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Research article

Impaired incorporation of fibronectin into the extracellular matrix during aging exacerbates the senescent state of dermal cells

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ABSTRACT

Fibronectin (Fn) is a ubiquitous extracellular matrix (ECM) glycoprotein that acts as an ECM scaffold organizer and is essential in many biological functions, including tissue repair, differentiation or cancer dissemination. Evidence suggests that the amount of Fn changes during aging. However, how these changes influence the aging process remains unclear. This study aims to understand Fn influence on cell aging. First, we assess the relative level of Fn abundance in both different biopsies of skin donors and replicative senescence cellular model. In skin biopsies, we observed that Fn level decreases with aging in the reticular dermis, while its expression remains relatively stable in the papillary dermis, likely to sustain the dermis-epidermis junction. During replicative senescence, in BJ skin fibroblasts, while intracellular Fn increases, we found that secretion and Fn fibrils formation are less effective. Reduced Fn fibrils leads to disorganization of the ECM. This could be explained by the expression of different Fn isoforms observed in the secretome of senescent cells. Surprisingly, the knockdown of Fn delays the onset of senescence while cultivating cells onto a Fn-coated support promotes it. Taken together, these new insights on the role of Fn during aging may emerge new therapeutic strategies on aged-related diseases.

1. Introduction

The skin, the largest organ in the human body, serves as the body's first line of defense [1]. It is composed of three layers: the epidermis, dermis, and hypodermis. The dermis, situated between the epidermis and hypodermis, is a connective tissue mainly consisting of an extracellular matrix (ECM) produced by fibroblasts [2,3]. In the elderly, the skin becomes thin and fragile due to complex changes, often summarized as reduced dermal collagen and impaired cellular proliferation [4]. As aging progresses, the dermis loses thickness, with fewer and disorganized fibers, and the architecture between the papillary and reticular dermis becomes disrupted [5].

Among the components of these connective tissues, Fibronectin (Fn), a multi-domain protein, plays a crucial role as a master organizer of ECM assembly. Synthesized by cells as a soluble dimer, Fn assembles into insoluble fibrils at the cell surface helped by integrin receptors and

interacts with various ECM proteins like collagen, to form this complex network [6]. Fibronectin along with Types I and III collagen, is predominantly expressed in the dermis, while Type IV and type VII collagen are mainly localized in the lamina with type VII also partially present in the dermis. While Fn has been shown to influence the distribution of Types IV and VII collagen [7–9], the localization suggests that fibronectin's role in the dermal extracellular matrix (ECM) is closely associated with Types I and III collagen, facilitating the incorporation and stability of these ECM components [10–12]. This ECM network acts as a reservoir for growth factors, and remodeling Fn matrices affects the bioavailability of these factors, thereby altering cell behavior [13]. Besides its role in ECM assembly, Fn regulates a wide range of cellular functions essential for development, including cell migration, growth, differentiation, and tissue repair through direct interactions with cell surface integrin receptors [14,15]. This interaction between Fn and integrin receptors, like $\alpha 5 \beta 1$ integrin, is also crucial for the assembly of

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Fn fibrils which play key roles in cell adhesion [16]. Over the past decade, Fibronectin has been discovered to play a multifaceted role in the aging process, with its functions varying depending on the forms and secretion levels of Fibronectin, and the specific tissues where it is expressed [17–19]. Recent studies demonstrated that senescent cells favor age-related disease progression by creating a pro-inflammatory environment known in aging as senescence-associated secretory phenotype (SASP) [20,21]. SASP-related changes influence ECM turnover and have a notable effect on cell functions by altering collagen deposition or by increasing the stiffness of the ECM as aging progresses [22,23]. Whether the Fibronectin extracellular matrices in aging are also drivers of the senescent phenotype is yet to be explored.

Regarding skin tissue, it has been suggested that a lack of Fn in the dermis could lead to scar tissue formation [24]. Comparisons of Fibronectin expression between fetal and adult skin show a complete rearrangement during tissue development, with Fibronectin present throughout the entire fetal skin but only in the basement membrane zone in adult skin [25]. Temporospatial variations in Fibronectin expression have been observed during aging [26], but the regulation of Fn expression during this process is still debated [27]. Fn exists in multiple isoforms due to alternative splicing, and the complexity of its expression regulation, combined with its diverse isoforms, makes description challenging [28–30]. Additionally, Fn expression is regulated at various levels, including mRNA expression, protein expression, and secretion levels. Some studies report a decrease in Fn expression during aging, while others note an increase, with differences potentially depending on the epitope studied [31–33]. This raises questions about the organization of Fibronectin during aging. Despite numerous studies in the literature, the exact role and distribution of Fibronectin in aging skin remain unclear.

In this study we aim to highlight the status of Fn during aging by examining its expression in cells aged by replicative senescence and in skin biopsies from 20 different age donors. *In vivo*, we provide novel evidence that Fibronectin expression remained quite stable in the papillary dermis while its drastically reduced in reticular dermis. Detailed analysis *in vitro* on skin fibroblasts revealed that Fibronectin distribution in the extracellular matrix is altered in senescent cells with shortened Fibronectin fibrils. Furthermore, our results show evidence of a different Fibronectin fragment profile in senescent fibroblasts. Overall, our data provide new insights of Fibronectin assembly matrix as a key factor in aging process.

2. Material & methods

2.1. Skin donors

A series of skin biopsies were obtained from abdominal surgeries of 20 donors through BioEC® (France) and were divided into two groups based on the donor's ages. Young donors were between 24 and 29 years old ($27,33 \pm 1,75$ years old, $n = 10$) while old donors were between 56 and 60 years old ($58,11 \pm 1,99$ years old, $n = 10$). Biopsies were provided either mounted onto glass slides for immunohistochemistry analyses or as punches for protein analyses.

2.2. Cell culture

Human skin BJ fibroblasts are established from normal neonatal male foreskin and obtained from ATCC (#CRL-2522). Cells were cultivated, either directly on plate or onto a Fn-coated plate, in growth medium using Dulbecco's minimal essential medium (DMEM; Invitrogen) supplemented with 10 % fetal calf serum (FCS; Invitrogen). Cultures were incubated at 37 °C, 5 % CO₂ up until cells reached a senescent state around 65 cumulative population doublings (CPD) as previously described [34]. To characterize the senescence state, number of divisions was calculated according to the following formula where N_c represents the number of cells at confluence and N_s the number of

seeded cells: $DP = 3.32 \times (\log N_c - \log N_s)$. Subconfluent cultures were obtained by seeding 6×10^4 cells/cm². BJ cells were classified into young fibroblasts corresponding to early passage cells (below CPD 40) and old cells corresponding to late passage cells (up to CPD 55).

For knockdown experiments, early passage and late passage BJ cells were transfected at 80 % confluence using DharmaFECT transfection reagent (Dharmacon), according to the manufacturer's instructions. Briefly, cells were transfected with 1 μM siRNA Fibronectin (siFN1, Dharmacon) or 1 μM siRNA Control (siControl, Dharmacon) and harvested 2 days later.

