Aryl hydrocarbon receptor controls skin homeostasis, regeneration, and hair follicle cycling by adjusting epidermal stem cell function

Eva María Rico-Leo1,2 | Luis Francisco Lorenzo-Martín3 | Ángel Carlos Román1 | Xosé Ramón Bustelo3 | Jaime María Merino1,2 | Pedro María Fernández-Salguero1,2

1Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, Badajoz, Spain
2Instituto Universitario de Investigación Biosanitaria de Extremadura (INUBE), Badajoz, Spain
3Centro de Investigación del Cáncer and CIBERONC, CSIC-Universidad de Salamanca, Salamanca, Spain

Correspondence
Eva María Rico-Leo, PhD and Pedro M. Fernández-Salguero, PhD, Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, Avenida de Elvas s/n, 06071 Badajoz, Spain.
Email: evaricoleo@unex.es (E.M.R.) and pmfersal@unex.es (P.M.F.)

Funding Information
Consejería de Economía, Ciencia y Agenda Digital, Junta de Extremadura, Grant/Award Numbers: GR18006, IB160210, IB20014; Ministerio de Ciencia e Innovación, Grant/Award Numbers: PID2020-114644GB-I00, SAF2017-82597-R

Abstract
Skin integrity requires constant maintenance of a quiescent, yet responsive, population of stem cells. While interfollicular epidermal progenitors control normal homeostasis, hair follicle stem cells residing within the bulge provide regenerative potential during hair cycle and in response to wounding. The aryl hydrocarbon receptor (AhR) modulates cell plasticity and differentiation and its overactivation results in severe skin lesions in humans. However, its physiological role in skin homeostasis and hair growth is unknown. Reconstitution assays grafting primary keratinocytes and dermal fibroblasts into nude mice and 3-D epidermal equivalents revealed a positive role for AhR in skin regeneration, epidermal differentiation, and stem cell maintenance. Furthermore, lack of receptor expression in AhR−/− mice delayed morphogenesis and impaired hair regrowth with a phenotype closely correlating with a reduction in suprabasal bulge stem cells (α6lowCD34+). Moreover, RNA-microarray and RT-qPCR analyses of fluorescence-activated cell sorting (FACS)-isolated bulge stem cells revealed that AhR depletion impaired transcriptional signatures typical of both epidermal progenitors and bulge stem cells but upregulated differentiation markers likely compromising their undifferentiated phenotype. Altogether, our findings support that AhR controls skin regeneration and homeostasis by ensuring epidermal stem cell identity and highlights this receptor as potential target for the treatment of cutaneous pathologies.

KEYWORDS
AhR, cell differentiation, epidermal stem cells, hair follicle, quiescence, regeneration, skin homeostasis

Significance statement
Aryl hydrocarbon receptor (AhR) has novel roles in skin homeostasis, regeneration, and hair cycling by controlling epidermal stem cells (EpdSCs) functions. During hair growth, AhR deficiency delays entry into anagen likely through loss of the self-renewal capacity and multipotentiality of AhR−/− hair follicle EpdSCs. The authors reinforce the idea that AhR plays important roles in skin homeostasis and propose this receptor as a target for the treatment of cutaneous pathologies, skin lesions, and skin regeneration. The feasibility to manipulate the...
INTRODUCTION

The skin is a complex organ whose main function is to provide protection from external aggressions and dehydration. When this barrier is disturbed, various cell types, signaling factors and matrix interactors coordinate to reestablish skin integrity. Key players in this process are tissue-resident stem cells (SCs), which have a self-renewal capacity needed for skin homeostasis and hair regeneration, thus ensuring proper tissue maintenance and repair. Skin development is governed by functional interactions between the epithelium and the mesenchyme, ultimately promoting the formation of the epidermis, hair follicles (HFs) and sebaceous glands. Mature epidermis is constantly renewed through a balance between basal cell proliferation and suprabasal cell differentiation that results in a highly organized stratified epithelium. Epidermal stem cells (EpdSCs) of the proliferative interfollicular epidermis (IFEs) represent one of the SC niches of the skin that nevertheless do not contribute to HF formation under homeostatic conditions. EpdSCs express Keratin 5 and 14 (KRT5/14), which together with integrins α6β4 and α3β1, constitute the first group of markers useful to characterize skin epithelial progenitor cells. Additionally, p53-family member p63 plays an important role in regulating stratification and maintenance of IFEs SCs. Moreover, the terminal differentiation program of the epidermis is driven by different families of transcription factors including AP1, AP2, KLFs (Kruppel-like factor), and Notch.

The HF is an additional source of EpdSCs mainly located in the bulge and hair germ regions. In addition to the core structural markers mentioned above, these cells express CD34, KRT15, SOX9 (SRY Box9), LGR5 (leucine-rich repeat-containing G-protein coupled receptor 5), TCF3/4 (T-cell factor), NFATc1 (nuclear factor of activated T-cells, calcineurin-dependent), and tissue-resident SCs (heterogeneous) constitute the second group of markers useful to characterize epidermal progenitors. 

AhR IN SKIN HOMEOSTASIS VIA EPIDERMAL STEM CELLS

MATERIALS AND METHODS

2.1 Mice

AhR+/+ and AhR−/− mice (C57BL6/N × 129/Sv background) were generated by homologous recombination in embryonic SCs as described previously. Immunodeficient mice (Hsd: Athymic Nude-Foxn1nu) were purchased (Envigo) and housed under specific-pathogen-free conditions. All work involving mice was performed in accordance with the National and European legislation (Spanish Royal Decree RD53/2013 and EU Directive 86/609/CEE as modified by 2003/65/CE, respectively) for the protection of animals used for research. Experiments using mice were approved by the Bioethics Committee for Animal Experimentation of the University of Extremadura (Registry 109/2014) and by the Junta de Extremadura (EXP-20160506-1). Mice had free access to water and rodent chow.

