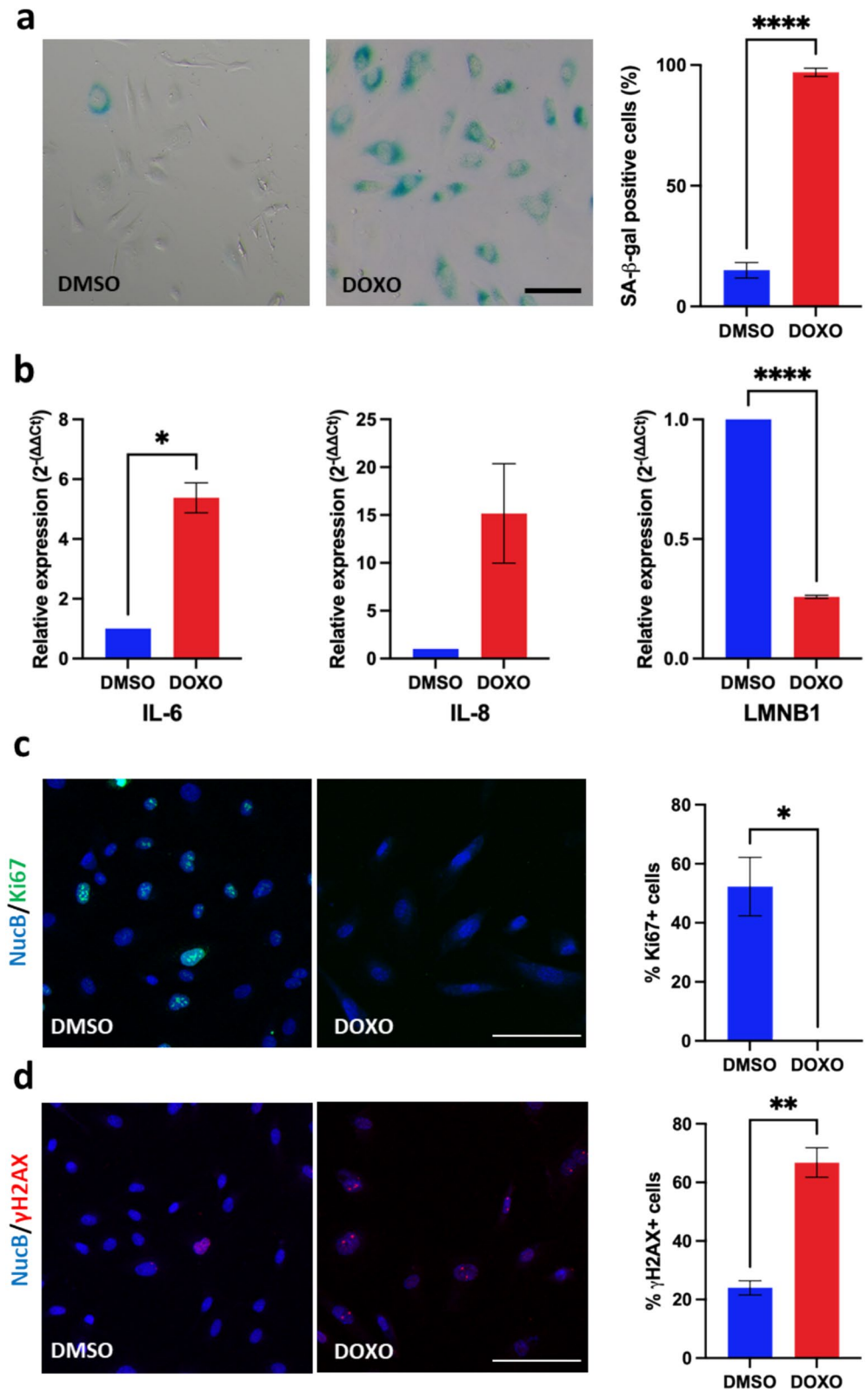
RT-qPCR analysis revealed a significant transcriptional upregulation of structural ECM components, including COL4A1, FBN2, and LAMA4A, in doxorubicin- HUVECs compared to DMSO-treated controls (Fig. 6). Additionally, genes involved in ECM regulation, such as GPC5 and LOXL1, were also significantly upregulated. While most genes showed an upward trend, not all exhibited statistically significant differences between senescent and non-senescent cells. These findings confirm that core components of the matrisome, including those encoding collagens and laminins, are upregulated in senescent endothelial cells, aligning with the enriched pathways identified in the GO analysis.