2.3. Adhesion assay

For adhesion assays, 96 wells-plates were coated with 10 μg/mL Fn purified in the laboratory as previously described [35]. Then, coated wells were incubated with 1 % Bovine Serum Albumin (BSA, Sigma Aldrich) in phosphate-buffered saline (PBS, Invitrogen) at 37 °C for 1 h and with RPMI glutaMAX (ThermoFisher), 0.07 % sodium bicarbonate, 0.2 % BSA. Adherent cells in T75 cm² flask were detached, re-suspended in adhesion buffer and seeded at 2×10^5 cells/cm². After 30 min at 37 °C in a 5 % CO₂ atmosphere, non-adherent cells were removed by three washes in PBS and the remaining adherent cells were fixed with 1 % glutaraldehyde (Sigma Aldrich). After three more washes in PBS, the cells were stained for 30 min in 0.1 % crystal violet (Sigma Aldrich) in H₂O, rinsed three times with water, and solubilized in 0.1 % SDS. Absorbance was measured with a microplate reader at 595 nm. Assays were performed three times in triplicate.

2.4. SA-β-galactosidase assays

Detection of SA-β-galactosidase (SA-β-gal) was performed in subconfluent cultures exhibiting 80 % confluence, as previously described by Dimri et al., 1995 [36]. Briefly, detection of SA-β-gal activity was done after fixation of cells by incubation in the fixation solution (2 % formaldehyde/0.2 % glutaraldehyde) for 5 min. Then, cells were incubated with the chromogenic β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl P3-D-galactoside, Sigma Aldrich) in a buffer (40 mM citric acid; sodium phosphate, pH 6.0; 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide; 150 mM NaCl; 2 mM MgCl₂). A blue color develops in some cells within 2 h, but staining is maximal after 12–16 h. After the staining, the cells are washed with PBS and viewed by bright field or phase contrast microscopy. The proportion of cells positive for SA-β-gal activity was determined by calculating the ratio between blue cells and uncolored cells in 5 fields.

2.5. Immunofluorescence microscopy

Immunofluorescence labeling of Fibronectin in early and late passage of BJ cells was carried out according to standard procedures [37]. Cells were seeded onto glass coverslips in 24-well plates at 2×10^4 cells/well and incubated for 24–48 h. Then, cells were fixed with 4 % paraformaldehyde in PBS and washed with PBS containing 0.5 % BSA (Bovine Serum Albumin; Sigma Aldrich). They were permeabilized with 0.1 % Triton X100 in PBS. Fibronectin was localized by indirect immunofluorescence using a monoclonal anti-cellular Fibronectin antibody (F6140; Sigma Aldrich) diluted at 1:50 or the anti-Fibronectin Frag antibody (MAB88916, Merck Millipore, 1:100), to detect the cell attachment Fn fragment, into PBS buffer containing 2 % BSA. For skin biopsies, immunofluorescence method was performed on frozen sections. Briefly, 5 μm-thick cryostat sections were air-dried, fixed in acetone for 20 min. After washing with a PBS solution, biopsies were incubated with PBS-1% BSA for 30 min, sections were then incubated with the anti-cellular Fibronectin antibody the anti-α5β1 integrin antibody (MAB1969, Merck Millipore, 1:200) or anti-α5 Integrin chains antibody (AB1928, Merck Millipore, 1:1000) for 1 h at room temperature.

After washing, the coverslips of each assay were incubated with the appropriate fluorescent secondary antibodies, Alexa Fluor® 568-conjugated anti-mouse antibodies (Invitrogen) or Alexa Fluor® 488-conjugated anti-rabbit antibodies (Invitrogen). Actin cytoskeleton was stained with FITC-phalloidin (P5282, Sigma Aldrich). Cell nuclei were stained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride, D9542, Sigma Aldrich). Coverslips were mounted in Prolong® Gold Antifade Reagent (P36930, Invitrogen) and examined with laser-scanning confocal microscopy (LSM710, Zeiss). Controls, in which primary antibodies were replaced with PBS, were negative.

To quantitatively analyze the amounts of $\alpha 5\beta 1$ integrin and Fibronectin, fluorescence images were collected by laser scanning confocal microscopy (LSM710, Zeiss). Briefly, for each acquisition, sections were scanned at the appropriate excitation/emission wavelengths depending on the Alexa-Fluor employed, with the laser intensity, confocal aperture, gain and black-level settings kept constant. The fluorescence intensity at the selected areas, linearly correlated with the number of pixels, was quantitatively analyzed using the standard imaging analysis software of an LSM710 system (Zen software).

2.6. Preparation of Alexa Fluor® 568-fibronectin solution

The coupling reaction was performed by the dilution of 5 μ L of Alexa Fluor® 568 conjugate in 1 mL of a 2 mg/mL Fn solution dialyzed at pH 9.0. The mixture was incubated during 1h at room temperature in the dark. Non-conjugated and excess dye were removed by running the concentrated reaction mix through a desalting column (Sephadex G-25, G2580 column, Sigma Aldrich) with PBS buffer.

2.7. Two-dimensional gel electrophoresis

Conditioned medium was prepared by growing BJ cells at confluence in 10 % FBS supplemented medium. Cells were washed with PBS and incubated in serum-free medium for 24 h. The conditioned medium was centrifuged to remove cellular debris and then was subjected to ethanol precipitation using one volume of the sample to three volumes of absolute ethanol previously cooled at -20°C . After centrifugation (20 000 g, 20 min, 4°C), the pellet obtained was air-dried to remove any residual ethanol and then dissolved in a rehydration buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 2 % (v/v) Pharmalytes, 60 mM DTT and bromophenol blue). The first dimension was performed using ReadyStrips IPG strips (pH_{linear} 4–7; length 17 cm; BioRad, Life Science Group, Marnes la Coquette, France) and the Multiphor II isoelectric focusing system (Amersham Biosciences, Orsay, France). Strips were rehydrated actively and were then focused over 37 h for a total of 58 000 V.

The second dimension was performed using the Protean II XL system (BioRad, Life Science Group, Marnes la Coquette, France). Strips were then placed on top of SDS-PAGE 7 % (w/v) and migration was done under 1500 V h in running buffer (50 mM Tris-HCl, pH 8.8, 100 mM glycine, 0.1 % SDS). Then, proteins into the polyacrylamide gel were electrotransferred to perform a western blotting.

2.8. RNA isolation and RT-PCR analysis

Extraction of total RNA from cultured cells was achieved using the RNeasy midi kit (Qiagen) according to the manufacturer's instructions. Total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad). RNA levels were analyzed by real-time quantitative TaqMan RT-qPCR (ThermoFisher), following the manufacturer's instructions. The relative mRNA quantification was calculated via the $\Delta\Delta\text{Ct}$ method with normalization of each sample to the average change in cycle threshold value of the control genes. Reactions were performed with the LightCycler® 480 Instrument (Roche) in 384-multiwell plates. The list of the selected genes and the control genes (*Gapdh*, *Rpl13a*, *Gusb*) were listed in Table 1.

Table 1

List of the selected and control genes tested for RT-PCR analysis.

