

Rapamycin Alleviates Age-Related Senescence in Human Facial Dermal Fibroblasts by Modulating Autophagy

Xuan Wei

Southwest Medical University

Fei-fei Cao

Bohao Stomatology

Long-yu Zhang

Suining Central Hospital

Ming-yang Chen

Zunyi Medical University

Cheng Zeng

Southwest Medical University

Sen Yang

Suining Central Hospital

Lijuan guo

663418587@qq.com

Southwest Medical University

Research Article

Keywords: rapamycin, human facial skin fibroblasts, intrinsic aging, cellular senescence, autophagy, mTOR signaling

Posted Date: September 10th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10880328/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Rapamycin Alleviates Age-Related Senescence in Human Facial Dermal Fibroblasts by Modulating Autophagy

Xuan Wei^{1,2#} MM, Fei-fei Cao^{6#} Dr, Long-yu Zhang³ MM, Ming-yang Chen⁷ MM, Cheng Zeng¹ MM, Sen Yang^{3,*} PhD, Lijuan Guo^{1,2,4,5*} PhD.

1 School of Stomatology, Southwest Medical University, Luzhou 646000, Sichuan, China.

2 Department of Medical Cosmetology, Suining Central Hospital, Suining 629000, Sichuan, China.

3 Department of Oral and Maxillofacial Surgery, Suining Central Hospital, Suining 629000, Sichuan, China.

4 University of Electronic Science and Technology of China, Chengdu, China

5 Institute of Electronic and Information Engineering of UESTC in Guangdong, University of Electronic Science and Technology of China, Guangdong, China

6 Bohao Stomatology, Guiyang, Guizhou 550002, China.

7 School of stomatology, Zunyi Medical University, Zunyi, China

#Co-first authors:

Fei-fei Cao, Bohao Stomatology, Guiyang, Guizhou 550002, China.

*Corresponding authors:

Sen Yang, Department of Oral and Maxillofacial Surgery, Suining Central Hospital, No. 27 of Dongping North Road, Hedong New District, Suining, Sichuan Province, 629000, China.

Email: ys13880435413@163.com.

Lijuan Guo, Department of Medical Cosmetology, Suining Central Hospital, No. 27 of Dongping North Road, Hedong New District, Suining, Sichuan Province, 629000, China.

Email: 663418587@qq.com.

Abstract

Introduction: Intrinsic skin aging is associated with reduced autophagic activity and dermal fibroblast senescence. Although rapamycin has shown anti-aging effects in stress-induced senescence models, its effects on naturally aged human skin fibroblasts and their age dependence remain unclear.

Objective: This study investigated the effects and underlying mechanisms of low-dose rapamycin on senescence-associated phenotypes and autophagy signaling in human facial skin fibroblasts (HSFs) from women of different ages.

Methods: An age-stratified model of physiological skin aging was established using primary HSFs isolated from discarded facial skin tissues of healthy female donors aged 20–29 years (Group 1), 30–39 years (Group 2), and 40–49 years (Group 3). After optimization, exponentially growing HSFs were treated with 0.1 nmol/L rapamycin for 48 h. Cellular senescence was assessed by SA- β -gal staining, and cell-cycle distribution was analyzed by flow cytometry. Immunofluorescence, quantitative real-time PCR, and Western blotting were used to assess mTOR and the autophagy-related markers Beclin-1 and LC3B at the mRNA and protein levels.

Results: Basal expression of autophagy-related markers progressively declined with increasing donor age. Treatment with 0.1 nmol/L rapamycin significantly reduced the proportion of SA- β -gal-positive cells across all age groups and increased the proportion of cells in the G0/G1 phase, accompanied by a reduction in the proportion of cells in the S phase. At the molecular level, rapamycin suppressed mTOR expression and increased the mRNA and protein expression of Beclin-1 and LC3B, indicating activation of autophagy-related signaling. Notably, the response to rapamycin varied among the age groups. HSFs derived from women aged 40–49 years exhibited the greatest increases in autophagy-related markers and the most pronounced improvement in senescence-associated phenotypes.

Conclusions: Low-dose rapamycin may alleviate age-related senescence in human facial skin fibroblasts by inhibiting mTOR signaling and enhancing autophagy-related activity. Among the age groups examined, fibroblasts derived from women aged 40–49 years showed the greatest responsiveness to rapamycin. These findings provide an in vitro basis for the future development of age-stratified and locally targeted interventions for facial skin aging.

Keywords: rapamycin; human facial skin fibroblasts; intrinsic aging; cellular senescence; autophagy; mTOR signaling

1. Introduction

Skin, the largest organ of the human body, undergoes a complex aging process driven by both intrinsic biological aging and extrinsic environmental factors. Skin aging may also affect systemic health through inflammatory and metabolic pathways^[1]. Degenerative alterations in the dermal microenvironment are central to facial skin aging. As the principal cellular component of the dermis, human facial skin fibroblasts (HSFs) play an indispensable role in maintaining extracellular matrix (ECM) homeostasis and organizing the collagen fiber network^[2]. Importantly, the ECM not only provides structural support for the skin but also actively regulates cellular behavior. With advancing age and age-related changes in the endocrine environment of women, the balance between collagen and elastic fibers produced by HSFs becomes progressively disrupted, resulting in continuous deterioration of the dermal ECM. In turn, the degraded ECM further impairs the biosynthetic capacity of fibroblasts, thereby establishing a vicious cycle of ECM disruption and cellular dysfunction. This progressive deterioration of the dermal microenvironment constitutes a major pathological basis for the loss of skin elasticity and structural integrity^[3, 4].

Autophagy is a highly conserved intracellular degradation and recycling system that removes damaged macromolecules and organelles and thereby maintains cellular metabolic homeostasis^[5]. Autophagy may counteract fibroblast aging through multiple homeostatic mechanisms. Basal autophagy facilitates the clearance of intracellular waste and the recycling of nutrients required for tissue maintenance and repair, whereas selective autophagic processes, including mitophagy, contribute to mitochondrial quality control and the regulation of senescent-cell survival^[6]. A decline in autophagic activity therefore represents more than a failure of intracellular clearance; it also promotes metabolic dysfunction and the acquisition of a senescence-associated secretory phenotype (SASP), characterized by the excessive secretion of proinflammatory mediators and matrix metalloproteinases^[7]. Through paracrine signaling, these factors spread throughout the dermal microenvironment, accelerating local ECM degradation and disrupting signaling pathways in neighboring cells. Consequently, localized cellular senescence may develop into broader structural degeneration of the skin. Emerging

evidence suggests that basal autophagic activity in HSFs does not decline at a constant rate but instead exhibits age-dependent and stage-specific changes^[8]. Such impairment compromises the clearance of damaged organelles and further accelerates fibroblast senescence. Accordingly, the precise activation of autophagy during specific age-related windows has emerged as a promising strategy for restoring cellular homeostasis and delaying skin aging^[9].

Autophagic activity is principally inhibited by the mechanistic target of rapamycin (mTOR) signaling axis. mTOR complex 1 (mTORC1) primarily integrates nutrient availability with cellular growth and anabolic metabolism, whereas mTOR complex 2 (mTORC2) participates in the regulation of metabolism, survival, and cellular responses to environmental stress. These two complexes interact extensively within the broader mTOR signaling network^[10]. Rapamycin, a classical inhibitor of mTORC1, can partially mimic the biochemical effects of caloric restriction by shifting cellular metabolism from an anabolic state toward maintenance and repair. Through this mechanism, rapamycin promotes autophagy-related processes and contributes to the restoration of intracellular proteostasis^[11]. Rapamycin has also been reported to enhance cellular defenses against DNA damage, thereby exerting protective effects in the context of aging^[12]. In addition, interactions between autophagy and mTORC2-related signaling may contribute to the regulation of fibroblast senescence^[13].

Pharmacological interventions targeting cellular senescence are generally divided into senolytics, which selectively eliminate senescent cells, and senomorphics, which suppress or modify detrimental senescence-associated phenotypes without directly inducing cell death. As a prototypical mTOR inhibitor, rapamycin acts primarily by remodeling autophagy-related activity and cellular metabolism rather than by directly eliminating senescent cells, making its mechanism more consistent with that of a senomorphic agent. By inhibiting mTOR signaling, rapamycin may restore autophagy-related activity and delay the progression of cellular senescence^[14,15]. However, systemic administration of conventional rapamycin is associated with adverse effects, including immunosuppression. Therefore, improving its clinical applicability for facial anti-aging interventions requires strategies that enable safe, low-dose, and locally targeted modulation within the dermal microenvironment.

Previous studies investigating the relationship between rapamycin and skin autophagy have predominantly relied on models of acute exogenous injury, such as hydrogen peroxide-

induced oxidative stress and ultraviolet B-induced photoaging. However, the age-related evolution of autophagy in naturally aging female facial HSFs and the potential differences in their responsiveness to very low concentrations of rapamycin remain insufficiently characterized. Our previous work provided preliminary evidence that 0.1 nmol/L rapamycin may exert anti-senescence effects^[16]. In the present study, we established an age-stratified model using facial HSFs derived from women aged 20–49 years and confirmed 0.1 nmol/L as the intervention concentration. We subsequently investigated the effects of rapamycin on senescence-associated phenotypes and the autophagy-related molecules Beclin-1 and LC3B across different age groups, with particular attention to the transition toward perimenopause. By using naturally aged primary human fibroblasts rather than an acute stress-induced model, this study was designed to better reflect the heterogeneity and age-dependent characteristics of physiological skin aging. We further examined whether the effects of low-dose rapamycin differed among age groups and whether HSFs derived from women aged 40–49 years exhibited greater responsiveness to the intervention. These findings may provide an experimental basis for the future development of age-stratified and locally targeted strategies for facial skin aging (Figure 1).

2 Materials and methods

2.1 Ethics Approval and Consent to Participate and Materials and Reagents

All procedures in this research adhered strictly to the ethical guidelines outlined in the Declaration of Helsinki. The Medical Ethics Committee of Suining Central Hospital granted approval for harvesting and utilizing leftover facial skin specimens to isolate primary fibroblasts (Approval No.: LLSLH20200051). Before their scheduled elective surgeries, all tissue donors provided written informed consent.

Chengdu Kangborui Biotechnology Co., Ltd. (Chengdu, China) supplied the rapamycin (RAPA). Cell culture components, including alpha-modified minimum essential medium (α -MEM) and Dulbecco's modified Eagle's medium (DMEM), were sourced from Gibco (USA), while HyClone (USA) provided the fetal bovine serum (FBS). We procured the senescence-associated β -galactosidase (SA- β -gal) staining kit from Chengdu Ruiya Technology Co., Ltd. (Chengdu, China). NuSep (USA) was the supplier for phosphate-buffered saline (PBS), trypsin, and the penicillin-streptomycin solution. The Cell Counting Kit-8 (CCK-8) and RNAiso Plus

reagent were acquired from APExBIO (USA) and Takara Bio (Japan), respectively.

