Stimulation of Stem Cell Niches and Tissue Regeneration in Mouse Skin by Switchable Protoporphyrin IX-Dependent Photogeneration of ROS In Situ
--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60859R3
Full Title:	Stimulation of Stem Cell Niches and Tissue Regeneration in Mouse Skin by Switchable Protoporphyrin IX-Dependent Photogeneration of ROS In Situ
Section/Category:	JoVE Biology
Keywords:	Skin Regeneration; Burn Healing; hair follicle; Stem cells; Reactive Oxygen Species; Photodynamic Treatment; Protoporphyrin IX
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Madrid, Spain

1 **TITLE:**
2 **Stimulation of Stem Cell Niches and Tissue Regeneration in Mouse Skin by Switchable**
3 **Protoporphyrin IX-Dependent Photogeneration of Reactive Oxygen Species In Situ**
4
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29 **KEYWORDS:**
30 reactive oxygen species (ROS), protoporphyrin IX, photogeneration, mouse skin, stem cells,
31 tissue regeneration, hair follicle, burn healing
32

33 **SUMMARY:**
34 The aim of this protocol is to induce transient in vivo production of nonlethal levels of
35 reactive oxygen species (ROS) in mouse skin, further promoting physiological responses in
36 the tissue.
37
38 **ABSTRACT:**
39 Here, we describe a protocol to induce switchable in vivo photogeneration of endogenous
40 reactive oxygen species (ROS) in mouse skin. This transient production of ROS in situ
41 efficiently activates cell proliferation in stem cell niches and stimulates tissue regeneration
42 as strongly manifested through the acceleration of burn healing and hair follicle growth
43 processes. The protocol is based on a regulatable photodynamic treatment that treats the
44 tissue with precursors of the endogenous photosensitizer protoporphyrin IX and further

45   irradiates the tissue with red light under tightly controlled physicochemical parameters.
46   Overall, this protocol constitutes an interesting experimental tool to analyze ROS biology.

47

48   **INTRODUCTION:**
49   Reactive oxygen species (ROS) are the result of the chemical reduction of molecular oxygen
50   to form water, and include singlet oxygen, superoxide anion, hydrogen peroxide and the
51   hydroxyl radical[1–3]. ROS have a very short lifespan due to their extremely chemical reactive
52   nature. In aerobic organisms, ROS are incidentally formed inside the cells as a major leaky
53   by-product of aerobic respiration (electron transport chain) in the mitochondria. Transient
54   accumulation of high levels of ROS in the cell results in an oxidative stress condition that
55   may provoke the irreversible inactivation of proteins, lipids and sugars and the introduction
56   of mutations in the DNA molecule[2–5]. The gradual accumulation of oxidative damage in cells,
57   tissues and whole organisms steadily increases with time and has been associated with the
58   induction of cell death programs, several pathologies, and the ageing process[2–4,6].

59

60   Aerobic organisms have steadily evolved efficient molecular mechanisms to tackle excess
61   ROS accumulation in cells and tissues. These mechanisms include members of the
62   superoxide dismutase (SOD) protein family, which catalyze superoxide radical dismutation
63   into molecular oxygen and hydrogen peroxide, as well as different catalases and
64   peroxidases which use the antioxidant pool (glutathione, NADPH, peroxiredoxin,
65   thioredoxin[7,8]) to catalyze the subsequent conversion of hydrogen peroxide to water and
66   molecular oxygen.

67

68   However, several reports support the role of ROS as key components of molecular circuits
69   that regulate critical cell functions, including proliferation, differentiation and mobility[2–4].
70   This concept is further supported by the initial identification and characterization of
71   dedicated ROS-producing mechanisms in aerobic organisms, including lipoxygenases
72   cyclooxygenases and NADPH oxidases[9,10]. In this sense, ROS exhibit an active role during
73   vertebrate embryo development[11–13] and key roles for these molecules in the regulation of
74   specific in vivo physiological functions have been reported in different experimental
75   systems, including the differentiation program of hematopoietic progenitors in
76   *Drosophila*[14], healing induction in zebrafish, or tail regeneration in *Xenopus* tadpoles[15]. In
77   mammals, ROS have been involved in the self-renewal/differentiation potential of neural
78   stem cells in a neurosphere model[16] and in the deregulation of intestinal stem cell function
79   during colorectal cancer initiation[17]. In the skin, ROS signalling has been associated with
80   epidermal differentiation and the regulation of the skin stem cell niche and the hair follicle
81   growth cycle[18,19].

82

83   In this perspective, a major experimental limitation to determine the physiological roles of
84   ROS in biological systems, both in normal or pathological conditions, is the lack of adequate
85   experimental tools to induce controlled production of these molecules in cells and tissues,
86   accurately resembling their physiological production as second signalling messengers. At
87   present, most experimental approaches involve the administration of exogenous ROS,
88   mostly in the form of hydrogen peroxide. We have recently implemented an experimental