Gene ID	Symbol	Gene description
633	<i>Bgn</i>	Biglycan
1281	<i>Col3a1</i>	Collagen type III alpha 1 chain
1462	<i>Vcan</i>	Versican
3037	<i>Has2</i>	Hyaluronan synthase 2
8985	<i>Plod3</i>	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3
5033	<i>P4ha1</i>	Prolyl 4-hydroxylase subunit alpha 1
4312	<i>Mmp1</i>	Matrix metalloproteinase 1
5327	<i>Plat</i>	Plasminogen activator, tissue type
3688	<i>Itgb1</i>	Integrin subunit beta 1
2252	<i>Fgf7</i>	Fibroblast growth factor 7
7040	<i>Tgfb1</i>	Transforming growth factor beta 1
7431	<i>Vim</i>	Vimentin
6687	<i>Spg7</i>	Matrix AAA peptidase subunit, paraplegin
3910	<i>Lama4</i>	Laminin subunit alpha 4
1634	<i>Dcn</i>	Decorin
2239	<i>Gpc4</i>	Glypican 4
960	<i>Cd44</i>	CD44 molecule
1289	<i>Col5a1</i>	Collagen type V alpha 1 chain
2006	<i>Eln</i>	Elastin
3339	<i>Hspg2</i>	Heparan sulfate proteoglycan 2
4015	<i>Lox</i>	Lysyl oxidase
5118	<i>Pcolce</i>	Procollagen C-endopeptidase enhancer
4323	<i>Mmp14</i>	Matrix metalloproteinase 14
7076	<i>Timp1</i>	TIMP metalloproteinase inhibitor 1
6382	<i>Sdc1</i>	Syndecan 1
2260	<i>Fgfr1</i>	Fibroblast growth factor receptor 1
7048	<i>Tgfb2</i>	Transforming growth factor beta receptor 2
1490	<i>Ctcf</i>	CCN2, cellular communication network factor 2
3643	<i>Insr</i>	Insulin receptor
3912	<i>Lamb1</i>	Laminin subunit beta 1
7422	<i>Vegfa</i>	Vascular endothelial growth factor A
3371	<i>Tnc</i>	Tenascin C
1307	<i>Col16a1</i>	Collagen type XVI alpha 1 chain
1291	<i>Col6a1</i>	Collagen type VI alpha 1 chain
2200	<i>Fbn1</i>	Fibrillin 1
3921	<i>RpsA</i>	Ribosomal protein SA
4017	<i>Loxl2</i>	Lysyl oxidase like 2
871	<i>Serpinh1</i>	Serpin family H member 1
4313	<i>Mmp2</i>	Matrix metalloproteinase 2
3673	<i>Itga2</i>	Integrin subunit alpha 2
1956	<i>Egfr</i>	Epidermal growth factor receptor
2263	<i>Fgfr2</i>	Fibroblast growth factor receptor 2
59	<i>Acta2</i>	Actin alpha 2
5119	<i>Chmp1a</i>	Charged multivesicular body protein 1A
1282	<i>Col4a1</i>	Collagen type IV alpha 1 chain
4811	<i>Nid1</i>	Nidogen 1
3915	<i>Lamc1</i>	Laminin subunit gamma 1
7057	<i>Thbs1</i>	Thrombospondin 1
1277	<i>Col1a1</i>	Collagen type I alpha 1 chain
1295	<i>Col8a1</i>	Collagen type VIII alpha 1 chain
2335	<i>Fn1</i>	Fibronectin 1
5351	<i>Plod1</i>	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1
4060	<i>Lum</i>	Lumican
6678	<i>Sparc</i>	Secreted protein acidic and cysteine rich
9509	<i>Adams2</i>	ADAM metalloproteinase with thrombospondin type 1 motif 2
3678	<i>Itga5</i>	Integrin subunit alpha 5
2247	<i>Fgf2</i>	Fibroblast growth factor 2
1839	<i>Hbegf</i>	Heparin binding EGF like growth factor
5829	<i>PXN</i>	Paxillin
857	<i>CAVI</i>	Caveolin 1
1294	<i>COL7A1</i>	Collagen type VII alpha 1 chain
2597	<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase
23 521	<i>RPL13A</i>	Ribosomal protein L13a
2990	<i>GUSB</i>	Glucuronidase beta

2.9. Western blotting

Whole cell extracts were obtained from cultured cells and tissues with RIPA buffer (20 mM Tris, 150 mM NaCl, and 1 % NP-40), supplemented with a cocktail of protease inhibitors (Roche). The lysates were centrifuged at 15,000 g for 15 min at 4°C to pellet the cellular debris and the protein concentration of the supernatant was determined by the BCA method (Sigma Aldrich). Total protein (30 μ g) was separated on sodium

dodecyl sulfate–polyacrylamide gel. The gels were electrotransferred onto a Hybond nitrocellulose membrane (GE Healthcare, Velizy-Villacoublay, France) for 75 min at room temperature. Western blotting was done with anti-Fibronectin (F6140), anti-actin (A5441, Sigma Aldrich), and anti-phosphoFAK (3283, Cell Signaling Technologies) antibodies in a 0.1 % TBS- Tween (TBST) 0.1 % (v/v) containing 1 % BSA at 1:1000 dilution. After primary antibody incubation, membranes were washed in TBST 0.1 % and incubated for 1 h at RT with an anti-rabbit (Dako) or anti-mouse (Dako) IgG horseradish peroxidase-conjugated antibody in TBST 0.1 % containing 2 % non-fat dry milk (w/v) at a 1:10 000 dilution. After four additional washes in TBST 0.1 %, blots were developed using enhanced chemiluminescence (GE Healthcare). Images were captured with the Odyssey system (LI-COR). Densitometric analysis was performed using ImageJ Software.

2.10. Apoptosis proteome profiler arrays

To investigate if Fibronectin enhances senescence, we performed an Apoptosis Proteome Profiler Arrays (R&D Systems), according to the manufacturer's instructions. The arrays were incubated overnight with siControl and siFN1 BJ lysates at 4 °C. The blots were detected using an enhanced chemiluminescence. Images were captured with the Odyssey system (LI-COR). Densitometric analysis of the arrays were performed using ImageJ Software.

2.11. Statistical analysis

Results, expressed as mean \pm S.E.M were compared by analysis of variance followed by Student's t-test for unpaired data. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Decreased Fibronectin levels in dermis of old donors

Fibronectin has been previously shown to be highly expressed, and to exert its function, in EMC [38,39], but whether Fibronectin expression levels changed in skin dermis during aging is currently unknown. To assess the changes in Fibronectin levels we performed the immunostaining of Fibronectin in skin biopsies of young and old donors. As shown in Fig. 1A, Fibronectin staining is different in epidermal and dermal tissues independently of the age of the donor. A very low detection of Fibronectin was observed in the epidermal section while the staining intensity is high in the dermal section. A gradient of dermal Fibronectin intensity was observed in dermis of young donors as well as in the dermis of old donors. Most of the Fibronectin immunostaining was present in papillary dermis while the reticular dermis was less immunoreactive for Fibronectin (Fig. 1A). Interestingly, a small but significant decrease (≈ 10 %) of Fibronectin levels was measured in old skins compared to young skins (Fig. 1B). This down-regulation of Fibronectin expression was further confirmed by western blotting. Our results show that Fibronectin protein level decreased by almost 40 % with age donor skins compared to young donor skins even if a heterogeneity in Fibronectin expression between donors is observed. (Fig. 1C and D). These data demonstrate that Fibronectin is differently expressed in the dermis section and in the old donors.

