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Original Article

Dissecting the Impact of α-MSH-MC1R-cAMP Signaling on UVA-Induced Stress in Fibroblasts – Implications for Regulation of Cutaneous Photoaging

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ABSTRACT: Alpha-melanocyte-stimulating hormone (α-MSH) is a key endocrine mediator of photoprotection in the ultraviolet (UV)B-induced skin response. Its effects are mediated by the melanocortin-1 (MC1R) receptor and include increased eumelanin synthesis but also direct cytoprotective actions. Previous studies have shown that individuals with the red hair and fair skin phenotype, carrying loss-of-function *MC1R* alleles, exhibited accelerated dermal photoaging. The underlying mechanism remains poorly understood. Here, we investigated the impact of α-MSH-MC1R-cAMP signalling on UVA-induced stress in key cells of dermal photoaging. Primary human dermal fibroblasts from adult donors were genotyped for common *MC1R* variants and analysed in both 2D monolayer and 3D spheroid culture models. UVA-induced oxidative stress and induction of genes related to the DNA damage response and senescence-associated secretory phenotype were assessed by flow cytometric analysis, real-time PCR, Western immunoblotting and ELISA. Functional MC1R signalling was evaluated by cAMP assays, with receptor expression examined by RT-PCR and Western immunoblotting. UVA exposure induced oxidative stress, and upregulation of p21, heme oxygenase-1, Sirtuin 1, interleukin-6 and -8. Although MC1R expression and functional cAMP signalling were confirmed, α-MSH treatment did not attenuate these UVA-induced stress responses. In accordance with this, pharmacological activation of canonical cAMP signalling did not alter the examined UVA-induced oxidative stress and inflammatory cell responses. Our findings do not provide evidence for a direct UVA-photoprotective effect of the α-MSH-MC1R axis in adult dermal fibroblasts. However, they do not exclude that a functional α-MSH-MC1R-cAMP signalling axis in other cell types of the skin is important in preventing photoaging.

Keywords: α-MSH, MC1R, UVA, human dermal fibroblasts, skin photoaging

INTRODUCTION

Photoaging is part of the complex and multifaceted process of skin aging [1], which results in a combination of intrinsic and extrinsic factors. Intrinsic factors include the natural, biological processes of aging such as genetic predisposition, hormonal changes, and the gradual decline in cellular function. Extrinsic factors, on the other hand, include ultraviolet (UV) radiation from sunlight or artificial UV sources, fine particulate matter, smoking,

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and other environmental stressors that accelerate skin aging. The combined impact of these extrinsic factors is referred as the exposome [2]. Extrinsic skin aging shares many characteristics with intrinsic, or chronological aging. Common features include increased production of reactive oxygen species (ROS), collagen degradation via matrix metalloproteases (MMPs), and the development of the senescence-associated secretory phenotype (SASP) including proinflammatory cytokines such as interleukin (IL)-6 and -8 [3-6]. Uncontrolled ROS production represents a critical link between intrinsic and extrinsic aging, leading to telomere shortening, mitochondrial dysfunction, and DNA damage. Subsequent DNA damage response (DDR) signalling, i. e., induction of p21, or p53, may result in cell cycle arrest and metabolic reprogramming [3,7-11].

Notably, UVA is more effective than UVB in generating ROS and penetrates deeply into the dermis, damaging dermal fibroblasts, which play a central role in photoaging. These dermal fibroblasts not only produce extracellular matrix components that maintain connective tissue but also interact with endogenous stem cell niches and with quiescent, mitotic, and postmitotic cells [4].

Endocrine and neuroendocrine signals are key regulators of pathways involved in both intrinsic and extrinsic skin aging [12]. Among these hormones, alpha-melanocyte-stimulating hormone (α-MSH) is a tridecapeptide produced within the skin through processing of proopiomelanocortin (POMC) [13,14]. UVB irradiation of the skin induces POMC expression and processing into β-endorphin (β-ED) and melanocortin peptides such as α-MSH in epidermal cells. The cutaneous stress-induced POMC system is regarded as an analogue of the classical hypothalamic-pituitary-adrenal axis [15]. Research over the last decades has extended this concept towards an even more complex cutaneous photo-neuro-endocrine system with additional players, e. g. enkephalins. This cutaneous system communicates in a bidirectional manner with the central nervous, endocrine, and immune systems, in order to control body homeostasis [16-18].

Once released, α-MSH binds with high affinity to the melanocortin 1 receptor (MC1R), a seven transmembrane, G protein-coupled receptor. Owing to their high expression of MC1R, melanocytes serve as the primary cellular targets of α-MSH following UVB exposure [13]. MC1R signalling triggered by α-MSH or agonistic peptides activates canonical cAMP signalling, increases tyrosinase expression, and shifts the pheomelanin-eumelanin ratio toward photoprotective eumelanin synthesis [19].

The human *MC1R* gene is highly polymorphic. Some variants are linked to red hair, fair skin, freckling and reduced tanning capacity [19,20], and some are associated with higher risks of melanoma and non-melanoma skin cancer. Importantly, eumelanin induction is not the only photoprotective mechanism of α-MSH. Studies have identified direct mechanisms in melanocytes, including reduction of UVB-induced DNA damage and apoptosis via enhanced nucleotide excision repair [21-23] as well as indirect antioxidative effects such as catalase upregulation and redistribution [24] and activation of nuclear factor erythroid-derived 2-like 2 (Nrf2), a transcription factor controlling key antioxidant and phase II enzymes including heme oxygenase-1 (HO-1) [25].

Moreover, MC1R is expressed in other skin cell types, including fibroblasts and endothelial cells, where it mediates "non-pigmentary" effects such as cytoprotection and immunomodulation. This evidence suggests that α-MSH acts as an intrinsic anti-stress natural response modifier in skin biology orchestrating protective responses against environmental and cellular stress [13-18].

Interestingly, epidemiologic studies have shown that major diminished-function *MC1R* variants are associated with an increased risk for dermal photoaging [26,27]. After adjusting for possible confounders such as demographic and phenotypic factors and sun exposure intensity, the presence of diminished-function *MC1R* variants, e. g., D84E, R142H or R151C, was identified as a significant risk factor for severe photoaging with the risk increasing more than fivefold when two major diminished-function alleles were present. Similar findings have been reported by others [28]. The underlying mechanism remains unclear, but one hypothesis is the expression of functional MC1Rs in dermal fibroblasts, which may directly protect from UVA-induced stress.

MATERIALS AND METHODS

Cell culture

Normal adult human dermal fibroblasts (aHDFs) purchased from PromoCell (Heidelberg, Germany) from eight independent donors (mean age: 50.8 years, male-female ratio: 3:5; donor sites: head and trunk) were routinely cultured in RPMI 1640 medium (Merck, Darmstadt, Germany) with supplements as outlined before [29]. Cells were used between passages 2 and 8. HBL melanoma cells were a gift from Dr. G. E. Ghanem (Laboratory of Oncology and Experimental Surgery, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium) and were cultured in Ham's F10 medium plus 2.5 mg/l trypsin (Merck). aHDFs carrying the homozygous *MC1R* mutations R151C/R151C and R160W/R160W were generously provided by Dr. Z. Abdel-Malek (University of Cincinnati, Dermatology, OH, USA). The genotype of these cells was confirmed by

Taqman SNP genotyping assay. Normal human melanocytes were obtained from Tebu-bio, Offenbach, Germany, and cultured as previously described [25].

