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Effects of Repeated UVA Irradiation on Human Skin Fibroblasts Embedded in 3D Tense Collagen Matrix

Wongnapa Nakyai[1], Marion Tissot[2], Philippe Humbert[2,3], François Grandmottet[4], Jarupa Viyoch*[1] and Céline Viennet*[2]

[1]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand
[2]Engineering and Cutaneous Biology Laboratory (LIBC), UMR 1098 INSERM EFS BFC, University of Bourgogne Franche-Comté, Besançon, France
[3]Department of Dermatology, University Hospital, Besancon, France
[4]Faculty of Agriculture Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand

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ABSTRACT

Skin photoaging is caused by cumulative UVA exposure that leads to dermal matrix alterations associated with impaired fibroblast functions. In this study, we evaluated the effects of repeated UVA irradiation on mechanically stressed fibroblasts which were embedded in 3D tense collagen matrix. By comparison to 2D monolayer culture, we investigated the expressions of alpha-smooth muscle actin (α-SMA) cytoskeleton and α2 subunit of integrin receptors, as well as the collagen metabolism, focusing to MMP-1 and collagen type-I expressions. We found that UVA exposure reduces collagen levels in both culture conditions. However, concerning integrin α2 and α-SMA expression, UVA irradiation had no effect on 2D culture, whereas in tense 3D culture, it had an inhibitory effect. In UVA-irradiated 3D culture, fibroblasts acquired elongated shape and lost their dynamic interaction with collagen fibers through a decrease in integrin α2 and α-SMA. Fibroblast responses to UVA irradiation were different in 2D *versus* 3D environment, highlighting the importance of collagen environment in the regulation of mechanical activities. The behavior of fibroblast upon mechanical stimulation closely mimics stressed extracellular environment. The model of UVA-irradiated fibroblasts cultured in tense 3D collagen gel illustrated the *in vivo* situation of both mechanically stressed and photoaged human skin.

INTRODUCTION

Human skin is constantly exposed to the ultraviolet (UV) radiation that mediates a complex biological response and causes not only changes of skin appearance such as sunburn, pigmentation and wrinkle but also the risk of skin cancers (1). In photoaged skin, major changes are seen in dermal tissue and are characterized by the degradation and disorganization of the extracellular matrices (ECMs). Such damages result largely from dysfunction of fibroblasts and unbalanced production of ECM proteins and matrix metalloproteinases (MMPs) including MMP-1, which is predominantly produced by dermal fibroblasts. Many studies have focused on effects of UVA rays on skin because of their high penetration property and their role in the photoaging process (2–5). Successive UVA irradiation contributes to the histological features of photoaged skin. As experimental UV exposure is difficult to perform in humans for ethical reasons, *in vitro* studies using UV-irradiated fibroblasts cultured in two-dimensional (2D) monolayers were reported by many investigators (6–8). They described that UV rays inhibit type-I collagen synthesis through interfering TGFβ/smad pathway (9,10), activate MMPs through the release of pro-inflammatory/immunosuppressive cytokines (11,12) and modulate organization of cell cytoskeleton through alteration of binding capacity between collagen and integrin receptors (13,14). Although some 2D culture data could be correlated to *in vivo* observations, it is now accepted that cells cultured in tense three-dimensional (3D) collagen matrix mimic better the *in vivo* situations (15,16). Fibroblasts in 3D collagen matrix present a branched cytoplasm or dendritic shape that is similar to fibroblasts in dermal tissue whereas fibroblasts cultured in 2D usually exhibit a spindle shape (6,17,18). The dendritic shape of fibroblasts results from the embedment of cells into the collagen network and, thus, the cross-linking with the collagen fibrils. Such interactions develop the tensional force surrounding the tensed or restrained collagen matrix and, hence, turn on the mechanism of remodeling and synthesis of ECM and cytoskeletal components (19–21). The functional linkage between type-I collagen fibers and actin cytoskeleton is mediated by cell surface receptors such as integrins. Expressions of integrin alpha 2 (α2) and α-smooth muscle actin (α-SMA) provide essential mechanical support for fibroblasts. On the contrary, cells in 2D cultures have a low capacity to generate mechanical force and to trigger signaling of biosynthesis and ECMs remodeling. The difference of cell morphology and behavior between 2D and 3D cultures suggests the functional importance of the extracellular environment in synthetic capacity and cell–ECMs interaction.

Few studies have addressed the functional consequences of UV on mechanically stressed fibroblasts (22,23). The skin in its physiological state is constantly exposed to tensional forces. Thus, it is necessary to have an appropriate model to examine the mechanism of photoaging caused by chronic UV exposure. In the present work, we have evaluated the effects of repeated

*Corresponding authors' emails: celine.viennet@univ-fcomte.fr (Céline Viennet), jarupav@nu.ac.th or jarupaviyoch4@yahoo.com (Jarupa Viyoch)

UVA irradiation using a dermal equivalent model in which fibroblasts were embedded in tense collagen matrix and therefore develop tension (21,24). By comparison to the monolayer, we investigated the expressions of α-SMA cytoskeleton and α2 subunit of integrin receptors, as well as the collagen metabolism, focusing to MMP-1 and collagen type-I expressions.

MATERIALS AND METHODS

Isolation of human dermal fibroblasts. Dermal fibroblasts were isolated from facial skin of 54-year-old woman who underwent cosmetic surgery, using explant technique. The cell isolation protocol was approved by the Institutional Review Board of Naresuan University (No. 242/57). Briefly, skin tissues (2 mm diameter) were cut using a biopsy punch, placed in a 25 cm^2 flask and incubated for 30 min in 37°C humidified incubator containing 5% CO_2. After that, Dulbecco's modified Eagle's medium (DMEM; Pan Biotech, Dominique Dutscher, Brumath, France) supplemented with 10% fetal bovine serum (FBS; Pan Biotech) and antibiotics (1% penicillin (100 U mL^{-1})–streptomycin (0.1 mg mL^{-1}) solution; Pan Biotech) were added, and skin biopsies were incubated at 37°C with 5% CO_2 for 2–3 weeks until fibroblasts migrated and reached approximately 80% confluence. Fibroblasts were then trypsinized using trypsin (0.05%)–EDTA (0.02%) solution (Pan Biotech) and maintained in DMEM supplemented with 10% FBS and antibiotics in a 37°C humidified incubator containing 5% CO_2. They were cultured to 80% confluence and then subcultured. They were used between passage 3 and 5 for the following experiments (Fig. 1).

