Femtosecond-laser stimulation induces senescence of tumor cells in vitro and in vivo

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Abstract: Tumor cells present anti-apoptosis and abnormal proliferation during development. Senescence and stemness of tumor cells play key roles in tumor development and malignancy. In this study, we show the transient stimulation by a single-time scanning of tightly focused femtosecond laser to tumor cells can modulate the stemness and senescence in vitro and in vivo. The laser-induced cellular senescence and stemness present distinct transitions in vitro and in vivo. The cells 1.2 mm deep in tumor tissue are found with significant senescence induced by the transient photostimulations in 100-200 µm shallow layer in vivo, which suppresses the growth of whole tumor in living mice.

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1. Introduction

Cellular senescence is generally a stress-responsive cell-cycle arrest program that terminates the further expansion of malignant cells in tissue and thus restrains tumorigenesis, which is implicated in cancer and in aging [1]. But senescence has recently been recognized as a double-edged sword for tumor development. It induces immune surveillance against pre-malignant cells by a potent tumor-suppressive mechanism, while, however, accumulation of senescent cells in aged tissues blocks tissue renewal and leads chronic inflammation associated with age-related diseases to finally form cancer [2]. In tumors, cellular senescence does not conflict with stemness but can contribute to the generation of cancer cells with high stemness. The cell signaling molecules in senescence, for example, Ras-type oncogene proteins including activated Ras, Raf or MEK, induce senescent G1-phase cell-cycle arrest, which is also stably maintained by global epigenetic reprogramming, suggesting an interplay between senescence and stemness [3,4]. Hence, manipulation of cell senescence is of great significance for cancer research and therapy.

Laser technology is able to deliver precise energy to biological systems at predefined spatiotemporal coordinates noninvasively, and thus naturally suitable for photobiomodulation. Recently, the technology of optogenetics enables molecule-specific photobiomodulation by transfecting the optogenetic genes into cells and animals to introduce photosensitivity of specific targeted molecules to light [5]. The optogenetic photomodulation of cancerization and cancer immunotherapy brings the potential of optogenetic cancer therapy [6,7]. However, the invasive gene engineering to introduce optogenetics into human beings makes a barrier to clinical applications due to the biosafety issue and ethical concerns [8,9].

To this challenge, direct photobiomodulation technology has been developed. In theory, most cells cannot respond to light stimulation with any specific biological processes except photodamage. Laser or light irradiation generates thermal effect, oxidative stress, and direct breakdown of molecular bonds in cells at infrared, visible, and ultraviolet bands respectively [10]. The idea using continuous-wave (CW) low-level laser irradiation (LLLI) to restrain photodamage in a moderate range and maintain cells viable, is found able to initiate some cell processes like cell repair and even therapy effect [11]. Hence the idea is also named as low-level laser therapy (LLLT) and applied to a series of diseases therapy in clinic and even tumor therapy [12–14]. Signaling pathways including TGF-β and ERK can be activated by long-term irradiation of CW lasers nonspecifically to initiate cell repair and proliferation and thus prompt wound healing
and tissue regeneration [15,16]. LLLT also presents some anti-inflammatory effect at cellular and tissue level [17]. Therefore, some studies reported LLLT could help for side effects and complications caused by cancer therapy [18,19]. Nevertheless, application of LLLT was limited by the modulation efficiency and depth in vivo.

It has been found the tightly focused femtosecond laser could excite intracellular Ca$^{2+}$ release in diverse types of cells [20], which was firstly reported at 2002 [21]. This technology was used in neurons for the intercellular Ca$^{2+}$ propagation [22]. We found the femtosecond-laser stimulation could precisely release and deplete the intracellular Ca$^{2+}$ store in endoplasmic reticulum [23]. The Ca$^{2+}$ rise pattern could further activate ERK pathway in a controllable manner [24]. Since Ca$^{2+}$ signaling is the universal second messenger in cells to regulate all cell processes [25], we recently further developed a method to control the store-operated Ca$^{2+}$ channel specifically by femtosecond laser [26]. Those results indicate the femtosecond laser holds the ability to activate cell processes. Even more remarkably, calcium (Ca$^{2+}$) plays a major role in many key cellular processes as the universal second messenger in cells, which control a series of “hallmarks of cancer” and regulate senescence through nuclear factor of activated T cells (NFAT) and NF-κB pathways for balancing between proliferation and cell cycle arrest [27–29]. Therefore, it is possible to influence cellular senescence by laser-regulated Ca$^{2+}$ signaling.

