IFSCC 2025 full paper (IFSCC2025-265)

MAMs Interactions: The Mitochondrial Hub Unlocking Cellular Rejuvenation

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1. Introduction

Mitochondria are essential organelles in eukaryotic cells, primarily responsible for energy production and maintaining cellular metabolism. The Endoplasmic Reticulum (ER), the largest organelle, plays a critical role in protein synthesis, folding, transport, lipid synthesis, carbohydrate metabolism, and calcium storage [1] . Mitochondria-associated membranes (MAMs) are key contact sites between mitochondria and the ER, involved in various cellular processes and signaling pathways [2-4] . These MAMs, comprising 4% to 20% of the mitochondrial surface, facilitate inter-organellar communication and content exchange, impacting their structure and function. MAMs are stabilized by tethering proteins, maintaining a 10 to 80 nm gap between the membranes [5] . MAMs play vital roles in multiple cellular functions [1,5]. These include calcium transfer, lipid movement and processing, mitochondrial behavior regulation, control of reactive oxygen species (ROS), cell death processes, and cellular recycling through autophagy [6]. Additionally, MAMs provide essential support for inflammatory responses and help fight viral infections [7]. As communication centers between cellular organelles, MAMs facilitate two-way exchange of both metabolites and signals. This exchange is crucial for regulating organelle function, coordinating cellular processes, and maintaining metabolic balance. Research has shown that changes in both the quality and quantity of MAMs are characteristic of various age-related diseases [3, 8-10]. Conditions such as diabetes, heart diseases, neurodegeneration, and cancer have been connected to MAM dysfunction [11].
The aging process significantly impacts MAMs through several mechanisms that compromise their structure and function. Oxidative stress, primarily from mitochondrial energy production, causes damage to proteins, particularly those involved in communication between the endoplasmic reticulum and mitochondria [12-15]. These changes particularly affect MAMs lipids composition, which is essential for their structural stability and signaling abilities, leading to disrupted calcium movement, lipid processing, and cellular recycling processes. The contact points between the endoplasmic reticulum and mitochondria should maintain a dynamic 'kiss-

and-run' nature rather than being static, allowing for controlled metabolic exchanges. However, with aging, these contact sites tend to increase in number, potentially leading to excessive metabolite transfer between the organelles, which can ultimately result in cellular dysfunction [16-18].

While the exact relationship between MAM changes and age-related diseases isn't fully understood, these alterations may accelerate cellular aging, suggesting MAMs could be key regulators in the aging process. What's becoming clear is that MAM changes are early indicators of cellular dysfunction. Monitoring these changes could potentially help track cellular aging and enable earlier detection of age-related diseases [8, 9, 11, 14,19].

However, studying MAMs presents significant technical challenges. Despite advances in technology, observing and isolating MAMs remains difficult, making it hard to study how various factors affect them. The analysis of mitochondria-associated membranes (MAMs) in skin tissue and dermal explants has not been investigated in the context of aging progression.

To further investigate the impact of aging on MAMs, we conducted a comparative analysis between senescent primary fibroblasts—induced through replicative exhaustion or oxidative stress exposure—and their non-senescent counterparts. We assessed the effects of these aging treatments on MAMs using proximity ligation analysis (a technic that allows to visualize and quantify protein interactions, and proximity between proteins and organelles in cell), cholesterol level measurements, intra-mitochondrial calcium quantification, mitochondrial respiration quantification, and atomic force microscopy. We examined the hypothesis that age-related modifications in mitochondria-associated membranes, characterized by altered organelle spacing, decreased membrane plasticity, and impaired signaling cascades, represent a critical determinant in cutaneous fibroblast aging.

2. Materials and Methods

Skin tissue procurement

Skin surgical discards were obtained from abdominoplasty or mammoplasty procedures performed for cosmetic purposes. (Declaration no. agreement DC-2024-6561). Fibroblast were isolated as previously published [20].

Assessment of mitochondria-ER membrane interactions by In Situ Proximity Ligation Assay (PLA).