For the immunofluorescence assays, HuaAn Biotechnology Co., Ltd. (Hangzhou, China) furnished the necessary reagents, which included washing and blocking buffers, DAPI, Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L), and primary antibodies targeting vimentin, keratin, mTOR, Beclin-1, and LC3B. Finally, primary antibodies for GAPDH and β -actin, along with their corresponding secondary antibodies, were purchased from Proteintech Group, Inc.

2.2 Skin Tissue Collection and Donor Eligibility Criteria

Normal facial skin tissues were obtained from specimens that would otherwise have been discarded during elective facial plastic or maxillofacial surgical procedures, including blepharoplasty and rhytidectomy. To minimize the influence of extrinsic skin aging, particularly photoaging, and thereby better characterize intrinsic aging, strict inclusion and exclusion criteria were applied.

The exclusion criteria were as follows: (1) severe clinical photoaging or signs of chronic sun damage at the tissue-harvesting site; (2) a history of chronic skin diseases, such as psoriasis or atopic dermatitis, or a history of cutaneous malignancy; (3) cosmetic or aesthetic procedures at the donor site within the preceding 12 months, including chemical peeling, laser resurfacing, botulinum toxin injection, or hyaluronic acid filler injection; (4) long-term topical use of retinoids or systemic treatment with immunosuppressive agents or glucocorticoids; and (5) a history of long-term smoking or severe systemic metabolic disease. Written informed consent was obtained from all tissue donors.

2.3 Isolation, Culture, and Immunofluorescence Characterization of HSFs

Freshly harvested facial skin tissues underwent processing no later than 4 hours post-collection. We washed the samples thrice with sterile phosphate-buffered saline (PBS) and carefully dissected away the epidermis and subcutaneous fat. The remaining dermal sections were diced into approximately 1-mm³ cubes and plated evenly in α -MEM supplemented with 10% fetal bovine serum (FBS). The tissue fragments were then incubated at 37°C in a 5% CO₂ humidified atmosphere, and we changed the culture medium every 2 to 3 days.

Third-passage (P3) HSFs were seeded onto sterile glass coverslips (5×10^4 cells/mL) and allowed to attach. To prevent non-specific binding, the cells were fixed with 4%

paraformaldehyde and blocked using PBS containing 1% goat serum for 30 minutes at room temperature. This was followed by an overnight incubation at 4°C in a humidified environment with primary antibodies against vimentin and keratin. After PBS washes, the samples were incubated with matching fluorescent secondary antibodies for 1 hour at room temperature in complete darkness. For nuclear visualization, the cells were counterstained for 5 minutes with DAPI (100 ng/mL) in the dark, and images were subsequently recorded using a fluorescence microscope (Figure 2A and B)

2.4 Optimization of the Rapamycin Treatment Concentration and Cell Viability Assessment

To evaluate cell survival and proliferation, we utilized the Cell Counting Kit-8 (CCK-8) following the guidelines provided by the manufacturer. Log-phase primary HSFs were detached using trypsin and distributed into 96-well plates (3×10^3 cells/100 μ L complete medium per well). These plates were left overnight in a 37°C, 5% CO₂ humidified incubator to ensure proper cell adherence and stabilization.

Following recovery, the existing medium was removed and substituted with fresh medium supplemented with varying rapamycin concentrations (0, 0.1, 0.5, 1, 5, and 10 nmol/L). We monitored cellular viability at specific intervals (0, 12, 24, 48, and 72 hours) post-treatment. For the viability measurements, 10 μ L of the CCK-8 solution was introduced into each well, followed by a 2-hour incubation period at 37°C protected from light. A microplate reader was employed to record the optical density at 450 nm. Every treatment group consisted of five biological replicates. The optimal drug concentration and exposure time identified here were applied throughout the remainder of the study (Figure 2C).

2.5 Senescence-Associated β -Galactosidase Staining

Senescence-associated β -galactosidase (SA- β -gal) staining was performed to evaluate cellular senescence. HSFs in the logarithmic growth phase were assigned to six groups according to donor age and treatment condition: Group 1 control, Group 1 plus rapamycin, Group 2 control, Group 2 plus rapamycin, Group 3 control, and Group 3 plus rapamycin. Cells in the treatment groups were cultured in complete medium containing 0.1 nmol/L rapamycin for 48 h, whereas the corresponding control cells were maintained under identical culture conditions without rapamycin.

After treatment, SA- β -gal staining was performed according to the manufacturer's instructions. Briefly, the cells were fixed with the fixation solution for 15 min and then washed with PBS. Each sample was subsequently incubated with 1 mL of freshly prepared SA- β -gal staining solution containing 10 μ L of staining solution A, 10 μ L of staining solution B, 930 μ L of staining solution C, and 50 μ L of X-gal solution. The cells were incubated overnight at 37°C in a CO₂-free incubator in the dark. After incubation, the cells were washed with PBS and examined under a light microscope. Cells exhibiting blue-green cytoplasmic staining were considered SA- β -gal positive (Figure 3).

2.6 Flow Cytometric Analysis of Cell-Cycle Distribution

To assess cell cycle progression, HSFs exposed to 0.1 nmol/L rapamycin for 48 hours were harvested via mild trypsinization, yielding sample sizes of roughly 1×10^6 cells. Following centrifugation (1,000 rpm, 5 min), the cellular pellets underwent two washes with chilled PBS. For fixation, the samples were vortexed gently while ice-cold 70% ethanol was added drop by drop to prevent clumping, and then stored overnight at 4°C.

After 24 hours, the fixative was removed via centrifugation, and the cells were rinsed twice in PBS. The pellets were resuspended in 500 μ L of staining buffer containing RNase A (100 μ g/mL) and kept at 37°C for 30 minutes to degrade intracellular RNA. Subsequently, propidium iodide (PI, 50 μ g/mL) was introduced, and the mixture was incubated in the dark at room temperature for another 30 minutes.

Finally, a flow cytometer was utilized to quantify the DNA content, allowing for the determination and statistical comparison of the G0/G1, S, and G2/M phase fractions across all experimental cohorts (Figure 4).

2.7 Immunofluorescence Analysis of Intracellular LC3B Expression

Untreated HSFs were cultured in standard medium, while the experimental groups received 0.1 nmol/L rapamycin for a 48-hour period. Following this exposure, we aspirated the medium and rinsed the cells mildly with PBS. Fixation was achieved by applying 4% paraformaldehyde for 15 minutes at ambient temperature.

To block non-specific antigenic sites, we washed the samples with PBS and applied a 1% goat serum or BSA solution for 30 minutes at room temperature. After discarding the blocking buffer, the samples were kept overnight at 4°C in a moist environment with a 1:200 dilution of

the primary anti-LC3B antibody.

On the following day, extensive PBS washes were performed to clear any unattached primary antibodies. The cells were then exposed to a suitable fluorophore-conjugated secondary antibody for 1 hour at room temperature, protected from light. Nuclear counterstaining was performed using DAPI. Finally, the coverslips were mounted, and fluorescence microscopy was employed to capture images for analyzing the punctate formation, fluorescence intensity, and spatial distribution of LC3B (Figure 5)

2.8 Reverse Transcription–Quantitative Polymerase Chain Reaction Analysis

Following the supplier's guidelines, we utilized the RNAiso Plus reagent to isolate total RNA from the HSFs across all experimental conditions. This purified RNA was immediately converted into complementary DNA (cDNA) via a standard reverse transcription kit.

For the quantitative PCR assays, the thermal cycling parameters were programmed as follows: an initial 2-minute denaturation phase at 95°C, succeeded by 40 cycles comprising 95°C for 5 seconds (denaturation) and 60°C for 31 seconds (annealing and extension). To verify the amplification specificity of the targeted products, a melting-curve evaluation was conducted post-amplification.

Finally, target mRNA expression levels were quantified relative to the internal control, GAPDH, applying the $2^{-\Delta\Delta C_t}$ analytical approach (Figure 6) (Table 1).

2.9 Western Blot Analysis

To extract total intracellular protein, HSF cultures from all treatment conditions were lysed in a standard buffer. We cleared the crude lysates of cellular debris via centrifugation (12,000 rpm, 10 minutes, 4°C) and recovered the clarified supernatants for downstream assays.

Western blot analysis was employed to evaluate the protein abundance of mTOR, Beclin-1, LC3B, and β -actin. Equivalent protein loads were resolved via gel electrophoresis prior to being electro-transferred onto membranes. These blots underwent overnight probing at 4°C with specific primary antibodies targeting the proteins of interest. Following several wash steps, a 1-hour room temperature incubation was performed using appropriate secondary antibodies (either goat anti-mouse or goat anti-rabbit). A standard chemiluminescence imaging system was utilized to capture the immunoreactive bands.

Finally, ImageJ software (version 1.41; NIH, Bethesda, MD, USA) was applied to conduct

densitometric quantification, where target signal intensities were strictly normalized against the β -actin internal control (Figure 7).

3. Statistical Analysis

Every in vitro assay was conducted in triplicate at a minimum, with the resulting quantitative outcomes expressed as the mean $\pm$ standard deviation (SD). To evaluate statistical variances across multiple cohorts, we applied a one-way analysis of variance (ANOVA). Statistical significance was established at a two-tailed threshold of $P < 0.05$.

4.results

4.1 Cell Culture, Morphological Observation, and Immunofluorescence Characterization

After approximately 1 week of primary culture, numerous cells were observed migrating radially from the edges of the facial dermal tissue explants and adhering to the culture surface. After the first passage (P1), the cells increased in size and predominantly exhibited a regular spindle-shaped morphology. The cells grew in an interlacing arrangement and formed a characteristic network-like pattern, consistent with the typical in vitro morphology of fibroblasts (Figure 2A).

To confirm the identity and purity of the isolated cells, immunofluorescence staining was performed using cell-type-specific markers. Strong red fluorescence corresponding to the mesenchymal marker vimentin was detected in the cytoplasm, whereas no detectable fluorescence was observed for the epithelial marker keratin. Cell nuclei were counterstained blue with DAPI (Figure 2B). These findings confirmed that the isolated and cultured primary cells were human facial skin fibroblasts with high purity.

4.2 Optimization of the Rapamycin Treatment Concentration

Before evaluating the potential anti-senescence effects of rapamycin (RAPA) on human facial skin fibroblasts (HSFs), we first sought to determine an appropriate and well-tolerated in vitro treatment condition. The CCK-8 assay was used to assess the viability of HSFs derived from the youngest donor group (Group 1, 20–29 years) after exposure to different concentrations of RAPA (0, 0.1, 0.5, 1, 5, and 10 nmol/L) for up to 72 h.

Higher concentrations of RAPA (0.5–10 nmol/L) produced a mild, concentration-dependent reduction in the CCK-8 signal, consistent with the growth-inhibitory effects associated with mTOR suppression. By contrast, 0.1 nmol/L RAPA was well tolerated by the

cells. At 48 h, the CCK-8 signal in cells treated with 0.1 nmol/L RAPA was significantly higher than that in the untreated control group (**P < 0.001; Figure 2C).