2.2 Primary cultures and cell lines

Primary Ker and dermal fibroblasts (DFs) were isolated from newborn AhR+/+ and AhR−/− mice at 2 to 3 days of age. Pups were
sequentially washed in povidone, sterile water and 70% ethanol in phosphate-buffered saline (PBS). Legs and tail were removed, and the complete skin was dissected using forceps. Skins were incubated in Cnt-07 medium (CELLnTEC) containing 3.6 units/mL Dispase II (Roche), 200 units/mL penicillin and 200 μg/mL streptomycin at 4°C overnight. Next, the epidermis was separated from the dermis and incubated in Accutase medium (CELLnTEC) for 30 minutes at 37°C. After digestion, cell suspensions were homogenized, filtered through a 100 μM mesh (Corning) and centrifuged at 300g for 5 minutes at 4°C. Subsequently, cell precipitates were resuspended in Cnt-07 medium at the desired density for each assay. DFs were obtained from mice dermises following protocols used in our laboratory. Phoenix packaging cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 37°C in a 5% CO₂ atmosphere.

2.3 | Colony formation assays

Ker were obtained from 2-month-old AhR+/+ and AhR−/− mouse skins essentially as described for the isolation of epidermal cells by flow cytometry (see below). Aliquots of $5 \times 10^4$ and $25 \times 10^5$ cells were seeded in Eagle’s minimum essential medium (EMEM) containing 4% Chelex-treated FBS and 0.2 mM CaCl₂. After 8 hours, Ker were grown in CnT-07 medium. Ten days later, dishes were washed with PBS, fixed in 5% formaldehyde, and stained with 1% crystal violet for 5 minutes to visualize colony formation. Colony size and number were scored under a microscope. Data were obtained from three separate experiments performed in duplicate.

2.4 | Skin and hair cycle analysis

Skins from AhR+/+ and AhR−/− mice were examined at different postnatal days (P10, P12, P21, P25, P48, P75, and P100) in order to follow all stages of hair cycling. After sacrificing the mice, dorsal skins were shaved with an electric clipper in situ to allow easier tissue sectioning and observation. Biopsies were collected from the same region of each mouse and genotype, fixed at 4°C in 4% paraformaldehyde and processed for hematoxylin-eosin (H&E) staining and immunohistochemistry (IHC) as described below.

2.5 | Hair recovery assays

AhR+/+ and AhR−/− mice at 18 days of age (HFs synchronized in telogen), were anesthetized in 2.5% isoflurane (kept along the procedure) and their dorsal skins shaved with an electric hair clipper. In some assays, mice were also treated with depilatory cream (Veet) for 2 to 4 minutes after shaving. Hair recovery was monitored daily by taking pictures from a fixed distance using a digital camera (Canon PowerShot SX620 HS). At day 6 after hair removal, mice from each genotype were sacrificed and skin biopsies collected for further histological studies. Experiments were made in triplicate using 5 to 6 mice per genotype and time.

2.6 | Wound-healing assays

AhR+/+ and AhR−/− mice at 8 weeks of age (second telogen phase) were anesthetized by i.p. injection of a mixture of Ketamine:Valium:Atropine (0.25:2.0:0.1 mg/mL; 100-120 μL/25 g body weight). An area of 4 to 6 cm² of dorsal hair was removed with depilatory cream (Veet). After disinfection by topical application of povidone, a full thickness wound 1 cm in length was performed in the upper region of the depilated dorsal skin. Wound healing was assessed by taking daily pictures from a fixed distance using a ruler for wound closure calculation. Images were processed and analyzed with the Image J software as the percentage of remaining wound with respect to the initial wound area. Experiments were performed four times using 3 to 4 mice per genotype and time.

2.7 | Skin reconstitution assays

Skin grafting was performed by transplanting freshly isolated Ker and DFs from 2 to 3 days old neonatal AhR+/+ and AhR−/− mice into the back of nude Hsd: Athymic Nude-Foxn1nu mice. Both isolated cell types were mixed and resuspended in 150 μL of CnT-07/DMEM medium using 1 full epidermis per 4 × 10⁶ DFs for each nude mouse transplanted. Nude mice were anesthetized by i.p. injection of a mix of Ketamine:Valium:Atropine (0.25:2.0:0.1 mg/mL; 100-120 μL/25 g body weight), controlling their body temperature with thermal blankets. After disinfection by topical application of povidone, a 1 cm in diameter layer of skin was removed from the upper region of their dorsal skin. Next, a silicon chamber (Renner GmbH) was implanted in the skin-depleted area and the Ker: DFs mixture was added to the chamber. At day 7 after surgery, mice were anesthetized and chambers removed. Skin and hair reconstitution was monitored by taking photographs at different times. After 2 months, biopsies of the grafted area were collected and fixed in 4% paraformaldehyde at 4°C for subsequent histological processing. Experiments were made in triplicate using 3 to 4 mice per genotype and time.

2.8 | Skin equivalents

For the establishment of 3-D skin models in vitro, the 3-D Culture Kit (CELLnTEC) was used following the manufacturer’s instructions. Briefly, Ker from neonatal AhR+/+ and AhR−/− mice were seeded into polycarbonate inserts (Nunc) in CnT-PR medium for 2-D growth. After 48 hours, cells were switched to CnT-PR-3D barrier for 16 hours and then cultured at the air-liquid interface for 16 days. For phenotypic rescue assays, Ker were transiently transduced with expression vectors containing a small hairpin RNA for AhR (shAhR) or...
the coding sequence for the wild type protein (AhR) (Stanford University Medical Center). In brief, constructs LMP-shAhR, pBABE-AhR or empty vectors LMP + pBABE were transfected in Phoenix cells by calcium phosphate precipitation, allowing virus production for 48 and 72 hours. Keratinocyte cultures were exposed to two rounds of overnight viral infections (24 and 48 hours) after being seeded into the inserts. The next day, cells were switched to CnT-PR-3D medium and cultured for 12 additional days as described above. Skin equivalents were processed for RNA analysis and histological examination following the manufacturer’s instructions (CELLnTEC). Transfection efficiency and AhR expression were analyzed by RT-qPCR.

2.9 | H&E staining

Deparaffinized and rehydrated sections of tissues were incubated with Harris hematoxylin for 10 minutes at room temperature. After washing with tap water, eosin solution was added for 30 seconds. A final washing step was performed and tissues were dehydrated, mounted with Dibutylphthalate polystyrene xylene (DPX) medium (Sigma-Aldrich), and observed in an Olympus BX51 microscope.

2.10 | Masson’s Trichrome staining

Once deparaffinized and hydrated, skin sections were stained with the Masson’s Trichrome Kit (Bio Optica) following the manufacturer’s instructions. Next, samples were dehydrated, mounted with DPX medium (Sigma-Aldrich) and observed in an Olympus BX51 microscope. Quantitative analyses of images were performed using an ARIOL 4.0 scanner (Leica Microsystems) equipped with a SL50 Slide loader and an Olympus BX61 microscope with multi-bay stage.