3D spheroid model of dermal fibroblasts

Spheroids of aHDFs were generated as reported before [30]. Briefly, spheroids consisting of 6,000 aHDFs were grown for 2 days in 96-well plates using methylcellulose solution and RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. An appropriate number of spheroids was removed from the 96 well plate and transferred to 3.5 cm dishes for further experimental settings. The spheroids were then deprived from FBS, treated with α-MSH and irradiated with UVA under exactly the same conditions as outlined below.

Cell treatment and UVA irradiation

α-MSH, vitamin C and β-ED were all obtained from Merck, NDP-α-MSH and KdPT from Abbexa (Cambridge, UK). Forskolin (FSK) was purchased from Tocris (Wiesbaden, Germany). For all functional experiments aHDFs were seeded at a density of 3 x 10^5 (or 5 x 10^5 for RNA analysis) cells into 3,5 cm (or 6 cm for RNA analysis) diameter tissue culture dishes into normal cell culture medium. Prior to stimulation, cells were deprived from FBS for 24 h followed by incubation with the peptides (α-MSH, β-ED, KdPT) or agents (vitamin C, forskolin) for further 24 h. Directly before UVA irradiation, peptides or agents were additionally given at dosages indicated. Irradiation of aHDFs with UVA was performed in serum-free RPMI 1640 medium without phenol red using the UVA lamp Sellamed 3000 with a measured emission spectrum of 335-420 nm and a peak emission wavelength of ~380 nm (Sellas, Ennepetal, Germany). Cells were irradiated in a distance of ≈20 cm. The emitted energy in W/cm^2 of the UVA source was monitored and calibrated by a photometer (Waldmann UVA700 Sensor VARIOCONTROL, Villingen-Schwenningen, Germany). Given the energy of 75 mW/cm^2, cells were irradiated for 133 sec to achieve 10 J/cm^2.

RNA extraction and quantitative/semi-quantitative RT-PCR

Total RNA was prepared from cells using the Innuprep RNA Kit (AnalytikJena, Jena, Germany). cDNA synthesis was performed employing the Revert Aid™ cDNA Kit (ThermoFisher Scientific, Darmstadt, Germany). The primer sequences of all analysed gene products are listed in Supplementaty Table 1 and 2. Semi-quantitative RT-PCR was performed using the following cycles: 1 × 94˚C/3 min; 35 × 94˚C/10 sec; 60˚C/30 sec; 72˚C/30 sec; 72˚C/5 min. Real-time RT-PCR analysis was conducted using the qTower 2.2 from Analytik Jena. Quantification of gene expression of each sample was done by the $2^{-\Delta\Delta}CT$ method using RPL23 as an internal standard and depicted as n-fold induction with the reference condition set as 1.

Western Immunoblotting

Protein samples were prepared by adding hot 2×Laemmli buffer to cell pellets followed by heating at 95°C for 5 minutes. Lysates were then separated by SDS-PAGE followed by immunoblotting as described before [29]. The following antibodies were used: phosho-p38, p38, sirtuin 1, p21, p53, HO-1 and MC1R. Equal protein loading was assured by anti α-tubulin, β-actin or GAPDH. Chemiluminescene analysis was conducted using the GBox ChemiXX9 from Syngene, Schwerte, Germany. Further information on the used antibodies is given in Supplementaty Table 3.

Detection of reactive oxygen species

The redox-sensitive fluoroprobe 5-[and-6]-chloromethyl-2',7' dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA) was obtained from Molecular Probes (Invitrogen). After irradiation with UVA, cells were loaded with CM-H_2DCFDA (1 µM) for 45 min at 37°C, 5% CO_2. Cells were washed, harvested and analysed by Cytek NL-3000 using SpectroFlo software (Cytek Biosciences, Amsterdam, Netherlands). For each sample, 20,000 events were collected.

Determination of cAMP

For the detection of intracellular cAMP, aHDFs were deprived from FBS as described above (Cell treatment and UVA irradiation). Cells were then preincubated with the phosphodiesterase inhibitor isobutyl methylxanthine (Merck) at 0.1 mM for 30 min. Subsequently, cells were left unstimulated (negative control) or stimulated with α-MSH or FSK for 20 minutes. FSK served as positive control. Intracellular cAMP levels were measured by a commercially available enzyme immunoassay (Amersham Pharmacia Biotech, Freiburg, Germany).

MC1R gene sequencing

Sequencing of *MC1R* was performed using the Taqman SNP genotyping assay (ThermoFisher). Each *MC1R* SNP was generated via the custom design tool. 15 ng DNA was used for the analysis including the major diminished-function *MC1R* variants· p.V60L (rs1805005), p.D84E

(rs1805006), p.V92M (rs2228479), p.R142H (rs11547464), p.R151C (rs1805007), p.I155T (rs1110400), p.R160W (rs1805008), p.R163Q (rs885479) [26,27] and p.F196L (s3212366). Variant receptors with reduced cell surface expression and impairment in cAMP coupling (loss-of-function) are: V60L, D84E, R151C, I155T, R160W, R163Q [31]. Analyses of *MC1R* variants were performed according to the manufacturer's protocol using the following RT-PCR program: 1 × 95˚C/10 min; 40 × 92˚C/15 sec; 60˚C/60 sec; 4˚C/ cool down.

Luminex Multiplex ELISA

aHDFs were cultivated and treated as described. After 6 h and 24 h following UVA irradiation, cell culture supernatants were collected and stored at -80°C until analysis. Luminex multiplex-ELISAs of IL-6 and IL-8 performed with undiluted supernatants (each sample in duplicates) using the Bio-Plex 200 system with HTF (#171000205, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturers' protocol.

Statistical analysis

All experiments were performed at least three times. Expression levels were calculated as means ± SD, and deviations from normality assessed using the Shapiro-Wilk test. Statistically significant differences between mean values were determined using the Mann-Whitney U-test, Kolmogorov-Smirnov test, or one-way ANOVA. Differences were considered as significant when $p<0.05$.

RESULTS

Effects of α-MSH and related peptides on UVA-induced generation of ROS in 2D cell cultures of aHDFs

We first examined if α-MSH can suppress UVA-mediated generation of ROS in 2D cultures of dermal fibroblasts. We specifically used primary adult human dermal fibroblasts (aHDFs), as many previous studies relied on neonatal, foreskin-derived, or unspecified dermal fibroblasts [32-35]. Using primary adult human fibroblasts is crucial because photoaging is an age-related phenomenon, and adult cells more accurately reflect the cellular and molecular context of dermal photoaging *in vivo*. Neonatal fibroblasts may differ in oxidative stress responses, senescence pathways, and MC1R signalling, which could limit the relevance of prior findings to adult skin. To exclude *MC1R* mutations that would mask biological effects of α-MSH all primary cells were genotyped for the most common *MC1R* variant alleles (Fig. 1A).