In this study, we report the transient stimulation by a tightly focused femtosecond laser to tumor cells induces cell senescence in vitro and in vivo. The stemness and senescence of tumor cells can be directly modulated by a single-time short flash of femtosecond-laser activation noninvasively. We propose this method can work as a photobiomodulation technology for tumor and benefit cancer research and therapy.

2. Methods and materials

2.1. Preparation of PC3 cells

Human prostate cancer cell line PC3 cells were cultivated in Roswell Park Memorial Institute-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 1% (v/v) penicillin/streptomycin at 37 °C with 5% CO$_2$. Cells were seeded on the bottom of 35 mm glass-bottom (0.17 mm thick) dishes, and another 2 mL medium was added 1 hour later when the cells were adherent. Cells were cultured for another 12 hours before experiments. Cells were stained with Fluo-4/AM (final concentration 2 µM, Thermo Fisher Scientific, F14202) for 30 minutes at 37 °C for the indication of intracellular Ca$^{2+}$. For in vivo experiments, PC3 cells with stable expression of green fluorescent protein (GFP) were cultured under the same condition before tumor implantation.

2.2. Immunofluorescence microscopy

Immunofluorescence microscopy of cells was performed following the protocol below. In brief, cells were fixed with 4% paraformaldehyde (Beyotime) and permeabilized with 0.1% Triton X-100 (Beyotime) to allow the primary antibody to diffuse into cells. After being blocked with 1% bovine serum albumin (Sigma-Aldrich), cells were incubated with primary antibodies at 4 °C overnight followed by incubation with secondary antibodies for 2 hours at room temperature.

Immunofluorescence microscopy of frozen tissue section (10 µm thick) was performed according to the following protocol. Tumor sections were fixed with 4% paraformaldehyde (Beyotime) and permeabilized with 0.3% Triton X-100 (Beyotime). After being blocked with 5% bovine serum albumin (Sigma-Aldrich), sections were incubated with primary antibodies at 4 °C overnight followed by incubation with secondary antibodies for 2 hours at room temperature.

The immunofluorescence microscopy works based on the immune-reaction of specific antibody (primary antibody) against the antigen in the sample. After washing out the residual primary antibody, the anti-antibody with a fluorophore (secondary antibody) could combine with the
primary antibody such that the targeted protein (antigen) in the sample was fluorescently labeled with ultrahigh specificity. Antibodies for immunofluorescence were as follows (with the dilution ratio): primary antibodies: anti-SOX2 mouse antibody (abcam, ab79351, 1:250), anti-Oct4 rabbit antibody (abcam, ab181557, 1:250), anti-p21 rabbit antibody (abcam, ab109520, 1:1000), anti-CDKN2A/p16INK4a mouse antibody (abcam, ab16123, 1:1000). Secondary antibodies: Goat Anti-Rabbit IgG H&L-Green (Alexa Fluor 488) (Abcam, ab150077, 1:1000) and Goat Anti-Mouse IgG H&L-Red (Alexa Fluor 555) (Abcam, ab150114, 1:1000).

2.3. Animal model of subcutaneous tumor mice

8-9 weeks male BALB/c nude mice, weighing approximately 20 g were purchased from Charles River, and maintained in a specific pathogen-free (SPF) environment. To establish the subcutaneous tumor model, 100 µL cell suspension with the concentration of 10⁷/mL PC3-GFP cells in phosphate buffer saline was implanted into the axilla of right hindlimb subcutaneously per mouse. Since the day of tumor implantation, we measured tumor volume every three days for 3 weeks. The blood vessels within tumor were stained with PE anti-mouse CD105 antibody (0.1 mg/mL, 100 µL, BioLegend, 120408) by intravenous injection.