After treatment, fixed and permeabilized cells (5 x 10^4 in glass-bottom 35 mm cells) were incubated overnight at 4 °C with a binary mixture of anti-IP3RI (1/500 dilution) and VDACI (1/500 dilution) primary antibodies. Thereafter, after two washes with TBS-Tween (tris-buffered saline polysorbate 20) 0.05%, cells were incubated (1 h at 37 °C) with the complementary secondary antibody. The proximity ligations were then performed according to the manufacturer's protocol. Preparations were mounted in mounting medium containing DAPI (4',6-diamidino-2-phenylindole). Fluorescence was analyzed with an inversed fluorescent microscope.Results were expressed as number of blobs per nucleus. A minimum of 10 images were taken per sample, and three independent series were performed for each treatment.

Cholesterol quantification

Intracellular cholesterol levels were quantified using the Filipin III cell-based cholesterol assay. The average fluorescence intensity was quantified, with a minimum of 10 images per condition, each being performed in quadruplicate.

Mitochondrial calcium quantification

Calcium dye loading was performed one hour before imaging. 10 µL of Stock Rhod-2AM calcium dye (1 mg/mL) in DMSO was mixed with 10 µL of pluronic F-127 (20% DMSO) and vortexed. Rhod-2AM fluorophores were excited by lasers emitting at wavelengths 581 nm. The dish was observed live for 5 min.

Evaluation of mitochondrial function

Mitochondrial respiration was evaluated using a Seahorse XF96 extracellular flux analyzer. A total of 4×10^4 cells were plated on an XF96 cell-culture microplate. On the following day, the medium was substituted with XF assay medium, and sequential injections of 1 µM oligomycin A, 1 µM FCCP, and 2 µM antimycin/rotenone were administered. Measurements were normalized by a direct imaging of the cells via hoescht-stained nuclei.

Gastrodia elata orchid extract preparation and characterization

The plant underwent a washing and drying process. The extraction phase involves the use of 40% ethanol as a solvent, conducted at room temperature over a period of four days. Following extraction, the resulting solution is subjected to filtration, effectively removing any solid particles and impurities. The filtrate is then concentrated through vacuum evaporation. The concentrate is subsequently dissolved in a 50% butylene glycol solution. This is followed by microfiltration to further refine the solution.

Statistical analysis

Results are presented as the mean ± standard deviation (SD). Data distribution was assessed using the Shapiro-Wilk test ($\alpha<0.1$). Normally distributed data were compared using paired or unpaired Student t-tests.

3. Results

Effects of replicative ageing on MAMs

To evaluate the impact of ageing on MAMs, we initially quantified MAMs in young primary fibroblasts (passage 6) and fibroblasts aged through replicative senescence (passage 31). We used the Proximity Ligation Assay (PLA) which is a molecular technique that uses pairs of antibodies linked to complementary DNA oligonucleotides which, when in close proximity (<40 nm), can interact and generate an amplified fluorescent signal, enabling visualization and quantification of protein-protein interactions or proximity in situ. We used one antibody directed to VDAC (mitochondrial protein) and IP3R2 key components of the calcium transfer machinery between ER and mitochondria. Results from proximity ligation assays indicated that replicative senescence increased the number of MAMs (Figure 1A, +78%, $p<0.01$). Following tert-butyl hydroperoxide (t-BHP), treatment, a powerful oxidant at 50 µM for 30 minutes, both senescent and non-senescent cells exhibited an increase in MAMs abundance, with senescent cells displaying a sustained elevation in MAM numbers over time (data not shown), (Figure 1A, +100%, $p<0.01$). Our data suggest that with senescence MAMs became static leading to ER and mitochondria dysfunction.