3.2. In vitro replicative senescence is correlated to a reduction of fibronectin incorporation into the ECM

To better characterize the diminution of Fibronectin level in dermis during aging, we used a model of cellular aging. As ECM dermis is mainly produced by fibroblasts, we have cultivated normal human BJ fibroblasts until cells reached a senescent state. Different senescent markers such as cumulative population doublings levels, SA- β -gal activity and mRNA expression levels of SASP markers (Fig. S1) as well as

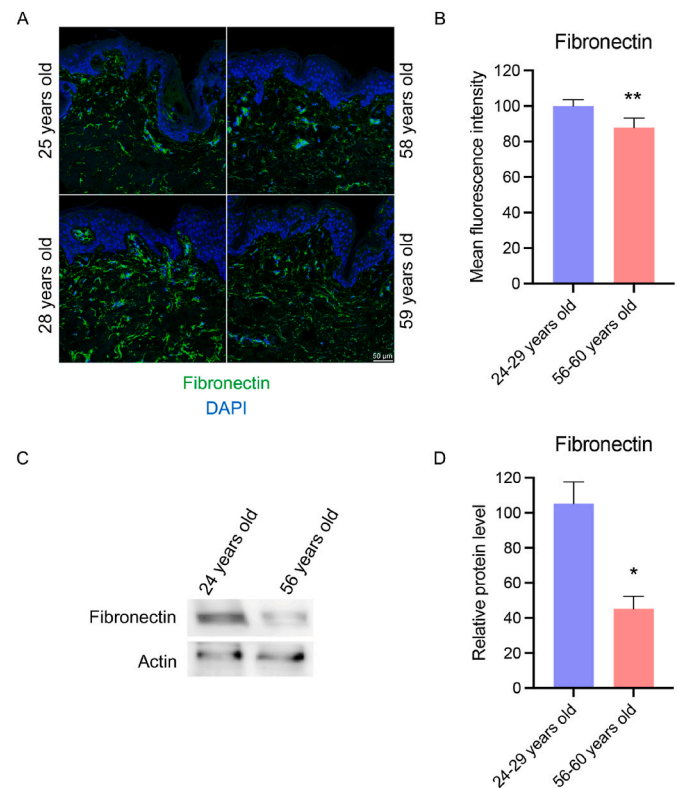


Fig. 1. Effect of aging on dermal fibronectin expression. Immunofluorescent staining (A) and relative immunofluorescence intensity (B) of fibronectin (green, with anti-cellular Fn antibody) expressed in skin biopsies from young donors (24–29 years old) and older donors (56–60 years old). Cell nuclei were stained with DAPI (blue). Staining was examined with confocal microscopy. Scale bar is 50 μ m, n = 6. Fibronectin protein levels presented as Western blot (C) and quantified via densitometric analysis (D) in skin biopsies from young (24 years old) and older donors (56 years old). n = 4. *p value < 0.05; **p value < 0.005.

metallopeptidases MMPs levels obtained by gelatin zymography (Fig. S2) were measured to confirm the senescent stage of the late passage cells. To compare the levels of Fibronectin between early and late passage BJ cells, we initially performed gene expression analysis. As previously described in the literature [40], we also detected elevated amounts of mRNA of Fn in senescent cells compared to young cells (Fig. 2A). We then compared the amount of Fibronectin and protein levels in early passage and late passage BJ cells. Western blotting analysis shows that in late passage cells, the amount of Fibronectin protein increased significantly by 25 % ($p < 0.05$) compared to the early passage cells (Fig. 2B and C).

In vitro, we showed an increased level of Fn in fibroblasts in a senescent state while *in vivo* we observed a decrease of Fibronectin levels in the dermis of old donors compared to the young ones. To better understand these results, we estimated *in vitro* the levels of Fn in the ECM through immunofluorescence analysis. The study involved young and senescent cultures of BJ fibroblasts using an anti-Fibronectin antibody solution targeting the cellular fragment of the Fibronectin. As shown in Fig. 2D and E, Fibronectin fibrils were less detected in ECM of the late passage cells suggesting that the incorporation of Fibronectin into insoluble multimeric fibrils is less efficient for old cells than for the young ones. In senescent culture, cells also appeared more disorganized where Fibronectin matrix seemed randomly deposited (Fig. 2D and E). Taken together, these *in vitro* results indicate that replicative senescence in BJ cells leads to a decrease in Fibronectin secretion and fibril formation in the ECM, despite high Fibronectin mRNA and protein levels in these senescent cells, suggesting an intracellular accumulation of Fn.

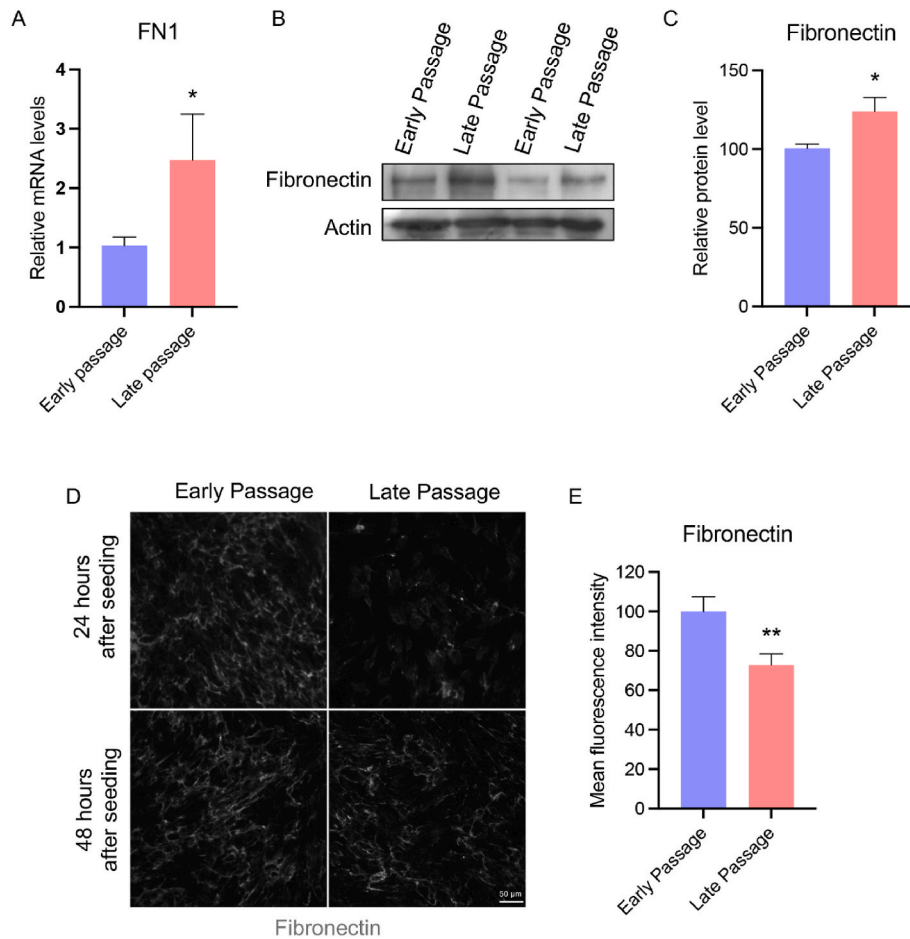


Fig. 2. Effect of cellular aging of fibronectin expression levels and extracellular matrix distribution in BJ fibroblasts.

Relative *fibronectin* (FN1) mRNA levels in BJ cells from early and late passage of BJ cells (A). Fibronectin protein levels presented as Western blot (B) and quantified via densitometric analysis (C) in BJ cells from early and late passage cultures. Immunofluorescent staining (D) and relative immunofluorescence intensity (E) of Fibronectin (with anti-cellular Fibronectin fragment antibody) expressed in BJ cells from early and late passage. Staining was examined with confocal microscopy. Scale bar is 50 μ m. n = 4. *p value < 0.05; **p value < 0.005.

3.3. Aging modify $\alpha 5\beta 1$ receptor expression *in vivo* but not its distribution

Given that integrin receptors and more especially $\alpha 5\beta 1$ integrin is, in part, responsible for Fibronectin fibrils formation at cell surface [41] we questioned if expression of this receptor could be modified during aging. Fluorescence microscopy shows that, like Fibronectin, $\alpha 5\beta 1$ integrin levels are significantly reduced in old donors and are mostly expressed in human dermis (Fig. 3A and B). In contrast to the expression of Fibronectin, which is mainly localized in papillary dermis, $\alpha 5\beta 1$ integrin was present in papillary dermis as well as in reticular dermis. But no significant modification in the localization of $\alpha 5\beta 1$ integrin in the dermis was observed during aging.