Based on these findings, treatment with 0.1 nmol/L RAPA for 48 h was selected as the standard intervention condition for all subsequent experiments investigating age-associated cellular phenotypes.

4.3 Effects of Rapamycin on Senescence-Associated phenotypes in HSFs derived from women of different ages

Cellular senescence was evaluated by senescence-associated β -galactosidase (SA- β -gal) staining. Among the untreated control groups, the proportion of SA- β -gal-positive cells, characterized by blue-green staining, progressively increased with donor age. HSFs in Group 1 exhibited a predominantly elongated and regular morphology, whereas cells in Group 3 showed more pronounced senescence-associated morphological alterations, including reduced cell size and increased intercellular spacing (Figure 3A).

Following treatment with 0.1 nmol/L rapamycin for 48 h, the proportion of SA- β -gal-positive cells decreased in all three age groups, accompanied by an overall attenuation of senescence-associated morphological changes. Quantitative analysis further confirmed the reduction in SA- β -gal positivity after rapamycin treatment (Figure 3B and C). The decreases were most pronounced in Groups 2 and 3, suggesting that rapamycin effectively alleviated age-associated senescence phenotypes in HSFs, with greater effects observed in cells derived from the older donor groups.

4.4 Effects of Rapamycin on Cell-Cycle distribution in HSFs derived from women of different Ages

To characterize the cell-cycle changes associated with the anti-senescence effects of rapamycin, flow cytometry was performed to determine the cell-cycle distribution of HSFs in each age group. In the DNA-content histograms, the prominent left peak represented cells in the G0/G1 phase, the region between the two peaks represented cells in the S phase, and the right peak represented cells in the G2/M phase.

Following treatment with 0.1 nmol/L rapamycin for 48 h, the G0/G1 peak became sharper and more prominent in all three age groups, whereas the area corresponding to the S and G2/M phases was reduced (Figure 4A). Quantitative analysis further showed that rapamycin

treatment increased the proportion of cells in the G0/G1 phase and decreased the proportion of cells in the S phase relative to the corresponding untreated controls. The increase in the G0/G1-phase population reached statistical significance in Group 1 ($P < 0.05$) and Group 2 ($P < 0.01$) (Figure 4B). These findings indicate that rapamycin altered cell-cycle distribution by promoting G0/G1-phase accumulation and limiting cell-cycle progression into the DNA synthesis phase.

4.5 Effects of Rapamycin on LC3B expression in HSFs derived from women of different ages.

LC3B puncta are commonly used as an indicator of autophagosome accumulation. Immunofluorescence analysis showed that LC3B fluorescence intensity increased in HSFs from all three age groups following treatment with 0.1 nmol/L rapamycin for 48 h compared with the corresponding untreated controls. The increase was relatively modest in Group 1 but was more pronounced in Groups 2 and 3 (Figure 5A).

Quantitative analysis of LC3B fluorescence intensity and punctate accumulation further confirmed significant increases after rapamycin treatment within each age group (Figure 5B and C). In the between-group comparison, only the difference between Group 3 and Group 1 reached statistical significance. Overall, Group 3 exhibited the most prominent increase in LC3B-associated fluorescence after treatment. These findings suggest that rapamycin enhanced LC3B-associated autophagy-related activity in HSFs, with a more pronounced response observed in cells derived from the oldest donor group.

4.6 Effects of Rapamycin on mTOR and Autophagy-Related Gene expression in HSFs derived from women of different ages.

To characterize age-associated transcriptional changes in the mTOR/autophagy signaling axis, the basal mRNA expression levels of Beclin-1, LC3B, and mTOR were first evaluated in HSFs from the three age groups. Basal Beclin-1 mRNA expression remained relatively stable across the three groups (Figure 6A). By contrast, basal LC3B mRNA expression exhibited an age-associated decline. Compared with Group 1, LC3B mRNA expression was significantly lower in Group 2 ($P < 0.01$) and Group 3 ($P < 0.05$) (Figure 6B), suggesting a reduction in basal autophagy-related activity with increasing donor age.

In parallel, basal mTOR mRNA expression was significantly elevated in HSFs derived

from the older donor groups. The highest level was observed in Group 2, which differed significantly from Group 1 ($P < 0.0001$), whereas mTOR expression remained elevated in Group 3 compared with Group 1 ($P < 0.01$) (Figure 6C).

To further investigate the molecular effects of rapamycin, RT-qPCR was used to assess the expression of key genes involved in the mTOR/autophagy signaling pathway. Compared with the corresponding untreated controls, rapamycin treatment reduced mTOR mRNA expression in all three age groups (Figure 6F). In contrast, the mRNA expression levels of the autophagy-related genes Beclin-1 and LC3B were generally increased following rapamycin treatment (Figure 6D and E).

The magnitude of transcriptional activation of the autophagy-related genes differed among the age groups. In particular, rapamycin markedly increased Beclin-1 mRNA expression in Groups 2 and 3 ($P < 0.001$). Collectively, these findings indicate that rapamycin modulated the expression of mTOR and autophagy-related genes, with more pronounced transcriptional responses observed in HSFs derived from the older donor groups.

4.7 Effects of Rapamycin on Autophagy-Related Protein Expression in HSFs derived from women of different ages

To further validate the transcriptional findings, Western blotting was performed to determine the protein expression levels of mTOR, Beclin-1, and LC3B in HSFs from the three age groups. Consistent with the RT-qPCR results, basal mTOR protein expression was significantly elevated in HSFs derived from the older donor groups. The highest level was observed in Group 2, which was significantly higher than that in Group 1 ($P < 0.001$), while mTOR protein expression also remained elevated in Group 3 compared with Group 1 ($P < 0.01$) (Figure 7B3).

By contrast, the basal expression of the autophagy-related proteins showed an age-associated decline. Basal Beclin-1 protein expression was significantly lower in Group 3 than in Group 1 ($P < 0.05$) (Figure 7B1). Although basal LC3B protein expression remained relatively stable in Groups 1 and 2, it was significantly reduced in Group 3 compared with both Group 1 and Group 2 ($P < 0.01$ for both comparisons) (Figure 7B2). These results indicate that the expression of key autophagy-related proteins was reduced in HSFs derived from the oldest donor group.

Treatment with 0.1 nmol/L rapamycin for 48 h significantly reduced mTOR protein expression in all three age groups compared with the corresponding untreated controls ($P < 0.0001$) (Figure 7C3). Rapamycin treatment also increased LC3B protein expression across the three age groups (Figure 7C2). No significant treatment-induced changes in Beclin-1 protein expression were observed in Groups 1 and 2; however, Beclin-1 expression was significantly increased in Group 3 following rapamycin treatment ($P < 0.01$) (Figure 7C1).

Collectively, the mRNA and protein expression profiles suggest that rapamycin downregulated mTOR expression while enhancing the expression of autophagy-related molecules. The more pronounced changes observed in Group 3 further suggest that HSFs derived from the oldest donor group may exhibit greater molecular responsiveness to rapamycin-mediated regulation of the mTOR/autophagy axis.

5. Discussion

Developing effective strategies to mitigate intrinsic facial skin aging remains an important focus in aesthetic dermatology. Previous studies investigating the anti-aging effects of rapamycin have largely relied on acute exogenous stress models, such as H_2O_2 -induced oxidative stress or UVB exposure^[17]. Intrinsic aging, however, is characterized by the progressive accumulation of intracellular damage and the gradual deterioration of cellular homeostasis, which differs fundamentally from the rapid disruption induced by acute stress^[18, 19]. To investigate the age-dependent effects of rapamycin in the context of intrinsic aging, the present study established an age-stratified model using primary human facial skin fibroblasts^[20,21]. HSFs isolated from healthy female donors were categorized into three age groups: 20–29 years, 30–39 years, and 40–49 years. These groups represent important physiological stages from early adulthood to the transition toward perimenopause, during which endocrine and cellular metabolic changes may occur, and are consistent with the 10-year age intervals commonly used in clinical cohort studies^[22,23].

During dose optimization, the higher tested concentrations of rapamycin (0.5–10 nmol/L) caused a modest, concentration-dependent decrease in the CCK-8 signal, consistent with the growth-suppressive effects of mTOR inhibition. In contrast, 0.1 nmol/L rapamycin was well tolerated and produced a significantly higher CCK-8 signal after 48 h than that observed in untreated control cells. This regimen was therefore selected for subsequent experiments, as it

provided a suitable low-dose condition for evaluating the effects of rapamycin on age-associated cellular phenotypes without causing evident cytotoxicity or marked growth suppression^[24,25].

Cellular senescence is commonly accompanied by increased senescence-associated β -galactosidase activity and degenerative morphological alterations. In the present study, the proportion of SA- β -gal-positive HSFs progressively increased with donor age, accompanied by more pronounced senescence-associated morphological changes. Treatment with low-dose rapamycin reduced SA- β -gal positivity in all three age groups, with more prominent effects in Groups 2 and 3. These observations are consistent with the emerging concept of senomorphics, which modulate the adverse phenotypes of senescent cells without necessarily inducing their elimination. Senomorphic agents are thought to attenuate the detrimental effects of senescent cells by improving intracellular homeostasis and suppressing components of the senescence-associated secretory phenotype (SASP), rather than directly inducing apoptosis^[26,27]. In contrast to senolytic agents, which selectively eliminate senescent cells^[28,29], low-dose rapamycin may exert a more moderate regulatory effect on cellular homeostasis. Nevertheless, because SASP components and apoptosis were not directly measured in the present study, the classification of rapamycin as a senomorphic intervention in this model should be regarded as a mechanistically plausible interpretation that requires further experimental confirmation.

Alterations in cell-cycle regulation are closely associated with cellular senescence. Flow cytometric analysis showed that rapamycin treatment increased the proportion of HSFs in the G0/G1 phase while reducing the proportion of cells in the S phase. Persistent growth-promoting signaling, including mTOR signaling, during the early stages of irreversible cell-cycle arrest may promote cellular hypertrophy and the development of a highly secretory senescent phenotype^[30]. The observed G0/G1-phase accumulation following rapamycin treatment may therefore reflect a shift toward a less proliferative cellular state. One possible interpretation is that controlled suppression of growth-related signaling allows cells to enter a quiescence-like state rather than progressing toward a more pronounced senescent phenotype. However, the reversibility of this state was not directly examined, and additional experiments involving cell-cycle regulatory proteins and recovery after drug withdrawal are required to distinguish reversible quiescence from irreversible senescence-associated arrest.