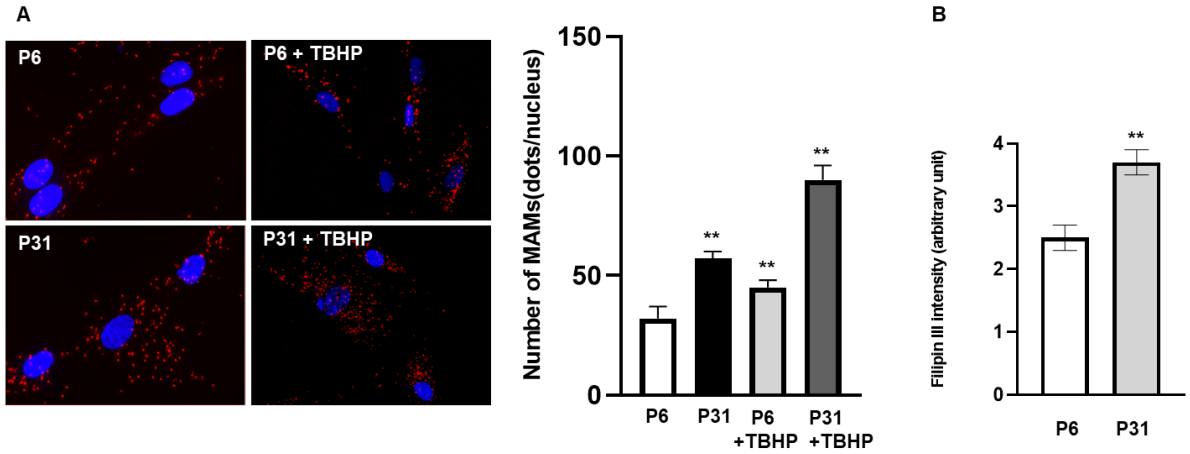

Figure 1. Effects of replicative ageing on MAMs. (A) Quantification of MAMs using proximity ligation assays. (B) Quantification of cholesterol levels using Filipin III labelling. Results are presented for young fibroblasts (P6) and fibroblasts aged through replicative senescence (P31). Statistical differences were analysed using unpaired Student t-test and are presented with: **: $p<0.01$

To further investigate this effect, we quantified cholesterol. Replicative senescence led to a significant increase in cholesterol levels (Figure 1B, +48%, $p<0.01$). MAMs function as crucial platforms mediating cholesterol trafficking from mitochondria to the plasma membrane. When these membranes contact sites become static, resulting in excessive ER-mitochondrial contact points, mitochondrial cholesterol accumulation occurs, compromising organelle functionality.

Effects of oxidative stress-induced ageing on MAMs

In addition to replication-induced ageing, we examined the effect of another senescence-inducing treatment: oxidative stress. For this purpose, primary fibroblasts from a donor at passage 3 were subjected or not to a treatment with 50 μM t-BHP for 30 minutes. Quantification of MAMs contact points (Figure 2A) revealed that, compared to untreated controls, a 30 minute treatment with t-BHP increased MAMs contact points by +56% ($p<0.01$). We developed an orchid extract from Gatrodia Elata which stimulates mitochondrial respiration. Treatment with orchid-derived bioactive compounds modulates ER-mitochondrial contact sites, resulting in reduced tethering points between these organelles nearly to non- t-BHP treated fibroblasts (Figure 2A). This modification promotes enhanced MAM plasticity and dynamics, effectively decreasing the static nature of these membrane contact sites and potentially optimizing their functional capacity. Oxidative stress also led to higher cholesterol levels after 30 min of t-BHP (Figure 2B, +67%, $p<0.01$). Treatment with orchid extract , which reduces t-BHP -induced contact points, restores proper MAMs fluidity, thereby leading to enhanced cholesterol export to plasma membranes, resulting in decreased cholesterol levels in mitochondrial membranes. This effect is more pronounced than in control conditions, suggesting that MAM flexibility is indeed crucial for MAMs to function as a metabolic hub for cholesterol.