Monolayer (2D) culture and UVA irradiation. Fibroblasts were seeded at a density of 5×10^4 cells/dish into 60 mm petri dishes and cultured for 24 h in DMEM supplemented with 10% FBS. They were then maintained for 24 h in DMEM with 1% FBS and then washed with phosphate-buffered saline (PBS; Pan Biotech) and covered with thin layer of PBS prior to UVA irradiation. The UVA source was a bank of eight fluorescent black light lamps (F15T8; Sylvania, Danvers, MA). The peak emission was at 370 nm and was checked using a UV radiometer IL-1700 with UVA filter (Dexter Industrial Green, Newburyport, MA). The UVA source was positioned at ~ 17 cm above the cell culture petri dish, emitting a UV intensity of 2.2 W cm^{-2} for 45 min (intensity of 6 J cm^{-2}). The intensity of UVA used in this study was modified from the intensity used in our previous study (5 J cm^{-2}, (25)), according to the difference in UVA lamp source and culture condition. After UVA exposure, cells were maintained in DMEM supplemented with 10% FBS and antibiotics. UVA radiation process was performed three times at an interval of 48 h. Fibroblasts were further cultivated for 24 h after the last irradiation, and then cells and cell-free supernatants were collected. Control cells were cultured under the same conditions but without UVA exposure.

Tense collagen lattice (3D) culture and UVA irradiation. Tense collagen lattices were prepared according to previous studies (24,26). Briefly, lattice mixture solution consisted of DMEM medium (0.8 X), FBS (9% v/v), NaOH (0.005 N), acid-extracted type-I collagen (0.6 mg mL^{-1}) (Institut de Biotechnologies Jacques Boy, Reims, France), cells suspension (8×10^5 cell mL^{-1}), $NaHCO_3$ (0.3%), penicillin (200 U mL^{-1}) and streptomycin (0.2 mg mL^{-1}). It was then poured into 60 mm petri dishes containing a sterilized nylon mesh ring (Sefar Nitex 03-150/50, Heiden, Switzerland). After collagen gel polymerization at 37°C, 3 mL of DMEM supplemented with 10% FBS and antibiotics was added. After 5 days of culture period, incubation time required for the development of a 3D architecture (27) collagen lattices were irradiated following the same protocol as for monolayers. Control collagen lattices were cultured under the same conditions but without UVA exposure.

Cell number and cell cycle analysis. At 24 h after the last UVA exposure, cells cultured in monolayers were trypsinized. For collagen lattices, they were incubated with collagenase A (0.5 mg mL^{-1}; Roche Diagnostics; Sigma-Aldrich, St Quentin Fallavier, France) at 37°C for 90 min. The collected cells were count using the trypan blue exclusion method and then were centrifuged to obtain cell pellets. Both 2D- and 3D collected cells were washed twice with PBS, fixed in 1 mL of cold 70% ethanol and stored overnight at 4°C. Then, they were resuspended in PBS containing 1 mg mL^{-1} propidium ionide and 100 U mL^{-1} RNase (Sigma-Aldrich). Cell cycle distribution was determined by FACS analysis (FC500 flow cytometer, Beckman Coulter; Roissy, France). Each experimental condition was performed in triplicate.

Quantification of type-I procollagen and MMP-1—ELISA for protein levels. Cells-free supernatants of monolayer and tense collagen lattice cultures were collected at 24 h after the last UVA irradiation. Protease-inhibitor cocktail (10% v/v; Sigma-Aldrich) was added to supernatants which were then stored at −80°C until analysis. Cells were trypsinized and counted with Trypan blue. MMP-1 and type-I procollagen secreted in the culture medium by fibroblasts were quantified using ELISA kits (respectively, Human MMP-1 ELISA Kit, Thermofisher, Courtaboeuf,

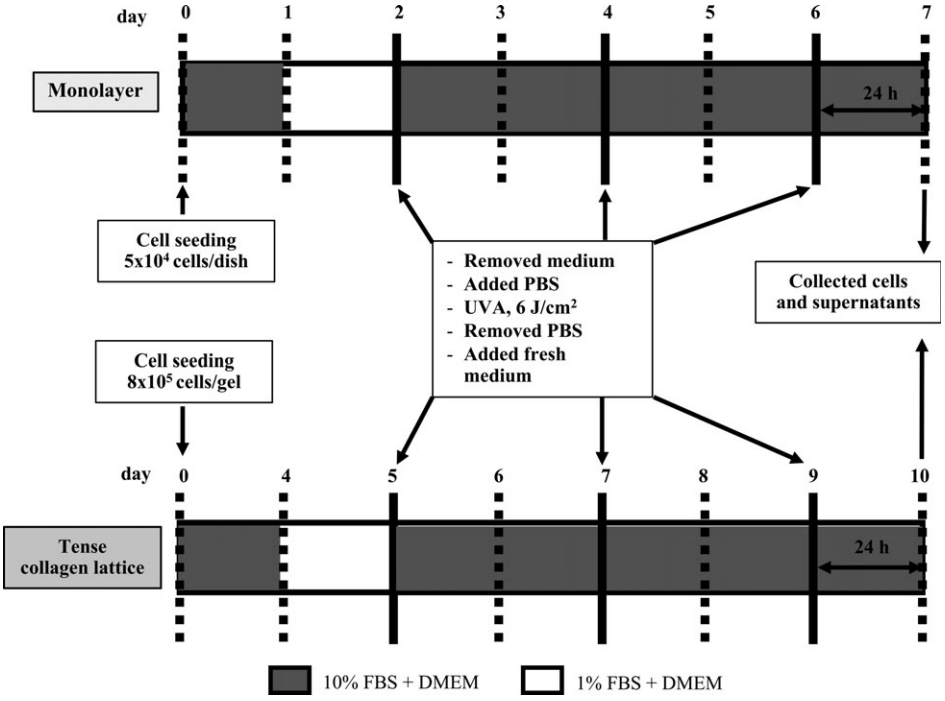

Figure 1. Schematic representation of the protocol used.