The potential implications of these cellular effects may extend beyond individual fibroblasts. Aging of the skin on the face is a coordinated process that involves many layers of tissue. More and more evidence shows that interactions between the dermis and epidermis are important for keeping the skin healthy^[31]. SASP factors secreted by senescent fibroblasts may promote extracellular matrix degradation and disturb the homeostasis of neighboring epidermal cells through paracrine signaling. This process may also be influenced by matricellular proteins such as cellular communication network factor 1 (CCN1)^[32]. Accordingly, attenuation of senescence-associated phenotypes in HSFs may help preserve the dermal microenvironment and normal dermal–epidermal communication. However, the present study did not directly assess SASP production, extracellular matrix remodeling, or epidermal responses. Therefore, the proposed tissue-level benefits of low-dose rapamycin remain a hypothesis that should be evaluated using multicellular or three-dimensional skin models.

The molecular findings suggest that the response to rapamycin may vary according to donor age. Under basal conditions, LC3B mRNA expression decreased with increasing age, whereas mTOR mRNA expression was elevated in the older donor groups. At the protein level, Group 3 exhibited lower basal expression of Beclin-1 and LC3B together with persistently elevated mTOR expression. These findings suggest an age-associated alteration in the balance between mTOR signaling and autophagy-related machinery. Nevertheless, because autophagic flux was not directly measured, reductions in LC3B or Beclin-1 expression should be interpreted as evidence of altered autophagy-related molecular activity rather than definitive evidence of impaired autophagic flux.

Following rapamycin treatment, mTOR mRNA and protein expression decreased, whereas LC3B expression generally increased at both the transcriptional and protein levels. Beclin-1 mRNA expression was also increased, particularly in Groups 2 and 3, while a significant increase in Beclin-1 protein expression was detected only in Group 3. Immunofluorescence analysis further demonstrated increased LC3B-associated fluorescence and punctate accumulation after treatment. Together, these results indicate that low-dose rapamycin modulated the expression of mTOR and key autophagy-related molecules. The molecular response was generally more pronounced in HSFs derived from the older donor groups, particularly Group 3, suggesting that cellular responsiveness to rapamycin may depend

on the age-associated molecular state of the cells.

The heightened response observed in Group 3 may be related to the physiological changes that occur in women aged 40–49 years. This period may include the transition toward perimenopause, during which declining estrogen levels may influence the PI3K/AKT/mTOR signaling axis and cellular metabolic homeostasis^[33-35]. The elevated basal mTOR expression and reduced Beclin-1 and LC3B expression observed in Group 3 may therefore represent a state in which mTOR/autophagy-associated homeostasis is more substantially altered and consequently more responsive to pharmacological intervention. However, estrogen levels and the phosphorylation status of PI3K/AKT/mTOR pathway components were not measured in the present study. The proposed association between perimenopausal endocrine changes and the enhanced rapamycin response should therefore be considered speculative. Future studies incorporating hormonal measurements and direct assessment of mTOR pathway activity are needed to clarify this relationship^[36,37].

These age-dependent differences have potential implications for the development of targeted interventions for skin aging. Conventional anti-aging strategies often apply uniform interventions across individuals without considering age-related differences in cellular metabolism and stress responses. The present findings suggest that age stratification may be relevant when evaluating interventions directed at the mTOR/autophagy axis. In particular, cells derived from older donors may exhibit a greater molecular response to low-dose rapamycin. Nevertheless, these findings were obtained in an in vitro model and cannot yet be directly extrapolated to clinical treatment. Additional studies are required to determine whether age-dependent differences in cellular responsiveness are reproduced in intact human skin and whether they translate into meaningful clinical benefits.

Several limitations should be acknowledged. First, the study relied on a two-dimensional cell-culture system, which does not reproduce the three-dimensional extracellular matrix, mechanical tension, cellular heterogeneity, or complex dermal–epidermal communication present in human skin^[38,39]. Second, although rapamycin treatment increased the expression of the autophagy-related markers LC3B and Beclin-1, autophagic flux was not directly evaluated using late-stage autophagy inhibitors such as bafilomycin A1. Consequently, the observed accumulation of LC3B cannot distinguish between increased autophagosome formation and

impaired autophagosome degradation. In addition, reverse-validation experiments involving siRNA-mediated knockdown of key autophagy-related genes were not performed, limiting the strength of the causal conclusions.

Third, the long-term effects and safety of sustained low-dose rapamycin treatment were not evaluated. Whether the observed senescence-associated phenotypes recur after treatment withdrawal also remains unknown because long-term kinetic and washout experiments were not conducted^[40,41,42]. Finally, the primary HSFs were obtained from discarded facial skin tissue collected from a limited anatomical region^[43]. Basal autophagy-related activity and aging characteristics may differ among anatomical sites, particularly between chronically sun-exposed and relatively protected skin. Future studies should therefore include HSFs derived from multiple facial regions and employ three-dimensional skin equivalents or organotypic models to determine the generalizability and tissue-level relevance of the present findings^[44,45].

6. Conclusion

In conclusion, this study established an age-stratified model of intrinsic cellular aging using primary human facial skin fibroblasts derived from women of different ages. Low-dose rapamycin attenuated senescence-associated phenotypes to varying degrees, and these effects were accompanied by changes in mTOR expression and autophagy-related molecular markers. Notably, the cellular response to rapamycin differed among the age groups, indicating an age-dependent pattern of treatment responsiveness. These findings provide experimental evidence for understanding intrinsic facial skin aging from an age-stratified perspective and support further investigation of individualized anti-aging strategies targeting autophagy-related pathways.

Reference

1. Furman, D., Auwerx, J., Bulteau, A. L., Church, G., Couturaud, V., Crabbe, L., Davies, K. J. A., Decottignies, A., Gladyshev, V. N., Kennedy, B. K., Neretti, N., Nizard, C., Pays, K., Robinton, D., Sebastiano, V., Watson, R. E. B., Wang, M. C., & Woltjen, K. (2025). Skin health and biological aging. *Nature aging*, 5(7), 1195–1206. <https://doi.org/10.1038/s43587-025-00901-6>
2. Cole, M. A., Quan, T., Voorhees, J. J., & Fisher, G. J. (2018). Extracellular matrix

regulation of fibroblast function: redefining our perspective on skin aging. *Journal of cell communication and signaling*, 12(1), 35–43. <https://doi.org/10.1007/s12079-018-0459-1>

3. Herranz, N., Gallage, S., Mellone, M., Wuestefeld, T., Klotz, S., Hanley, C. J., Raguz, S., Acosta, J. C., Innes, A. J., Banito, A., Georgilis, A., Montoya, A., Wolter, K., Dharmalingam, G., Faull, P., Carroll, T., Martínez-Barbera, J. P., Cutillas, P., Reisinger, F., Heikenwalder, M., ... Gil, J. (2015). Erratum: mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. *Nature cell biology*, 17(10), 1370.

<https://doi.org/10.1038/ncb3243>

4. Südel, K. M., Venzke, K., Mielke, H., Breitenbach, U., Mundt, C., Jaspers, S., Koop, U., Sauermann, K., Knussman-Hartig, E., Moll, I., Gercken, G., Young, A. R., Stäb, F., Wenck, H., & Gallinat, S. (2005). Novel aspects of intrinsic and extrinsic aging of human skin: beneficial effects of soy extract. *Photochemistry and photobiology*, 81(3), 581–587.

<https://doi.org/10.1562/2004-06-16-RA-202>

5. Dikic, I., & Elazar, Z. (2018). Mechanism and medical implications of mammalian autophagy. *Nature reviews. Molecular cell biology*, 19(6), 349–364.

<https://doi.org/10.1038/s41580-018-0003-4>

6. Lu, Y., Pan, G., Wei, Z., Li, Y., & Pan, X. (2024). Role of fibroblast autophagy and proliferation in skin anti-aging. *Experimental gerontology*, 196, 112559.

<https://doi.org/10.1016/j.exger.2024.112559>

7. Wang, B., Han, J., Elisseeff, J. H., & Demaria, M. (2024). The senescence-associated secretory phenotype and its physiological and pathological implications. *Nature reviews. Molecular cell biology*, 25(12), 958–978. <https://doi.org/10.1038/s41580-024-00727-x>

8. Kang, H. T., Lee, K. B., Kim, S. Y., Choi, H. R., & Park, S. C. (2011). Autophagy impairment induces premature senescence in primary human fibroblasts. *PloS one*, 6(8), e23367. <https://doi.org/10.1371/journal.pone.0023367>

9. Choi, M. S., Chae, Y. J., Choi, J. W., & Chang, J. E. (2021). Potential Therapeutic Approaches through Modulating the Autophagy Process for Skin Barrier Dysfunction. *International journal of molecular sciences*, 22(15), 7869.

<https://doi.org/10.3390/ijms22157869>

10. Weisman R. (2016). Target of Rapamycin (TOR) Regulates Growth in Response to

Nutritional Signals. *Microbiology spectrum*, 4(5), 10.1128/microbiolspec.FUNK-0006-2016.
<https://doi.org/10.1128/microbiolspec.FUNK-0006-2016>

11. Roark, K. M., & Iffland, P. H., 2nd (2025). Rapamycin for longevity: the pros, the cons, and future perspectives. *Frontiers in aging*, 6, 1628187.
<https://doi.org/10.3389/fragi.2025.1628187>

12. Kell, L., Jones, E. J., Gharahdaghi, N., Wilkinson, D. J., Smith, K., Atherton, P. J., Simon, A. K., Cox, L. S., & Alsaleh, G. (2026). Rapamycin Exerts Its Geroprotective Effects in the Ageing Human Immune System by Enhancing Resilience Against DNA Damage. *Aging cell*, 25(2), e70364. <https://doi.org/10.1111/accel.70364>

13. Bernard, M., Yang, B., Migneault, F., Turgeon, J., Dieudé, M., Olivier, M. A., Cardin, G. B., El-Diwany, M., Underwood, K., Rodier, F., & Hébert, M. J. (2020). Autophagy drives fibroblast senescence through MTORC2 regulation. *Autophagy*, 16(11), 2004–2016.
<https://doi.org/10.1080/15548627.2020.1713640>

14. Chung, C. L., Lawrence, I., Hoffman, M., Elgindi, D., Nadhan, K., Potnis, M., Jin, A., Sershon, C., Binnebose, R., Lorenzini, A., & Sell, C. (2019). Topical rapamycin reduces markers of senescence and aging in human skin: an exploratory, prospective, randomized trial. *GeroScience*, 41(6), 861–869. <https://doi.org/10.1007/s11357-019-00113-y>

15. Saqcena, M., Patel, D., Menon, D., Mukhopadhyay, S., & Foster, D. A. (2015). Apoptotic effects of high-dose rapamycin occur in S-phase of the cell cycle. *Cell cycle (Georgetown, Tex.)*, 14(14), 2285–2292. <https://doi.org/10.1080/15384101.2015.1046653>

16. Tang, Y., Yang, S., Qiu, Z., Guan, L., Wang, Y., Li, G., Tu, Y., & Guo, L. (2024). Rapamycin Attenuates H₂O₂-Induced Oxidative Stress-Related Senescence in Human Skin Fibroblasts. *Tissue engineering and regenerative medicine*, 21(7), 1049–1059.
<https://doi.org/10.1007/s13770-024-00660-2>