Given the differences in Fibronectin levels observed between biopsies from young and aged donors, as well as similar findings in the cellular environment of cultured cells, we aimed to investigate $\alpha 5\beta 1$ integrin expression levels in BJ fibroblasts undergoing replicative senescence. As cell cultures seeded without Fn-coating showed no $\alpha 5\beta 1$ expression (data not shown), we decided to seed the cells onto Fibronectin-coated glass cover and measured $\alpha 5\beta 1$ expression by confocal microscopy. As shown in Fig. 3C, Fibroblasts from young and senescent cultures expressed $\alpha 5\beta 1$ integrin at similar levels. Early passage cells exhibited a pattern clearly distinguishable from that of cell-cell fibrillar adhesions. Integrin $\alpha 5\beta 1$ was distributed in thin dots with some elongated labeling resembling small fibrils (Fig. 3C, arrows). Some focal contacts could also be observed. On the contrary, in late passage cells, the presence of thin and short Fibronectin fibrils (Fig. 3C,

arrowheads) indicated a reduced Fibronectin assembly at the substratum level compared to the one observed at cell-cell interaction level. Differences in shape in senescent cells indicate a defect in Fibronectin fibril assemblies correlated with change in distribution patterned of $\alpha 5\beta 1$ integrin.

3.4. The adhesive capacity of BJ fibroblasts is decreased during replicative senescence

Given that $\alpha 5\beta 1$ integrin expression decreases *in vivo* during aging and its distribution *in vitro* is slightly different, we investigated the capacity of cells to adhere on a Fn-coating. Regardless of their senescent state, adherence increases to 90 % in young cultures and 80 % in old cultures on a Fn-coating compared to the control without coating. This demonstrates that both young and old fibroblast cultures recognize and adhere to the Fn-coating (Fig. 4A). However, late passage cells adhered 10–15 % less than early passage cells on any support, even in the presence of Fn-coating. This suggests that senescent fibroblasts may be less able to recognize and bind to the Fn-coating.

3.5. Premature senescence induced by exogenous Fn

Given our demonstration that reduced secretion of Fibronectin is associated with ECM remodeling in old BJ cells, we assessed the potential relevance of Fibronectin in inducing or protecting cells against senescence. Cultivating BJ cells on a Fn-coating accelerated the entry of

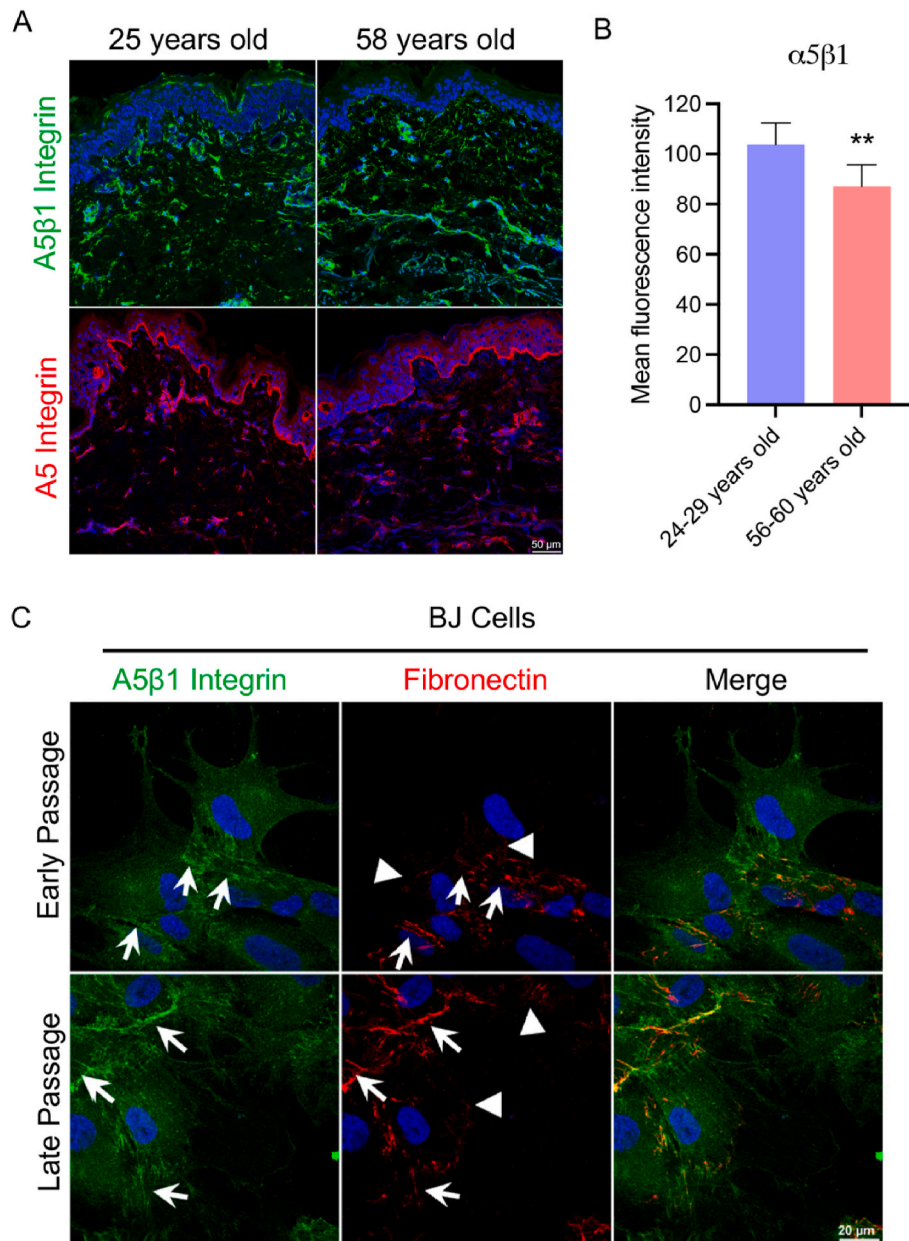


Fig. 3. $\alpha 5\beta 1$ integrin expression levels and localization in biopsy donors and cultured fibroblast cells.

Immunofluorescent staining (A) and relative immunofluorescence intensity (B) of $\alpha 5\beta 1$ integrin (green) and $\alpha 5$ integrin (red) in skin biopsies from young (24–29 years old) and older donors (56–60 years old). Immunofluorescent staining of $\alpha 5\beta 1$ integrin (green) and fibronectin in BJ cells from early and late passage (C). Arrowheads indicated indicate fibronectin which is not in fiber form while arrows indicate fibers of fibronectin. In all samples, cell nuclei were stained with DAPI (blue) and examined with confocal microscopy. Scale bar is 50 μm . $n = 6$. Scale bar is 20 μm . $n = 4$. ** p value < 0.005.

cell senescence (Fig. 4B). FACS analysis also showed that cultivating cells on Fibronectin significantly increased the percentage of cells in the G0-G1 phase compared to the control group suggesting cell cycle arrest and early entry into senescence (Fig. 4C). To determine whether Fn exogenous expression leads to the induction of premature senescence, early passages cells seeded on Fn were analyzed for the levels of metalloproteinases (MMPs) and SASP markers expression. As shown in Fig. 4D, we observed an elevation in metalloproteinases, *Mmp1* and *Mmp14*, and senescent markers, *Tgfb1* and *Vimentin*, mRNA levels in the young BJ cells seeded on Fn-coating, compared with control without coating. Altogether, these data demonstrate that *in vitro*, Fn is sufficient to promote premature senescence in skin fibroblasts.