2.11 | Whole mount and label-retaining cell identification

To analyze label-retaining cells (LRCs), whole mounts of mouse tail epidermis were used.21 10-day-old AhR+/+ and AhR−/− mice were injected with four doses of BrdU (50 mg/Kg of body weight, Sigma-Aldrich) every 12 hours and animals were sacrificed after 70 days. Tail skin was incubated in PBS containing 5 mM EDTA at 37°C for 4 hours, the epidermis was then gently peeled-off and fixed in 4% paraformaldehyde for 2 hours at room temperature. To detect LRCs, epidermal sheets were permeabilized in 0.2% Triton X-100, blocked with 0.2% PBS-gelatin and denatured in 2 N HCl for 20 minutes at 37°C. After washing, samples were incubated overnight at 4°C with a BrdU-Alexa 647 conjugated monoclonal antibody (Invitrogen) in a 1:50 dilution. After washing the excess of antibody, sheets were incubated with Hoechst 33258 (Sigma-Aldrich) and mounted in PBS-glycerol (1:1). Fluorescence was analyzed with an Olympus FV1000 confocal microscope. Thirty optical sections were acquired with an increment of 1 μm and the intensity of labeling was quantified using the Image J Fiji software.

2.12 | Immunofluorescence and IHC

For immunofluorescence, skin sections (3 μm) from hair cycle analyses were manually deparaffinized and rehydrated in PBS. Antigen unmasking was performed in citrate buffer at pH 6. After washing in PBS containing 0.05% Triton X-100 (PBS-T), nonspecific epitopes were blocked by incubation for 1 hour at room temperature in PBS-T containing 0.2% gelatin and 3% bovine serum albumin (BSA) (PBS-T-G-B). Sections were incubated overnight at 4°C with the following primary antibodies diluted in PBS-T-G-B: anti-AhR (Thermo Fisher Scientific, 1:100) and anti-K14 (Calbiochem, 1:200). Following washing in PBS-T, sections were incubated for 1 hour at room temperature with Alexa-488 or Alexa-633 labeled secondary antibodies diluted in PBS-T-G-B. After additional washing, sections were dehydrated and mounted in PBS-glycerol (1:1). Tissue sections (2 μm) from 3-D assays were deparaffinized and gradually rehydrated using a Discovery Ultra automated equipment (Roche). After antigen unmasking in citrate buffer pH 6, sections were blocked and incubated with anti-K14 antibody (BioLegend, 1:300) diluted in PBS-T containing 0.2% gelatin. Following washing, sections were incubated with Alexa-488 labeled secondary antibody and mounted in VectaShield. Samples were visualized using an Olympus FV1000 confocal microscope (Olympus). Fluorescence analysis was done using the FV10 software (Olympus). DAPI (4’,6-Diamidino-2-phenylindole) was used to stain cell nuclei.

For IHC, tissue sections (2 μm) from skin reconstitution and 3-D assays were deparaffinized and gradually rehydrated using a Discovery Ultra automated equipment (Roche). After antigen unmasking in Tris-EDETA pH 8, sections were incubated for 45 minutes in PBS-T containing 0.3% H2O2 to block endogenous peroxidase activity. Following washing in PBS-T, unspecific binding was blocked by incubation in PBS-T containing 2 mg/mL gelatin (PBS-T-G) and 0.1 M lysine. Then, the following primary antibodies diluted in PBS-T-G were added: anti-Ki67 (Master Diagnostica, 1:50), anti-Cyclin D1 (Ventana, 1:100), anti-Keratin 14 (BioLegend; previously Covance PRB-155P) and anti-Involucrin (BioLegend; previously Covance PRB-140C). Following washing in PBS-T-G, sections were incubated for 1 hour at room temperature with the corresponding biotin-conjugated secondary antibody (OminiMap). After a final washing step in PBS-T-G, the streptavidin peroxidase complex was added, and the presence of target proteins revealed with diamobenzidine. Next, nuclei were counterstained with Harris hematoxylin and sections dehydrated and mounted using VectaShield. Samples were visualized using an Olympus BX51 microscope.

2.13 | Fluorescence-activated cell sorting

To isolate SCs from mouse skin, adult male mice of different ages were used. Animals were euthanized and shaved with an electric hair clipper. After removing the skin, subcutaneous fat was scraped-off with a scalpel and the remaining skin placed (dermis side down) in 0.25% trypsin-EDTA (Gibco) at 4°C overnight. To obtain epithelial single cell suspensions, HF s and epidermal cells were gently scraped-off
from trypsinized skins with a scalpel, resuspended in cold PBS containing 2% FBS and filtered through 100 μm strainers (Falcon). After centrifugation at 300g for 10 minutes and 4°C, precipitates were resuspended in cold MEM containing 15% FBS and filtered through 40 μm strainers (Falcon). Cells were then incubated with biotinylated anti-CD34 antibody (eBioscence, 1:50) diluted in 15% FBS-MEM for 30 minutes at 4°C. After washing in 2% FBS-PBS, cell suspensions were incubated with the avidin-APC reagent (Becton-Dickinson Pharmingen, 1:300) and phosphoerythrin labeled anti-CD49 antibody (AbD Serotec, 1:200) for 30 minutes at 4°C. Finally, cell suspensions were washed and incubated with DAPI. FACS analyses and cell purification were performed on a FACS Aria III cytometer (Becton-Dickinson). Data obtained were analyzed using FlowJo_V10_CL software.

2.14 | RNA purification and real-time RT-qPCR

Total RNA from skin was isolated using Trizol Reagent (Life Technologies) and purified with the High Pure RNA isolation kit following the manufacturer’s instructions (Roche).22 Total RNA from flow cytometry-isolated SCs was purified using the microRNeasy kit (Qiagen). Total RNA from 3-D epidermal cultures was extracted using the miRNeasy kit (Qiagen), which includes a first step of digestion with guanidine phenol-thiocyanate solution (Qiazol). Reverse transcription was done using random priming and the iScript Reverse Transcription Super Mix (Bio-Rad). Real-time PCR (qPCR) was performed using SYBR Select Master Mix (Life Technologies) in a Step One Thermal Cycler (Applied Biosystems).18,23 Gapdh was used to normalize gene expression (ΔCt) and 2−ΔΔCt to calculate changes in mRNA levels with respect to untreated or wild type conditions. Primer sequences used are indicated in Supplementary Table 1.