89 approach to switch on a transient, nonlethal in vivo production of endogenous ROS in the
90 mouse skin, based on the administration of precursors of the endogenous photosensitizer
91 protoporphyrin IX (PpIX; e.g., aminolaevulinic acid or its methyl derivative
92 methylaminolevulinate) and further irradiation of the sample with red light to induce the in
93 situ formation of ROS from intracellular molecular oxygen (**Figure 1**). This photodynamic
94 procedure may be efficiently used to stimulate resident stem cell niches, thus activating the
95 regenerative programs of the tissue[19,20] and opening the way for new therapeutic
96 modalities in skin regenerative medicine. Here, we present a detailed description of the
97 protocol, showing representative examples of stimulation of stem cell niches, measured as
98 an increase in the number of long-term 5-bromo-2'-deoxyuridine (BrdU) label retaining cells
99 (LRCs) in the bulge region of the hair follicle[19,21], and subsequent activation of regeneration
100 programs (acceleration of hair growth and burn healing processes) induced by transient,
101 nonlethal ROS production in the skin of C57Bl6 mouse strain.
102
103 **PROTOCOL:**
104
105 All mouse husbandry and experimental procedures must be conducted in compliance with
106 local, national, international legislation and guidelines on animal experimentation.
107
108 **1. Induction of hair growth, burn induction and identification of long-term BrdU LRCs in**
109 **the tail skin epithelium wholemounts**
110
111 NOTE: Use 10-day or 7-week old C57BL/6 mice, preferably littermates, for the experimental
112 designs described below. In all the experimental procedures, animals will be anesthetized
113 by 3% isoflurane inhalation or euthanized by cervical dislocation as indicated.
114
115 1.1. Induction of hair growth in the back skin of mice in the second telogen (resting) phase
116 (about day 50 post-natal)
117
118 1.1.1. Anesthetize mice with inhalation of 3% isoflurane. Confirm full deep anaesthesia by
119 lack of a pedal reflex (firm toe pinch). Shave two independent side by side regions of the
120 back skin in each single mouse using hair clippers and depilatory cream (**Table of Materials**).
121 Use the left side for the control and the right side for treatment.
122
123 NOTE: Check that, after shaving, the subjacent back skin is pinkish and not grey/black, an
124 indicator of melanogenesis and entrance into the anagen (growing) phase in this mouse
125 strain.
126
127 1.1.2. Wash thoroughly with PBS to remove all cream remains and proceed to induction of
128 transient in situ production of nonlethal ROS levels as described in section 2.1.
129
130 1.1.3. Record hair follicle growth through daily acquisition of high-resolution images of the
131 control and treated back skin areas in each animal (e.g., using an HD camera coupled to a
132 5–20x binocular lens).

1.2. Induction of 2nd degree burn lesions in the back skin of mice in the second telogen (resting) phase (about day 50 post-natal)

1.2.1. Anesthetize mice. Shave the whole back skin region in each single mouse using hair clippers and depilatory cream and wash thoroughly with PBS to remove all cream remains.

1.2.2. Preheat a brass bar (1 cm in cross-section) at 95 °C by immersing in boiling water and then apply on the central region of the dorsal back skin surface of each mouse for 5 s.

1.2.3. Just after burn generation, intraperitoneally inject the animals with 1 mL of physiological solution (0.9% NaCl) on an electric blanket to prevent dehydration. Let the animals recover for 24 h and proceed to induction of transient in situ production of nonlethal ROS levels as described in section 2.3.

1.2.4. Record burn wound progression through daily acquisition of high-resolution images of the control and treated back skin areas in each animal (e.g., using an HD camera coupled to a 5–20x binocular lens).

1.3. Generation and identification of long-term BrdU LRCs in the tail skin epithelium

1.3.1. Inject 10/14-day old littermates intraperitoneally (no anaesthesia) once a day for 4 consecutive days with 50 mg/kg bodyweight BrdU dissolved in PBS. After the labelling phase, allow mice to grow for 50–60 days before any treatment.

1.3.2. Proceed as described in section 2.3 for the induction of transient in situ nonlethal ROS production in the tail skin at different times before the preparation of tissue wholemounts.

1.3.3. To prepare wholemounts of tail epidermis, euthanize mice by cervical dislocation and clip the tails with surgical scissors.

1.3.3.1. Use a scalpel to make a straight longitudinal incision all along the tail and peel the whole skin as a single piece from the backbone. Incubate the peeled skin in 5 mM EDTA in PBS in 5 mL tubes for 4 h at 37 °C and carefully separate intact sheets of epidermis from the dermis using forceps.

1.3.3.2. Fix the tissue in 4% formaldehyde in PBS for at least 72 h at room temperature (RT) and proceed for BrdU detection using appropriate antibodies.

1.3.3.3. Use fluorescence/confocal microscopy to identify and quantify LRCs in each experimental condition, including light controls and photodynamic treatments at different times before the preparation of tissue wholemounts, as previously described in detail[19,20].

NOTE: Fixed epidermal sheets may be stored in PBS containing 0.02% sodium azide at 4 °C

for up to three months. Fixed epidermal sheets may be used for immunolocalization of required proteins following standard histological sections procedures.

2. Induction of transient production of nonlethal ROS levels in mouse skin

NOTE: To induce transient production of nonlethal ROS levels in mouse skin, a photodynamic treatment using a precursor of the endogenous photosensitizer PpIX, in this case, methyl-aminolevulinate (mALA), and red light will be used.

2.1. To switch on transient ROS production for the induction of hair growth in the back skin, prepare the animals as indicated in section 1.1.

2.1.1. Apply ~25 mg of mALA in the form of topical cream (**Table of Materials**) on the right region, keeping the left side as an internal control avoiding inter-individual differences. Incubate for 2.5 h in the dark, wash off the excess cream thoroughly with PBS.