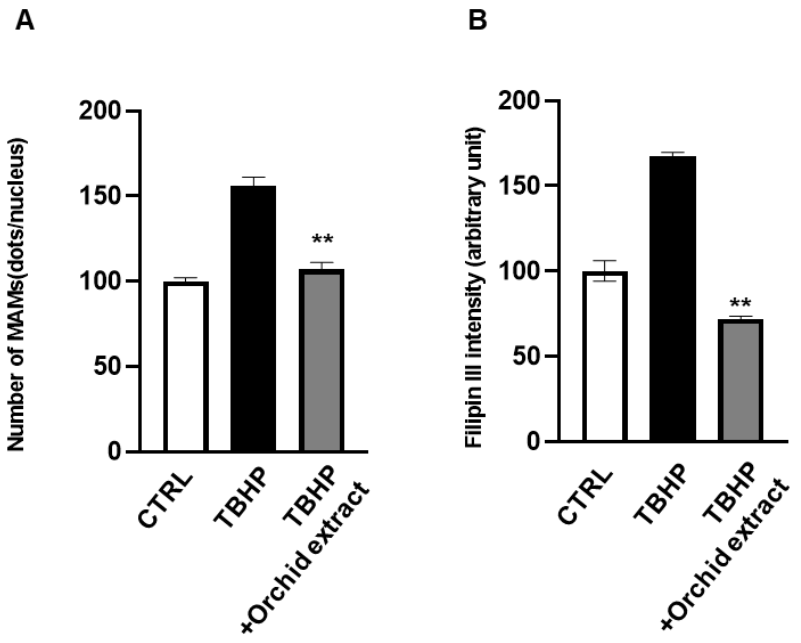

Figure 2. Effects of oxidative stress-induced ageing on MAMs. (A) Relative quantification of MAMs using proximity ligation assays. Fibroblast ((grey bars) were treated with 0.1 % Orchid extract (Gastrodia elata) for 48 h before t-BHP treatment (50 μM, 30 min) (B) Quantification of cholesterol levels using Filipin III labelling. Relative quantifications are normalised to the untreated control at the same timepoint. Results are presented for fibroblasts (P6) that were aged or not through oxidative stress. Statistical differences were analysed using unpaired Student t-test and are presented with: **: $p<0.01$.

Effects of oxidative stress-induced ageing on mitochondria functioning

Mitochondria serve as crucial dynamic calcium buffers in cellular calcium homeostasis, acting as both spatial and temporal regulators of intracellular calcium signals through their ability to rapidly uptake Ca2+ via the mitochondrial calcium uniporter (MCU) and release it through the Na+/Ca2+ exchanger (NCLX), thereby modulating the amplitude and spatiotemporal patterns of cytosolic calcium waves and influencing various calcium-dependent cellular processes including energy metabolism, cell death pathways, and signal transduction and with the Endoplasmic reticulum. The oxidative stress treatment also had a direct effect on intra-mitochondrial calcium levels (Figure 3A). Compared to respective untreated controls, a 30-minute t-BHP treatment increased it by +51% ($p<0.01$), but this increase is prevented by Orchid treatment. Excessive mitochondrial calcium accumulation can trigger detrimental cascades of events, including the opening of the mitochondrial permeability transition pore (mPTP), leading to mitochondrial swelling, loss of membrane potential, reactive oxygen species (ROS) production, impaired ATP synthesis, release of pro-apoptotic factors such as cytochrome c, and ultimately cell death through either apoptotic or necrotic pathways.