17. Papaccio, F., D Arino, A., Caputo, S., & Bellei, B. (2022). Focus on the Contribution of Oxidative Stress in Skin Aging. *Antioxidants (Basel, Switzerland)*, 11(6), 1121.
<https://doi.org/10.3390/antiox11061121>

18. Chen, J., Liu, Y., Zhao, Z., & Qiu, J. (2021). Oxidative stress in the skin: Impact and related protection. *International journal of cosmetic science*, 43(5), 495–509.
<https://doi.org/10.1111/ics.12728>

19. Wong, Q. Y. A., & Chew, F. T. (2021). Defining skin aging and its risk factors: a systematic review and meta-analysis. *Scientific reports*, 11(1), 22075.
<https://doi.org/10.1038/s41598-021-01573-z>
20. Lephart, E. D., & Naftolin, F. (2021). Menopause and the Skin: Old Favorites and New Innovations in Cosmeceuticals for Estrogen-Deficient Skin. *Dermatology and therapy*, 11(1), 53–69. <https://doi.org/10.1007/s13555-020-00468-7>
21. Lago, J. C., & Puzzi, M. B. (2019). The effect of aging in primary human dermal fibroblasts. *PloS one*, 14(7), e0219165. <https://doi.org/10.1371/journal.pone.0219165>
22. Cappola, A. R., Auchus, R. J., El-Hajj Fuleihan, G., Handelsman, D. J., Kalyani, R. R., McClung, M., Stuenkel, C. A., Thorner, M. O., & Verbalis, J. G. (2023). Hormones and Aging: An Endocrine Society Scientific Statement. *The Journal of clinical endocrinology and metabolism*, 108(8), 1835–1874. <https://doi.org/10.1210/clinem/dgad225>
23. Raine-Fenning, N. J., Brincat, M. P., & Muscat-Baron, Y. (2003). Skin aging and menopause : implications for treatment. *American journal of clinical dermatology*, 4(6), 371–378. <https://doi.org/10.2165/00128071-200304060-00001>
24. Fu, D., Yin, J., Huang, S., Li, H., Li, Z., & Chong, T. (2018). Rapamycin Inhibits the Growth and Collagen Production of Fibroblasts Derived from Human Urethral Scar Tissue. *BioMed research international*, 2018, 7851327. <https://doi.org/10.1155/2018/7851327>
25. Demidenko, Z. N., Zubova, S. G., Bukreeva, E. I., Pospelov, V. A., Pospelova, T. V., & Blagosklonny, M. V. (2009). Rapamycin decelerates cellular senescence. *Cell cycle (Georgetown, Tex.)*, 8(12), 1888–1895. <https://doi.org/10.4161/cc.8.12.8606>
26. Wong, Q. Y. A., & Chew, F. T. (2021). Defining skin aging and its risk factors: a systematic review and meta-analysis. *Scientific reports*, 11(1), 22075.
<https://doi.org/10.1038/s41598-021-01573-z>
27. Zhang, L., Pitcher, L. E., Yousefzadeh, M. J., Niedernhofer, L. J., Robbins, P. D., & Zhu, Y. (2022). Cellular senescence: a key therapeutic target in aging and diseases. *The Journal of clinical investigation*, 132(15), e158450. <https://doi.org/10.1172/JCI158450>
28. Chaib, S., Tchkonja, T., & Kirkland, J. L. (2022). Cellular senescence and senolytics: the path to the clinic. *Nature medicine*, 28(8), 1556–1568. <https://doi.org/10.1038/s41591-022-01923-y>

29. Gasek, N. S., Kuchel, G. A., Kirkland, J. L., & Xu, M. (2021). Strategies for Targeting Senescent Cells in Human Disease. *Nature aging*, *1*(10), 870–879.
<https://doi.org/10.1038/s43587-021-00121-8>
30. Blagosklonny M. V. (2023). Cellular senescence: when growth stimulation meets cell cycle arrest. *Aging*, *15*(4), 905–913. <https://doi.org/10.18632/aging.204543>
31. Salzer, M. C., Lafzi, A., Berenguer-Llargo, A., Youssif, C., Castellanos, A., Solanas, G., Peixoto, F. O., Stephan-Otto Attolini, C., Prats, N., Aguilera, M., Martín-Caballero, J., Heyn, H., & Benitah, S. A. (2018). Identity Noise and Adipogenic Traits Characterize Dermal Fibroblast Aging. *Cell*, *175*(6), 1575–1590.e22. <https://doi.org/10.1016/j.cell.2018.10.012>
32. Quan, T., Qin, Z., Robichaud, P., Voorhees, J. J., & Fisher, G. J. (2011). CCN1 contributes to skin connective tissue aging by inducing age-associated secretory phenotype in human skin dermal fibroblasts. *Journal of cell communication and signaling*, *5*(3), 201–207.
<https://doi.org/10.1007/s12079-011-0144-0>
33. Lephart, E. D., & Naftolin, F. (2021). Menopause and the Skin: Old Favorites and New Innovations in Cosmeceuticals for Estrogen-Deficient Skin. *Dermatology and therapy*, *11*(1), 53–69. <https://doi.org/10.1007/s13555-020-00468-7>
34. Yu, T., Zhang, D., Yang, Y., Jiang, Y., Wang, J., Li, J., Zhang, Y., Xie, R., & Hong, X. (2025). 17 β estradiol activates autophagy and attenuates homocysteine mediated inflammation in endothelial cells through PI3K AKT MTOR signaling. *Scientific reports*, *15*(1), 24270. <https://doi.org/10.1038/s41598-025-08797-3>
35. Wang, Z., Li, X., Liu, X., Yang, Y., Yan, Y., Cui, D., Meng, C., Ali, M. I., Zhang, J., Yao, Z., Long, Y., & Yang, R. (2025). Mechanistic insights into the anti-fibrotic effects of estrogen via the PI3K-Akt pathway in frozen shoulder. *The Journal of steroid biochemistry and molecular biology*, *249*, 106701. <https://doi.org/10.1016/j.jsbmb.2025.106701>
36. Farkas, E., Goldblatt, A., Nehorayan, I., Lefkowitz, R. B., Fleshner, L., Tepper, K., & Marmon, S. (2026). Topical estrogen for skin aging: A systematic review of safety and efficacy. *Journal of the American Academy of Dermatology*, *94*(1), 212–215.
<https://doi.org/10.1016/j.jaad.2025.08.050>
37. Goryszewska-Szczurek, E., & Wacławik, A. (2026). Estradiol-17 β , a key embryonic signal, regulates gene expression and cellular functions in the porcine trophoblast. *Biology of*

reproduction, 114(6), 1813–1828. <https://doi.org/10.1093/biolre/ioag077>

38. Lombardi, F., Augello, F. R., Ciafarone, A., Ciummo, V., Altamura, S., Cinque, B., & Palumbo, P. (2024). 3D Models Currently Proposed to Investigate Human Skin Aging and Explore Preventive and Reparative Approaches: A Descriptive Review. *Biomolecules*, 14(9), 1066. <https://doi.org/10.3390/biom14091066>

39. Yao, Y., Zhang, Z., Zhang, J., Gao, Y., Li, X., Yang, K., Si, N., Chen, Z., Gu, Z., & Yin, N. (2026). In vitro modelling of extracellular matrix changes during skin aging: from static 2D to 3D dynamic microphysiological systems. *Microsystems & nanoengineering*, 12(1), 70. <https://doi.org/10.1038/s41378-026-01170-y>

40. Bitto, A., Ito, T. K., Pineda, V. V., LeTexier, N. J., Huang, H. Z., Sutlief, E., Tung, H., Vizzini, N., Chen, B., Smith, K., Meza, D., Yajima, M., Beyer, R. P., Kerr, K. F., Davis, D. J., Gillespie, C. H., Snyder, J. M., Treuting, P. M., & Kaeberlein, M. (2016). Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice. *eLife*, 5, e16351. <https://doi.org/10.7554/eLife.16351>

41. Mannick, J. B., Del Giudice, G., Lattanzi, M., Valiante, N. M., Praestgaard, J., Huang, B., Lonetto, M. A., Maecker, H. T., Kovarik, J., Carson, S., Glass, D. J., & Klickstein, L. B. (2014). mTOR inhibition improves immune function in the elderly. *Science translational medicine*, 6(268), 268ra179. <https://doi.org/10.1126/scitranslmed.3009892>

42. Leontieva, O. V., & Blagosklonny, M. V. (2017). While reinforcing cell cycle arrest, rapamycin and Torins suppress senescence in UVA-irradiated fibroblasts. *Oncotarget*, 8(65), 109848–109856. <https://doi.org/10.18632/oncotarget.17827>

43. Solé-Boldo, L., Raddatz, G., Schütz, S., Mallm, J. P., Rippe, K., Lonsdorf, A. S., Rodríguez-Paredes, M., & Lyko, F. (2020). Single-cell transcriptomes of the human skin reveal age-related loss of fibroblast priming. *Communications biology*, 3(1), 188. <https://doi.org/10.1038/s42003-020-0922-4>

44. Solé-Boldo, L., Raddatz, G., Schütz, S., Mallm, J. P., Rippe, K., Lonsdorf, A. S., Rodríguez-Paredes, M., & Lyko, F. (2020). Single-cell transcriptomes of the human skin reveal age-related loss of fibroblast priming. *Communications biology*, 3(1), 188. <https://doi.org/10.1038/s42003-020-0922-4>

45. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2023).

Hallmarks of aging: An expanding universe. *Cell*, 186(2), 243–278.

<https://doi.org/10.1016/j.cell.2022.11.001>

Acknowledgments

We would like to express our gratitude to all those who contributed to this work.

Funding Statement

This study was supported in part by the National Natural Science Foundation of China 346 (81600819), Provincial Science and Technology Plan Project of Sichuan Provincial Department of Science and Technology (2021SNZY001); Science and Technology Research Project of Sichuan Administration of Traditional Chinese Medicine (2024MS643); Sichuan Provincial National Natural Science Foundation of China(81600819), Provincial Science and Technology Plan Project of Sichuan Provincial Department of Science and Technology (2021SNZY001); Science and Technology Research Project of Sichuan Administration of Traditional Chinese Medicine (2024MS643); Sichuan Provincial Science and Technology Support Program (2022SNZY001); Suining Municipal Health and Science Technology Program-Guiding Project (25ZDJB03).

Data Availability Statement

All data are in the manuscript and/or supporting information files.

Author Contributions

Writing - original draft :Xuan Wei, Lijuan Guo, Zhiming He

Conceptualization: Sen Yang, Xuan Wei, Fei-fei Cao

Formal analysis: Zhiming He. Fei-fei Cao

Funding Acquisition: Lijuan Guo, Sen Yang

Investigation: Lijuan Guo, Tiwei Fu, Panpan Liang

Methodology: Lijuan Guo. Xuan Wei

Project administration: Sen Yang.

Supervision: Sen Yang.