France; Human Procollagen type-I C-peptide (PIP) ELISA Kit, Takara Bio, St Germain-en-Laye, France), according to the manufacturer's instructions. Each experimental condition was performed in triplicate, and each supernatant was quantified in triplicate. Protein levels of type-I procollagen and MMP-1 were double-normalized over cell number and then non-UVA-irradiated 2D culture corresponding to 100%.

Quantification of type-I procollagen and MMP-1—qPCR for mRNA levels. Cell pellets were collected from monolayers and tense collagen lattices at 24 h after the last UVA irradiation. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. Next, cDNA was performed from 1 µg of total RNA using the High Capacity RNA-to-cDNA kit (ThermoFisher, Applied Biosystems, Courtaboeuf, France). MMP-1 and type-I collagen genes were quantified using quantitative real-time PCR. Taqman® Gene Expression Assay kits (Thermofisher, Applied Biosystems) were used to assess MMP-1 (Hs00233958_m1), COL1A1 (Hs01076777_m1) and β-actin (Hs99999903_m1) gene expressions. β-actin was used as housekeeping gene. The PCR mixture comprised 2 µL of cDNA sample, 10 µL of TaqMan universal PCR master mix, 1 µL of each of TaqMan gene expression assay and RNase-free water. Reactions were performed and monitored using the Biorad CFX96 Real-Time PCR System. Thermal cycling conditions were initiated with 2 min at 50°C, followed by 10 min at 95°C, and then 40 cycles of 95°C for 10 s (denaturation) and 60°C for 1 min (annealing/extension). For quantification, gene expression levels were calculated by the comparative CT method (also known as the $2^{-\Delta\Delta CT}$). The mRNA data were normalized to the β-actin. The expression value of non-UVA-irradiated cells in 2D was arbitrary set to 1. Each experimental condition was performed in triplicate, and each cDNA was quantified in duplicate.

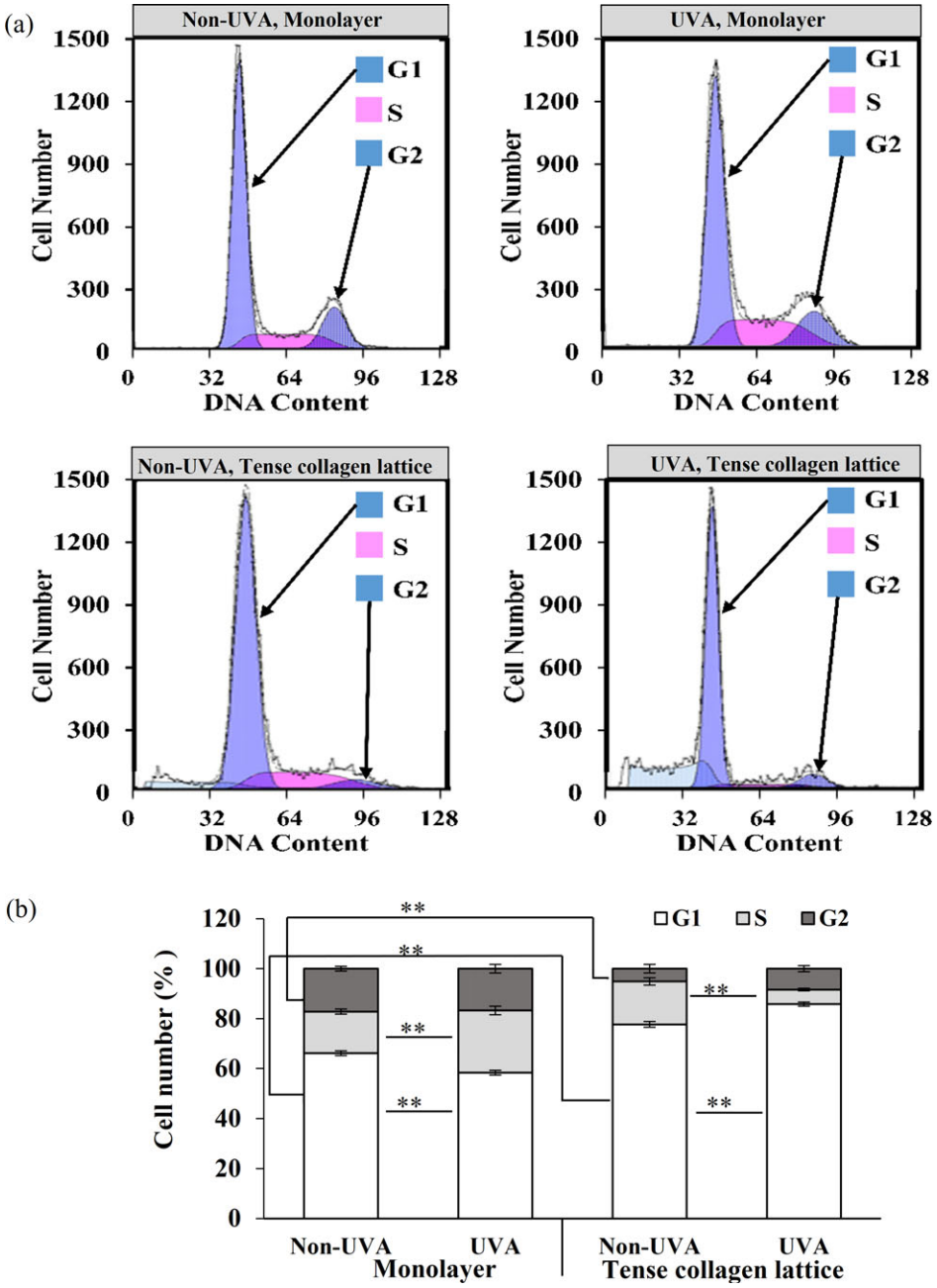

Figure 2. Effects of repeated UVA irradiation on the cell cycle of fibroblasts cultured in 2D and 3D at 24 h after the last UVA exposure. (a) Cell cycle histograms from the flow cytometric assay. (b) Cell cycle distribution of fibroblasts cultured in monolayers (2D) and tense collagen lattices (3D). Means ± SD, $n = 3$, $^{**}P < 0.01$