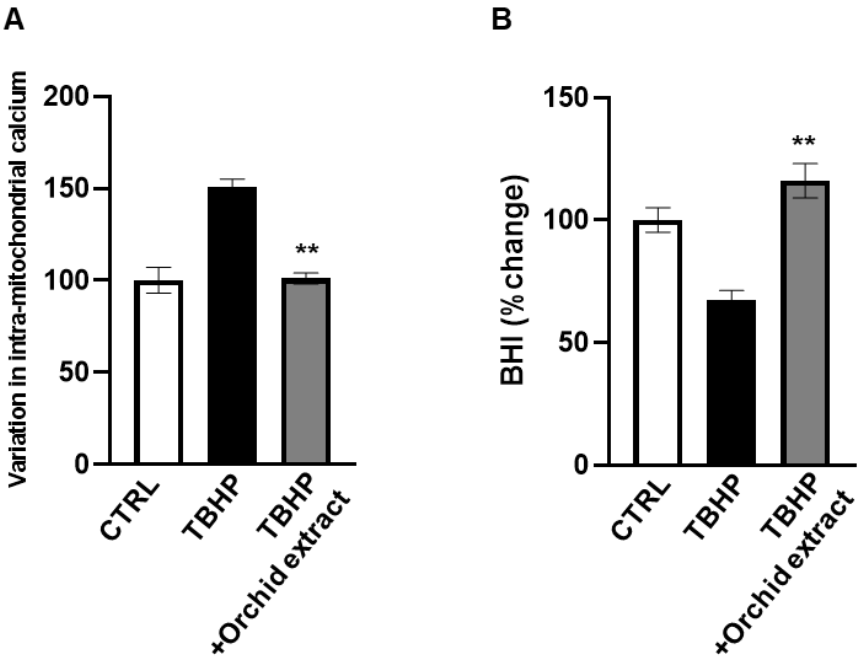

Figure 3. Effects of oxidative stress-induced ageing on mitochondrial function (A) Relative quantification of intra-mitochondrial calcium levels using Rhod-2, AM labelling. (B) Mitochondrial respiration and BHI measurement. BHI = (ATP-linked × reserve capacity)/(proton leak × non-mitochondrial respiration), Student t-test and are presented with: **: $p<0.01$.

To further explore the consequences of oxidative stress-induced we assessed mitochondrial function by analysing various aspects of cellular respiration, with a particular emphasis on the Bioenergetic Health Index (BHI). The Bioenergetic Health Index (BHI) is a quantitative measure of mitochondrial function and cellular bioenergetic health, calculated from cellular oxygen consumption rate (OCR) measurements that assess key parameters of mitochondrial function, including basal respiration, ATP-linked respiration, maximal respiration, and reserve capacity, relative to proton leak and non-mitochondrial respiration, with the formula: BHI = (ATP-linked × reserve capacity)/(proton leak × non-mitochondrial respiration), where a higher index indicates better bioenergetic health and cellular resilience to stress.

Compared to untreated fibroblasts, those subjected to oxidative stress- exhibited significant decrease in all respiratory evaluated parameters. Consequently, when calculating the BHI, which combines all parameters except basal respiration, a 33% reduction ($p=0.01$) was observed, indicating mitochondrial dysfunction (Figure 3B). However, the BHI was increased by 16 % compared to control cells when the cells were pre-treated with the orchid extract suggesting that when MAMs flexibility is increased, calcium and cholesterol are well handled and mitochondria function is optimal.

Effects of Orchid extract on collagen synthesis

The increase in MAMs and enhanced contact points between mitochondria and ER with senescence or oxidative stress leads to dysregulation of calcium homeostasis and mitochondrial

respiration, resulting in ER dysfunction. Since collagen synthesis occurs in the ER and requires ATP, this disruption impacts collagen production. We treated skin explants with our orchid extract at a dose previously shown to protect mitochondrial function and endoplasmic reticulum integrity while preserving MAM flexibility. We observed a 56% increase in type I collagen synthesis (Figure 4).