2.15 | Expression microarrays

Total RNA was purified using the microRNeasy kit (Qiagen) from FACs-isolated cells in RLT buffer. RNA quality was determined using an Agilent 2100 Bioanalyzer and only samples having RNA integrity numbers (RIN) higher than 8 were used. Gene expression analysis was carried out using the Mouse Gene 2.0 ST Array (Affymetrix) and only samples having RNA integrity containing 15% FBS and filtered through 40 μm strainers (Falcon). Cells were then incubated with biotinylated anti-CD34 antibody (eBioscence, 1:50) diluted in 15% FBS-MEM for 30 minutes at 4°C. After washing in 2% FBS-PBS, cell suspensions were incubated with the avidin-APC reagent (Becton-Dickinson Pharmingen, 1:300) and phosphoerythrin labeled anti-CD49 antibody (AbD Serotec, 1:200) for 30 minutes at 4°C. Finally, cell suspensions were washed and incubated with DAPI. FACS analyses and cell purification were performed on a FACS Aria III cytometer (Becton-Dickinson). Data obtained were analyzed using FlowJo_V10_CL software.

2.16 | Statistical analyses

Quantitative data are shown as mean ± SD. Comparison between experimental conditions was done using GraphPad Prism 6.0 software (GraphPad). The unpaired Student’s t test was used to analyze differences between two experimental groups. The Mann-Whitney nonparametric statistical method was used to compare rank variations between independent groups (*P < .05; **P < .01, ***P < .001).

3 | RESULTS

3.1 | Mice lacking the aryl hydrocarbon receptor show histological abnormalities in the skin and alterations in hair cycle

At the macroscopic level, mice lacking AhR do not show any marked phenotypic alteration in the skin or hair. However, since its activation by agonistic ligands can cause severe skin lesions,27 we analyzed if AhR is required for skin and HF homeostasis by performing a detailed histological analysis of the back skin of AhR+/+ and AhR−/− mice (Figure 1). During HF morphogenesis, on days 10 and 12 after birth, skin thickness and the number of pilosebaceous units were similar between wild type and AhR-null mice. Similar results were observed on day 21 during the resting phase (telogen), at which the synchrony between both genotypes was maintained (Figure 1A). However, the number of hair shafts crossing the epidermis at early stages of the cycle up to 21 days was lower in AhR−/− mice, to be progressively normalized between both genotypes from day 25, when the first anagen phase had already started (Figure 1B). Surprisingly, differences in the spatial distribution of HFs were found at that developmental time between both genotypes. Thus, whereas most HFs of AhR+/+ mice were already invading the hypodermis, closely located in the dermis or in the limiting area (Figure 1A, middle and upper panels; Supplementary Figure 1A). A more detailed analysis of the position of the hair bulb confirmed the existence of a delay in anagen subphases during hair cycle progression in AhR−/− mice (Figure 1D). Nevertheless, differences in the active phase of early hair growth were compensated later-on as the second cycle progressed. The dermis did not reveal relevant changes in collagen content of the connective tissue among both genotypes, with the exception of day 10, when a significant increase was found in AhR−/− mice (Supplementary Figure 1A,B). Consistently, the percentage of area of the dermis occupied by fibroblasts was markedly lower in AhR−/−
Figure 1  Hair-cycle abnormalities in mice lacking aryl hydrocarbon receptor (AhR). A, Representative H&E images of back skin sections of male AhR+/+ and AhR−/− mice. Note anagen follicles in AhR−/− mice at 25 days (arrowheads). Scale bar = 200 μm. Groups of 5 to 6 mice were used and three sections were examined for each age and genotype. B, Quantification of the number of hair shafts crossing the epidermis per field. At least 10 fields were counted for each age and mouse genotype. The decrease in hair follicle shafts in AhR−/− skin is highlighted with red arrows and the normalization between both genotypes past 25 days in blue line (AhR+/+) and red line (AhR−/−). C, Detailed analysis of the hair cycle. Percentage of follicles in catagen (upper), telogen (middle) and anagen (lower) at the specified ages. At least 20 follicles were analyzed per time point and genotype. D, Analysis of hair follicles in anagen stage, according to the different subphases described.9 Sets of 20 anagen follicles were analyzed for each subphase and genotype. Data are shown as mean ± SD. (*P < .05; **P < .01; ***P < .001)
mice, and this pattern was maintained until the beginning of the second hair cycle (Supplementary Figure 1C). These results revealed a morphological alteration in the epidermal and dermal compartments of the skin and a delay in hair cycle progression in absence of AhR.

We then confirmed that AhR was in fact expressed in the skin during hair development by IHC. As shown in Supplementary Figure 2A, besides its expression in the dermis and the panniculus carnosus, the receptor was also located in the IFEs and HFs, thus supporting its role in hair cycle (Supplementary Figure 2B).

3.2 | Impaired hair growth in AhR deficient mice

To further study the role of AhR in hair growth, we next analyzed the response to depilation of AhR+/+ and AhR−/− male mice during the telogen to anagen transition (see the Methods). Such treatment induced a faster and more robust hair regrowth in wild type than in AhR-null mice, suggesting a more efficient anagen entry (Figure 2A). This outcome occurred independently of gender, although differences were more noticeable in males (data not shown). Skin biopsies harvested 7 days after depilation reinforced these results, revealing a higher number of follicles in AhR+/+ than in AhR−/− anagenic mice (Figure 2B,C). These data suggested that AhR positively regulates the telogen-to-anagen phase transition and hair growth.

Although HF cells are not essential for skin regeneration upon injury, re-epithelialization in hair-bearing skin occurs faster and more efficiently than in nonbearing areas. We next investigated whether the decreased ability of AhR deficient mice to support hair growth was accompanied by a reduction in their wound healing potential in vivo. To do that, longitudinal wounds were made in the back of 8-week-old AhR+/+ and AhR−/− mice synchronized in the second telogen resting stage. However, quantification of wound-healing competence did not reveal significant differences between both genotypes in their ability to close this type of skin wounds (Supplementary Figure 3A,B).