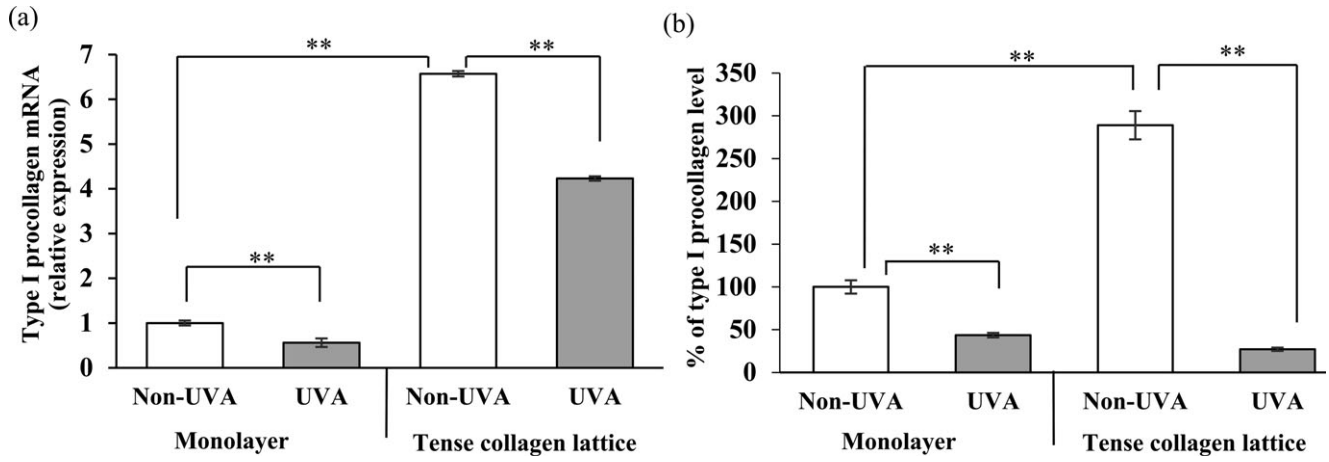

Figure 3. Quantification of (a) type-I collagen mRNA expression by qPCR. The mRNA data were normalized to the β-actin. The expression value of non-UVA-irradiated cells in 2D was arbitrary set to 1. (b) Type-I procollagen protein production by ELISA. The secreted type-I procollagen level of the non-UVA-irradiated cells in 2D was normalized to 100%. Data are mean ± SD, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$.

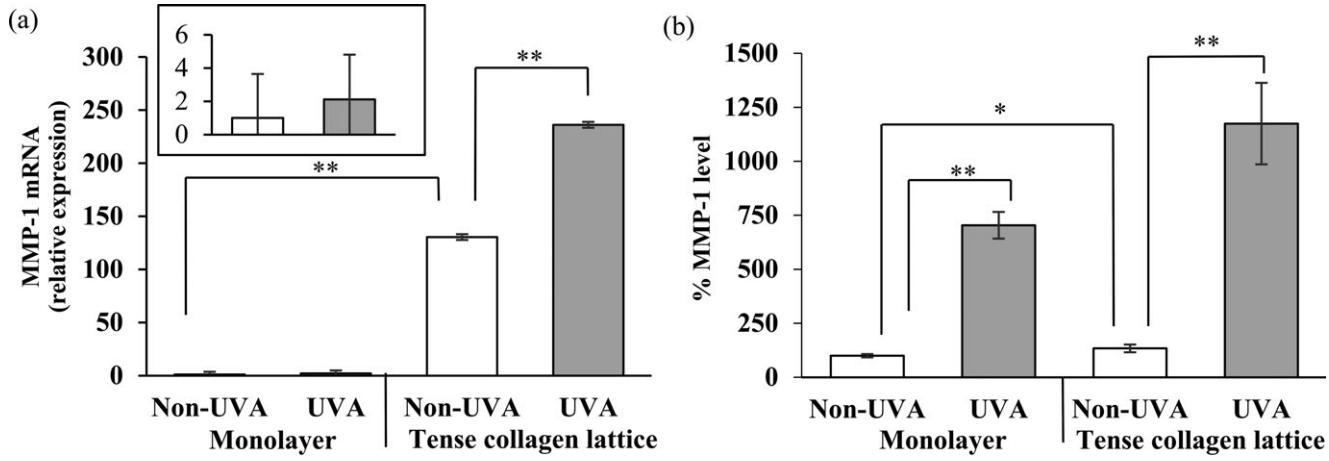

Figure 4. Quantification of (a) MMP-1 mRNA expression by qPCR. The mRNA data were normalized to the β-actin. The expression value of non-UVA-irradiated cells in 2D was arbitrary set to 1. (b) MMP-1 protein production by ELISA. The secreted MMP-1 level of the non-UVA-irradiated cells in 2D was normalized to 100%. Data are mean ± SD, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$.

Determination of α-SMA and integrin α2—Microscopy. At 24 h after the last UVA exposure, fibroblasts grown in monolayers and collagen lattices were fixed with 3% paraformaldehyde (PFA) in PBS and permeabilized with 0.1% Triton X-100 in PBS. Then, they were treated with 3% BSA in PBS for blocking nonspecific binding sites and incubated at 4°C overnight with mouse anti-α-SMA monoclonal antibody (Sigma-Aldrich) diluted 1:100 in PBS containing 1% BSA and 0.1% Triton X100 or mouse anti-integrin α2 monoclonal antibody (Clinisciences, Santa Cruz, Nanterre, France) diluted 1:30 in PBS containing 1% BSA and 0.1% Triton X100. Samples were then incubated at room temperature for 1 h with goat anti-mouse antibody conjugated with FITC diluted 1:75 (Sigma-Aldrich). To identify cell nuclei, cells were double-stained with Hoechst solution (Sigma-Aldrich) for 10 min at room temperature. Samples were mounted on glass slides with Fluoromount™ medium (Sigma-Aldrich) and observed under a fluorescence microscope (Axiocam MRc5; Carl Zeiss, Munich, Germany). The mean fluorescence intensity per cell was quantitated using Image J software, and the obtained values were normalized to non-UVA group, which was set to 100%.