NOTE: The production of PpIX in the back skin should be tested in situ by its red fluorescence under blue light (407 nm) excitation.

2.1.2. Anesthetize the animals.

2.1.3. Irradiate the whole back skin with an adequate red-light source (**Table of Materials**) for a total dose of 2.5–4 J/cm^2. Keep mice on an electric blanket until their complete recovery and proceed as described in step 1.1.3.

NOTE: Irradiance should be adjusted by manipulating the distance between the light source and the tissue and measured using a power energy meter (**Table of Materials**). The experiment is considered finished when full hair growth is observed in any of the independent shaved regions of each animal. The whole procedure involves just a single photo treatment.

2.2. To switch on transient ROS production for healing improvement of 2nd degree burn lesions, prepare the animals as indicated in section 1.2.

2.2.1. Apply ~25 mg of mALA in the form of topical cream all along the burned surface, encompassing about 4 mm of adjacent tissue. Incubate for 2.5 h in the dark, wash off the excess cream thoroughly with PBS.

2.2.2. Anesthetize the animals.

2.2.3. Irradiate the whole back skin with an adequate red-light source for a total dose of 2.5–4 J/cm^2. Keep the mice on an electric blanket until their complete recovery and proceed as described in step 1.2.4.

221 NOTE: The experimental procedure for each animal is considered finished when full burn
222 healing is observed. The whole procedure involves just a single photo treatment.

223

224 2.3. To switch on transient ROS production in tail skin, prepare mice as indicated in section
225 1.3, apply ~25 mg of mALA in the form of topical cream all along the dorsal tissue area and
226 proceed as described in section 2.1 for back skin. Perform photo treatments and
227 correspondent light controls 24 h, 48 h, or 72 h before animal euthanasia and further
228 extraction of tail skin whole mounts.

229

230 NOTE: In all experimental designs, assay the ROS-dependence of the process by using
231 antioxidant ROS scavengers (e.g., daily inoculation of 100 mg/kg bodyweight N-acetyl-
232 cysteine by intraperitoneal injection of a 20 mg/mL solution in PBS, pH 7.2, starting 5 days
233 before mALA treatments or, alternatively, two doses of 100 mg/mL ascorbic acid in 50%
234 ethanol, spaced 30 min, topically applied on the skin in the time interval between the mALA
235 treatments and red-light irradiation).

236

237 **3. ROS detection in the skin**

238

239 3.1. Ex vivo evaluation of ROS production in tail skin after photodynamic treatment using
240 hydroethidine

241

242 NOTE: Hydroethidine is a non-fluorescent molecule that reacts specifically with ROS to
243 produce fluorescent dye 2-hydroxyethidium (hET).

244

245 3.1.1. Incubate whole tail skins, obtained as described in section 1.3.3, for 3 h at 37 °C in 5
246 mM EDTA in PBS solution (control samples) or additionally containing 2 mM mALA
247 (photodynamic treatment samples).

248

249 3.1.2. In all cases, add hydroethidine to a final concentration of 3.2 µM from a 25 mg/mL
250 stock in dimethyl sulfoxide (DMSO) and incubate for 1 h in the dark at RT.

251

252 3.1.3. Stretch the tail skin samples using the flat sides of a pair of scalpels over a glass surface
253 and irradiate with 636 nm red light at a 10 J/cm^2 fluence.

254

255 3.1.4. Proceed immediately to separate the epidermis from the dermis and to fix the
256 epidermal sheets as described in section 1.3.3.

257

258 3.1.5. Evaluate the hET red emission under a fluorescence/confocal microscope using green
259 exciting light, capture high quality images and proceed with further analysis.

260

261 NOTE: Use hET staining of tissue samples in the absence of photodynamic treatment as a
262 negative control for hET autoxidation.

263

264 3.2. In vivo detection of ROS production in the back skin after induction of hair growth and

265 burn healing followed by photodynamic treatment

266

267 NOTE: This step is performed using 2',7'-dichlorodihydrofluorescein diacetate (DHF-DA), a
268 cell permeant non-fluorescent compound that, after cleavage by intracellular esterase
269 enzymes, specifically reacts with ROS giving the 2',7'-dichlorofluorescein (DCF) fluorescent
270 dye.

271

272 3.2.1. Use animals prepared for induction of hair growth (section 1.1) or burn healing
273 (section 1.2). Just before topical mALA cream treatments (see sections 2.1 and 2.2), topically
274 dispense 100 μL of 1 mg/mL in 50% ethanol of DHF-DA on all target control/treated skin
275 areas, let the skin fully absorb the material and proceed forward with topical mALA cream
276 application.

277

278 3.2.2. Incubate treated animals for 4 h in the dark, wash the topical mALA cream thoroughly
279 off the skin with PBS and dispense a second dose of 100 μL of DHF-DA solution on the skin.

280

281 3.2.3. Incubate treated animals for 50 min in the dark, anesthetize and irradiate the whole
282 back skin with a fluence ranging from 2.5 to 4 J/cm^2 of 636 nm red light using a LED lamp.

283

284 3.2.4. Immediately after irradiation, evaluate ROS levels generated in the skin using an in
285 vivo imaging system (**Table of Materials**). Set the filter box for 445–490 nm excitation and
286 515–575 nm emission, capture high quality images and proceed with further analysis.