Visualization: Lijuan Guo.

Writing – review & editing: Lijuan Guo, Sen Yang.

technical support: Long-yu Zhang , Ming-yang Chen

Investigation: Cheng Zeng

All authors participated in this article.

Ethics approval and consent to participate

Details regarding ethical standards, ethics approval (Approval No.: LLSLH20200051), and informed consent are provided in the “Ethics Approval and Consent to Participate” section of the Methods.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Figure1

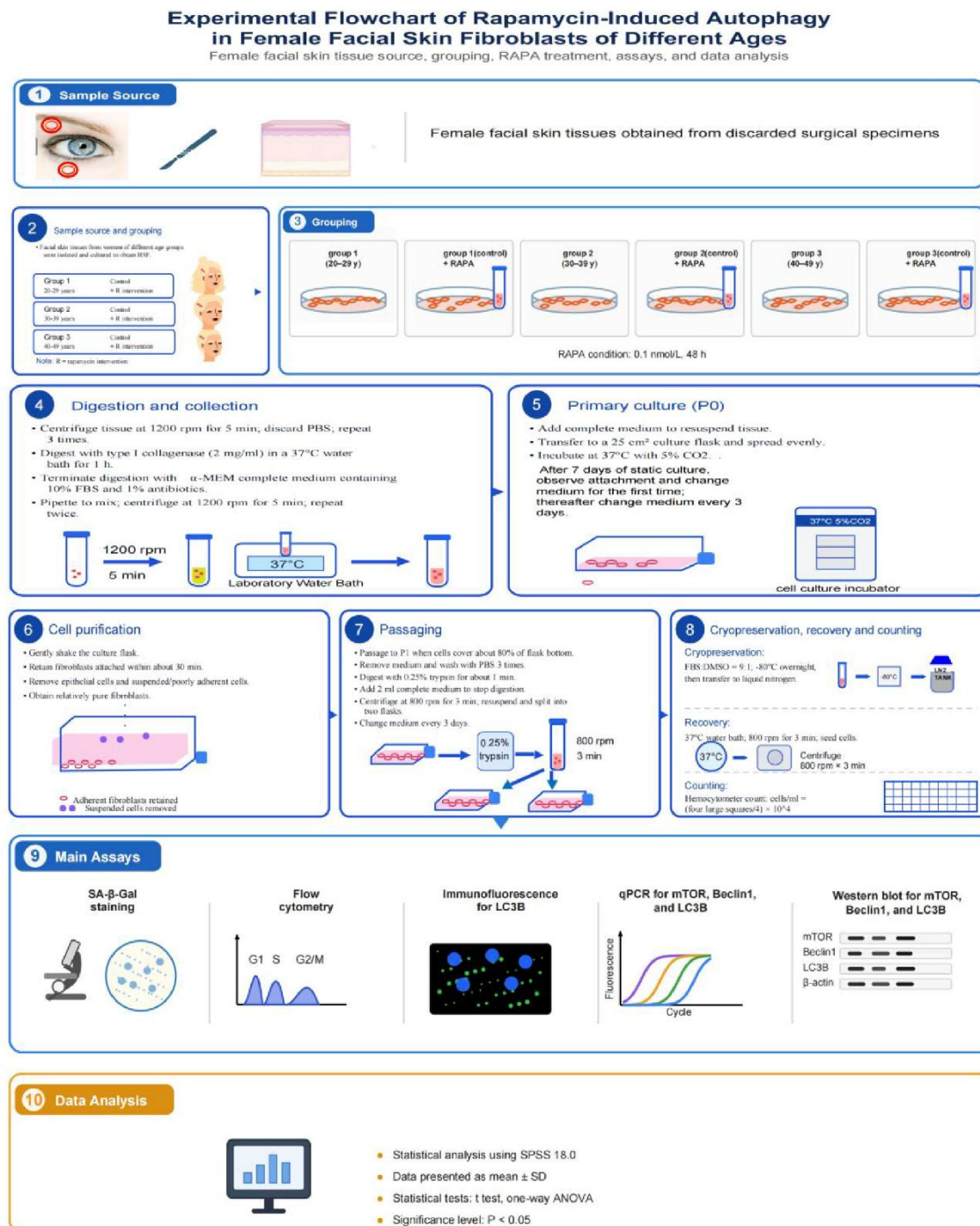


Figure 1 Schematic overview of the experimental design used to evaluate the effects of rapamycin on autophagy-related activity and senescence-associated phenotypes in human facial skin fibroblasts (HSFs).

(1) Sample collection and primary cell culture: Facial skin tissues were obtained from discarded surgical specimens from female donors. Primary HSFs from donors of different ages were isolated by type I collagenase digestion and subsequently cultured.

(2) Age stratification and rapamycin treatment: Purified HSFs were categorized into three groups according to

donor age: Group 1, 20–29 years; Group 2, 30–39 years; and Group 3, 40–49 years. Based on the results of preliminary dose-optimization experiments, cells in each age group were treated with 0.1 nmol/L rapamycin for 48 h, with corresponding untreated cells serving as controls.

(3) Assessment of senescence-associated phenotypes and autophagy-related molecular changes: Cellular senescence was evaluated using senescence-associated β -galactosidase (SA- β -gal) staining and flow cytometric analysis of cell-cycle distribution. LC3B expression and intracellular localization were assessed by immunofluorescence staining, while the mRNA and protein expression levels of mTOR and the autophagy-related molecules Beclin-1 and LC3B were determined by RT-qPCR and Western blotting, respectively.

(4) Data analysis: Statistical analyses were performed to evaluate the effects of low-dose rapamycin on senescence-associated phenotypes and autophagy-related molecular markers in HSFs derived from women of different ages.

Figure 2

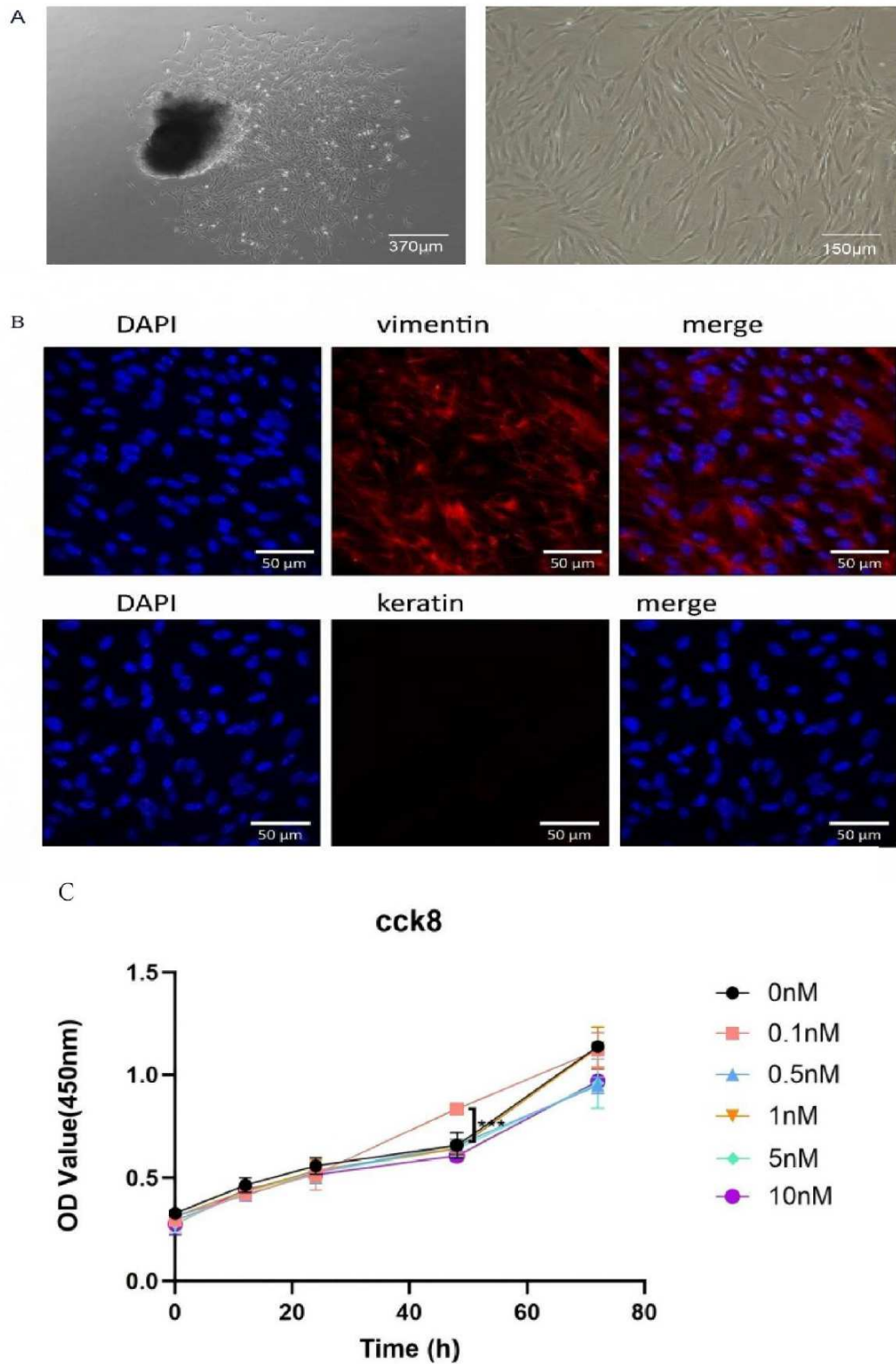


Figure 2 Morphological and immunofluorescence characterization of human facial skin fibroblasts (HSFs) and the effects of different rapamycin concentrations on cell viability.

(A) Morphological characteristics of primary HSFs. Representative bright-field images show primary cells

migrating outward from the edges of small tissue explants during the initial stage of culture (left) and passaged HSFs displaying the typical elongated spindle-shaped morphology and radial or whorl-like growth pattern (right).

(B) Immunofluorescence characterization of HSFs. The isolated cells showed strong positive staining for the mesenchymal marker vimentin and negative staining for the epithelial marker keratin, confirming their mesenchymal fibroblast identity. Nuclei were counterstained with DAPI (blue).

(C) Effects of rapamycin on HSF viability. HSFs were exposed to rapamycin at concentrations ranging from 0 to 10 nmol/L for up to 72 h, and cell viability was assessed using the CCK-8 assay. After 48 h of treatment, the CCK-8 signal was significantly higher in cells treated with 0.1 nmol/L rapamycin than in untreated control cells ($P < 0.001$), indicating good cellular tolerance under this treatment condition. At the higher concentrations tested, CCK-8 signals were comparable to or lower than those of the untreated control group, with no statistically significant differences ($P > 0.05$).