Accordingly, all subsequent experiments were performed using primary cells without detectable *MC1R* polymorphic alleles, derived from eight independent donors (n=8). Serum starvation (< 1% FBS) was essential for UVA-induced ROS generation in the cells. Under these conditions, a physiological UVA dose (10 J/cm^2) boosted intracellular ROS, whereas co-treatment with the antioxidant vitamin C (10 µM) blocked the increase (Fig. 1B). In contrast, α-MSH (10^{-6} M, applied under the same conditions as vitamin C, did not suppress UVA-induced ROS (Fig. 1B). Variation of the incubation protocol - use of a shorter preincubation period (1 h instead of 24 h) or application of lower α-MSH doses that mimic peripheral blood concentrations (10^{-8} and 10^{-10} M; n=3) - likewise failed to attenuate the pro-oxidative effects of UVA (Supplementary Fig. 1). We next tested whether other POMC-derived peptides could modulate ROS production in aHDFs. These included (i) NDP-α-MSH, a superpotent analogue that is clinically approved for the treatment of erythropoietic protoporphyria [36], (ii) KdPT, a C-terminal tripeptide derivative of α-MSH with preserved anti-inflammatory activity but without MC1R binding [37], and (iii) β-endorphin (β-ED), that typically reduces intracellular cAMP. None of these peptides affected UVA-induced ROS generation (Fig. 1B; Supplementary Fig. 2). Incubation of aHDFs with α-MSH, KdPT, or β-ED *per se* did not alter basal ROS levels, whereas vitamin C slightly but not significantly decreased intracellular ROS (Fig. 1C). Collectively, these data indicate that intracellular ROS induction – a central signalling event of the UVA-mediated cellular stress response and a key driver of dermal photoaging – is not modulated by α-MSH or related peptides. In line with this, UVA exposure rapidly increases phosphorylation of p38, a stress-responsive kinase. This UVA response was clearly detectable 10 and 30 min post-irradiation (Fig. 1D) and declined almost to basal level within 60 min (data not shown). Treatment with α-MSH did not alter this stress response.

We further examined as to whether UVA irradiation under the given conditions can induce other markers of oxidative stress in 2D cell cultures of aHDFs. Measuring malondialdehyde as marker of lipid peroxidation using a commercial ELISA and lysates of up to 10^6 cells did show any signal. In addition, and in accordance with earlier reports from others [38], UVA irradiation failed to generate γH2AX as an indicator of DNA double-strand breaks (data not shown). We did not examine 8-oxo-deoxyguanosine since recent findings on dermal fibroblasts irradiated with UVA even three times a day for 4 consecutive days likewise did not reveal any induction of this oxidative DNA marker [39].

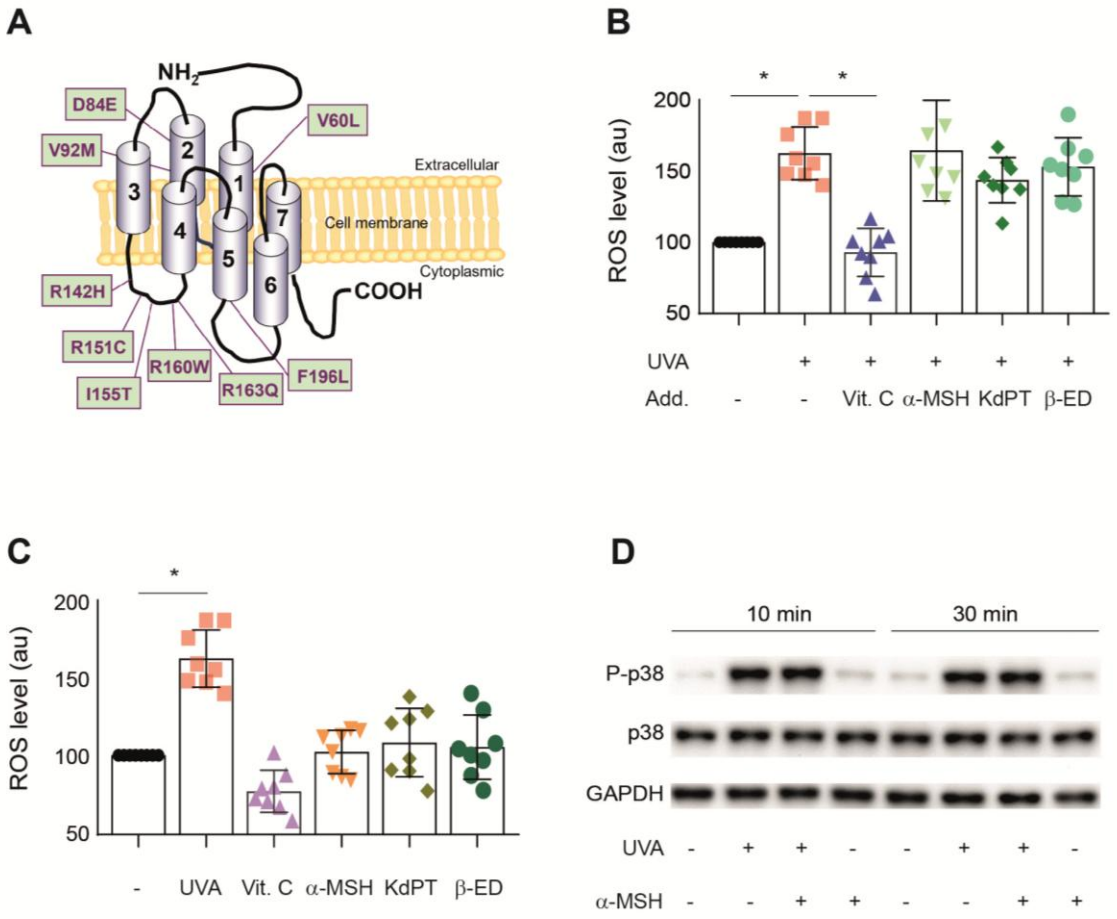

Figure 1. Impact of UVA, α-MSH and related peptides on UVA-mediated intracellular ROS generation and phosphorylation of p38 in 2D cell culture of aHDFs. (A) Examined and excluded *MC1R* mutations of our study. **(B)** Effect of UVA irradiation (10 J/cm^2) alone and in combination with α-MSH (10^{-6} M), KdPT (10^{-6} M), β-ED (10^{-6} M), and vitamin C (Vit. C; 10 µM). ROS was measured by FACS analysis using the redox-sensitive fluoroprobe CM-H_2DCFDA. n=8 independent donors; *p<0.01, evaluated by one-way ANOVA **(C)** Impact of the above peptides *per se* (each 10^{-6} M; n=8 independent donors) on UVA-induced ROS generation; *p<0.01, evaluated by one-way ANOVA. **(D)** Protein phosphorylation of p38 in response to UVA, α-MSH, and in combination, shown as a representative Western immunoblot of n=5 independent donors.

Impact of α-MSH on UVA-induced DDR and SASP gene expression in 2D aHDF cultures

We next investigated whether α-MSH could modulate UVA-induced expression of DDR and SASP genes in aHDFs (n=8). The panel included DDR-related genes (p16, p21, p53), the oxidative stress marker HO-1 [40], and typical markers of the SASP, i. e., IL-6, IL-8, MMP1 and MMP3 [5,6].

Quantitative real-time PCR revealed a significant time-dependent induction of HO-1 (2h: 19.2±7.5; 6h: 27.9±13.5), p21 (1h: 1.8±0.4; 2h: 2.7±0.6; 6h: 2±0.5; 24h: 1.9±0.3), IL-6 (0.5h: 1.5±0.5; 1h: 2.7±0.6; 2h: 2.6±1.6), and IL-8 (0.5h: 2±0.6; 1h: 6±3.6; 2h: 5.1±4) mRNA after UVA irradiation (10 J/cm^2) compared to sham-irradiated controls shown as n-fold induction ± means (Fig. 2A-D). HO-1 was the most strongly upregulated transcript and showed an almost 40-fold induction (Fig. 2A). In contrast, no consistent changes were observed for p16, p53, MMP1, and MMP3 (Supplementary Fig. 3A-D).