287

288 NOTE: Use DHF-DA staining of tissue samples in the absence of photodynamic treatment as
289 negative control for DHF-DA autoxidation.

290

291 **REPRESENTATIVE RESULTS:**
292 The topical administration of the mALA precursor in the mouse back and tail skin results in
293 a significant accumulation of PpIX in the whole tissue and, noticeably, in the hair follicle, as
294 demonstrated by the reddish-pink fluorescence of this compound under blue light (407 nm)
295 excitation (**Figure 2A,C**). Subsequent irradiation of treated tissue with red light (636 nm) at
296 a fluence of 2.5–4 J/cm^2 promotes transient production of ROS in the tissue, particularly in
297 the bulge region of the hair follicle (**Figure 2B,D**).

298

299 Switching on nonlethal ROS production in mouse skin in vivo promotes a significant increase
300 in the number of LRCs, categorized as somatic stem cells, in the bulge region of the hair
301 follicle two days after photo treatments (**Figure 3**, left panels). Notably, the increase in the
302 number of LRCs is transient, restoring to normal levels 6 days after treatments (**Figure 3**,
303 right panel). As this region is one of the main stem cell niches in mouse skin, a transient
304 induction of cell proliferation in this region mainly reflects the functional activation of the
305 bulge niche and of the resident stem cell programs of proliferation and differentiation[22,23].

306

307 The ROS-dependent activation of the bulge hair follicle niche is further associated with
308 physiological responses in the skin. Thus, transient ROS production notably accelerates the

309   skin healing process after a 2nd degree burn (**Figure 4A,B**). Quantification of the gradual
310   reduction of the damaged/scab skin area demonstrates the robustness and statistical
311   significance of the wound healing acceleration process induced by PpIX-dependent
312   transient ROS production in the tissue (**Figure 4C**). In the same way, nonlethal ROS levels
313   strongly promote hair growth after shaving during the second coordinated telogen (**Figure
314   5A**), a phase during which the hair follicle is refractory to respond to growth stimuli[22,23],
315   constituting an adequate way to evaluate the potential of new compounds and/or
316   processes to stimulate hair growth. Notably, the use of antioxidant compounds like ascorbic
317   acid (AA) results in a statistically significant reduction in the number of animals showing
318   accelerated hair growth (**Figure 5B**). In addition, ROS production in the skin after photo
319   treatments, quantified by the fluorescent emission of DHF in the skin, is also significantly
320   reduced by antioxidant compounds (**Figure 5C**). Together, these results demonstrate that
321   ROS production after PpIX-based photo treatments is strictly required to induce a
322   physiological response in the tissue.
323
324   **FIGURES LEGENDS:**
325   **Figure 1: Theoretical background for the controlled switching on of endogenous
326   photodynamic production of ROS in situ in cells and tissues using the heme biosynthetic
327   pathway**. (**A**) Schematic representation of the basic photochemical reactions resulting in
328   molecular oxygen excitation during photodynamic treatments. Upon absorption of light
329   with the appropriate λ, a photosensitizer molecule (PS) in the ground state S_0 undergoes a
330   transition to an excited singlet state S_1. Since any excited state is energetically less
331   preferable than the ground state, the molecule returns to S_0 after a short period of time.
332   Most PS have a high quantum efficiency for the transition from S_1 to the triplet state T_1,
333   generally characterized by a relative long lifetime. Activated PS in the excited triplet state
334   can react with other molecules via two different pathways. A type I photochemical reaction
335   is the transfer of electrons to adjacent molecules to form radical species; these radicals are
336   likely to react with molecular oxygen to produce ROS, including superoxide anion ($\bullet O_2^-$),
337   hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$). A type II photochemical reaction
338   represents the dominant process for most PS employed in PDT. During this reaction, the
339   transfer of energy (not electrons) to molecular oxygen (whose configuration in the ground
340   state is the triplet, $^3O_2$) drives the formation of the non-radical but highly reactive singlet
341   oxygen ($^1O_2$). The photoproducts formed during these reactions trigger a cascade of
342   biochemical events resulting in an oxidative stress that finally causes cell death or that can
343   potentially stimulate cell growth. (**B**) 5-aminolevulinic acid (ALA) is a natural precursor in
344   the heme biosynthetic pathway, which involves both mitochondrial and cytosolic cellular
345   compartments. The ALA synthase enzyme activity is regulated by a negative feedback
346   control whereby free heme, the final product of this pathway, inhibits the synthesis of ALA
347   from glycine and succinyl CoA. The administration of exogenous ALA or its derivative methyl
348   aminolevulinate (mALA) bypasses the regulatory feedback system, so that downstream
349   metabolites, especially protoporphyrin IX (PpIX), are accumulated in the cell inducing
350   photosensitization. The rate-limiting characteristics of ferrochelatase, catalyst enzyme of
351   the iron insertion in PpIX, promote the accumulation of this endogenous PS compound. PBG
352   = porphobilinogen. This figure has been modified from Carrasco et al.[19].