Figure3

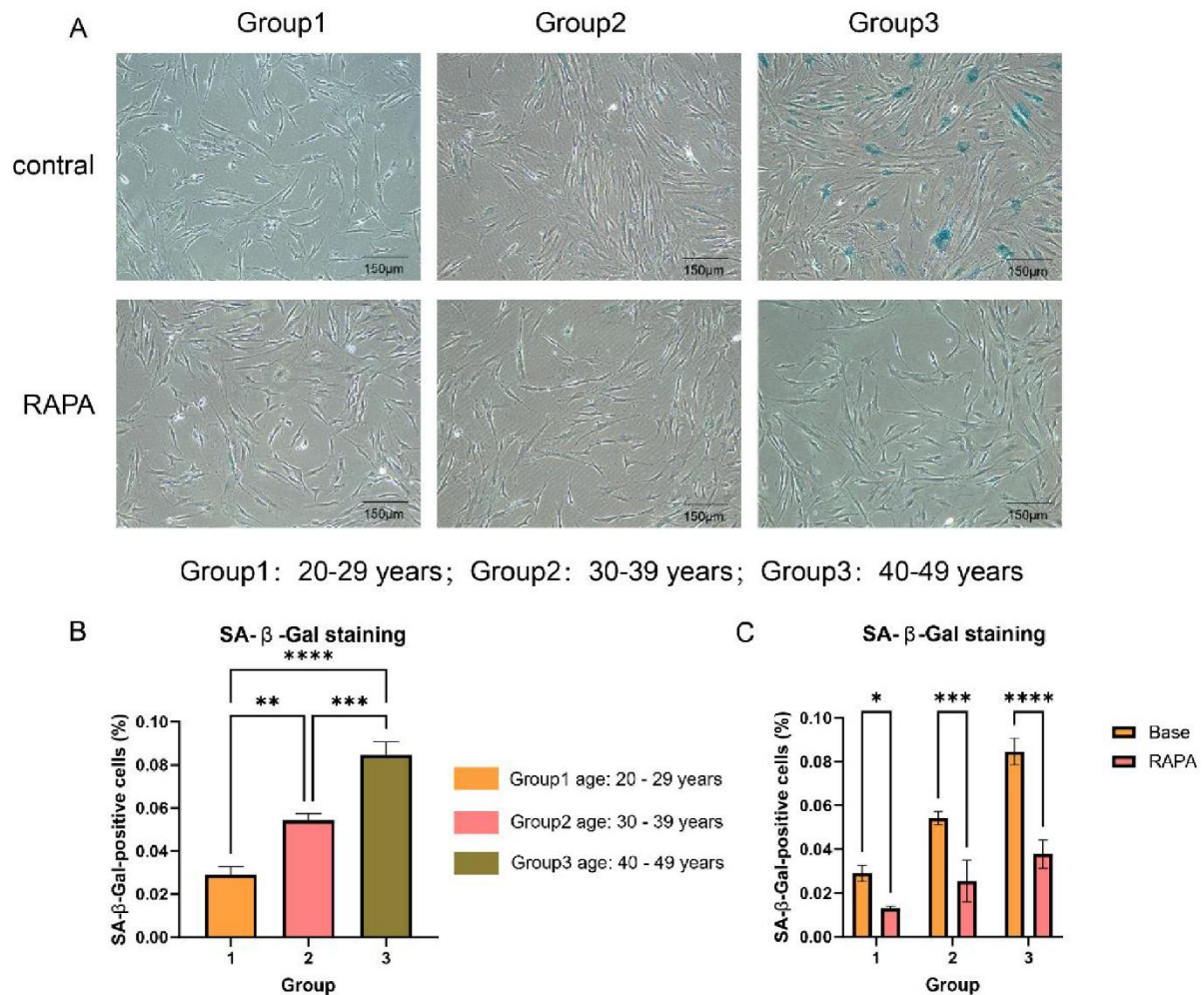


Figure 3 Effects of rapamycin on senescence-associated phenotypes in human facial skin fibroblasts (HSFs) derived from women of different ages.

(A) Representative SA-β-gal staining images. Representative microscopic images show HSFs from the three age groups—Group 1 (20–29 years), Group 2 (30–39 years), and Group 3 (40–49 years)—under untreated control conditions or following treatment with 0.1 nmol/L rapamycin (RAPA) for 48 h. Blue-green staining indicates SA-β-gal-positive senescent cells.

(B) Quantification of basal cellular senescence. Under untreated conditions, the proportion of SA-β-gal-positive cells progressively increased with donor age, indicating an age-associated increase in the senescent phenotype.

(C) Effects of rapamycin treatment on cellular senescence. Rapamycin significantly reduced the proportion of SA-β-gal-positive cells in all three age groups, with the most pronounced reduction observed in Group 3. Data are presented as the mean ± standard deviation from at least three independent experiments. $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$.

Figure4

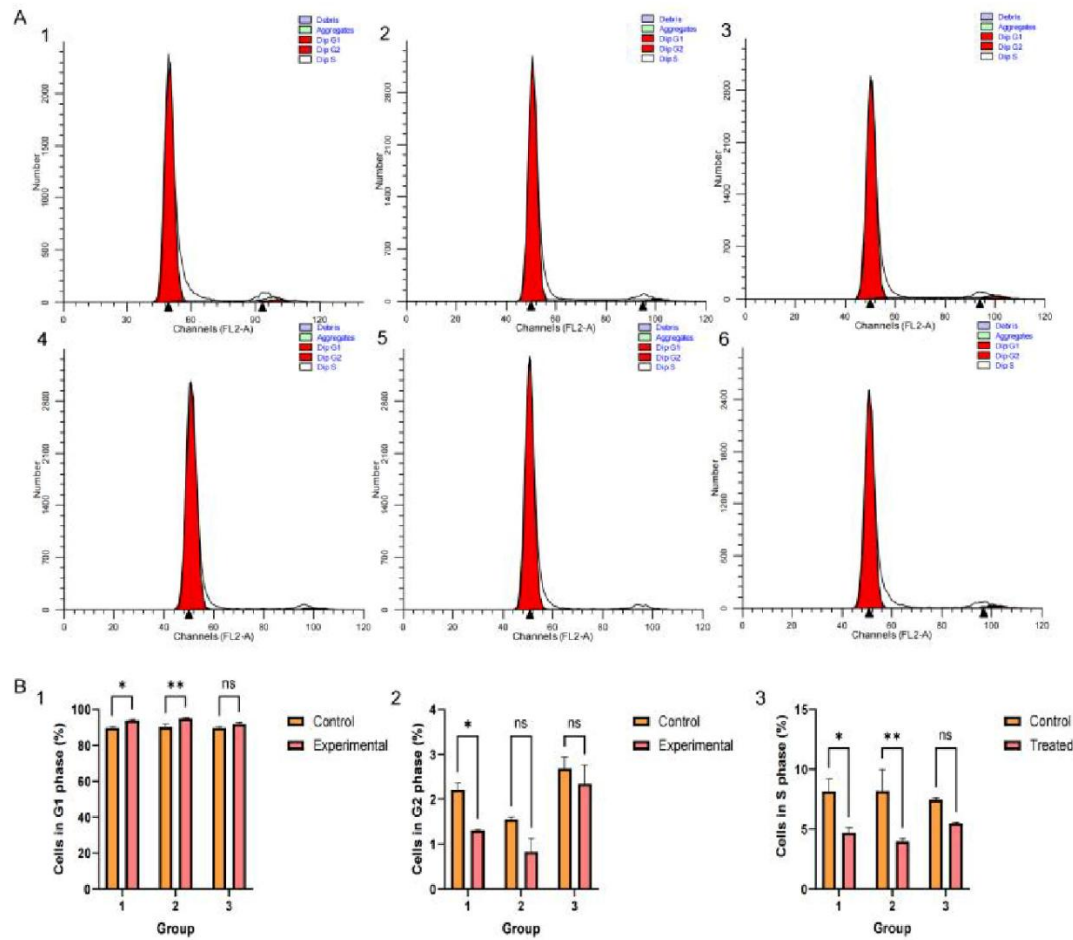


Figure 4 Effects of rapamycin on cell-cycle distribution in human facial skin fibroblasts (HSFs) derived from women of different ages.

(A) Representative flow cytometric profiles of cell-cycle distribution. Representative DNA-content histograms show the distribution of HSFs across the G0/G1, S, and G2/M phases under untreated control conditions or following treatment with 0.1 nmol/L rapamycin (RAPA) for 48 h.

(B) Quantitative analysis of cell-cycle distribution. The percentages of cells in the G0/G1, S, and G2/M phases were quantified for each experimental group. Compared with the corresponding untreated controls, RAPA treatment increased the proportion of cells in the G0/G1 phase and concomitantly decreased the proportion of cells in the S phase across the three age groups. Data are presented as the mean $\pm$ standard deviation from at least three independent experiments. $P < 0.05$, $P < 0.01$; ns, not significant.

Figure 5

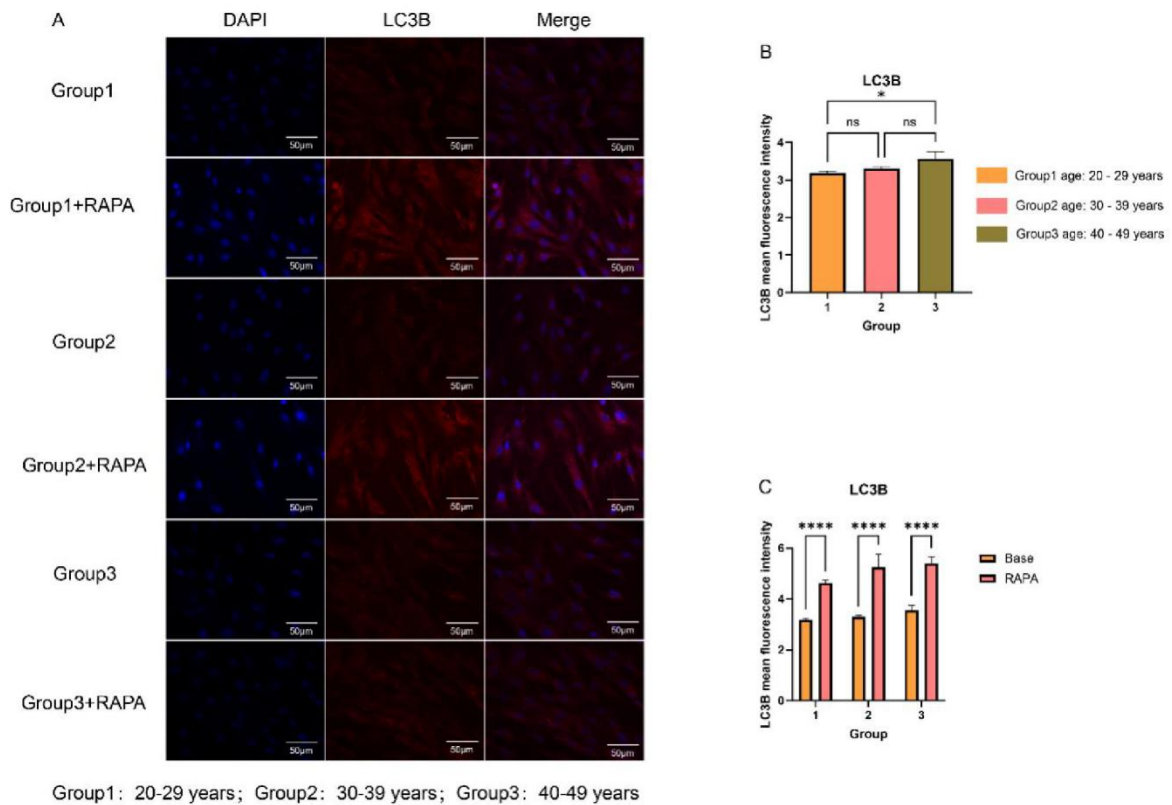


Figure 5 Effects of rapamycin on LC3B expression in human facial skin fibroblasts (HSFs) derived from women of different ages.