3.3 | AhR deficiency compromises skin and hair regeneration in vivo

Ker combined with DFs from neonatal mice can generate epidermis, hair, and sebaceous glands in immunosuppressed mice. We then decided to investigate whether lack of AhR could affect skin reconstitution in vivo. As shown in Figure 3A, nude mice transplanted with Ker and DF from AhR+/+ mice regenerated skin and hair earlier and more efficiently than those receiving both types of AhR-null cells. Thus, the area of the implantation site regenerating a complete hair-containing skin was only 7% in immunocompromised mice transplanted with Ker and DF from AhR−/− mice compared with those receiving AhR+/+ Ker and DF (100%). Histological analysis of the newly developed skins harvested 60 days after grafting evidenced those differences between genotypes: skins derived from AhR−/− cells displayed epidermis with fewer projections, thinner hypodermises, and a significant reduction in the number of pilosebaceous units (Figure 3B). The proliferative activity was similar in AhR+/+ and AhR−/− grafts as determined by Ki67 and Cyclin D1 immunostaining (Figure 3C). However, grafts from AhR+/+ cells displayed a more localized expression of both proteins in HFs (Figure 3C). Finally, immunohistochemical characterization of epidermal differentiation revealed similar expression levels of the basal cell layer marker K14 in both genotypes (Figure 3D). Nonetheless, expression of involucrin, a component of the cornified cell envelope and upper spinous layer, was found to be significantly reduced in AhR−/− skins (Figure 3D).

To discriminate the relative contribution of Ker (cell-autonomous) and the microenvironment (non-cell autonomous) on skin reconstitution, we combined AhR+/+ Ker with AhR−/− DFs and vice versa. Compared with the regeneration induced by wild type cells, when cells of opposed genotypes were combined skin growth was reduced and hair formation delayed to levels similar to those observed in mice transplanted with AhR−/− cells (Figure 4A). However, nude mice transplanted with AhR+/+ Ker and AhR−/− DF produced hairier skins than those grafted with the opposite cell combination (Figure 4B). In this case, the area of the hair-containing fully regenerated skin was reduced to 20% in mice transplanted with the combination Ker AhR+/+ plus DF AhR−/− with respect to those transplanted with Ker + DF from AhR+/+ mice (100%). When AhR was absent in the Ker fraction (Ker AhR−/− plus DF AhR+/+) the regeneration dropped to about 9% compared with that in the reference mice receiving wild type Ker and DF (100%). Histological analysis confirmed this result revealing a more reactive epidermis with thickening in discrete areas and a larger number of pilosebaceous units in AhR+/+ Ker + AhR−/− DF skins (Figure 4C). Ki67 and Cyclin D1 immunostaining was similar in both cell combinations although reconstituted skins with AhR+/+ Ker + AhR−/− DF contained more HFs than those reconstituted with AhR−/− Ker + AhR+/+ DF cells (Figure 4D). Finally, although no significant differences were found in K14 expression among both experimental conditions, involucrin was significantly downregulated in AhR−/− Ker + AhR+/+ DF skins, indicating a more differentiated status in grafts expressing AhR in the Ker compartment (Figure 4E). All together, these results support that the cell autonomous effects of AhR on Ker favors skin regeneration and that, when lost, regeneration is compromised even under an AhR positive microenvironment.

3.4 | AhR depletion inhibits epidermal stratification and differentiation in mouse skin equivalents

Since skins derived from AhR−/− epithelial cells showed altered involucrin expression, we next generated epidermal skin equivalents from primary Ker to further investigate the role of AhR on epidermal differentiation. This in vitro approach largely mimics the architecture of the epidermis and allows genetic and pharmacological manipulation. As shown in Figure 5A, AhR−/− epidermal skin equivalents displayed defects in stratification with an average reduction in epidermal thickness of 51% and an impaired spinous layer and lack of granular
FIGURE 2  Lack of aryl hydrocarbon receptor (AhR) impairs hair regrowth. A, AhR+/+ and AhR−/− mice were depilated at the first telogen phase and pictures taken at different times to monitor hair growth dynamics. At every time point, groups of 5 to 6 mice were used for each genotype. B, H&E images of skin sections 7 days after depilation. Tangential sections show the external root sheath (ORS) of hair follicles (arrowheads) and the pigmented axis of hair (asterisks). Scale bar = 1 mm (upper) and 200 μm (lower). Three technical replicates were performed for 5 to 6 AhR+/+ and AhR−/− mice. C, Quantification of the percentage of follicles in anagen phase in the skin sections analyzed in panel B. Data are shown as mean ± SD. (**P < .01)
FIGURE 3  Cells lacking aryl hydrocarbon receptor (AhR) have decreased skin regeneration and reduced hair growth potential after transplantation in immunodeficient mice. A, Representative images of regeneration dynamics of the skin and hair in nude mice transplanted with AhR+/+ and AhR−/− keratinocytes (Ker) and dermal fibroblasts (DF). Between six and eight nude mice were transplanted for each combination of Ker and DF. B, H&E of regenerated skin obtained 60 days after grafting. C, Immunohistochemistry analysis for Cyclin D1 and Ki67 in regenerated skins. D, Immunohistochemistry analysis for Keratin14 and Involucrin in regenerated skins. Five nude mice and three technical replicates were processed for each experimental condition shown in panels B-D.
FIGURE 4  Keratinocyte aryl hydrocarbon receptor (AhR) prevails over stromal AhR in skin regeneration. A, Comparison of skin regeneration in nude mice transplanted with the indicated combinations of keratinocytes and dermal fibroblasts 37 days postgrafting. B, Representative images of regeneration dynamics of skin and hair in nude mice transplanted with AhR+/+ keratinocytes and AhR−/− fibroblasts or vice versa. C, H&E of regenerated skin obtained 60 days after grafting. D, Immunohistochemistry analysis for Cyclin D1 and Ki67 in regenerated skins. E, Immunohistochemistry analysis for Keratin14 and Involucrin in regenerated skins. Between six and eight nude mice were transplanted for each combination of Ker and DF and five nude mice and three technical replicates were processed for each experimental condition shown in panels C-E.
FIGURE 5  Aryl hydrocarbon receptor (AhR) depletion induces defects in epidermal differentiation. A, H&E of AhR+/+ and AhR−/− keratinocytes 3-D cultures. Six cultures were established for each genotype and three technical replicates were analyzed. B, Immunohistochemistry for Cyclin D1 and Ki67 in the same cultures. C, mRNA level of the indicated differentiation genes in 3-D cultures obtained at day 16 determined by RT-qPCR using the oligonucleotides indicated in Supplementary Table 1. Six cultures were analyzed performing triplicate determinations for each gene shown. D, Immunohistochemistry and immunofluorescence analysis for Involucrin and Keratin 14, respectively, in AhR+/+ and AhR−/− keratinocyte 3-D cultures. Alexa 488-labeled secondary antibody was used. Six cultures were established for each genotype and three technical replicates were analyzed. E, mRNA level of p63 in the 3-D cultures indicated in panel C. Gapdh expression was used to normalize expression levels (2^{ΔΔCt}). Data are shown as mean ± SD. (**P < .01; ***P < .001). Scale bar = 50 μm.
stratum. Similarly, to the results obtained in vivo, AhR depletion limited the proliferative activity of Ker as revealed by reduced levels of Cyclin D1 and Ki67 (Figure 5B). Real-time PCR analysis showed that the mRNA levels of major differentiation markers, except for Krt5 and Krt14, were significantly decreased in absence of AhR (Figure 5C); as expected, Involucrin mRNA and protein expression were also reduced.
AhR-depleted epidermal skin equivalents (Figure 5C,D). Notably, the mRNA level of p63, a gene essential in epidermal differentiation and in maintaining epithelial progenitors, was also substantially reduced in AhR+/− epidermal skin equivalents (Figure 5E).