2.4. Photostimulation in vitro and in vivo

A microscopic system was established for simultaneous photostimulation and microscopy by coupling a femtosecond laser (BlueCut, Menlo) into a confocal microscope (FV1200, Olympus) as shown in Fig. 1 (a). The beam of femtosecond laser at 1030 nm was expanded to match the back aperture of objective (30×, oil immersion, N.A. = 1.05) for tight focusing and controlled by the galvo mirrors and a mechanical shutter for scanning in a predesigned region.

For in vitro photostimulation, the cells adherent on petri dishes (the bottom was a 0.17 mm glass slide) were observed by confocal microscopy and selected randomly by defining an arbitrary polygon region in the field of view (FOV) to suffered femtosecond-laser scanning point by point for a single time. In this scheme, the photostimulation was defined as a single frame of microscopy, which could be inserted in any time slot of a predefined confocal microscopy sequence for real-time continuous observation. In the Ca²⁺ study, cells were stimulated by the laser scanning in a predefined area (180 × 420 µm²) in the FOV (420 × 420 µm²) for a single frame (220 × 512 pixels, 2 µs/pixel, 0.8s) at 5.25 mW (L), 10.5 mW (M), and 21 mW (H) respectively. The average Ca²⁺ level was acquired by accessing the fluorescence intensity of each individual cell manually according to the cell morphology indicated by the intracellular Ca²⁺ and Fluo-4/AM. In the experiments of senescence and stemness, cells were stimulated by the laser scanning in the whole FOV (420 × 420 µm²) for a single frame (512 × 512 pixels, 2 µs/pixel, 1.1 s) at 10.5 mW (M). The total photostimulation area per dish was 5 mm².

For in vivo photostimulation, the mice were anaesthetized by Isoflurane on the microscope stage. The implanted subcutaneous tumor of PC3 cells with genetically labeled GFP was observed by confocal microscopy. The regions of the tumor at the horizontal X-Y planes were randomly selected and then photostimulated at 3 different depths of 100 µm, 150 µm and 200 µm under the epidermis the respectively. In the senescence and stemness experiments, the tumors were stimulated in succession frame by frame (420 µm × 420 µm, 512 × 512 pixels, 4 µs/pixel, 2.2 s/frame) at 18 mW in a region of 16 mm² per layer. In the study of tumor development, the tumors were stimulated in succession frame by frame (420 µm × 420 µm, 512 × 512 pixels, 4 µs/pixel, 2.2 s/frame) at 18 mW in a region of 30 mm² per layer. The laser propagation efficiency in the tumor tissue was estimated by the absorption and scattering model of multilayer cells and direct measurement of laser transmission efficiency of the skin. After 200 µm tissue attenuation, the total transmission efficiency was calculated as around 0.55 ~ 0.62. To compensate the attenuation of laser propagation in tissue, the laser power was increased to 18 mW, around two times of that in the in vitro case.
Fig. 1. Photostimulation to targeted PC3 cells in any predefined regions by femtosecond-laser. (a) Optical design for the femtosecond-laser stimulation to cells. The photostimulation was accomplished by the predefined scanning of femtosecond laser (1030 nm, 220 fs, 1 MHz) controlled by the shutter and galvo mirrors. The stimulation region could be defined as any arbitrary polygons. (b) The Ca\(^{2+}\) dynamics in the stimulated cells. Left: the baseline, peak, and last Ca\(^{2+}\) level in photostimulated cells (left region of the left dashed line) and control (right region of right dashed line). Green fluorescence: Fluo-4/AM. Right: the kinetic change of the Ca\(^{2+}\) levels after photostimulation at 5.25 mW (L, laser n = 64 cells from 3 fields, control n = 38 cells from 3 fields), 10.5 mW (M, laser n = 41 cells from 3 fields, control n = 60 cells from 3 fields), and 21 mW (H, laser n = 44 cells from 3 fields, control n = 63 cells from 3 fields). Zoom in: the damaged cells with little fluorescence of Fluo-4 and bright spots of bubbles on membrane. Insert: the definition of amplitude, peak time, and decay time of the Ca\(^{2+}\) pattern. The violin plots of amplitude (ΔF/F\(_0\)) (c), peak time (d) and decay time (e) of the Ca\(^{2+}\) kinetic dynamics at different photostimulation modes. (f) The ratio of cellular Ca\(^{2+}\) responses to different photostimulations. Data represent mean ± SEM. Scale bar: 100 µm.
3. Results