(A) Representative immunofluorescence images of LC3B. LC3B expression and intracellular localization were examined in HSFs from the three age groups under untreated control conditions or following treatment with 0.1 nmol/L rapamycin (RAPA) for 48 h. LC3B is shown in red, and nuclei were counterstained with DAPI (blue). Scale bar, 50 μm.

(B) Comparison of basal LC3B fluorescence intensity. Under untreated conditions, no statistically significant differences in mean LC3B fluorescence intensity were observed among the three age groups.

(C) Effects of rapamycin on LC3B fluorescence intensity. Semiquantitative analysis showed that RAPA treatment increased LC3B fluorescence intensity in all three age groups compared with the corresponding untreated controls. Data are presented as the mean ± standard deviation from at least three independent experiments. $P < 0.05$ and $P < 0.0001$; ns, not significant.

Figure6

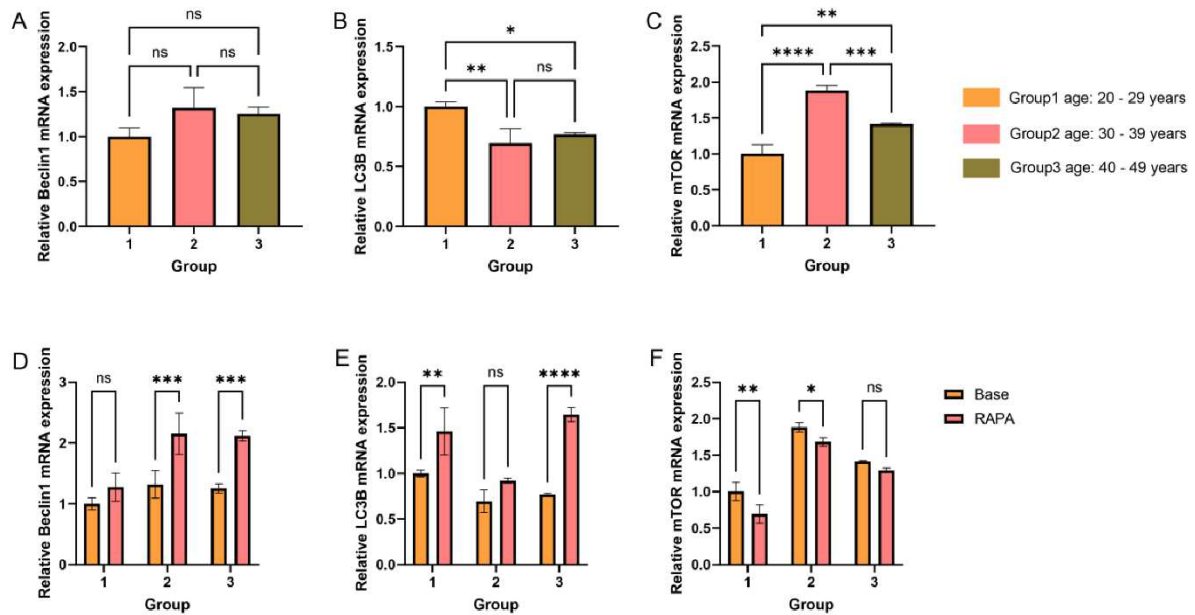


Figure 6 mRNA expression levels of mTOR and autophagy-related genes in human facial skin fibroblasts (HSFs) under basal conditions and following rapamycin treatment.

The mRNA expression levels of Beclin-1, LC3B, and mTOR in HSFs were determined by reverse transcription–quantitative polymerase chain reaction (RT-qPCR).

(A–C) Basal gene expression across the three age groups. The relative mRNA expression levels of Beclin-1 (A), LC3B (B), and mTOR (C) were compared among HSFs derived from the three donor age groups under untreated conditions.

(D–F) Transcriptional responses to rapamycin treatment. The relative mRNA expression levels of Beclin-1 (D), LC3B (E), and mTOR (F) were assessed after treatment with 0.1 nmol/L rapamycin (RAPA) for 48 h.

Compared with the corresponding untreated controls, RAPA treatment generally increased Beclin-1 and LC3B mRNA expression while reducing mTOR mRNA expression, with the magnitude of these changes varying among the age groups. GAPDH was used as the reference gene for normalization. Data are presented as the mean $\pm$ standard deviation from at least three independent experiments. $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$; ns, not significant.

Figure 7

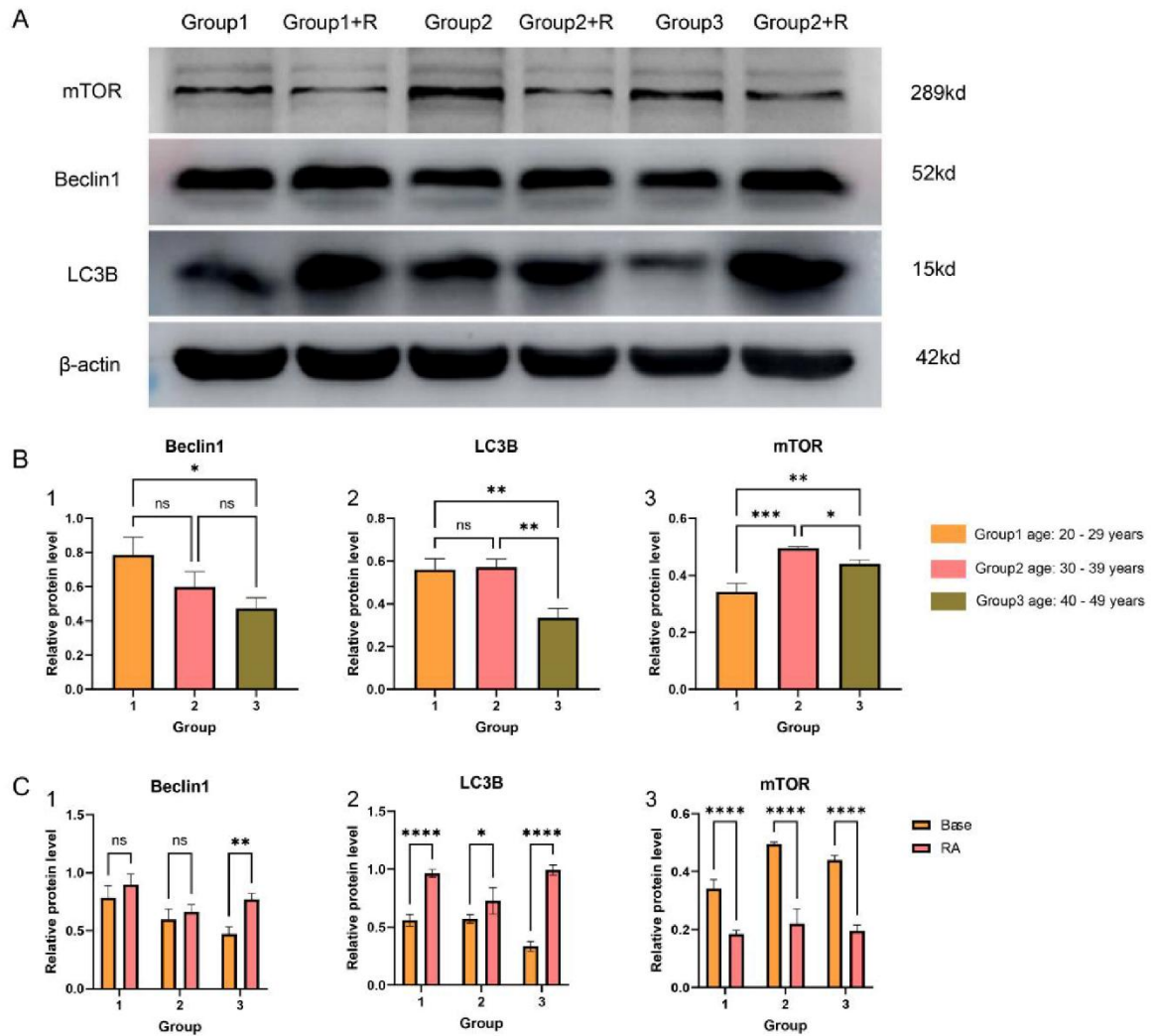


Figure 7 Effects of rapamycin on the protein expression of mTOR and autophagy-related molecules in human facial skin fibroblasts (HSFs).

(A) Representative Western blot images. Representative immunoblot bands show the expression of mTOR (289 kDa), Beclin-1 (52 kDa), and LC3B (15 kDa) in HSFs from the three age groups under untreated control conditions or following treatment with 0.1 nmol/L rapamycin (RAPA) for 48 h. β-Actin (42 kDa) was used as the loading control.

(B) Basal protein expression across the three age groups. Densitometric analyses were performed to compare the basal protein expression levels of Beclin-1 (B1), LC3B (B2), and mTOR (B3) among the three age groups.

(C) Changes in protein expression following rapamycin treatment. The relative protein expression levels of Beclin-1 (C1), LC3B (C2), and mTOR (C3) were compared between RAPA-treated cells and their corresponding untreated controls within each age group. Target protein expression was normalized to β-actin. Data are presented as the mean ± standard deviation from at least three independent experiments. $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$; ns, not significant.

Table 1

Gene name	Orientation	prodSize(bp)	Primer sequence(5'-3')
GAPDH	forward	114	ATCTTCCAGGAGCGAGATCC
	reverse		CTGCAAATGAGCCCCAGCCT
MTOR	forward	215	GAGATACGCTGTCATCCCTTTA
	reverse		CTGTATTATTGACGGCATGCTC
Beclin1	forward	235	ATCTAAGGAGCTGCCGTTATAC
	reverse		CTCCTCAGAGTTAAACTGGGTT
MAPILC3B	forward	86	GTCAGCGTCTCCACACCAATCTC
	reverse		TCCTGGGAGGCATAGACCATGTAC

Table 1 Reverse Transcription–Quantitative Polymerase Chain Reaction Analysis

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [qpcr.zip](#)
- [WB.zip](#)
- [IF.zip](#)
- [FC.zip](#)
- [file.zip](#)