Treatment with α-MSH (10^{-6} M) under these conditions failed to modify any of these UVA-induced gene responses. α-MSH alone likewise did not cause significant transcriptional changes (Fig. 2A-D; Supplementary Fig. 3A-D). Lower, physiologically relevant concentrations of α-MSH (10^{-8} and 10^{-10} M; n=5) and Vit. C showed no effect (Supplementary Fig. 4A-D). Notably, although vitamin C effectively suppressed UVA-induced ROS generation (Fig. 1B and C), it did not attenuate UVA-driven induction of HO-1, p21, IL-6, and IL-8 gene expression (Supplementary Fig. 4A-D). We have not examined as to whether vitamin C affected the UVA-induced increase in HO-1 and p21 protein as it did not alter the mRNA levels of these genes.

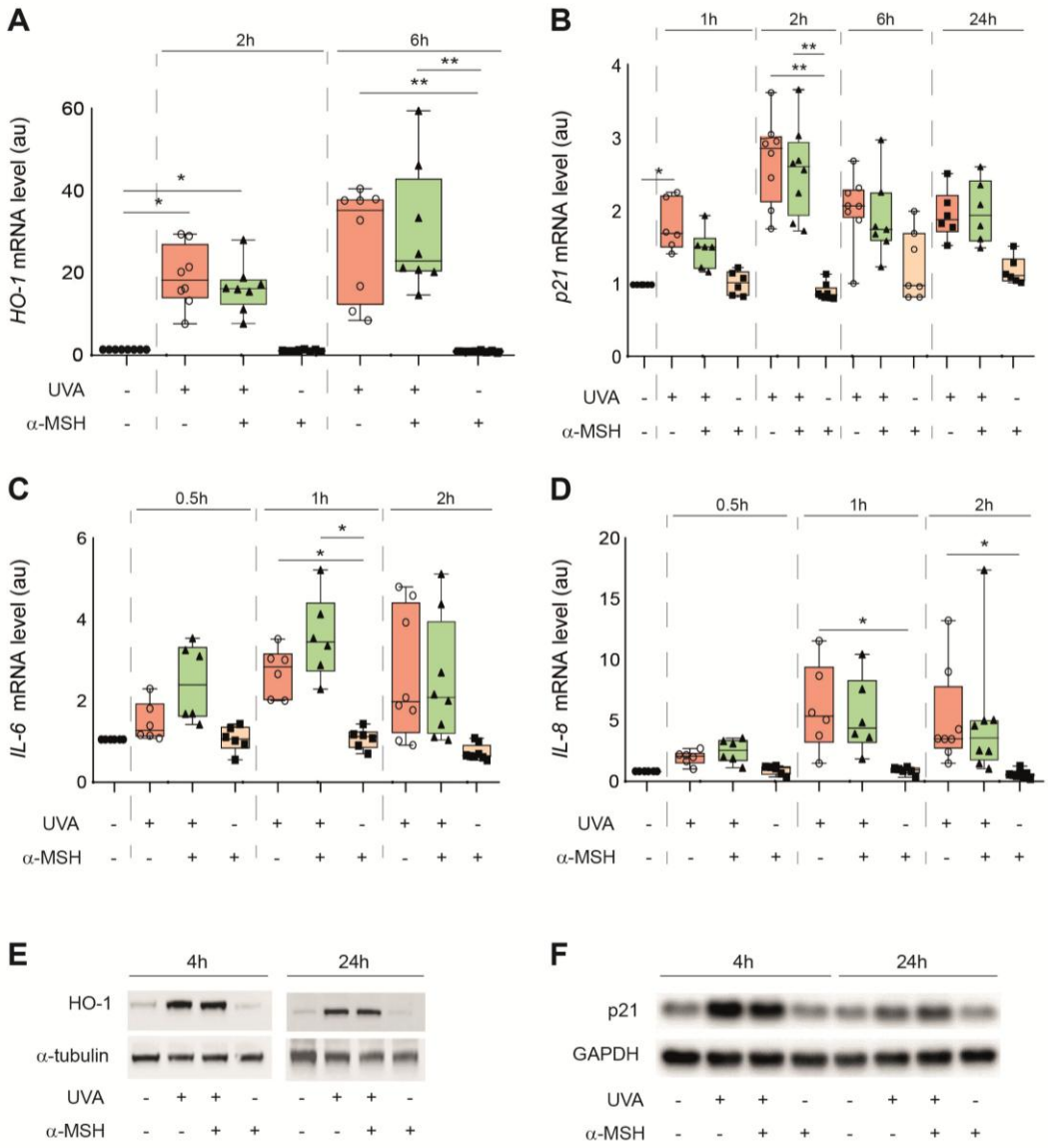

Figure 2. Time-dependent effects of UVA and α-MSH on gene expression in 2D cell cultures of aHDFs. Cells (n=8 independent donors) were treated with UVA (10 J/cm^2), α-MSH (10^{-6} M) or UVA plus α-MSH. Sham-irradiated cells served as controls. mRNA levels of HO-1 **(A)**, p21 **(B),** IL-6 **(C)** and IL-8 **(D)** at different time points after treatment were analysed by real-time RT-PCR analysis; *p<0.05 and **p<0.01, evaluated by one-way ANOVA. **(E,F)** Time-dependent protein analysis of HO-1 **(E)** and p21 **(F)** in cell lysates from aHDFs treated as described before. Protein expression of GAPDH and α-tubulin served as loading controls. Representative blots from at least n=3 independent donors are shown.

At the protein level, Western immunoblotting confirmed the strong, time-dependent induction of HO-1 and p21 at 4 h and 24 h post-irradiation, consistent with the mRNA results. Again, α-MSH treatment did not alter these effects (Fig. 2E, F). In line with the transcriptional data, p53 protein levels were not consistently modulated by UVA or α-MSH under the serum-deprived conditions used to enhance ROS generation (data not shown). We also examined by ELISA the secreted amounts of IL-6 and IL-8 in culture supernatants from aHDFs treated with UVA in presence and absence of α-MSH. There was a marked interindividual variation in the basal levels of IL-6 (18.39-1532.04 pg/ml) and IL-8 (15.8-3808,97 pg/ml) among the n=8 independent donors. UVA led to increased levels of IL-6 but (not IL-8) 6 and 24 h post-irradiation (Supplementary Fig. 5A, B). However, α-MSH did not alter this UVA response but increased *per se* the secreted amounts of IL-6.