3.1. Ca\textsuperscript{2+} kinetic patterns in PC3 cells by femtosecond-laser photostimulation

We established a system to simultaneously observe and stimulate PC3 cells by coupling a femtosecond laser (1030 nm, 220 fs, 1 MHz) to a confocal microscope and focusing it to a submicron spot (diameter < 1 µm). The photons at such long wavelength were generally absorbed by water due to low single-photon energy, but with little photochemical effect [30]. The ultrashort pulse width and relatively ultra-long pulse interval of the laser prevent thermal deposition and accumulation in cells [30]. The photostimulation was accomplished by a single-frame scanning of the femtosecond laser in a predefined region controlled by a mechanical shutter synchronized with galvomirrors (Fig. 1(a)). In this setup, the total FOV was 420 × 420 µm\textsuperscript{2}, mapping to 512 × 512 pixels, and scanned by lasers at a speed of 2 µs/pixel for microscopy. The photostimulation region could be defined as any arbitrary polygon areas in the FOV and coordinated by the galvo mirrors for accurate scanning inside. The total time of photostimulation was controlled by a mechanical shutter and usually defined as an integer multiple of a frame time. The incident laser energy on each cell was thus dependent on the projection area of cells adherent on the bottom slides in petri dishes. The cells were observed continuously by time-lapse confocal microscopy and the photostimulation was performed at a predefined time slot.

To show the femtosecond laser could stimulate cells effectively in a controllable manner, the Ca\textsuperscript{2+} response of cells as a readout of the photostimulation was investigated by laser scanning at different powers, as shown in Fig. 1(b). The cells were stained with Fluo-4/AM to indicate the Ca\textsuperscript{2+} kinetics and then stimulated by the laser scanning in a predefined area (180 × 512 pixels) in the FOV for a single frame (220 × 512 pixels, 0.8 s) at 5.25 mW (L), 10.5 mW (M), and 21 mW (H) respectively. Immediately the intracellular Ca\textsuperscript{2+} response was observed in those stimulated cells with distinct kinetic patterns. The cellular Ca\textsuperscript{2+} patterns were quantified as shown in Fig. 1(c-e). We compared the amplitude, peak time, and decay time, defined as in Fig. 1(b) under those three conditions. The fluorescence of most cells stimulated at mode H presented a rapid and significant increase but decayed very fast, suggesting extreme damage to cells such that the integrity and permeability of cytoplasm membrane was lost, Fluo-4 molecules leaked out, and little bubbles could be found in those cells (the insert in Fig. 1(b)). The violin plots of peak time and decay time of Ca\textsuperscript{2+} response indicate their variation at mode L was quite large compared with that at the other two modes respectively (Fig. 1(d) and (e)). Therefore, at 600 s, some cells in the L group still maintained bright Ca\textsuperscript{2+} signals. Synergistically, the efficiency of photostimulation at mode L defined by the cell ratio with effective Ca\textsuperscript{2+} responses over the total photostimulated cells was lowest as in Fig. 1(f). By contrast, mode M presented relatively uniform Ca\textsuperscript{2+} responses and the optimal balance between photostimulation efficiency and cell viability. In this regard, we used photostimulation at mode M for all following experiments.