We then tested the effects of Fibronectin knockdown on cell senescence with siRNA designed to target Fibronectin (siFN1). We first

confirmed the efficacy of Fibronectin silencing by Western Blot (Fig. 5A–C). Given that the Fibronectin- $\alpha 5\beta 1$ axis promotes cell migration and adhesion via the Focal Adhesion Kinase (FAK signaling pathway) we also assessed phospho-FAK levels in cells knockdown for Fn. As shown in Fig. 5A–C, we observed a decrease of both Fibronectin and phospho-FAK levels in both early and late passage cells transfected with siFN1. Next, we analyzed apoptosis markers expression in late passage cells knockdown for Fibronectin. Out of 35 proteins tested, five were found downregulated in absence of Fibronectin (Caspase 3, Bax, Fas, HIF-1 α and HSP27) (Fig. 5D and E). Taken together, these data suggest that Fn is involved in premature senescence of cells.

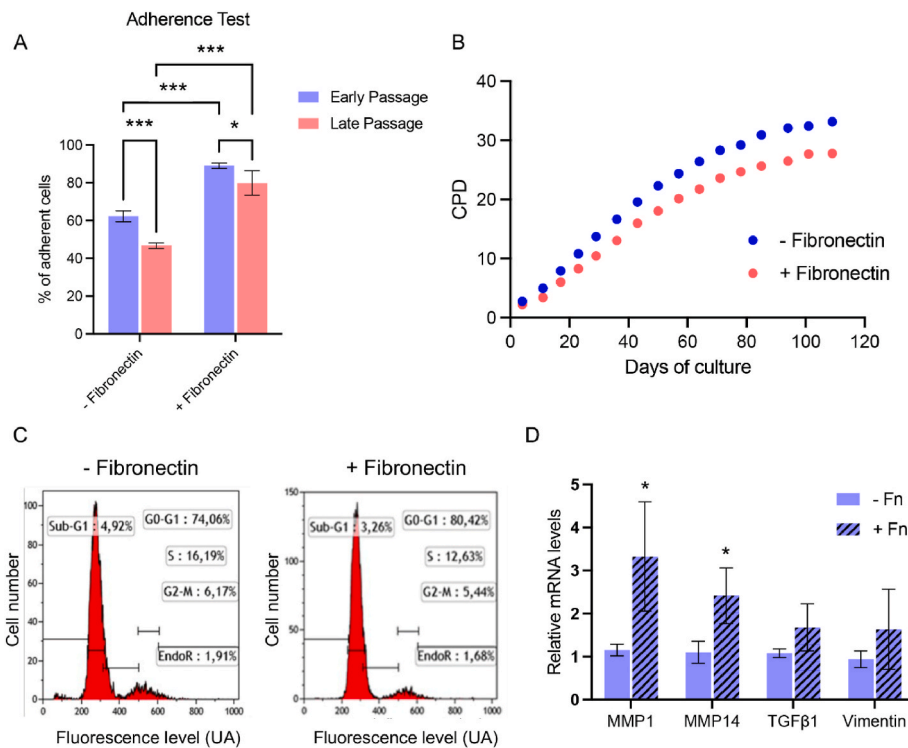


Fig. 4. Effects of Fibronectin-coating on skin fibroblast adhesion, proliferation, cell cycle distribution.

Percentage of early and late passage BJ cells remaining attached after being plated for 10 min on plastic or on 10 µg/mL Fibronectin coating (A). Decrease in replicative potential during serial passaging of BJ cells seeded or not on 10 µg/mL Fibronectin-coating. CPDs were calculated at each passage using the formula: $PD = 3.32 \times \log(Nc - Ns)$, with Nc, number of cells counted at confluence and Ns, the number of seeded cells (B). Flow cytometry analysis of G0/G1-phase arrest in late passage BJ cells coated or not with 10 µg/mL Fibronectin (C). *Mmp1*, *Mmp14*, *Tgfb1* and *Vimentin* mRNA levels in young BJ cells seeded or not on 10 µg/mL Fibronectin-coating (D). n = 3. *p value < 0,05; ***p value < 0.001.

3.6. Fibronectin matrix remodeling differs in senescent cells compared to young cells

Extracellular matrix is a highly dynamic structure, constantly remodeled through enzymatic and non-enzymatic processes. Fibronectin remodeling occurs through sequential steps which lead to a conversion of Fibronectin into a dense meshwork of interconnected fibrils and involved integrin receptors [41,42]. Therefore, variations of cell adhesion to Fibronectin-coated surfaces could modify the ECM organization.

Since we observed a less amount of secreted Fn in senescent cells compared to young ones, we wonder about the ability of cells to remodelate a Fn-coating. To answer that, we seeded early passage and late passage cells onto Alexa Fluor® 568-Fibronectin-coated cover glass and observed Fibronectin deposition using confocal microscopy at 4-, 24-, and 48-h post-seeding. At 4 h of culture, Fluorescent-Fibronectin was less abundant in early passage cells when compared to late passage cells (Fig. 6A) suggesting a rapid ECM remodeling with Alexa 568®-Fn degradation and newly secreted Fn incorporation into fibrils. This observation was confirmed at 24 and 48 h of culture. These results suggest that senescent cells failed to secrete and incorporate new Fn to fibrils.

3.7. Different fibronectin isoforms profile in senescent skin fibroblasts

The alterations of Fibronectin molecular status throughout senescence or aging might be associated not only with disturbances in the balance of Fibronectin production rate and secretion but also by the existence of different isoforms of Fibronectin. These Fn isoforms could induce different cell behaviors by an outside-in signaling pathway. Since it was shown that Fn isoforms can exist in human plasma [43], we are

investigating whether different isoforms are expressed by fibroblasts during aging *in vitro*. By using a 2-dimensional separation of the conditioned medium of fibroblasts we observed that Fibronectin isoforms produced and secreted by cells cultivated at early passage are a molecular weight about 260 kDa (Fig. 6B). Interestingly, identification of Fibronectin isoforms in conditioned medium from late passage cells showed a more complex isoform profile. It appeared that some isoforms with a molecular weight less important (about 150 kDa) were secreted (Fig. 6B). Collectively, these data suggest that the different pattern of Fn isoforms observed only in late passage cells may explain the previously mentioned altered incorporation of Fibronectin.

4. Discussion

Over the past 20 years, the expression of integrins and extracellular matrix proteins in the skin has been well characterized and discussed [44–46]. Our aim was to understand the discrepancies regarding Fibronectin expression during aging. These results collectively demonstrate, both *in vivo* and *in vitro*, that despite increased Fibronectin production during cell aging, its incorporation into the extracellular matrix scaffold is impaired. This observation aligns with Kumazaki et al., findings, who reported increased Fibronectin mRNA in aged skin fibroblasts obtained either through replicative senescence or biopsy from aged donors [47].