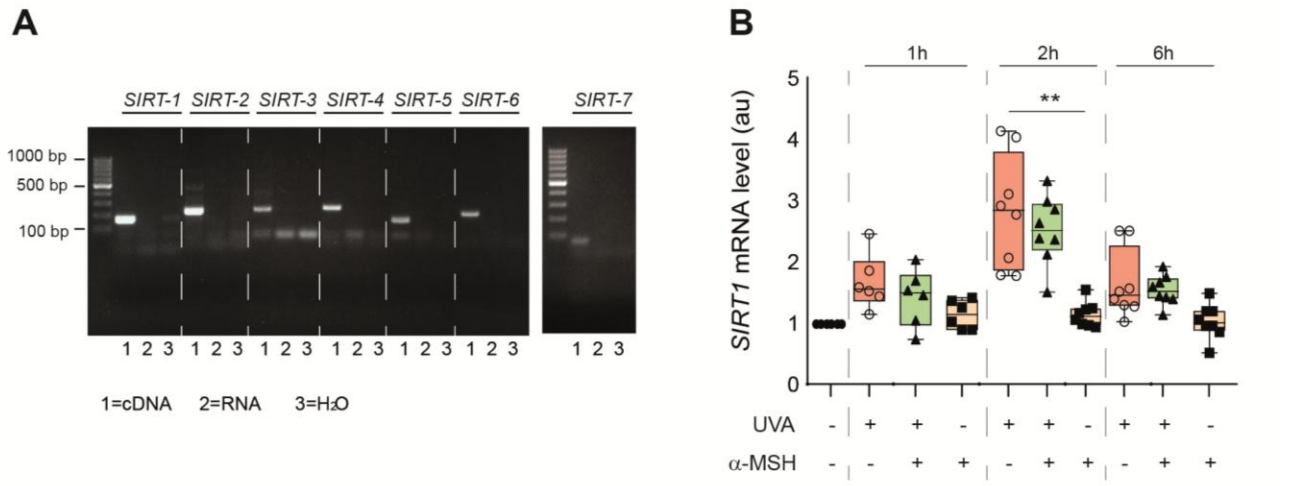

Figure 3. Expression pattern of sirtuins in 2D cell cultures of aHDFs and the impact of UVA and α-MSH on SIRT1 expression. **(A)** Endpoint-RT-PCR of SIRT1-7 expression analysis. A representative set derived from n=3 independent donors is depicted. H_2O (lane 2) and RNA of aHDFs (lane 3) served as negative and contamination controls for each detected amplicon of aHDFs (lane 1) **(B)** Time-dependent effect of UVA (10 J/cm^2), α-MSH (10^{-6} M), and combined treatment on SIRT1 mRNA expression in aHDFs (n=8 independent donors), determined by real-time RT-PCR. **p<0.01, evaluated by one-way ANOVA.

Together, our results demonstrate that UVA activates oxidative stress and a number of SASP-associated gene programs in aHDFs, whereas α-MSH does not modulate these responses.

Sirtuin expression in aHDFs: Effects of UVA and α-MSH

Mitochondria are both a major source and a primary target of UVA-induced ROS, linking oxidative stress directly to mitochondrial function. We therefore examined the expression and UVA responsiveness of Sirtuins (SIRTs), a family of mitochondrial proteins that regulate oxidative stress, metabolic homeostasis, and aging [41]. Given that mitochondrial dysfunction and ROS-driven signalling are central to dermal photoaging, understanding how SIRTs are expressed and regulated in aHDFs is particularly relevant. However, their role in dermal photoaging has remained until now poorly understood.

Endpoint-RT-PCR analysis showed that all seven SIRT members were detectable in aHDFs, though at various levels (Fig. 3A). Considering the amplification efficiency and amplicon size, SIRT1 and SIRT2 appear to be the most abundantly expressed Sirtuins in aHDFs. As shown by quantitative real-time PCR, UVA irradiation (10 J/cm^2) led to a time-dependent increase in SIRT1 transcripts, whereas none of the other SIRT members showed consistent regulation (Fig. 3B; Supplementary Fig. 6A-F). Treatment with α-MSH did not alter the basal expression of SIRTs or the UVA-induced upregulation of SIRT1 (Fig. 3B; Supplementary Fig. 6A-F).

Attempts to confirm this transcriptional change at the protein level were inconclusive, as none of the commercially available SIRT1 antibodies yielded specific or reproducible results. Taken together, these findings indicate that all SIRT members are expressed in aHDFs, but only SIRT1 is transcriptionally responsive to UVA irradiation, suggesting a possible role of this mitochondrial regulator in the fibroblast stress response associated with dermal photoaging. Importantly, this pathway does not appear to be influenced by α-MSH under the tested conditions.

MC1R expression and functional coupling in aHDFs

Since α-MSH did not alter UVA-mediated ROS generation, DDR signalling, or SASP-related responses in aHDFs, it was essential to prove whether MC1R is indeed expressed and functionally active in these cells. To address this, we compared aHDFs with normal human melanocytes, which are known to express the highest levels of MC1R in skin, and with HBL cells, a human melanoma cell line expressing wild-type and functional MC1R [42]. MC1R transcript levels were markedly lower in aHDFs compared with normal human melanocytes or HBL cells (n= 5 independent donors for aHDFs; mean ct values of ≈24, ≈18, and 19, respectively) (Fig. 4A). Consistently, MC1R protein levels were also lower in aHDFs but clearly present, as shown by Western immunoblotting of whole cell lysates (Fig. 4B). Importantly, α-MSH (10^{-6} M) stimulation significantly

increased intracellular cAMP levels in aHDFs compared to untreated controls (Fig. 4C). FSK, an adenylate cyclase activator, induced an even stronger response (Fig. 4C). Together, these data demonstrate that although MC1R expression is significantly lower in aHDFs than in melanocytes or melanoma cells, it remains present and functionally coupled to the canonical cAMP pathway.

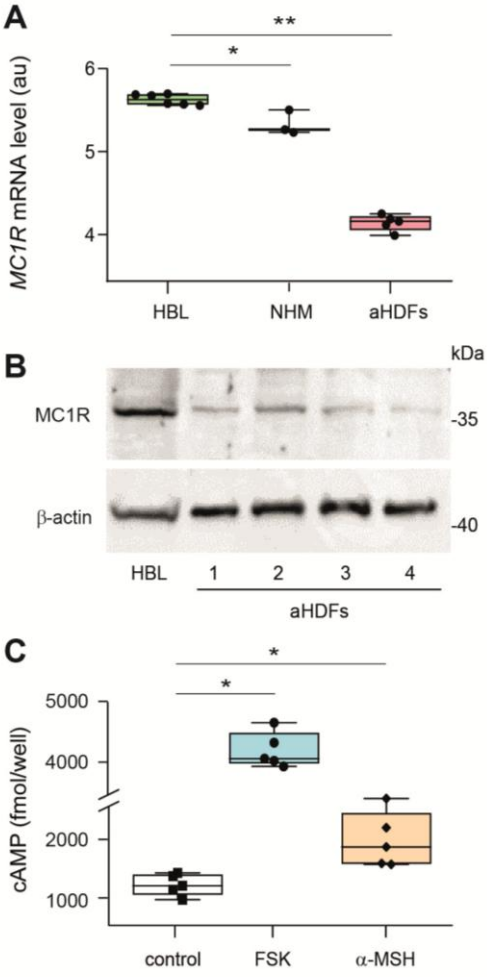

Figure 4. Detection of MC1R in aHDFs and functional coupling shown by cAMP induction. **(A)** Comparative mRNA expression of MC1R in aHDFs (n=5 independent donors) normal human melanocytes (NHMs, n=3 independent donors) and HBL melanoma cells (n=5 biological replicates), determined by real-time RT-PCR. Depicted are normalized ct values; *p<0.05 and **p<0.001, evaluated by the Mann-Whitney test. **(B)** Protein expression of MC1R in aHDFs (n=4 independent donors). Lysates from wild-type and functional MC1R-expressing HBL melanoma cells served as positive control. **(C)** Impact of α-MSH and FSK on intracellular cAMP levels. aHDFs (n=5 independent donors) were treated with FSK (1 µM) or α-MSH (10^{-6} M) for 20 min followed by determination of cAMP in cell lysates by ELISA; *p<0.01, evaluated by the Mann-Whitney test.