3.2. Senescence of cells induced by femtosecond-laser stimulation

We investigated the effect of femtosecond-laser stimulation (at mode M) on cell senescence. It should be noted cell senescence is associated with stem-cell functions, collectively referred to as ‘stemness’, to generate the potential to develop highly aggressive tumors [3]. Actually, cell stemness and senescence seem to be co-regulated by overlapping signaling networks [31]. Therefore, PC3 cells were photostimulated at Day 0 and measured both the senescence and stemness at Day 0, 1, and 3 respectively as designed in Fig. 2(a). Transcription factors Oct4 and Sox2 that regulate genes for the self-renewal and pluripotency of embryonic stem cells (ESC) together [32], are regarded as cancer stem cells markers [33]. Hence, we used the expression level of Oct4 and Sox2 to indicate the stemness of PC3 cells. The Day 0 group (3 fields × 2 dishes of cells) without photostimulation provided the immunofluorescence baseline of Oct4 and Sox2. The photostimulated cells (Laser group) and control cells (Control) in the same dish
(but > 1 mm far away from each other) were analyzed at Day 1 (3 fields × 3 dishes of cells) and Day 3 (3 fields × 3 dishes of cells) by immunofluorescence microscopy respectively. It could be found Oct4 and Sox2 did not change at Day 1 in those photostimulated cells compared with that at Day 0 and the control at Day 1 respectively (Fig. 2(b-e)). At Day 3, both Oct4 and Sox2 in the control and stimulated cells exhibited significant downregulation compared with that at Day 0. But no significant difference of Oct4 and Sox2 could be found between the control and stimulated cells at Day 3 (Fig. 2(c) and (e)). Therefore, the stemness of PC3 cells was not influenced by photostimulation in vitro.

We then measured the level of two typical senescence makers by immunofluorescence microscopy respectively, the cyclin-dependent kinase inhibitors p21 and p16, which are cell cycle inhibitors and anti-proliferative effectors [34], and therefore work as main tumor suppressor proteins (together with p53) to avert tumor formation [35,36]. As shown in Fig. 3(a) and (b), the p21 level in the photostimulated cells was significantly higher than that of control and at Day 0. But the p21 level at Day 3 did not exhibit further increase compared with Day 1 since cells with different cell fates exhibited distinct p21 dynamics. The p21 upregulated and was maintained at high levels for the senescent cell subpopulation [37]. We found only a slight
upregulation of p16 at Day 3 with no significant difference compared with that at Day 0, probably because the upregulation and accumulation of p16 was later than p21 [38]. Taken together, the photostimulation could induce senescence of tumor cells in vitro.

Fig. 3. The level of senescence markers after femtosecond-laser stimulation. (a) Representative immunofluorescence images of p21 (green) in the cells at Day 0 and the control and femtosecond-laser stimulated cells at Day 1 and 3 respectively. (b) The quantified p21 level from (a, Day 0: n = 3 fields × 2 dishes of cells, control and laser: n = 3 fields × 3 dishes of cells per group, P values were calculated by two-tailed t-test). (c) Representative immunofluorescence images of p16 (red) in the cells at Day 0 and the control and femtosecond-laser stimulated cells at Day 1 and 3 respectively. (d) The quantified p16 level from (d, Day 0: n = 3 fields × 2 dishes of cells, control and laser: n = 3 fields × 3 dishes of cells per group, P values were calculated by two-tailed t-test). Blue: DAPI fluorescence from nucleus. Data represent mean ± SEM. * P < 0.05. ** P < 0.01. *** P < 0.001. Scale bar: 100 µm.

3.3. Senescence of tumor cells induced by a femtosecond laser in vivo

We further assessed the senescence of tumor cells by femtosecond-laser stimulation in vivo. The subcutaneous tumor mouse model was established by implanting PC3-GFP cells (PC3 cells genetically labeled with GFP) in the armpit of the right hind leg of mice. After one week, the tumors reached a measurable size (about 110 mm³) and were stimulated by the femtosecond laser at 18 mW at Day 0. Similarly, tumor tissues in the Day 0 group (15 sections from 5 mice) without photostimulation worked for the immunofluorescence baseline. The tumors suffered photostimulation were analyzed at Day 3 (11 sections (Oct4) or 13 sections (Sox2) from 4 mice) as shown in Fig. 4(a).