To date, understanding Fibronectin status during aging is still challenging. Consequently, recent studies suggest that Fibronectin, as a therapeutic target, should be addressed at the appropriate location and time to maximize treatment effectiveness [21]. Bouissou et al., showed that the Fibronectin network was less developed in old human skin, with Fibronectin only revealed around capillaries and in the dermal-epidermal junction [48]. Our examination of biopsies from

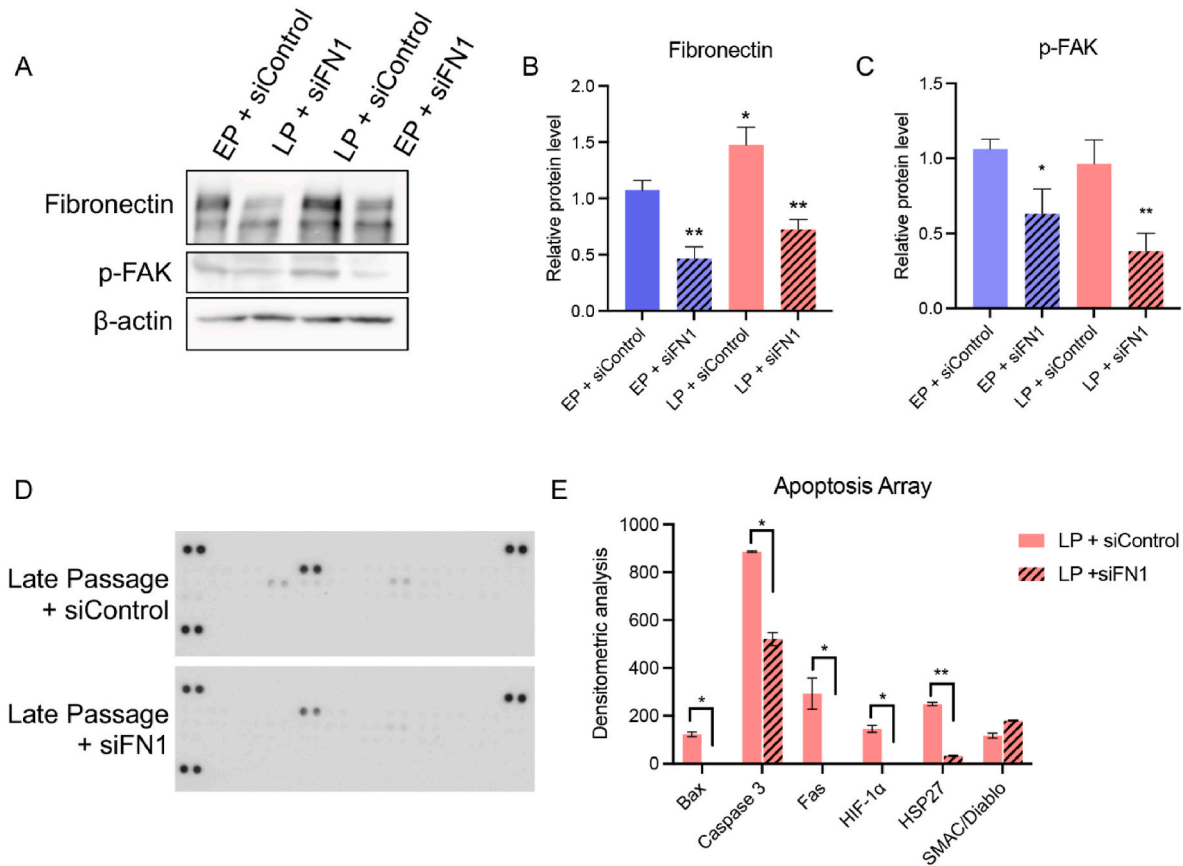


Fig. 5. Effect of Fibronectin knockdown on cell senescence.

Fibronectin and phospho-FAK protein levels in BJ cells knock-down for fibronectin (siFN1) presented as Western Blot (A) and quantified via densitometric analysis (B and C). Representative array results (D) and densitometric analysis (E) of apoptosis markers of siControl and siFN1 BJ cells from late passage. $n = 3$. * p value < 0.05 ; ** p value < 0.005 . EP, early passage; LP, late passage.

young and old donors showed a slight decrease in global dermis Fibronectin expression, more significant in the reticular dermis than in the papillary dermis. This distribution of Fibronectin in the dermis and its consistent expression in the papillary dermis underline its important role in the dermal-epidermal junction, independently of aging [25,49]. Decreased Fibronectin in the dermis, especially at the dermo-epidermal junction, can lead to more than just cosmetic changes like skin stiffness loss and wrinkle formation in old skin [50–52].

Our study also observed an elevated amount of Fn mRNA levels in cells during replicative senescence with senescent cells producing more Fibronectin proteins than young cells. Experiments using S^{35} methionine labeling highlighted this increase in Fibronectin production by senescent cells [53,54]. This observation was also seen *in vivo* by comparing Fibronectin expression in aortic endothelial cells from human donors of different ages [40].

Our *in vitro* studies on late passage BJ cells suggest that replicative senescence might lead to an accumulation of intracellular Fn and a decrease of Fn fibrils formation potentially due to ER stress induced by replicative senescence [55,56]. This hypothesis is supported by our data showing that late passage BJ cells incorporate Fn more slowly than early passage cells. Additionally, the presence of Fn isoforms with lower molecular weight in late passage BJ cells suggests that senescent fibroblasts might secrete different or misfolded Fn isoforms, resulting in poor detection of Fn in the ECM by immunohistochemistry.

Furthermore, our data demonstrated that old cells seeded on a Fibronectin-coating were less able to recognize and form Fibronectin fibers and/or degrade it. The difference in Fibronectin remodeling kinetics between young and old cells could be explained by the reduced adhesive capacity of old fibroblasts to adhere to a Fibronectin coating. In

dermis, we observed a decrease of about 15 % in $\alpha 5\beta 1$ expression with aging. Since $\alpha 5\beta 1$ is the main Fibronectin receptor, its reduced expression could be linked to the decreased Fibronectin fibrils in older dermis. Fibronectin fibril formation is induced by interactions with $\alpha 5\beta 1$ integrins on cell surfaces with soluble Fn [42,57–60]. Consequently, reduced $\alpha 5\beta 1$ levels leads to decreased modification of Fibronectin into its fibrillary form. These results align with the decreased number of dermal fibroblasts and their reduced functional activity observed during aging [61,62].

Our findings suggest that cultivating skin fibroblast cells on a Fn-coating accelerates the entry into senescence, supporting the idea that Fn plays a significant role in this process. We observed that Fn promotes the increase of senescent markers and metalloproteinases like MMP-1, which are induced by ROS and serve as markers for both ECM turnover and cellular senescence [63]. In metastatic cancer, endogenous Fn expression is highly promoted in epithelial cells entering senescence under oncogenic stimuli. These senescent cells, in turn, secrete SASPs to recruit fibrotic pro-inflammatory macrophages and fibroblasts [21]. Our data provides insight into the potential involvement of Fn in stress-induced premature senescence.