Pharmacological induction of canonical cAMP signalling fails to suppress UVA-induced ROS and DDR / SASP gene expression in aHDFs

The absence of a protective effect of the α-MSH/MC1R axis on UVA-induced ROS generation and stress-related gene expression prompted us to formulate a fundamental question: *could canonical cAMP signalling itself, when pharmacologically stimulated, provide UVA protection?* This was of particular interest given that FSK, a direct activator of adenylate cyclase, has been proposed as a topical drug candidate for UV protection based on its ability to mimic MC1R-mediated tanning response [43]. Moreover, many endogenous mediators, including catecholamines, opioids, melatonin and oxytocin, exert their biological effects by modulation of cAMP signalling, underscoring the far-reaching importance of this pathway. To address this, we treated aHDFs with FSK and examined its ability to counteract UVA-mediated oxidative stress and induction of DDR- and SASP-associated genes. FSK did not suppress UVA-induced ROS production (Fig. 5A)

On the contrary, FSK further increased the mRNA levels of SIRT1 and IL-6 compared to UVA alone. Real-time RT-PCR analysis further confirmed that FSK did not reduce the UVA-induced upregulation of p21, HO-1 and IL-8 mRNA in 2D cultures of aHDFs (n=6 independent donors). Importantly, FSK alone had no measurable effect on basal expression of these genes (Fig. 5B-F).

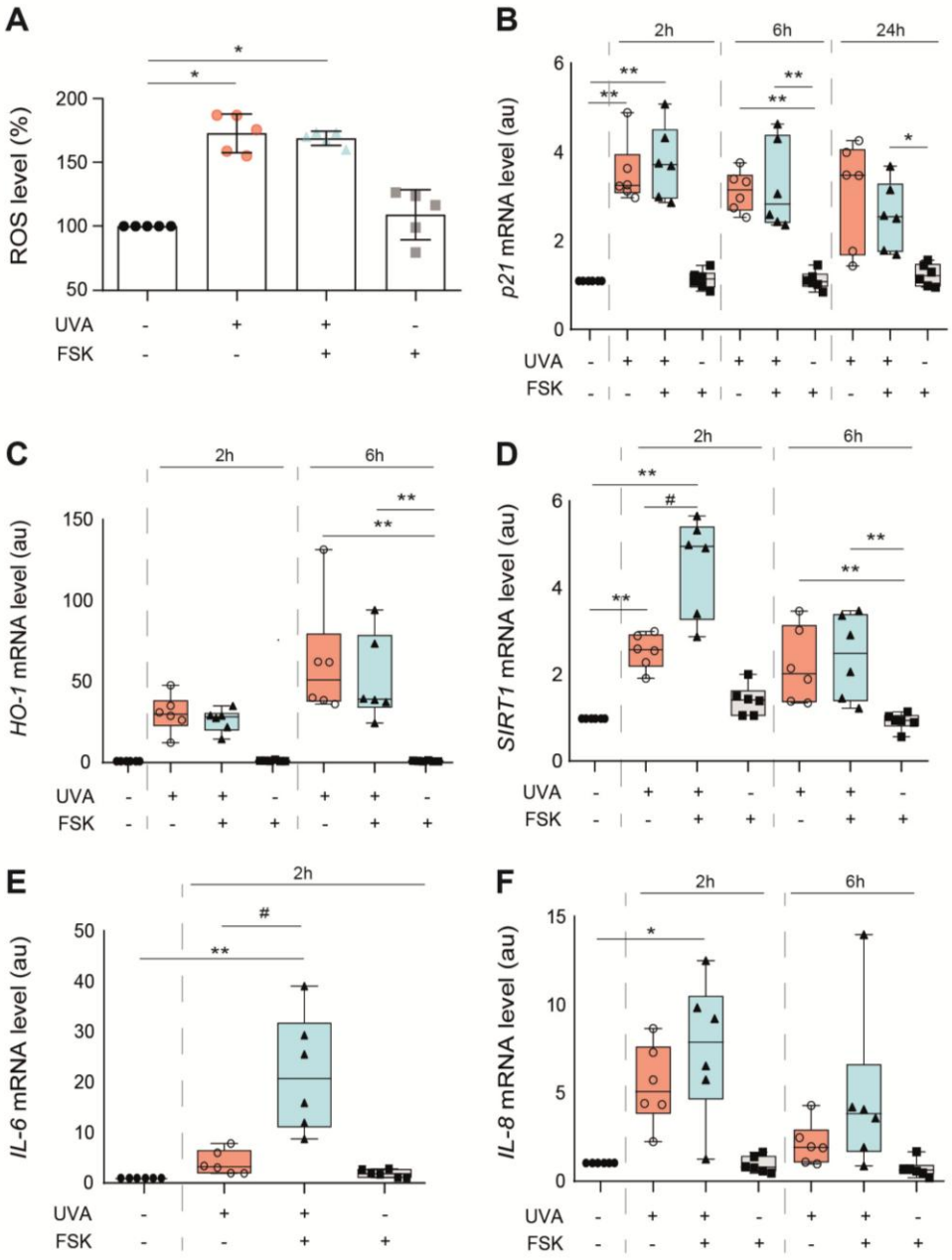

Figure 5. Impact of the artificial cAMP inducer FSK on UVA-mediated stress responses in 2D cell cultures of aHDFs. (A) Effect of UVA and FSK on intracellular ROS levels (n=5 independent donors), determined by FACS analysis using the redox-sensitive fluoroprobe CM-H_2DCFDA. As with α-MSH treatment, cells were pre-treated with FSK (1 µM) followed by UVA irradiation (10 J/cm^2); *p<0.001, evaluated by one-way ANOVA. **(B-F)** Time-dependent effects of FSK (1 µM) on UVA-mediated mRNA expression of p21 **(B)**, HO-1 **(C),** SIRT1 **(D)**, IL-6 **(E)** and IL-8 **(F)** in aHDFs (n=6 independent donors), determined by real-time RT-PCR; *p<0.05; **p<0.01; $^{\#}$p<0.05, evaluated by one-way ANOVA.

Impact of α-MSH and UVA on 3D spheroid cultures of aHDFs

In a final set of experiments, we validated our observations from 2D by using a more physiologically relevant model: 3D spheroid cultures of aHDFs [30]. Spheroids of 6000 cells were generated with methylcellulose as a carrier (Fig. 6A), and then subjected to serum deprivation, α-MSH treatment (10^{-6} M), and UVA irradiation (10 J/cm^2) under conditions corresponding to those used in 2D cell cultures. UVA irradiation of 3D spheroids resulted in a robust induction

of SIRT1, HO-1, and p21 as well as MMP1 and MMP3 mRNA (Fig. 6B-F). However, consistent with our findings in 2D cultures, α-MSH (10^{-6} M) neither modified these UVA-induced gene responses nor altered baseline expression when applied alone. Taken together, these findings are consistent with and further support our observations in the 2D cell culture model described above. However, we did not directly verify whether the MC1R-cAMP pathway remains fully functional in 3D spheroid cultures, *e.g.,* by measuring cAMP induction or the activation of downstream protein kinase A (PKA) targets.