Determination of α-SMA and integrin α2—flow cytometry. At 24 h after the last UVA exposure, cells were collected from monolayers and tense collagen lattices using the same method as described in cell cycle analysis. They were fixed with 3% PFA in PBS for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were then incubated at 4°C overnight with monoclonal anti-α-SMA antibody (diluted 1:250 in PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X100) conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich), or monoclonal anti-integrin α2 antibody (diluted 1:200 in PBS containing 1% BSA and 0.1% Triton X100) conjugated with FITC (Clinisciences, Abcore, Nanterre, France), or FITC isotype control. They were centrifuged and resuspended in 2 mM EDTA in PBS. Samples were analyzed by FACS (FC500 flow cytometer; Beckman Coulter). Each experimental condition was performed in triplicate.

Statistical analysis. For each condition, all quantitative data are expressed as mean ± SD. Student's unpaired *t*-test was used to compare groups, and $P < 0.05$ was considered significant.

RESULTS

Effect of UVA irradiation on cell cycle of fibroblasts cultured in monolayers (2D) and tense collagen lattices (3D)

Figure 2 shows the percentage of fibroblasts at different stages of the cell cycle of UVA-irradiated cultures and nonirradiated controls in 2D and 3D. Focusing on non-UVA-irradiated cells, the number of G2 cells in monolayers (17.18 ± 0.95% of total cell number) was markedly larger than those in lattices (5.14 ± 1.75%) while the number of G1 cells (66.14 ± 0.94%, 2D) was significantly lower (77.65 ± 1.15%, 3D). In both irradiated 2D and 3D cultures, the results showed that the proportion

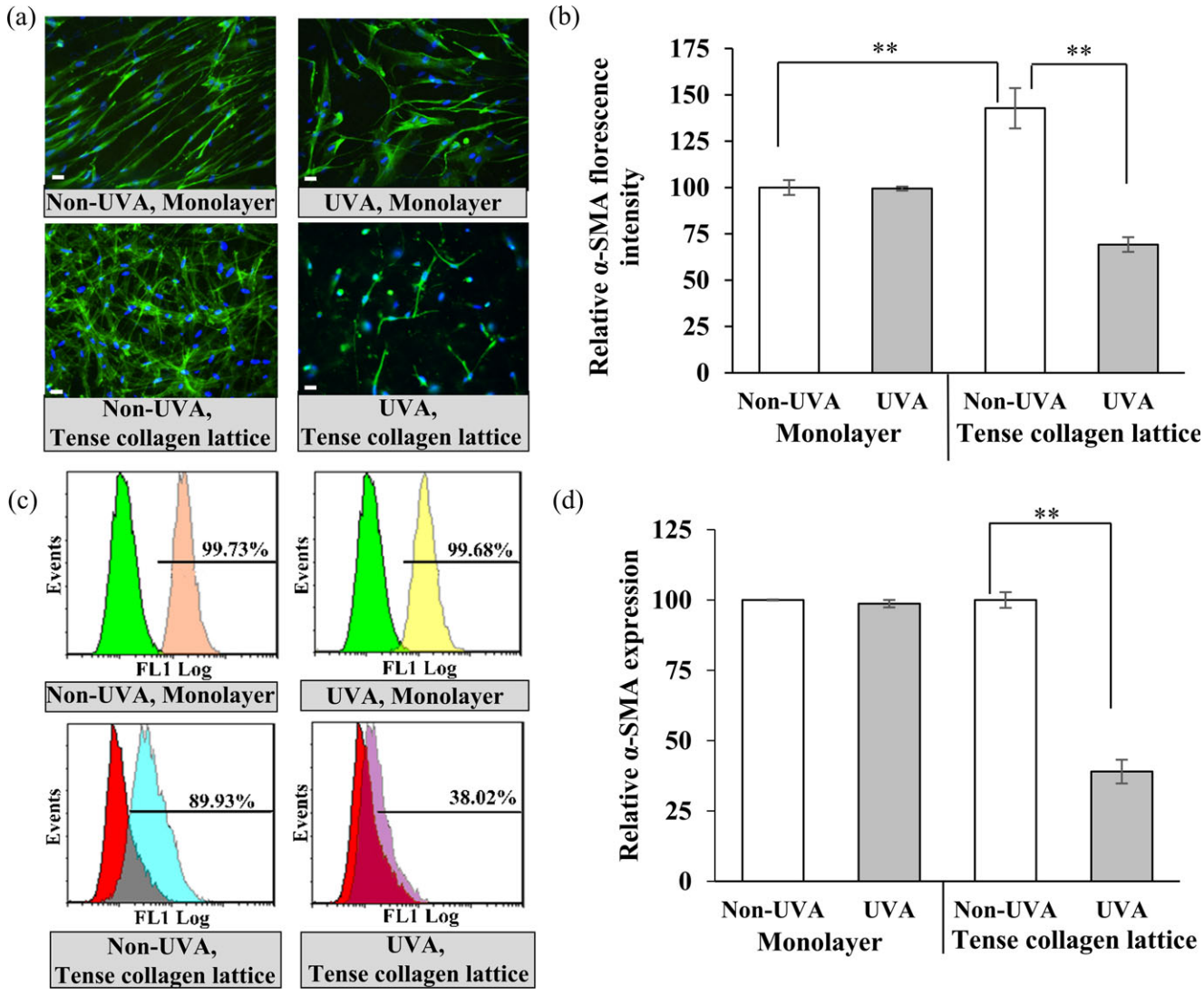

Figure 5. Alpha-smooth muscle actin (α-SMA) expression in fibroblast monolayer and fibroblast embedded collagen lattice cultures. (a) Representative fluorescent microscopy images for α-SMA (green) and nuclei (blue) immunostaining. Scale bar = 100 μm. (b) Quantification of α-SMA fluorescence in ×20 microscopy fields using image J software. The level α-SMA expression of the non-UVA-irradiated cells in 2D was normalized to 100%. (c) Flow cytometry histograms of α-SMA expression, in which the pale orange, yellow, light blue and purple colors represent non-UVA-irradiated cells for 2D, UVB-irradiated cells for 2D, non-UVA-irradiated cells for 3D and UVA-irradiated cells for 3D, respectively. Respective isotype controls are shown as light green (2D) and red (3D) lines in the histogram. (d) Quantification of α-SMA expression by flow cytometry. For each culture condition, the level of α-SMA expression in the repeated UVA-irradiated fibroblasts was normalized and compared with that of the non-UVA-irradiated fibroblasts. All data are expressed as mean ± SD, $n = 3$, $^{**}P < 0.01$.

of G2 cells (17.18 ± 0.95 for 2D, 5.14 ± 1.75 for 3D) was not significantly different from non-UVA-exposed cells (16.70 ± 1.75 for 2D, 8.37 ± 1.22 for 3D). However, in irradiated 3D cultures, most of cells accumulated in G1 phase (85.86 ± 0.83%) while the number of S cells declined (5.77 ± 0.51%) as compared to non-UVA-exposed cells (G1 phase: 77.65 ± 1.15%, S phase: 17.21 ± 1.52%).