353

354 **Figure 2: Photodynamic treatment with mALA and red light induces transient production
355 of ROS in the skin.** (**A**) Accumulation of endogenous PpIX after mALA topic treatment in
356 back skin. The left side in the same animal was used as control. (**B**) Left panel: PpIX-
357 dependent ROS (mALA+Light) production monitored by DHF-DA. Right panel: time-course
358 analysis of relative ROS production in back skin; the relative integrated density of DHF-DA
359 fluorescent emission of mALA+Light versus Light regions in each animal was quantified at
360 different times after irradiation and normalized as described in methodology. The mean ±
361 SE was represented (n = 4 for each time point). (**C**) Localization of PpIX in tail skin
362 (fluorescence microscopy images). (**D**) ROS production in tail skin after mALA+Light as
363 revealed by hET showing an increased and sustained accumulation in the bulge region of
364 the hair follicle. Representative confocal microscopy images (maximum projections) are
365 shown. Scale bar = 100 μm. This figure has been modified from Carrasco et al.[19].

366

367 **Figure 3: Switching on in situ ROS production in the skin promotes a significant increase
368 of stem cells in the bulge region of the hair follicle niche.** Left panels: representative
369 confocal microscopy images (maximum projections) showing the localization of BrdU label
370 retaining cells (LRC) in mouse tail skin whole mounts and the evident increase of LRC in the
371 bulge region of hair follicles 2 days after PpIX-based phototreatments. Right panel:
372 quantification of the number of LRC in the hair follicle bulge region. The mean + SE (n = 4)
373 is represented. Scale bar = 50 μm. This figure has been modified from Carrasco et al.[19].

374

375 **Figure 4: Switching on in situ ROS production in the skin accelerates burn healing.** (**A**) PpIX
376 production induced by mALA in burn injured regions in treated animals as compared to
377 control samples. (**B**) Burn healing evolution in mALA+Light treated and control animals. (**C**)
378 Time-course quantification of burned areas (left panel) showing accelerated burn healing in
379 mALA+Light treated animals; the mean + SE (n = 4) of unhealed area is represented. Area-
380 under-the-curve analysis (right panel) demonstrating statistical differences between both
381 time-course curves (p ≤ 0.06). This figure has been modified from Carrasco et al.[19].

382

383 **Figure 5: Switching on in situ ROS production in the skin stimulates hair growth.** (**A**) Top
384 row: Induction of hair growth during the refractory telogen phase by mALA+Light (right side
385 of dorsal skin) as compared to light control region (left side). Bottom row: Both ROS
386 production in the skin and the acceleration of hair growth induced by mALA+Light are
387 inhibited by ascorbic acid (AA) antioxidant treatment. (**B**) Quantification of the % of animals
388 showing accelerated hair growth in mALA-PT as compared to control region in the absence
389 or presence of the antioxidant AA (n = 4 in 3 independent experiments). (**C**) Quantification
390 of the ROS production inhibition in dorsal skin induced by AA during mALA-PT (n = 4). In all
391 cases, bars represent mean + SE. This figure has been modified from Carrasco et al.[19].

392

393 **DISCUSSION:**
394 Here, we present a methodology that allows a transient activation of endogenous ROS
395 production in vivo in mouse skin with physiological effects. The methodology is based on a
396 photodynamic procedure to induce a controlled and local stimulation of the endogenous

397   photosensitizer PpIX (**Figure 1B**). This experimental approach is an interesting tool to study
398   ROS biology in in vivo experimental systems constituting a significant advance over
399   methodologies using external ROS sources (usually hydrogen peroxide) and allowing
400   controlled and local production of ROS in the tissue/sample.
401
402   Given that aminolevulinate-based precursors are administered in excess to promote the
403   accumulation of PpIX inside the cells, a critical step in this methodology is the establishment
404   of an adequate light dose to induce transient production of ROS levels in the tissue below
405   the damage threshold but showing a strong stimulatory effect. Currently there are no
406   available technologies to directly quantify the exact amount of any type of ROS that is
407   produced in cells and tissues. In our methodology, it is still not possible to establish a direct
408   correlation between a given light dose, the exact amount of ROS produced, and a given
409   biological effect (e.g., cell death or cell proliferation). For this reason, the light dose
410   (fluence) for any particular experimental model should be established empirically by the
411   researcher using qualitative or semiqualitative parameters of choice for each situation. In
412   the case of mouse skin, we choose an easily measurable transition between cell death and
413   tissue damage and the induction of a significant and transient proliferative wave.
414
415   The methodology presented here has proven to be very effective in the improvement of
416   skin regeneration in different processes, including burn healing and hair follicle growth.
417   These observations pave the way for the implementation of therapeutic applications of this
418   technology in clinics for the treatment of incidental or chronic burns and wounds or for
419   different pathologies skin and, particularly, of the hair follicle involving a defective stem cell
420   functioning.
421

427

428   **DISCLOSURES:**
429   All commercial applications of the procedures described in this work are protected by a
430   CSIC-UAM patent (EP2932967A1) authored by EC, MIC and JE and licensed to Derma
431   Innovate SL for commercial exploitation. JE and JJM have an advisory position in Derma
432   Innovate SL.
433