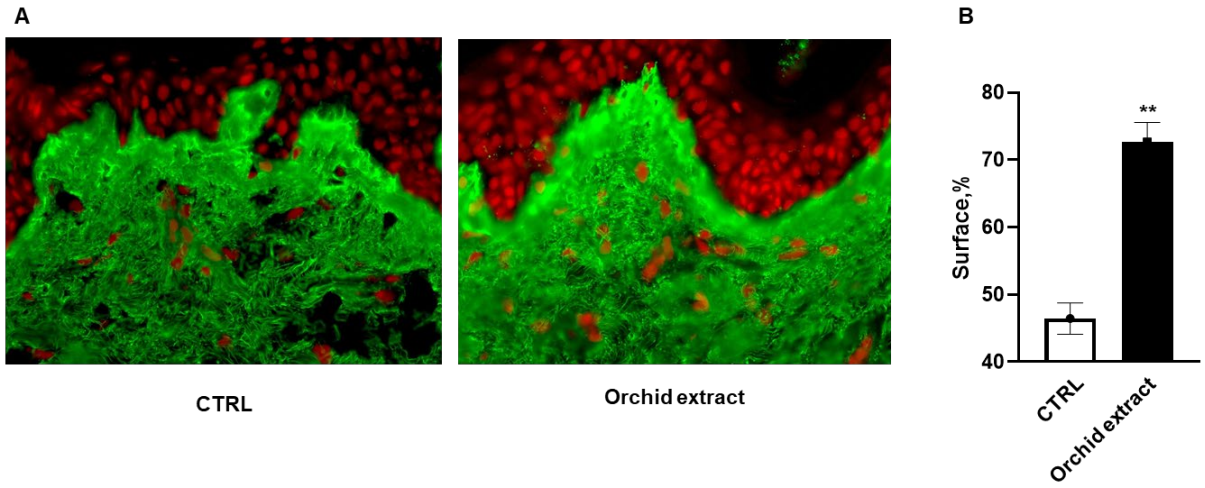

Figure 4. Relative quantification of the immunostaining of collagen I. images of immunofluorescent staining. (A) Type I human skin explants treated or not with 0.1% Orchid extract (B) Surface quantification. For statistical analysis, **: $p < 0.01$.

4. Discussion

The aging process affects skin deterioration through both genetic (intrinsic) and environmental (extrinsic) factors. While scientists have made significant advances in understanding how aging affects the extracellular matrix (ECM) degradation and fibroblast senescence, we still have limited knowledge about how mitochondria-associated membrane (MAM) changes influence fibroblast dysfunction during aging.

Studies have demonstrated that membrane contact sites between the endoplasmic reticulum and mitochondria (MAMs) and cellular senescence are crucial in aging-related processes. The connection between MAMs components and cellular senescence was initially established through various studies, with further support coming from experiments showing that artificially linking MAMS induces cellular senescence in pulmonary fibroblasts [16,17] . Another lab has shown that ITPR2 deficient mice display improved aging, associated with a decreased number of contacts between the mitochondria and the ER [16].

In our research using fibroblasts, which are crucial dermal cells responsible for ECM production and skin structure maintenance, we discovered that cellular aging increases MAMs contacts points, correlating with higher cholesterol concentrations. Additionally, we found that oxidative stress, which acts as both a signaling mediator and source of cellular damage in aging processes, increased MAMs contact points rapidly within 30 minutes. This effect persisted and was observable after 24 hours of exposure, accompanied by elevated mitochondrial calcium

levels. This was accompanied by increased cholesterol levels, compromised mitochondrial function.

The aging process is characterized by increased levels of reactive oxygen species (ROS) within cells, leading to widespread cellular damage affecting various organelles, including mitochondria and the endoplasmic reticulum. The impact of aging on MAM dynamics varies depending on the experimental context, cell type, and model system studied. Some research indicates decreased MAM tethering, while other studies show increased MAM contact sites in senescent cells, aligning with our observations in aged fibroblasts [21].

Our findings demonstrate that oxidative stress alone can trigger increased MAM contact sites, elevated mitochondrial calcium levels, and impaired mitochondrial function, creating a self-reinforcing cycle of cellular deterioration. The rapid response to oxidative stress, occurring within 30 minutes, suggests that any ROS-generating process could quickly affect MAMs and contribute to cellular senescence through calcium accumulation [22-24]. The resulting cholesterol integration into cellular membranes affects their physical properties, explaining the observed loss in mitochondrial respiration because mitochondria membranes are more rigid [24].

5. Conclusion

In conclusion, the observation that MAMs and cellular senescence play a crucial role in aging and age-associated diseases led to the hypothesis that MAMs may regulate aging at least partly through cellular senescence, and indeed, experimental data support that MAMs are key platforms controlling cellular senescence.

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