Discussion

This study analyzed publicly available RNA-seq datasets to identify gene expression changes in endothelial cells (ECs) undergoing senescence induced by replicative exhaustion, irradiation, or doxorubicin treatment. Despite the heterogeneity in experimental conditions, we identified common differentially expressed genes (DEGs) across senescence models, highlighting both shared and distinct transcriptional changes. These findings address the knowledge gap related to the heterogeneity of aging phenotypes, as outlined previously (Rattan 2024), emphasizing the diverse manifestations of aging at various levels, including cellular (senescence) and molecular levels (genes).

The shared DEGs often contribute to EC dysfunction rather than being direct markers of senescence. For instance, CD200, upregulated in senescent ECs, may impair immune clearance by suppressing local immune responses (Hoek et al. 2000; Kassiteridi et al. 2021) while SELP (P-selectin) promotes leukocyte adhesion (Zhang et al. 2016), chronic inflammation (Rombouts et al. 2014), and prothrombotic state (Costarelli et al. 2017). The upregulation of p53 target genes like CEACAM1 and SPATA18 suggests roles in vascular homeostasis (Ghavampour et al. 2018), mitochondrial quality control (Miyamoto et al. 2011), and DNA damage responses (Sappino et al. 2012; Dan et al. 2020), highlighting the interplay between senescence mechanisms and vascular dysfunction (Kleefeldt et al. 2019). Conversely, the consistent downregulation of SCML2 underscores its role in relieving p53 inhibition, thereby promoting senescence (Peng et al. 2023).

While transcriptional repression of DNA repair genes has been recognized as a hallmark and cause of senescence (Collin et al. 2018), previous studies have not emphasized the transcriptional changes associated ECM remodelling and matrisome in endothelial senescence, despite the well-documented changes that occur in ECM in senescence and aging (Mavrogonatou et al. 2019, 2023; Ewald 2019; Levi et al. 2020). Our results reveal significant transcriptional changes in ECM-related genes, such as COL4A1 and FBN2, which contribute to ECM stiffening (Annes et al. 2003; Hinz 2015; Chen et al. 2023) and impaired tissue repair (Brinckmann et al. 2010). Upregulation of LOXL1, LAMA4, and GPC5 further supports ECM


a
DMSO
DOXO
SA-β-gal positive cells (%)
****
b
Relative expression (2-(ΔΔCt))
*
IL-6
IL-8
LMNB1
****
c
NucB/Ki67
% Ki67+ cells
*
d
NucB/γH2AX
% γH2AX+ cells
**

◂**Fig. 5** Assessment of primary senescence markers in HUVECs induced to senescence by doxorubicin treatment. **a** Representative micrographs of β-galactosidase staining in cells treated with DMSO or DOXO (left) and quantification of the percentage of β-galactosidase-positive cells (right). Scale bar = 100 µm. **b** Relative mRNA expression of senescence-associated factors IL-6, IL-8, and LMNB1 in cells treated with DMSO or DOXO, as determined by RT-qPCR. **c** Representative immunofluorescence staining of Ki67 (green, left) and quantification of Ki67-positive cells (right). **d** Representative immunofluorescence staining of γH2AX (red, left) and quantification of γH2AX-positive nuclei. Scale bar = 50 µm. Statistical significance was determined using a Welch's t-test. The data are presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$)

remodeling, a hallmark of endothelial senescence. This remodeling disrupts vascular elasticity and function, linking senescence to age-related vascular diseases (Rickel et al. 2020; Schnellmann et al. 2022). While the transcriptional responses vary across models, the conserved enrichment of DEGs in pathways related to ECM organization, cholesterol metabolism, and DNA repair underscores common biological disruptions in endothelial senescence.