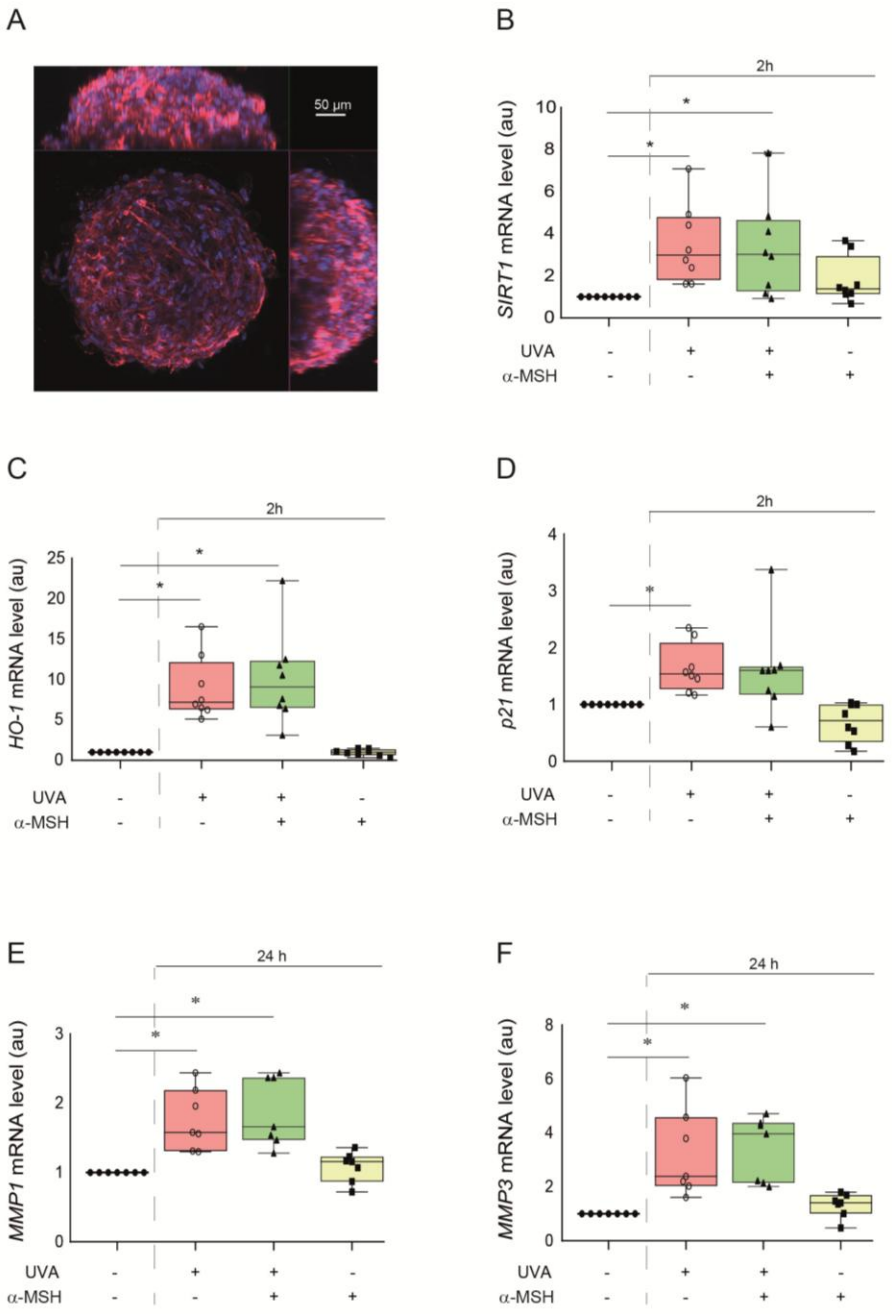

Figure 6. Impact of UVA and α-MSH on gene expression in 3D spheroid cultures of aHDFs. (A) Representative orthogonal view of a typical spheroid formed from 6000 aHDFs, followed by staining with wheat germ agglutinin (WGA)-TRITC (red) and Hoechst-33342 (blue). **(B-F)** Effects of α-MSH on UVA-mediated mRNA expression of SIRT1 **(B)**, HO-1 **(C),** p21 **(D),** MMP1 **(E)** and MMP3 **(F).** Spheroids made from aHDFs from n=7 independent donors were treated with UVA (10 J/cm^2), α-MSH (10^{-6} M), both stimuli and harvested at indicated time points for real-time RT-PCR analysis; *p<0.05, evaluated by one-way ANOVA.

DISCUSSION

In this work, we addressed the question of whether the α-MSH-MC1R-cAMP axis in aHDFs protects against UVA-induced stress responses, which - when repetitively induced in human skin – leads to dermal photoaging. Our findings based on an experimental setting in which aHDFs were treated with a single UVA exposure and treatment with α-MSH at different dosages in 2D and 3D models do not support this hypothesis. UVA-mediated generation of ROS was readily supressed by the antioxidant vitamin C but not by α-MSH (or related peptides). In this context, it is of interest to note that even a *direct* antioxidative capacity of α-MSH owing to oxidation of methionine residues within the amino acid sequence of this peptide has been postulated [44]. However, our data do not demonstrate such an effect of α-MSH when cells were stressed by UVA to induce intracellular ROS production. Importantly, expression of the MC1R was detectable in aHDFs. Treatment with α-MSH induced canonical cAMP signalling confirming functional coupling even at low MC1R protein levels in accordance with previous data on foreskin-derived neonatal HDFs [45]. Stimulation of these cells with FSK revealed that canonical cAMP signalling in aHDFs was intact - in full accordance with our data on α-MSH - and likewise neither reduced UVA-mediated generation of ROS nor induction of genes related to the UVA-induced stress, DDR and SASP. In support of this, treatment with β-ED, which signals via Gi, thereby reducing intracellular cAMP levels also did not alter UVA-mediated generation of ROS. Collectively, these data suggest that canonical cAMP signalling is not a key effector pathway suppressing UVA-induced responses, at least in aHDFs. To the best of our knowledge, we are not aware of any report indicating a natural hormone or compound inducing canonical cAMP signalling to be protective against UVA-induced stress in aHDFs. Interestingly, it was reported some time ago that α-MSH can reduce UVA-induced generation of ROS in HaCaT cells stably transfected with wild-type and functional *MC1R* (HaCaT-MC1R) [46]. In cells transfected with the Arg151Cys variant of *MC1R* (HaCaT-R(151)C), α-MSH failed to reduce UVA-induced ROS. PKA-dependent phosphorylation of the subunit A of NADPH oxidase (NoxA), was increased in HaCaT-MC1R cells compared to HaCaT and HaCaT-R(151)C cells. Moreover, pharmacological inhibition of PKA in HaCaT-MC1R cells led to a marked increase in ROS production after UVA irradiation. The discrepancies between our findings and those reported by Henri et al. [46] may reflect cell type-specific differences between aHDFs and the HaCaT cell line, *e.g.,* variation in the expression pattern of the NADPH oxidase (Nox) system [47] or in adenylyl cyclase isoforms.