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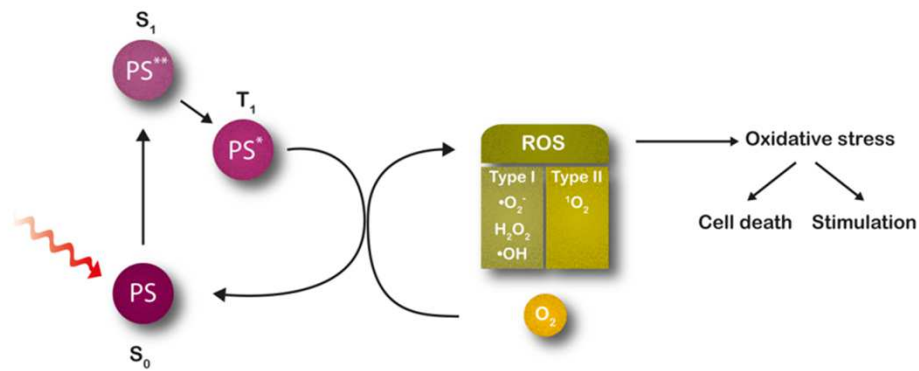
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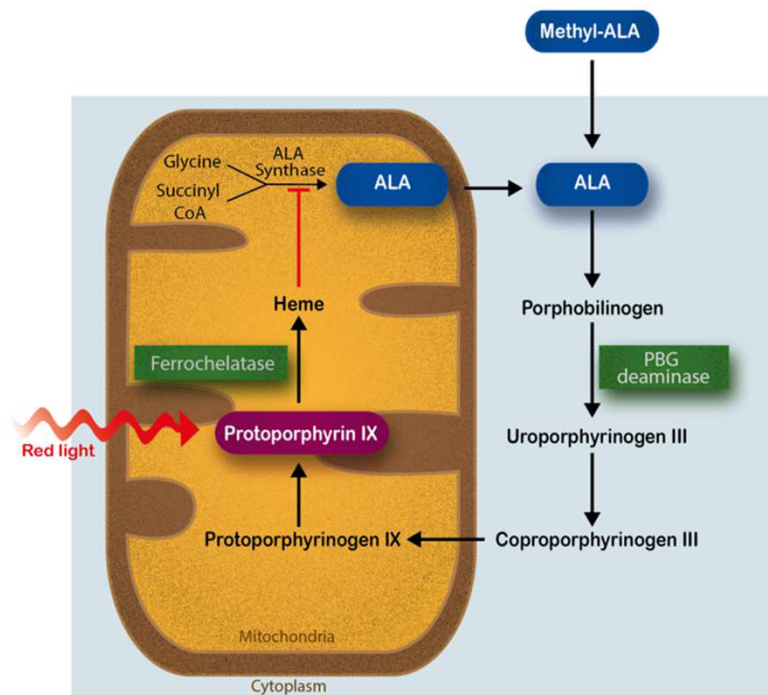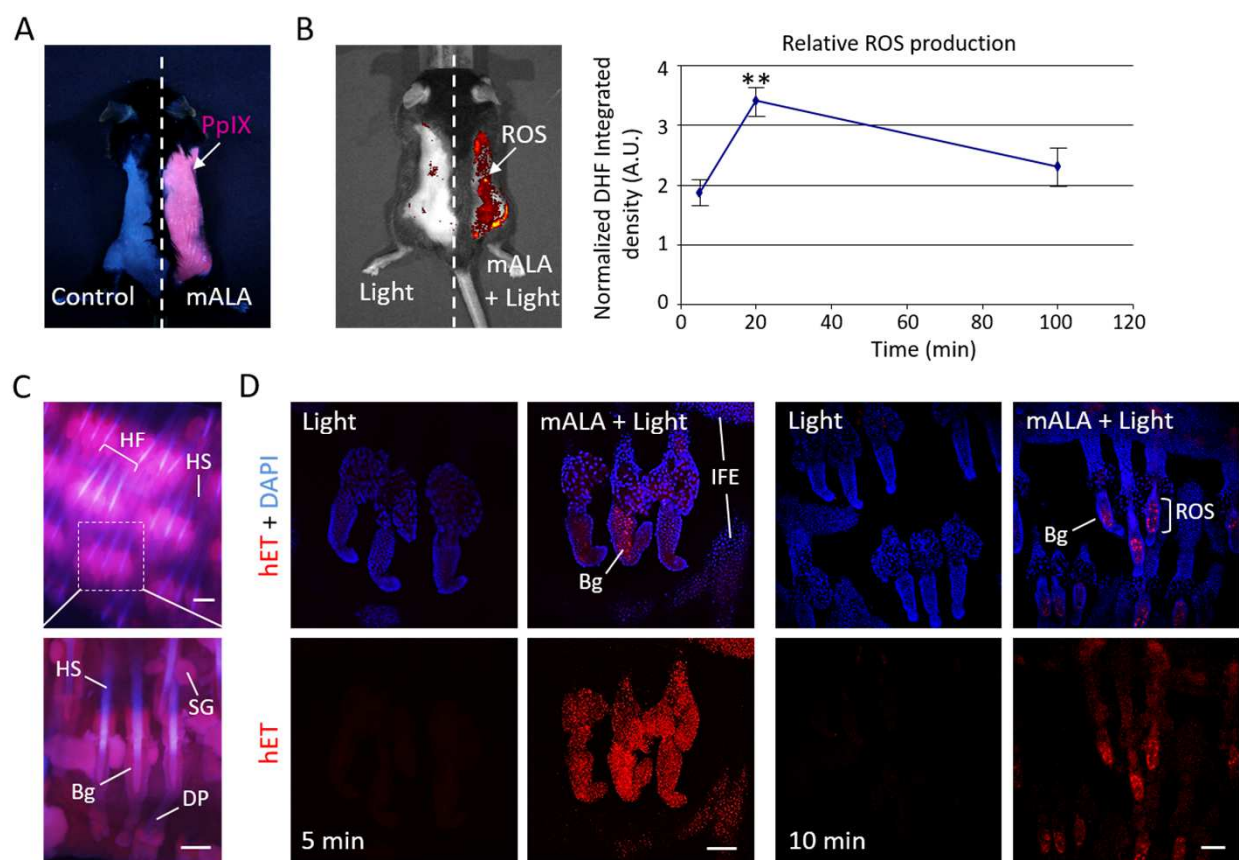
Figure 1   Click here to access/download;Figure;Jove Figure 1.pdf ±

Figure 1. Espada et al. 2019.