To confirm the positive effect of AhR on epidermal differentiation, we next performed phenotypic rescue experiments by infecting AhR+/+ or AhR−/− Ker with a short hairpin RNA or an AhR expression vector (respectively). Control epidermal skin equivalents were infected with the corresponding empty vector. As shown in Supplementary Figure 4A, AhR knockdown in wild type epidermal skin equivalents (+shAhR) drastically reduced transcript levels for early and late differentiation markers. In contrast, AhR−/− skin equivalents overexpressing the receptor (+AhR) significantly increased the expression of all differentiation markers analyzed (Supplementary Figure 4B). Infection efficiency in both experimental conditions was confirmed by measuring AhR mRNA levels (Supplementary Figure 4C,D).

3.5 Lack of AhR negatively affects EpdSCs properties

The skin phenotype of AhR deficient mice suggested that it could be due to defects in the number and/or functionality of EpdSCs, whose proliferation and mobilization are required for homeostasis and tissue regeneration.2,3 This prompted us to identify and characterize EpdSC populations in AhR+/+ and AhR−/− mice. We first analyzed the levels of known EpdSCs markers in the back skin of telogen mice of both genotypes. As shown in Figure 6A, with the exception of Lgr6, all markers analyzed were significantly downregulated in the absence of AhR, they included the hair cycling inducer Lgr5,43 the quiescence SC promoter NFATc44 and the HF stemness factors Lhx2 and Sox9.45,46

To further explore the effects of AhR depletion on EpdSCs functionality, clonogenic assays were performed47 using Ker from telogen phase skin of wild type and AhR-null mice. After 10 days in culture at low cell density (5 × 10^3), the number of colonies derived from AhR deficient cells was significantly smaller than that of wild type cells (Figure 6B,C). At increased cell density (25 × 10^3), no significant differences were found between both genotypes in terms of the total number of colonies generated (Figure 6B,C). Interestingly, AhR+/+ colonies were significantly larger in size (>1 mm) under both growing conditions, thus suggesting a higher proliferative potential (Figure 6C).

These findings support that AhR depletion results in a reduction in EpdSCs and sustain a positive role for the receptor in EpdSC proliferation.

Bulge cells are more quiescent than any other cell type within the follicle. This property allows their tracking after exposure to nucleotide analogs such as BrdU, since only low proliferative cells will retain the label.48 For that, neonatal AhR+/+ and AhR−/− mice were labeled with BrdU to detect the presence of LRCs in whole mounts of tail epidermis after 75 days. As shown in Figure 6D AhR deficiency caused a reduction in BrdU labeling with a significant decrease in the number of LRCs, suggesting that this receptor might modulate the quiescent state of these cells (Figure 6E).

3.6 AhR is needed to maintain HF bulge architecture and to control the quiescent vs active switching of EpdSCs

Two different populations of multipotent SCs have been described within the bulge: a first one attached to the basal lamina and a second, nonattached, called suprabasal. Both populations display high levels of CD34 but differ in their α6 integrin expression.13 Flow cytometry analysis using these markers confirmed that both populations (CD34^highα6^low and CD34^highα6^high) were present in AhR+/+ and AhR−/− mice (Supplementary Figure 5A). Nonetheless, quantification of each cell population revealed that AhR deficiency significantly depleted suprabasal CD34^highα6^low bulge cells and, interestingly, that such reduction was maintained during developmental stages of hair cycle from 2 to 4 months of age (Supplementary Figure 5B).

To better characterize these EpdSCs, we isolated mRNA from FACS-purified bulge cell populations at the second and third telogen resting stages, to then perform differential microarray gene expression studies. Statistical inference analysis revealed several functional categories which were differentially expressed upon AhR expression but independently of age (Figure 7). Thus, AhR−/− SCs overexpressed genes involved in keratinocyte differentiation and in the establishment of the skin barrier but repressed genes that regulate cell adhesion, TGFβ signaling and the immune response (Figure 7A,B, Supplementary Tables 2 and 3). Given this high functional overlap between gene profiles at 2 and 4 months of age, we performed a joint analysis of AhR+/+ vs AhR−/− CD34^highα6^low bulge cells. An

**FIGURE 6** Aryl hydrocarbon receptor (AhR) depletion represses epidermal stem cell markers and colony formation efficiency (CFE). A, Telogen phase skins of AhR+/+ and AhR−/− mice were harvested and mRNA expression of the indicated stem cells markers determined by RT-qPCR using the oligonucleotides indicated in Supplementary Table 1. Four mice of each genotype and triplicate technical replicates were used. Gapdh expression was used to normalize expression levels (2^−ΔΔCt). Data are shown as mean ± SD. (P < .05; **P < .001). B, Representative images of CFE of keratinocytes isolated from adult AhR+/+ and AhR−/− mice plated at two cell densities (5 × 10^4 and 25 × 10^4 cells/plate). Plates were grown in triplicate using keratinocytes from four mice of each genotype. C, Quantification of the number and size of colonies generated by both genotypes. Data are shown as mean ± SD. (P < .05; **P < .01; n.s., not statistically significant). D, AhR+/+ and AhR−/− neonatal mice were injected with BrdU and label retaining cells (LRCs) identified by immunofluorescence in whole mount tail epidermis after 70 days. At least six neonates of each genotype from different breeding were analyzed in triplicate immunofluorescences. Nuclei were stained with DAPI. Scale bar = 100 μm. Anti-BrdU conjugated to Alexa 647 was used. E, Quantification of LRCs/DAPI was done using Fiji Image J software following a published protocol.42 Data are shown as mean ± SD. (**P < .01)
AhR-dependent transcriptional program common to both ages was found (Figure 7C), and thus a combined dataset for both ages was used for further analyses.