Effect of UVA on type-I collagen mRNA expression and type-I procollagen protein production in monolayers (2D) and tense collagen lattices (3D)

The quantification of type-I collagen mRNA expression and type-I procollagen protein production is shown in Fig. 3. Both mRNA and protein levels (6.57 ± 0.06 fold change and 156.52 ± 8.96%, respectively) were markedly enhanced in collagen lattices as compared with monolayers (1.00 ± 0.06 fold change and 100.00 ± 7.77%). Both cell culture models repeated UVA exposure had inhibitory effect on type-I collagen mRNA and protein (2D: 0.55 ± 0.10 fold change and 43.58 ± 2.69%, 3D: 4.23 ± 0.06 fold change and 14.67 ± 1.05%).

Effect of UVA on MMP-1 mRNA expression and protein production in monolayers (2D) and tense collagen lattices (3D)

The quantification of MMP-1 mRNA expression and protein production is shown in Fig. 4. Both mRNA and protein levels (130.39 ± 2.69 fold change and 133.79 ± 17.69%, respectively) were enhanced in collagen lattices as compared with monolayers

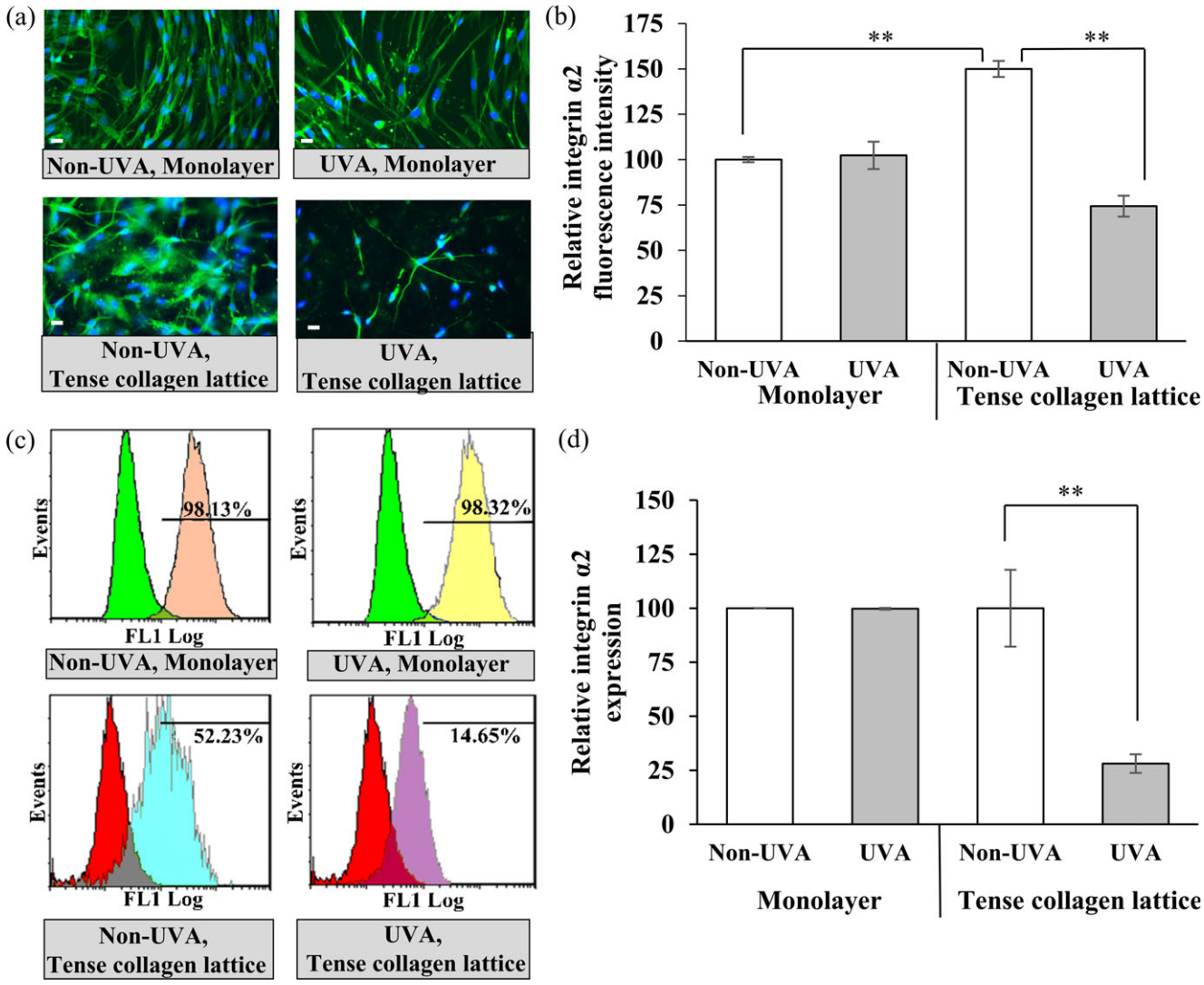

Figure 6. Integrin α2 expression in fibroblast monolayer and fibroblast embedded collagen lattice cultures. (a) Representative fluorescent microscopy images for integrin α2 (green) and nuclei (blue) immunostaining. Scale bar = 100 μm. (b) Quantification of integrin α2 fluorescence in ×20 microscopy fields using image J software. The level integrin α2 expression of the non-UVA-irradiated cells in 2D was normalized to 100%. (c) Flow cytometry histograms of integrin α2 expression, in which the pale orange, yellow, light blue and purple colors represent non-UVA-irradiated cells for 2D, UVB-irradiated cells for 2D, non-UVA-irradiated cells for 3D and UVA-irradiated cells for 3D, respectively. Respective isotype controls are shown as light green (2D) and red (3D) lines in the histogram. (d) Quantification of integrin α2 expression by flow cytometry. All data are expressed as mean ± SD, $n = 3$, $^{**}P < 0.01$