The matrisome comprises structural ECM proteins (collagens, fibronectins, laminins), ECM regulators, and secreted factors like metalloproteinases, their inhibitors, and growth factors embedded within the ECM. Its dynamic composition varies across tissues and changes during homeostasis, angiogenesis, and pathological conditions, including atherosclerosis (Ewald 2019; Xiao et al. 2023). Age-related ECM alterations impair angiogenesis, contributing to cardiovascular and cerebrovascular diseases and delayed wound healing (Xiao et al. 2023). Cellular senescence drives these changes, as senescent cells secrete pro-inflammatory and matrix-degrading factors, compounding reduced angiogenesis and declining vascular health in aging (Levi et al. 2020).

Notably, our findings highlight gaps in understanding ECM's dual role: while senescent cells remodel ECM through catabolic or fibrotic pathways, the ECM itself influences cellular behavior, potentially propagating senescence (Annes et al. 2003; Hinz 2015; Bai et al. 2017; Chen et al. 2023). For example, mutant mice expressing the collagenase-resistant type I collagen exhibit premature aging and increased vascular smooth muscle cell (VSMC) senescence (Vafaie et al. 2014). VSMCs cultured on mutant collagen show reduced replicative lifespan and higher senescence marker expression, including SA-βGal activity and upregulation of $p16^{INK4A}$ and $p21^{CIP1}$, becoming more susceptible to stress-induced senescence compared to those on wild-type collagen. Conversely, the proliferative capacity of senescent human diploid fibroblasts is restored by culturing them on ECM from young cells (Choi et al. 2011). Similarly, mesenchymal stem cells (MSCs) grown on ECM from young MSCs are protected from oxidative stress-induced senescence (Zhou et al. 2018; Burova et al. 2024). These findings highlight the need to further explore how senescent cells reshape their microenvironment and how ECM dynamics influence senescence and tissue aging. Finally, ECM remodeling varies by stimulus and cell type, with genes like collagens, fibrillin, and MMPs showing opposing expression patterns in different models (Levi et al. 2020). This context-dependent behavior reinforces the complexity of senescence-associated ECM changes. In conclusion, our study underscores the importance of ECM remodeling in endothelial senescence and its broad implications for vascular health. Future research should address context-specific ECM alterations and their contribution to aging and pathology progression.

Limitations and future directions

This study's reliance on publicly available RNA-seq datasets introduces inherent limitations, primarily due to the variability in experimental conditions. These variations may contribute to noise and reduce the resolution of observed transcriptional changes. However, RNA-seq, as a mature technology with a broad dynamic range, is well-suited for identifying global gene expression changes, and additional validation is not always required to establish robust transcriptomic conclusions. One of the strengths of integrating data from experiments conducted by different groups is the ability to identify common patterns, such as specific pathways consistently affected by senescence induction regardless of the stimulus. These shared pathways underscore the potential importance of these mechanisms in the senescence process, providing a more robust foundation for future research in this field. In this study, qPCR was employed strategically to validate a subset of key matrisome-related genes in the doxorubicin-induced model using three biological replicates, thereby strengthening the robustness