In addition, genes identified in Figure 7C coding for proteins involved in protein-protein interaction, were used to generate a functional network regulated by AhR. As shown in Supplementary Figure 6, among the different functional categories represented, a very well-defined cluster of overexpressed keratinocyte differentiation markers was identified in AhR−/− bulge cells.

We also employed a completely independent approach to analyze RNA microarray data from AhR+/+ and AhR−/− bulge SCs using transcriptional signature enrichment by GSEA. Since our results suggested an alteration of processes controlling stemness and differentiation, microarray data were analyzed in the context of known signatures for stem cell identity.49-51 We first used the transcriptional profile of epidermal progenitors driving regeneration of epidermal tissue.49 This report identified three gene sets whose enhanced expression was time-specific for epidermal progenitors and for their progeny undergoing early and late differentiation. Our GSEA revealed that epidermal progenitor and early differentiation signatures were repressed in AhR deficient cells whereas late differentiation-associated genes were upregulated (Figure 7D, Supplementary Tables 4-6). Likewise, molecular signatures of quiescent and activated SCs and their HF progeny50 were also downregulated in AhR deficient cells, including functional
categories of quiescent SCs encompassing survival and apoptosis genes. Notably, signatures including functional categories specific for cell migration and HF-SCs were repressed upon AhR depletion, supporting that this receptor is needed to maintain the stem cell phenotype (Figure 7E, Supplementary Table 7). Analyses using bulge and non-bulge signatures of genes expressed in anagen and in the telogen-anagen transition confirmed that AhR+/− SCs down-regulated bulge-specific genes (Figure 7F, Supplementary Table 8).

Finally, we assessed the conservation of the gene signatures found in AhR+/− mice that were relevant to stemness. In order to do this, we assessed if the downexpressed genes in AhR+/− bulge cells that were found in different EpdSCs gene signatures from GSEA were partially the same. We found that a robust set of genes characteristic of bulge cells (73) were repressed in the three different datasets (Figure 7G, Supplementary Table 9), representing bona fide targets of AhR in EpdSCs. Within this signature, we found genes directly related to different stemness models, like Lgr5, together with several transcription factors such as Sox9, Tcf3, Tbx1, or Id4. Based on the known association of AhR with some of the identified gene families, we selected representative genes that were present in these three datasets to be validated by RT-qPCR. Based on the known association of AhR with some of the identified gene families, we selected representative genes that were present in these three datasets to be validated by RT-qPCR. As shown in Figure 7H, most analyzed genes were repressed in EpdSCs, thus confirming the microarray data analyses. Altogether, these results propose that AhR depletion in HF EpdSCs represses stemness while inducing differentiation, thus negatively affecting the identity of the EpdSC compartment.

4 | DISCUSSION

The aryl hydrocarbon receptor modulates cellular plasticity and migration, and its stimulation by xenobiotics results in severe skin lesions such as contact hypersensitivity, dermatitis, and chloracne. From homeostatic and physiological points of view, AhR is recognized for having important roles in differentiation and, more recently, in stemness. Nevertheless, the role of AhR in normal skin physiology, regeneration and hair growth is still poorly understood. Additionally, the fact that AhR can be modulated by nontoxic ligands makes it a potential therapeutic target for human skin diseases. In this work, we have used primary cells and animal models to investigate the implication of AhR in skin homeostasis, HF cycling and tissue repair. Previous studies showed the existence of regenerative waves in murine hair and skin ultimately defining dynamic hair cycle domains that progress with age. In fact, lack of AhR caused a significant impairment in hair regrowth upon hair removal.

The phenotype of AhR-deficient mice may be due to an alteration in the EpdSCs compartment responsible for the maintenance of skin homeostasis, HF cycling and tissue repair. The expression of EpdSCs markers in the skin of telogen mice was significantly repressed in the absence of receptor, thus suggesting that AhR sustains proper EpdSCs numbers in the developing actively growing skin. Flow cytometry analysis of epidermal cells confirmed an imbalance between different SC niches in AhR+/− mice. Two distinct populations of SCs reside within the bulge: one that keeps basal lamina contacts (basal) and a second one that detaches from it (suprabasal). Both are multipotent and carry out terminal differentiation in vitro and in vivo despite differences in transcriptional signature, expression of integrin α6 and in the appearance of suprabasal cells only after the start of the first postnatal hair cycle. Interestingly, we found a decrease in the number of CD34high/α6low cells in AhR-null mice. Moreover, the fact that suprabasal bulge cells were deprived at different stages of the HF cycle further supports that AhR is required to maintain specific SC populations and SC gene expression profiles in the bulge. Indeed, bulge-specific gene signatures are maintained in anagen and telogen phases of the hair cycle and in basal and suprabasal bulge cell layers.

Although IFEs SCs control skin homeostasis, HF-SCs are activated upon wounding to support the re-epithelialization process. To check the contribution of AhR to that activation, we performed wound-healing assays in AhR+/+ and AhR+/− mice in the resting phase of the hair cycle. Our results revealed no significant differences between both genotypes, suggesting that other factors of the healing process might compensate the stem cell activity itself. In fact, we have previously reported that AhR+/− Ker have enhanced migratory capacity in vitro and in vivo wound healing assays.