(1.00 ± 2.64 fold change and 100.00 ± 7.65%). In 2D culture, repeated UVA exposure had no significant effect on MMP-1 mRNA expression (non-UVA: 1 ± 2.64 fold change, UVA: 2.12 ± 2.68 fold change), but stimulated MMP-1 protein secretion (non-UVA: 100.00 ± 7.65%, UVA: 703.81 ± 62.11%). In 3D culture, repeated UVA exposure activated both MMP-1 mRNA expression and protein secretion (non-UVA: 130.39 ± 2.69 fold change and 133.79 ± 17.69%, UVA: 236.11 ± 2.80 fold change and 1174.54 ± 188.85%, respectively).

Effect of UVA on α-SMA of fibroblasts cultured in monolayers (2D) and tense collagen lattices (3D)

Image J software was used to measure the fluorescence intensity per cell, and the mean fluorescence intensity per cell of the non-UVA group was adjusted to 100%. The quantitative analysis of fluorescent histological staining (Fig. 5b) showed that α-SMA expression was higher in non-UVA-irradiated 3D collagen lattices compared to non-UVA-irradiated 2D monolayers. In addition, interestingly, the repeated UVA exposure markedly suppressed α-SMA expression in 3D collagen lattices whereas it did not affect α-SMA expression in 2D monolayers. Such UVA effects were also observed using flow cytometry (Fig. 5d).

Effect of UVA on integrin α2 of fibroblasts cultured in monolayers (2D) and tense collagen lattices (3D)

The fluorescence intensity per cell was measured using Image J software, and the mean fluorescence intensity per cell of the non-UVA group was adjusted to 100%. The quantitative analysis of fluorescent histological staining (Fig. 6b) showed that higher integrin α2 expression was seen in non-UVA-irradiated cells

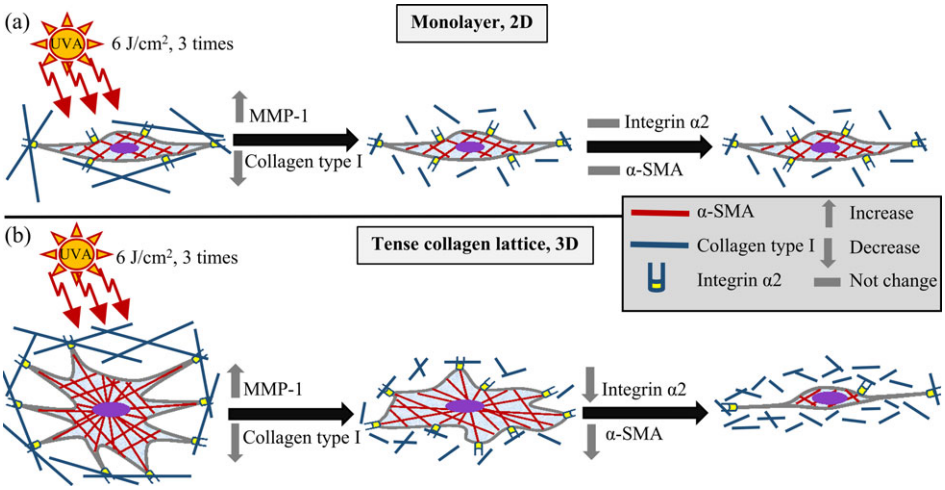

Figure 7. Illustration of the effect of repeated UVA irradiation on fibroblast cultured in (a) 2D, monolayer and (b) 3D, tense collagen lattice.

cultured in lattices, as compared with non-UVA-irradiated fibroblasts cultured in monolayers. In addition, the repeated UVA exposure markedly suppressed integrin α2 expression in 3D collagen lattices whereas it did not affect integrin α2 expression in 2D monolayers. Such UVA effects were confirmed using flow cytometry (Fig. 6d).

Effect of UVA on morphology and number of fibroblasts cultured in monolayers (2D) and tense collagen lattices (3D)

Fluorescent imaging of Figs. 5 and 6 was also used to analyze cell morphology. Control cells had a characteristic shape. Fibroblasts cultured in collagen lattices developed a dendritic morphology whereas those cultured in monolayers showed spindle shaped. After UVA exposure, the irradiated fibroblasts showed marked disorders of morphology. In irradiated monolayers, fibroblasts displayed flattened and satellite morphology but no significant difference in cell number compared with non-UVA-irradiated cells (non-UVA: 100.00 ± 10.96%, UVA: 82.06 ± 4.85%), whereas in irradiated collagen lattices, they appeared thin and elongated cytoplasm and underwent a decrease in the number as compared to non-UVA-irradiated cells (non-UVA: 100.00 ± 10.31%, UVA: 54.37 ± 3.82%).

DISCUSSION

Mechanical forces generated and sensed by cells are key regulators of a variety of biological processes. An abnormal mechanical environment can disrupt tissue homeostasis. Dermal fibroblasts sense the mechanical load of their environment and transfer the mechanical forces from the ECM to their cytoskeleton via the phenomenon of mechanotransduction (28).

Chronic sun exposure causes skin photoaging, a complex process that induces various skin changes such as fragmentation of collagen fibers. It is well established that fragmentation of the dermal collagenous ECM impacts skin mechanical properties and fibroblasts functions (29–31). Many of the works on UV-induced skin effects have used 2D cell culture models and have mainly studied cellular and molecular events, and structural changes (6,32,33). However, the UV response of fibroblasts upon mechanical stimulation remains poorly understood. In this study, we used a 3D dermal equivalent model in which fibroblasts are embedded in tense collagen matrix (34). As this model more closely approaches the physiological skin tension, it provided an opportunity to investigate responses of mechanically stressed fibroblasts to UV radiation.