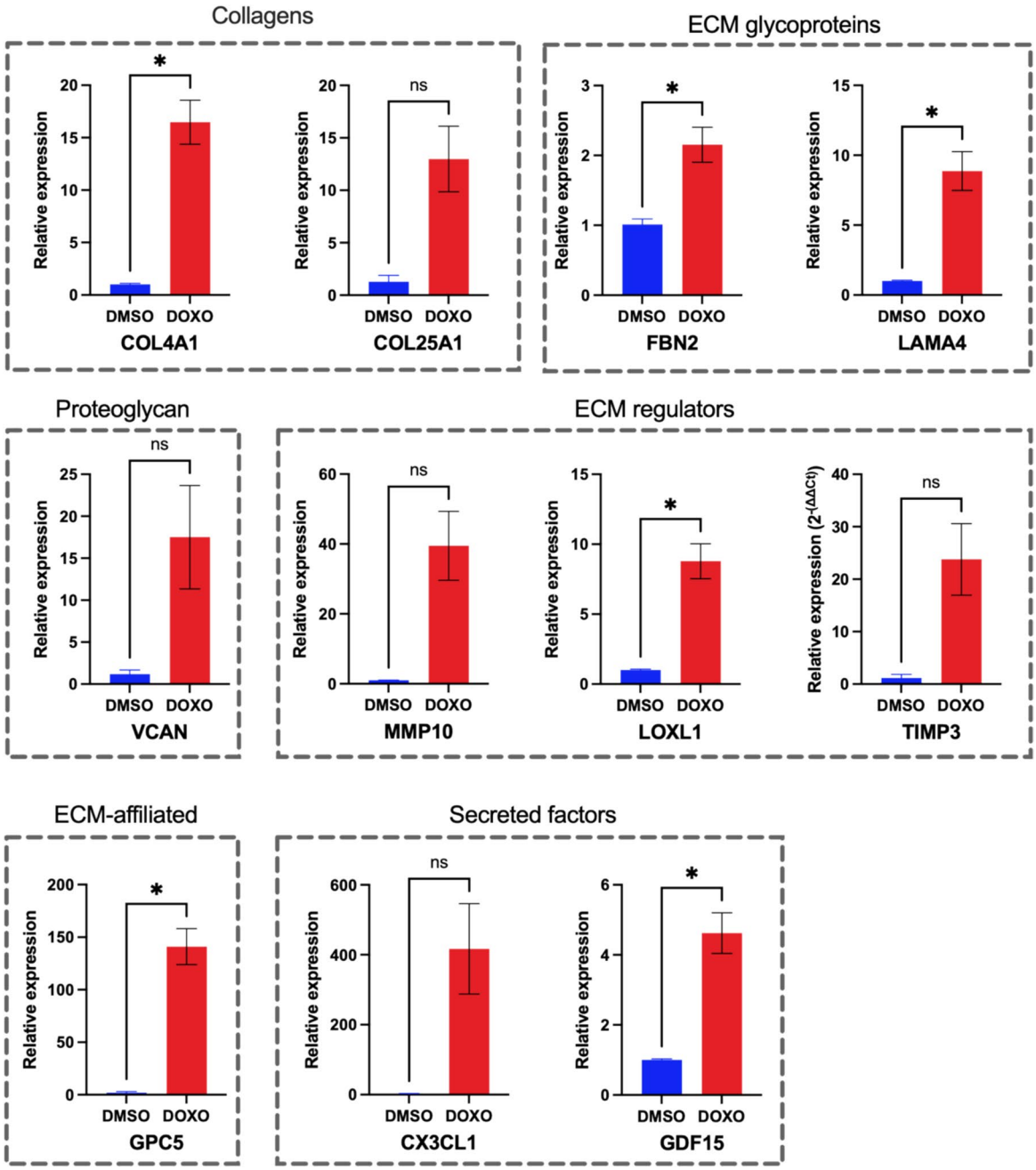


Fig. 6 Relative expression levels of collagens (COL4A1 and COL25A1), proteoglycans (VCAN), ECM glycoproteins (FBN2 and LAMA4A), ECM regulators (LOXL1, MMP10, and TIMP3), Secreted factors (CX3CL1 and GDF15), and ECM affiliated proteins (GPC5) in DMSO-treated (non-senescent) and doxorubicin-treated (senescent) endothelial cells. Expression levels were quantified using qPCR and analyzed according to the Pfaffl method. Data are presented as the mean ± SEM from three biological replicates. Asterisks (*) denote statistically significant differences ($p < 0.05$), while "ns" indicates no significant difference. Statistical significance was determined using a Welch's t-test

of conclusions drawn from the RNA-seq analysis. While qPCR validation was not performed for replicative senescence or irradiation-induced senescence, this does not undermine the broader validity of our findings. Because the changes detected in matrisome gene expression were senescence inducer-dependent, it would be necessary to validate a different group of genes for these other senescence models. This highlights that while specific genes may differ between models, the main biological processes remain conserved. Finally, while this study focuses exclusively on endothelial cells due to their critical role in vascular aging, we acknowledge the importance of other components of the vascular niche. Cell types such as smooth muscle cells and fibroblasts also undergo senescence and interact with endothelial cells. Evaluating these additional cell types in future studies would provide a more comprehensive understanding of vascular aging process.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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