A unique property of mouse HFs are the maintenance of older bulges alongside newly made ones. HF-SCs within the old bulges act as SC reservoirs capable of repairing wounded epidermis, whereas HF-SCs of new bulges participate in hair regeneration. Ablation of the cell-cell adhesion molecule Cdh1 in suprabasal SCs at the interface between the two bulges is sufficient to induce bulge loss. In agreement, it is probable that Cdh1 repression in AhR+/− primary Ker could contribute to reduce the pool of suprabasal SCs in absence of receptor. Another main feature of quiescent HF-SCs, its ability to retain BrdU labeling, was also reduced in AhR+/− mice. This finding could imply that wild type cells have higher self-renewal capacity but, on the other hand, it could also indicate that AhR+/− SCs are more committed to enter differentiation. Further studies will characterize HFs from both genotypes to demonstrate if, in absence of AhR, bulge SCs lose their self-renewal capacity in favor of differentiation. Yet, such hypothesis is supported by our microarray data analysis showing upregulation of late-differentiation genes in bulge SCs.
from AhR−/− mice. Furthermore, the reduced potential of AhR−/− cells to generate holocones from single SCs52 indicates that AhR sustains multipotentiality of EpdSCs. Even more, the faster and more effective regeneration of skin and hair in nude mice grafted with Ker and fibroblasts from AhR+/+ mice confirms the role of the receptor in regulating SCs functions. Notably, although the dermis is required for HF regeneration, AhR seems to have a cell-autonomous role in the epidermal component needed to reach an optimum regenerative efficiency. These data support the existence of AhR-dependent keratinocyte-autonomous mechanisms whose disruption compromises de novo generation of skin and hair.

In agreement, AhR depletion disturbed the genetic identity of EpdSC since they displayed a decreased expression profile of bulge-associated molecular signatures, including those determining their quiescent vs activated stage. Thus, the enrichment in late differentiation markers in AhR−/− EpdSCs likely cooperates to compromise their stemness properties. Indeed, well-known bulge-associated stemness genes were repressed whereas those involved in keratinocyte differentiation or in establishing the skin barrier were upregulated in AhR−/− EpdSCs. We have found a specific signature of 73 genes common to two different GSEAs of EpdSCs that were repressed in AhR−/− bulge cells. Thus, future work will analyze if this transcription factor binds consensus DNA binding elements in the promoter region of the identified set of genes in order to control their expression during skin differentiation. The potential role of epigenetic modifiers and transcriptional cofactors could be also assessed to find chromatin signatures in this set of genes eventually relevant to the stemness. Moreover, the presence of several transcription factors in this set of genes might be crucial to implement a deeper regulatory network of transcriptional targets whose activity is dependent on their expression levels.

It is worth noting the importance of EpdSCs in deciphering the molecular mechanisms underlying different types of skin cancer. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) represent the two most frequent types of nonmelanoma skin cancers.57 BCC is a slow-growing cancer, initially thought to derive from HFs due to its biochemical and histological features,58 but more recently ascribed to progenitor cells residing within the IFE.59 SCC appears to originate from cells undergoing differentiation similarly to IFE.60 Our findings demonstrating AhR-dependent activation of EpdSCs during skin regeneration could help understand the complex signaling pathways that take place in skin cancer.

Genetic depletion of AhR and rescue experiments in primary Ker grown in 3-D skin equivalents support the role of this receptor in epidermal differentiation. In agreement with our results, former studies have reported that AhR−/− mouse Ker have reduced expression of differentiation-related genes.28 On the opposite, AhR activation by the carcinogenic ligand TCDD induces the expression of epidermal genes causing aberrant differentiation26 whereas its pharmacological activation results in premature and anomalous differentiation with poor skin stratification. Altogether, these data are consistent with a promoting function of AhR in epithelial cell differentiation.23,61 Moreover, the AhR heterodimerization partner ARNT (aryl hydrocarbon receptor nuclear translocator) has also relevant functions in the maintenance of epidermal homeostasis by regulating epidermal differentiation genes ultimately avoiding abnormal cornification and desquamation of the skin.62 Consequently, the AhR-ARNT complex has major functions in maintaining a proper epidermal barrier to protect against toxic polycyclic aromatic hydrocarbons inducing severe skin lesions such as chloracne, acanthosis and hyperkeratosis.63 Expression analyses have identified different AhR/ARNT target genes relevant for cornification and skin barrier functioning whose dioxin-dependent activities are counteracted by the regulator of the Ker cell fate epidermal growth factor receptor.64

In summary, we report here that AhR has novel roles in murine skin homeostasis, regeneration and hair cycling through the control of EpdSCs functions. During hair growth, AhR deficiency delays entry into anagen likely through loss of the self-renewal capacity and multipotentiality of AhR−/− HF EpdSCs. Accordingly, EpdSCs have repressed bulge-associated signatures and aberrant upregulation of late-differentiation gene expression programs. Although the mechanisms by which AhR intervenes in these processes are not yet understood, we reinforce the idea that AhR plays important roles in skin homeostasis and propose that this receptor could be a potential target for the treatment of cutaneous pathologies, skin lesions and skin regeneration. The feasibility to manipulate the expression and/or activity of AhR in vitro and in vivo models that recapitulate the architecture of the epidermis provides an exceptional opportunity to validate selective molecular modulators useful in the treatment of skin pathologies.

5 | CONCLUSION

AhR plays important roles in the skin contributing to its regeneration and homeostasis by controlling the activities of Epidermal Stem Cells. The ever increasing number of non-toxic and non-harmful AhR ligands currently discovered and characterized, opens the exciting possibility of using such molecules to target AhR expression for the treatment of severe skin pathologies such as chloracne, psoriasis and several others.

ACKNOWLEDGMENTS

The authors acknowledge the support of the Servicio de Patología Molecular Comparada of the Centro de Investigación del Cáncer and CIBERONC (Universidad de Salamanca) and the Servicio de Técnicas Aplicadas a las Biociencias (STAB-SAIUEX) de la Universidad de Extremadura. This work was supported by grants to P.M.F.S. from the Spanish Ministry of Economy and Competitiveness (SAF2017-82597-R and PID2020-114846RB-I00) and from the Junta de Extremadura (GR18006, IB160210, and IB20014). E.M.R.L. was supported by the RTICC and the Junta de Extremadura. All Spanish funding is cosponsored by the European Union FEDER program.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

E.M.R.L.: conception and design, manuscript writing; L.F.L.M.: helped with the in vivo and in vitro experiments and discussed the results;
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