We identified the location of tumors by the green fluorescence of PC3-GFP cells and stimulated cells at 100 µm, 150 µm and 200 µm under the epidermis in succession frame by frame (420 µm × 420 µm, 2.2 s/frame) in a region of 16 mm² per layer. We measured the Oct4 and Sox2 level in the photostimulated and control (without photostimulation) regions at same depth. The Oct4 level of the tumor tissue after photostimulation at Day 3 showed significant upregulation than that of the control (Fig. 4(b) and (c)). Similarly, the Sox2 level was significantly higher than that of the control (but no significant difference compared with that at Day 0 as in Fig. 4(d) and (e)). The cells more than 1 mm deep under the photostimulation plane still presented significant
Fig. 4. The level of stemness markers in tumor tissue after femtosecond-laser stimulation in vivo. (a) The experimental scheme. The tumor was implanted 1 week before Day 0. The tumor biopsy was performed at Day 3 and before laser at Day 0 for immunofluorescence microscopy. (b) Left panel: the image of the whole tumor section. Scale bar: 1 mm. Dashed line: the photostimulation region by femtosecond-laser scanning. White boxes: the original areas for the zoom-in images in the right panel. Right panel: representative immunofluorescence images of Oct4 (red) in the tumor frozen sections of the tumors at Day 0 and the control and femtosecond-laser stimulated tumors at Day 3. Blue: DAPI fluorescence from nucleus. Green: GFP fluorescence from PC3 cells. Scale bar: 20 µm. (c) The quantified Oct4 level from (b, Day 3 n = 11 sections from 4 mice, Day 0 n = 15 sections from 5 mice, P values were calculated by one-tailed t-test). (d) Left panel: the image of the whole tumor section. Scale bar: 1 mm. Right panel: representative zoom-in immunofluorescence images of Sox2 (red) in the tumor frozen sections of the tumors at Day 0 and the control and femtosecond-laser stimulated tumors at Day 3. Blue: DAPI fluorescence from nucleus. Green: GFP fluorescence from PC3 cells. Scale bar: 20 µm. (e) The quantified Sox2 level from (d, Day 3 n = 13 sections from 4 mice, Day 0 n = 15 sections from 5 mice, P values were calculated by one-tailed t-test). Data represent mean ± SEM. * P < 0.05, ** P < 0.01.
different Oct4 and Sox2 level from that in control. This \textit{in vivo} result was different from that \textit{in vitro}, probably due to the \textit{in vivo} microenvironment of the tumor in which the PC3 cells could generate stemness for epithelial-mesenchymal transition after the activation of photostimulation.

We then studied the senescence of tumor cells \textit{in vivo} in this mouse model. The sections of the tumor tissue at Day 3 were immunostained with p21 and p16 respectively. As shown in Fig. 5(a-d), the p21 and p16 level of photostimulated tumor cells were both significantly higher than that of control. Notably, the tumor tissue more than 1 mm deep under the photostimulation plane showed overall significantly higher p21 and p16 signals than that of control (Fig. 5(a) and (c)). Therefore, the senescence of tumor cells could be induced by the transient photostimulation \textit{in vivo}. The