Figure 2

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Figure 2. Espada et al. 2019.

Figure 3   Click here to access/download;Figure;Jove Figure 3.pdf ⬇
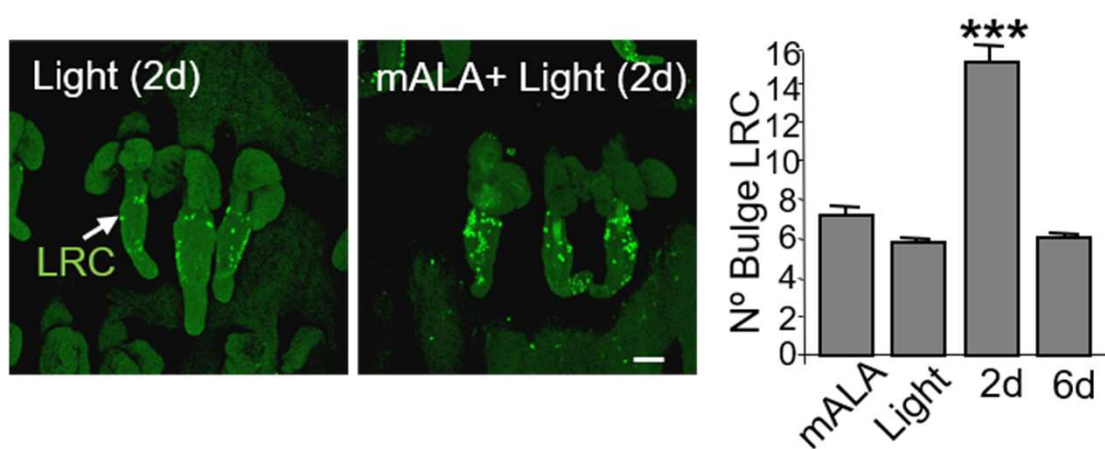
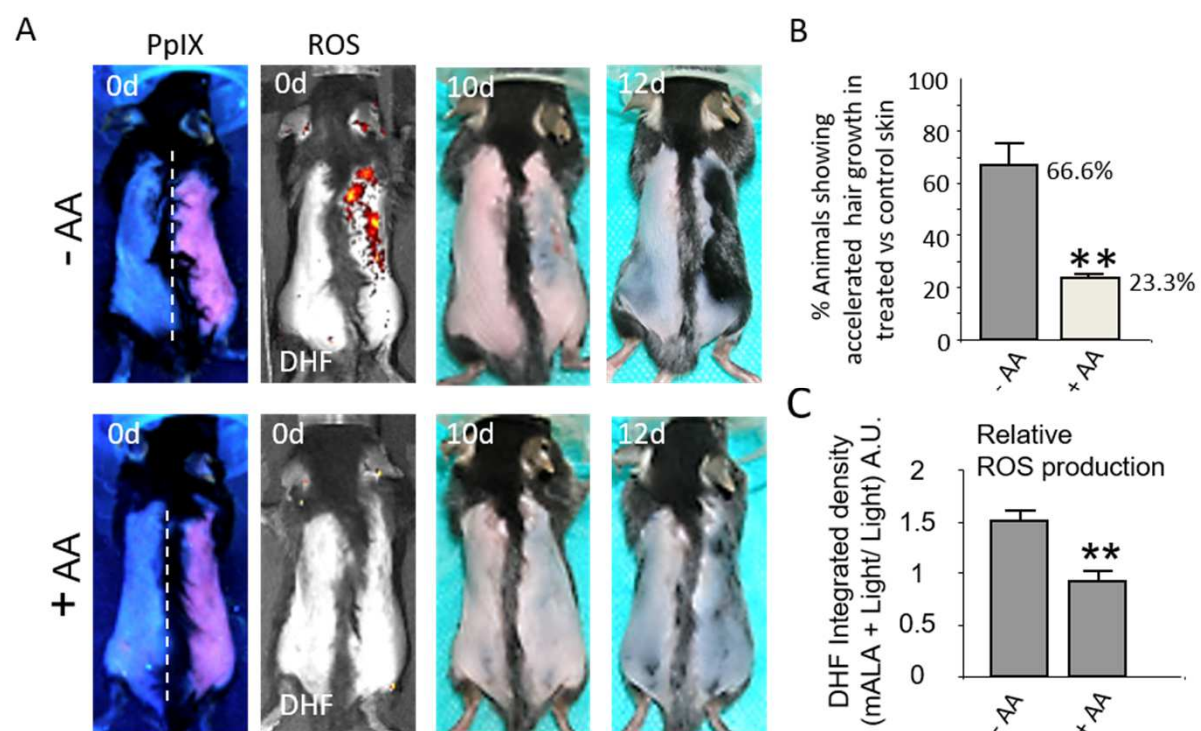Figure 3. Espada et al. 2019.
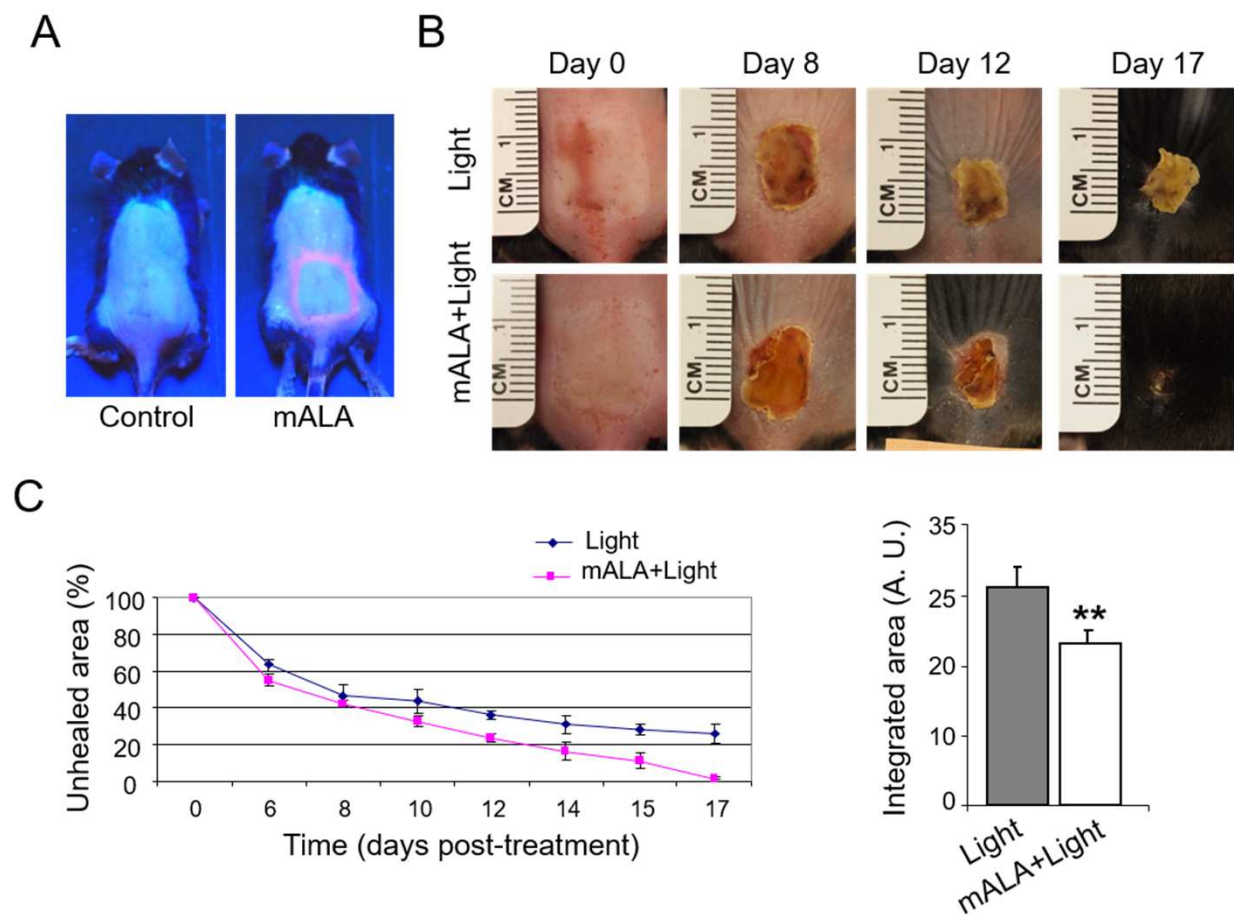
Figure 4. Espada et al. 2019.