One important factor that may contribute to the difference between UV responses of fibroblasts cultured in 2D and 3D is the cell division behavior. Dermal fibroblasts of healthy skin are found arrest in G0/G1 phase (27,35,36). Firstly, we compared the cell cycle progression profiles between 2D and 3D contexts. Our results indicated that the proportion of G1 phase cells of 3D cultures is significantly higher than 2D cultures, and about 80% of cells cultured in lattices are in G1 phase. Therefore, they confirmed data of previous studies suggesting that this difference of G0/G1 arrest is due to cellular interactions with ECM (27,37,38), which play an active role in cell cycle and growth (21,39). Secondly, we reported effects of UVA radiation, and no difference of cell number in G2 phase was observed between irradiated and nonirradiated cultures, for both 2D and 3D models. In our previous work, repeated exposure to UVA (5 J cm^{-2} × 3 times) induced a decline in G2 phase cell number coupled with a damaged cell morphology (a smaller area of cytoplasm) (25). The discrepancy may result from the difference of cell culture conditions. In the present study, DMEM with 10% FBS culture medium was used throughout the culture duration while serum-free DMEM was used previously. The growth factors in serum might slow the severity of cell damages after repeated UVA exposure. However, an accumulation of cells in G1 phase coupled with a decrease in cells in S phase was markedly found in repeatedly UVA-exposed cells compared with nonirradiated cells in 3D cultures. Generally, UV radiation induces G1-phase cell cycle arrest which allows DNA repair before replication, and thereby S-phase delay (40,41). Arrest of cells in the G1 phase modulates cell proliferation (42,43). Our earlier study indicated that the proliferation ability of fibroblasts from wrinkled facial skin (aged/photoaged fibroblasts) was lower than that of fibroblasts from nonwrinkled facial skin. Such lower proliferation concurred with reduced proportion of cells in S and G2 phases (44). Therefore, culture conditions influence the pattern of cell cycle transition. There is a correlation between the cell cycle profiles of UVA-irradiated 3D model and human aged/photoaged cells in dermis.

As we know, not only biochemical but also mechanical signals regulate collagen metabolism in dermal n-metissue (29,30). The interaction of dermal fibroblasts with extracellular collagen fibers generates mechanical resistance, thus, in turn leading to signal transduction cascades that modulate transmembrane and intracellular structures, gene expression and protein synthesis. Communication between fibroblasts and surrounding collagen is mediated by integrin transmembrane receptors, among them integrin $\alpha 2\beta 1$, and actin cytoskeleton, mainly α-SMA (45,46). As expected, we found that fibroblasts cultured in collagen lattices express highest levels of type-I procollagen, both mRNA and protein. Consistent with other findings, we found that UVA exposure reduces collagen levels in both culture conditions (8,30,31,47). However, the mechanism of UV action is complex. UV inhibits type-I collagen synthesis in dermal fibroblasts by interfering with the TGFβ/smad module (9,10). In photoaged skin, the fragmentation and disorganization of collagen fibers result from the impairment of fibroblast functions that includes upregulation of MMPs (31,48,49). Moreover, increased collagen cross-linking is induced by UVA (50). In our 3D culture model, the reduction in type-I collagen might be due to the lower capacity of fibroblasts to migrate and adhere to collagen fibers, and to the overproduction of MMP-1 mRNA and protein (48,49). However in 2D culture after UVA irradiation, MMP-1 mRNA did not correlate with protein. This may result from different contexts. After short-term UVA irradiation (6 J cm^{-2}), the synthetic pathway of MMP-1 metabolism at the pretranslational level was not affected, but the secretion of pre-existing MMP-1 protein pool was activated (51).

Previous studies have reported that mechanical stress influences cellular phenotype, ECM remodeling and cell-to-ECM interactions (28,52–54). Indeed, as confirmed in our study by fluorescence microscopic staining, the use of a 3D stressed model activated the expression of α-SMA, as well as it stimulated the activity of integrin α2 subunit in fibroblasts. α-SMA cytoskeleton proteins play a major role in fibroblast contractility and maintain cell shape (55,56). In 3D culture, fibroblasts showed a dendritic cell shape that better approaches the *in vivo* situation as compared to cells in 2D culture (17,39). When fibroblasts are embedded within a 3D collagen matrix, they move, form matrix adhesions and undergo morphological and cytoskeletal changes. The expression of α-SMA and integrin α2 upregulates fibroblast contractile activity and contributes to collagen lattice organization (55,57). Surprisingly, there is a lack of information on actin and integrin structures in photoaging. We compared the expression of α-SMA in UVA-irradiated cells to non-UVA-irradiated cells. In monolayer, we confirmed the data of our previous study and showed that α-SMA expression does not change (25). In tense collagen lattice, we demonstrated that α-SMA expression in UVA-irradiated cells is lower than in non-UVA-irradiated cell. Therefore, the inhibitory effect of UVA radiation on α-SMA expression was similar between tense and free-floating collagen lattices (58). Moreover, in 3D culture, it was clearly indicated that UVA irradiation induces obviously morphological changes from dendritic to elongated/flattened cell shape. Concerning integrin α2 expression, UVA irradiation had no effect on 2D culture, whereas in tense 3D culture, it had an inhibitory effect. In UVA-irradiated 3D culture, fibroblasts acquired elongated shape and lost their dynamic interaction with collagen fibers and number of cells spreading through a decrease in integrin α2 and α-SMA (59). Previous studies show that the decrease in integrin α2, coupled with elongated/flattened cell shape, leads to failure of cell growth/proliferation and migration (59–61). In addition, integrins are involved to regulate the cell cycle G1/S checkpoint signaling pathway, which allows cell to progress from G1 to S phase.

The mechanism of UV action on human skin fibroblasts is complex and has been largely documented under 2D culture conditions. As shown in Fig. 7, fibroblast responses to UVA irradiation were different in 2D *versus* 3D environment, highlighting the importance of collagen environment in the regulation of mechanical activities. The repeated exposure to UVA causes the impairment of fibroblast functions, such as overproduction of MMP-1 and suppression of collagen production, thus, leading to fragmentation and disorganization of collagen fibers. The phenomena in 3D environment lead to failure of interaction between cells and collagen fibrils, which in turn suppresses α-SMA and integrin α2 expression in fibroblasts. All the observations in UVA-irradiated fibroblasts cultured in tense 3D culture appeared coordinated and illustrated fibroblast synthetic capacities in both mechanically stressed and photoaged human skin.

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