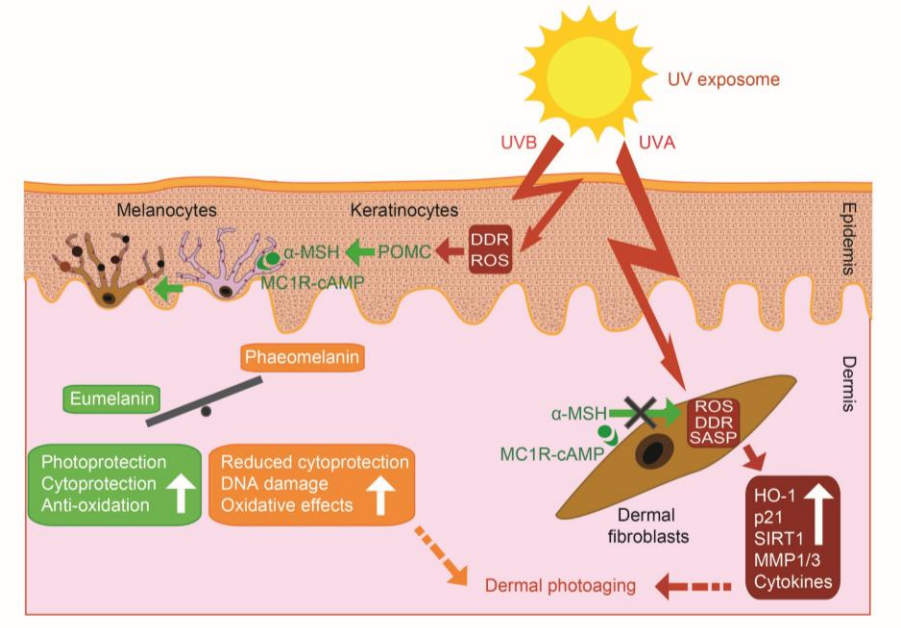

Figure 7. Model of our working hypothesis of the UVA/B light-induced stress response of the skin, the role of α-MSH-MC1R-cAMP signalling and its proposed impact on dermal photoaging. The scheme illustrates the impact of UVB and UVA irradiation (red colour) on the epidermal (left part) and dermal compartment (right part) of the skin with its key cell types. The modulatory effects of the α-MSH-MC1R-cAMP axis with its established protective effects and those examined in this work (green colour) is depicted.

The experimental focus of this work was not on aHDFs with defective α-MSH-MC1R signalling. Preliminary data from an exploratory set of two independent donors with MC1R loss-of-function (LOF) alleles revealed that UVA-mediated generation of intracellular ROS is not affected by α-MSH, e. g. in aHDFs with genetically confirmed R151C and R160W homozygosity. In both 2D cell cultures, UVA led to intracellular ROS generation, but α-MSH likewise did not alter this response (Supplementary Fig. 7A, B). In accordance with this UVA-induced upregulation of p21 and HO-1 was not affected by α-MSH (data not shown). Further studies are needed to confirm these findings in larger cohorts of aHDFs from donors with additional MC1R LOF alleles.

Our findings point towards an overarching role of the α-MSH-MC1R-cAMP axis within the epidermal compartment during photoaging. According to this working hypothesis (Fig. 7), a shift of eumelanin towards the pro-oxidative pheomelanin in individuals with loss-of-function *MC1R* alleles would result in increased ROS generation in response to UVB exposure. UVB-induced pheomelanin-derived ROS, including freely diffusible H_2O_2, would be generated within the epidermal compartment and elicit cellular stress responses in aHDFs, thereby promoting dermal photoaging. However, the possibility of protective effects of the α-MSH-MC1R-cAMP axis and cAMP-stimulating agents in individuals with hypomorphic MC1R alleles, e. g. p.D294, cannot be dismissed.

In this context, it is interesting to note that different antioxidative enzyme activities have been detected for peroxidase, catalase, superoxide dismutase, and glutathione peroxidase in melanocytes, keratinocytes, and dermal fibroblasts [48]. α-MSH has been demonstrated to counteract the suppressive effect of UVB irradiation on catalase expression in melanocytes [24]. Moreover, in both melanocytes and keratinocytes, α-MSH counteracted UVB-induced suppression of Nrf2-regulated genes that exert important antioxidative effects. These genes include HO-1, gamma-glutamate-cysteine synthetase, and glutathione-S-transferase P^i [25]. All of these α-MSH-regulated enzymes would create an antioxidative microenvironment within the epidermal compartment if MC1R is functional (Fig. 7). Thus, in individuals with dysfunctional MC1R alleles, impaired α-MSH-MC1R signalling would lead to increased oxidative stress within in the UVB-induced epidermis which is further exacerbated by increased concentrations of pro-oxidative pheomelanin.

In this context it is important to emphasize the limitation of antioxidants such as vitamin C as modulators of UV-mediated cellular responses. Albeit vitamin C was found to suppress UVA-mediated generation of intracellular ROS in aHDFs other read-outs related to the UVA-induced SASP were not altered by this oxidant. It is thus possible that these genes are regulated by UVA independently of ROS. Exposure to certain metals and natural compounds has been shown to induce HO-1 through ROS-independent mechanisms [49-51].

With regard to UVB-induced melanogenesis it further noteworthy to mention that melanin synthesis is an oxygen-consuming process in which antioxidations can inhibit melanogenesis. Thus, application of antioxidants such as vitamin C as the only strategy to prevent photoaging must be seen with caution.

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Limitations

In this study, we used primary cells which were characterized by a SNP genotyping for the most common allelic variants of *MC1R.* Among the wide range of detected variants, hypomorphic alleles (*e.g.,* p.D294H) were also identified. Such alleles may result in partial loss of function [52]**.** To detect such "leaky" mutations, complete sequencing of the *MC1R* gene would have been required in all primary cells we used in our study.

The findings of our work are limited to dermal fibroblasts. Consequently, the contributions of keratinocytes and melanocytes, particularly melanocytes, which express the highest levels of MC1Rs among cutaneous cell types, were not evaluated. However, both cell types are not primary targets of the UVA spectrum but are mainly affected by the more genotoxic UVB wavelength spectrum of the sun.

It is also noteworthy that our study in 2D cell cultures and 3D spheroid cultures of aHDFs has examined only a limited number of SASP-related genes at the RNA and protein levels.

Our data on the impact of α-MSH in 3D spheroid cultures of aHDFs must further take into account that we have not addressed the question as to whether the MC1R-cAMP pathway is intact. This could be tested by analysing intracellular cAMP amounts or downstream targets of induced protein kinase A, e. g. phosphorylation of the cAMP-response-element binding protein.

The statistical analysis of our data was primarily performed using different unpaired tests. Although several figures include samples from the same donors across multiple treatments and time points, suggesting a paired experimental design, we applied unpaired analyses as a conservative approach to ensure robust detection of significant differences between treatments. To further validate our findings, we also reanalysed the results using paired statistics (repeated-measures ANOVA), which, in general, did not alter the statistical outcomes and the interpretation of the results.

Finally, our experimental approach does not include data derived from a potential in vivo model of UVA-induced photoaging to examine the impact of α-MSH-MC1R signalling in fibroblasts. Importantly, murine skin is fundamentally different from human skin with MC1R-expressing melanocytes primarily located in hair follicles but not in interfollicular epidermis. More importantly, MC1R has not been reported to be expressed in dermal murine fibroblasts thus making a murine model for our study not suitable. Creating a human skin organ culture, furthermore, has the limitation of bystander effects of any epidermal or dermal cell targeted by UV exposure making interpretations of direct effects of the α-MSH-MC1R-cAMP axis in dermal fibroblasts exposed to UVA irradiation very difficult.

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Author Contributions

Conceptualization: MB, AS. Data curation: AS. Formal analysis: AS. Funding acquisition: MB, SG, JE. Investigation: MB, AS. Methodology: AS, NS, AW, BO. SN, VR. Project administration: MB, AS. Supervision: MB, LL, KS. Visualization: MB, AS, SN. Writing – original draft: MB, AS. Writing – review and editing: all authors. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data and material availability

Data of this study are available from the corresponding author upon request.

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