**Fig. 5.** The level of senescence markers in tumor tissue after femtosecond-laser stimulation \textit{in vivo}. (a) Left panel: the image of the whole tumor section. Scale bar: 1 mm. Dashed line: the photostimulation region by femtosecond-laser scanning. White boxes: the original areas for the zoom-in images in the right panel. Right panel: representative immunofluorescence images of p21 (red) in the tumor frozen sections of the tumors at Day 0 and the control and femtosecond-laser stimulated tumors at Day 3. Blue: DAPI fluorescence from nucleus. Green: GFP fluorescence from PC3 cells. Scale bar: 20 µm. (b) The quantified p21 level from (a, Day 3 n = 11 sections from 4 mice, Day 0 n = 15 sections from 5 mice). (c) Left panel: the image of the whole tumor section. Scale bar: 1 mm. Right panel: representative zoom-in immunofluorescence images of p16 (red) in the tumor frozen sections of the tumors at Day 0 and the control and femtosecond-laser stimulated tumors at Day 3. Scale bar: 20 µm. (d) The quantified p16 level from (c). (e) The quantified p21 and p16 level from (a and c) respectively versus depth. Data represent mean ± SEM. * P < 0.05. ** P < 0.01. *** P < 0.001. P values were calculated by one-tailed t-test (c and d) and two-tailed paired t test (e).
scanning at superficial layers in the tumor tissue of femtosecond laser could induce significant cell senescence at a depth of more than 1 mm in the tumor. We quantified the p21 and p16 level in the tumor tissue along depth. As shown in Fig. 5(e) and Supplement 1, at 1 mm, the p21 and p16 level of cells under the photostimulated region were still significantly higher than that of control, which remained the significantly different at 2 mm. The laser power of photostimulation was tuned to 15 mW and stimulation duration for 1.1 s and 2.2 s respectively, the p21 and p16 level changed accordingly (Supplement 1), further suggesting the photostimulation effect. We found the effective depth of photostimulation could achieve 1.2 mm by defining it as the full width at half maximum of the p16 and p21 level. Even more noteworthy is that senescent cells can acquire features of stemness partially through activation of Wnt to produce tumor-initiating cells (e.g., cancer stem cells) [39], consistent with the result of stemness upregulation by photostimulation in Fig. 4.

We finally verified the general influence of laser-induced senescence on the tumor development (6 mice per group). The subcutaneous PC3-GFP tumors were photostimulated by the femtosecond laser at 18 mW in a region of 30 mm$^2$ 100 µm, 150 µm and 200 µm below the epidermis for a single time respectively at Day 0 (1 week after tumor implantation). After that, the tumor volume was recorded and compared with that of control along the development. As shown in Fig. 6(a), the tumors suffered photostimulation showed significant slower development and

Fig. 6. The tumor development after photostimulation. (a) The tumor volume after photostimulation. (n = 6 tumors per group, P = 0.00043, two-way analysis of variance (ANOVA)). (b) The sections of the tumors with (right) and without (left) photostimulation. Red fluorescence: CD105 to indicate blood vessels. Green fluorescence: GFP from PC3 cells. Arrows: blood vessels in tumor tissue. Scale bar: 100 µm (c) The quantified total vessel area and mean area of continuous vessels in each frame. (d) The ROS level indicated by H2DCFDA by photostimulation at L and M mode respectively. Scale bar: 100 µm Data represent mean ± SEM. P values were calculated by one-tailed t-test.
smaller volume at Day 21 than that of control, consistent with the senescence effect. The tumor was resected and observed at Day 21 which presented loose distribution of blood vessels inside (indicated by CD105) as shown in Fig. 6(b). We quantified general distribution and morphology of blood vessels in the tumor tissue (Fig. 6(c) and Supplement 1). The total vessel area in the photostimulated tumor was a little smaller than that of control \((P = 0.0538)\), and the mean area of continuous vessels in the photostimulated tumors was significantly smaller \((P = 0.0348)\), indicating the loose and sparse distribution of vessels. We tested the generation of reactive oxygen species (ROS) in tumor cells after photostimulation, the general photodamage of laser that could influence tumor growth. As in Fig. 6(d), the PC3 cells did not show any ROS generation after photostimulation. This result further suggested the tumor growth was probably suppressed by laser-induced senescence.