Figure 5   Click here to access/download;Figure;Jove Figure 5.pdf ⬇

Figure 5. Espada et al. 2019.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2',7'-Dichlorofluorescin diacetate	Sigma Aldrich	D6883-50MG	
5'-bromo-2'-deoxiuridine	Sigma Aldrich	B5002-500MG	
Anti-Bromodeoxyuridine-Fluorescein	Roche	11202693001	
Depilatory cream (e.g., Veet)	Veet		
Dihydroethidium	Sigma Aldrich	37291-25MG	
In Vivo imaging system, e.g., IVIS Lumina 2	Perkin Elmer		
mALA in the form of topical cream, e.g.,METVIX Crema 160 mg/g	Galderma		
Power energy meter (e.g., ThorLabs Model PM100D)	ThorLabs		
Red light source, e.g., 636 nm Aktilite LED lamp	Photocure ASA		

Madrid, January 10th, 2020

Dear Editor,

We are sending you the revised version (3rd round) of our manuscript entitled "Stimulation of stem cell niches and tissue regeneration in mouse skin by a Protoporphyrin IX-dependent switchable photogeneration of ROS in situ" to be considered for publication in JoVE. We wish to thank the Editor for the new valuable comments and suggestions that have helped to improve the quality of our manuscript.

 Best regards,

Jesús Espada, PhD
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