Different isoforms of cellular Fibronectin have been previously described [29]. A single cell can produce many Fn variant mRNAs via alternative splicing at three possible sites: EDA, EDB, and IIICS. Different variants of Fibronectin protein seem to exist, and their status appears to change during normal human aging [33]. Measuring Fibronectin expression during aging is challenging and may reveal the existence of these isoforms. Discrepancies in Fibronectin amount measurements during aging could result from the specific monoclonal antibodies used, which target different domain epitopes. Our study shows that cells in

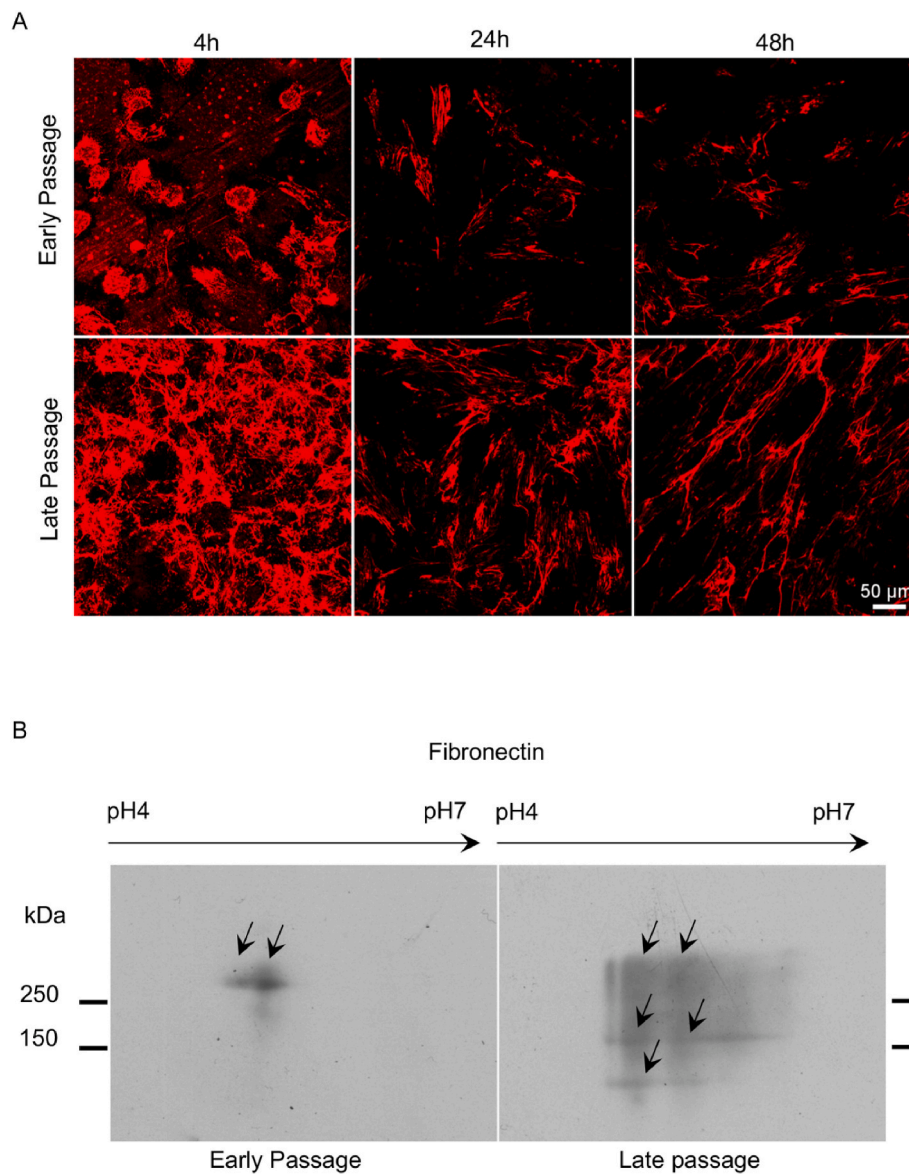


Fig. 6. Assessment of Fibronectin dynamics in BJ cells at early and late passage.

Immunostaining of coated fibronectin with BJ cells from early and late passage (A). At 4h, 24h and 48h after seeding, cells are fixed, and acquisition of fluorescence was done to estimate the remodeling of fibronectin by appearance of lysis areas or fibronectin fibers. Two-dimensional analysis of secreted fibronectin from early and late passage BJ cells (B).

replicative senescence express and secrete Fibronectin isoforms that differ by their pHi and molecular weight. These isoforms or degradation fragments could result from Fibronectin degradation by matrix-degrading enzymes like MMPs during cell aging [64], potentially contributing to a stress-induced senescence phenotype by activating inflammatory and proteolytic pathways. This could accelerate the onset of cellular senescence, as observed in the skin of older donors and in age-related diseases.

Accumulating evidence suggests that the secretome produced by senescent cells plays a key role in cellular senescence and physiological aging. There is a profound alteration in the secretome of senescent cells, including growth factors, cytokines, and extracellular matrix remodeling factors. Senescence has been closely associated with large focal adhesions (FAs), activated FA kinase (FAK) and aberrant integrin signaling [65]. However, how senescence alters cell-matrix adhesion and how this contributes to the senescent phenotype are still poorly understood. Our study suggested that knockdown expression of Fibronectin in senescent cells might delay the onset of senescence and decrease the activation of

senescence regulators. Senescent fibroblasts might express a Fibronectin variant that induces a senescent microenvironment.

The secretome of senescent cells could have various effects, either promoting or inhibiting aging, as shown for the tumor-suppressive and pro-tumorigenic properties of the senescent secretome [66–69]. Previous studies demonstrated that in age-related diseases, such as rheumatoid arthritis, some Fibronectin fragments exhibit proinflammatory activities by secreting cytokines and MMPs causing proteolytic damage [70–72]. In atherosclerosis, the ECM of atherosclerotic arteries contains abundant deposits of cellular Fn containing extra domain A (Fn-EDA); whereas the predominant form of Fn in plasma of healthy humans contains negligible amounts of EDA [73]. In 2024, a study identified a genetic variation in fibronectin that results in significantly lower fibronectin production and was found being protective against Alzheimer's disease [74]. Additionally, Labat-Robert proposed an age-dependent amplification loop of Fibronectin biosynthesis associated with the up-regulation of proteolytic enzymes that degrade Fn into fragments [75]. This degradation leads to further enhancement of Fn biosynthesis,

with harmful consequences in age-related diseases. Our *in vitro* observations on the production of various isoforms of Fn correlated to an increase of Fn production in senescent fibroblasts seem to support this hypothesis. Furthermore, our laboratory demonstrated that, in ovarian cancer, misfolded Fn amyloid-like aggregates promote a single-cell and sparse migration pattern of SKOV3 cancer cells [76] further confirming the important role of Fibronectin in aging, as well as the significance of its protein folding.

Recent advances in anti-aging therapy, particularly the development of senolytic drugs that selectively eliminate senescent cells, highlight promising perspectives for treating age-related diseases [77]. Our research, along with other studies [73,76], identifies Fn as an emerging SASP target with significant implications for therapies targeting age-related diseases.

Overall, our data reveal a distinct profile of Fibronectin fragments in senescent fibroblasts and an altered Fibronectin distribution into the ECM which appears to promote early entry of cells into a senescent state. These findings offer novel insights into the role of Fibronectin matrices in senescence and aging processes.

5. Conclusion

These results collectively show that aging increases Fibronectin expression in cells, both *in vitro* through replicative senescence and *in vivo* by comparing expression in skin from donors of different ages. However, Fibronectin is less present in the matrix environment of dermal cells. It would be of great interest to study the secretory mechanisms of Fibronectin and its turnover during aging.

CRediT authorship contribution statement

Luce Perié: Writing – original draft, Investigation, Formal analysis. **Cynthia Houël:** Validation, Investigation, Formal analysis. **Anne Zambon:** Validation, Investigation, Formal analysis. **Christelle Guere:** Investigation, Data curation. **Katell Vié:** Resources, Data curation. **Johanne Leroy-Dudal:** Data curation. **Charlotte Vendrely:** Data curation. **Rémy Agniel:** Validation, Investigation. **Franck Carreiras:** Data curation. **Cédric R. Picot:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest and competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yexcr.2024.114251>.

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