4. Discussion

Cellular senescence is implemented in response to severe cellular insults. It is a fail-safe program that protects organismic integrity by excluding potentially harmful cells from further expansion and also has a physiological function in tissue homeostasis during organ development [40]. The relationship of stemness and senescence of tumor cells is quite complex. Basically, the cell senescence restrains the development of tumor whereas stemness keeps the tumor potential of development, heterogeneity (defense against immunity), and metastasis [41]. However, in some recent studies, senescence of tumor cells can also induce or promote stemness [42]. In this study, we demonstrated the stemness and senescence of tumor cells could both be influenced by a single-time, short, and transient photostimulation of a tightly focused femtosecond laser. Here, the tumor tissue presented significant upregulation of both stemness and senescence after photostimulation \textit{in vivo}. This is plausible considering the overlapping of molecular signaling of regulating senescence and stem-cell functions. Key signaling components of cellular senescence machinery, such as p16, p21 and p53, have been reported to operate as critical regulators of stem-cell functions [43]. The stemness enhancement \textit{in vivo} might be involved in the initiation of epithelial-mesenchymal transition [44]. However, only the \textit{in vivo} microenvironment of tumor can provide cells with senescence-associated stemness to enable cells to escape from cell-cycle blockade. Here we did not further measure the p16 and p21 level after Day 3 mainly because the fast proliferation of tumor cells mixed the photostimulated cells and newly-generated cells and thus influenced the measurement of laser-induced upregulation of senescence markers. The photostimulated region could hardly be localized again. In the \textit{in vitro} cells, no stemness upregulation could be found after photostimulation.

The tumor senescence can suppress tumor development. In this study, the photostimulation for a short duration in a very localized small region at the surface of tumor (100-200 µm deep) introduced cell senescence at a depth of 1.2 mm and showed significant suppression to the tumor development. Even deeper cells still presented senescence upregulation (Fig. 5(e)). Even though the tumor heterogeneity was more and more significant at late stage, as a result of the stemness of tumor cells, the overall suppression to tumor development by cellular senescence induced by photostimulation could still be found at this subcutaneous tumor study. The laser-induced simultaneous upregulation of senescence and stemness did not conflict. The stemness of the tumor cells might initiate mutations to grow again and migrate to other organs. But still, the senescence of tumor cells is a quite effective and safe method for tumor therapy if the stemness could be controlled. Senescence has been shown to cancel the protumorigenic potential of cancerous lesions and contribute to the outcome of anticancer chemotherapy \textit{in vivo} [45]. These findings have profound implications for cancer therapy, and provide new insights into the mechanism of cancer cells plasticity [46].

Photostimulation by laser is always limited by the penetration depth in \textit{in vivo} applications. This physical limitation can hardly be broken. Here we used the femtosecond laser at 1030
nm which theoretically has limited improvement in the physical penetration depth in biological tissue. However, as shown in Fig. 5, although the femtosecond laser stimulated cells only around 100-200 µm below the skin surface, the cells more than 1 mm deep partially showed significant upregulation of senescence markers. This might be due to that the intercellular $\text{Ca}^{2+}$ propagation initiates senescence in the surrounding cells. Fortunately, the tumor tissue with abundant mesenchyme and gap junctions is quite suitable for $\text{Ca}^{2+}$ signaling propagation. In this study, we used cellular $\text{Ca}^{2+}$ kinetics as the readout of cellular response to photostimulation which indicated the representative molecular changes of cells induced by photostimulation and the stress level of the photostimulation. The cell senescence and stemness have been found able to be regulated by intracellular $\text{Ca}^{2+}$ signaling, the universal second messenger. We performed the immunofluorescence microscopy to measure those markers. The photostimulation at L mode could hardly initiate significant cellular senescence in vivo. The efficiency would be quite low. In contrast, the H mode might still induce senescence in cells surrounding the laser scanning lines with a moderate efficiency. But the cells at the laser focus would be greatly damaged and even dead by the high laser power, without upregulation of senescence. The M mode in this study is a relatively optimal choice. However, there might exist some powers that could lead a better photostimulation result.

5. Conclusion

In this study, we report a biophotomodulation method to induce cellular senescence by a fast single-time femtosecond laser photostimulation in vitro and in vivo. The photostimulation depth could achieve 1.2 mm in vivo by photostimulation in the shallow layer at 200 µm. Cell stemness was also involved and influenced. The growth of subcutaneous tumor showed suppression after a single-time photostimulation. Our results thus provide a powerful noninvasive tool for the research of senescence of tumor cells and hold good potential for tumor therapy.

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Data availability. Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

Supplemental document. See Supplement 1 for supporting content.

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