The combined effects of Map3k1 mutation and dioxin on differentiation of keratinocytes derived from mouse embryonic stem cells

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Epithelial development starts with stem cell commitment to ectoderm followed by differentiation to the basal keratinocytes. The basal keratinocytes, first committed in embryogenesis, constitute the basal layer of the epidermis. They have robust proliferation and differentiation potential and are responsible for epidermal expansion, maintenance and regeneration. We generated basal epithelial cells in vitro through differentiation of mouse embryonic stem cells (mESCs). Early on in differentiation, the expression of stem cell markers, Oct4 and Nanog, decreased sharply along with increased ectoderm marker keratin (Krt) 18. Later on, Krt 18 expression was subdued when cells displayed basal keratinocyte characteristics, including regular polygonal shape, adherent and tight junctions and Krt 14 expression. These cells additionally expressed abundant Sca-1, Krt15 and p63, suggesting epidermal progenitor characteristics. Using Map3k1 mutant mESCs and environmental dioxin, we examined the gene and environment effects on differentiation. Neither Map3k1 mutation nor dioxin altered mESC differentiation to ectoderm and basal keratinocytes, but they, individually and in combination, potentiated Krt 1 expression and basal to spinous differentiation. Similar gene-environment effects were observed in vivo where dioxin exposure increased Krt 1 more substantially in the epithelium of Map3k1+/− than wild type embryos. Thus, the in vitro model of epithelial differentiation can be used to investigate the effects of genetic and environmental factors on epidermal development.

The development of the epidermis is a consecutive multi-step process that commences at the differentiation of stem cell to surface ectoderm, which in turn differentiates to the keratinocytes1,2. Keratinocytes are the principal constituents of the epidermis, composed of four layers, basal, spinous, granular and cornified envelop. Cells in each layer have distinct morphological and biochemical properties and gene expression3. Typically, the basal keratinocytes express keratins (Krt) 5 and Krt 14, the spinous keratinocytes express Krt 1 and Krt 10, and the granular and cornified keratinocytes express Involucrin, Loricrin and Filaggrin4. Only the basal keratinocytes are capable of proliferation, and they serve as the reservoir to supply and replenish cells in the suprabasal layers.

Disruption of the differentiation program of the epidermis leads to developmental defects5–9. Skin abnormalities, such as neurofibromatosis, xeroderma pigmentosum, epidermolysis bullosa, and the most common psoriasis and atopic eczema, are costly congenital disorders that often require lifetime health care10. Mutations of genes associated with epithelial differentiation account for some anomaly cases, whereas environmental factors acting through epigenetic modulations are also known to be an etiology for the diseases11–14. Up to date, the genetic and environmental etiology for many skin disorders has remained poorly understood.

MAP3K1, also known as MEK Kinase 1 (MEKK1), is a member of the MAP3K superfamily, and plays highly specific roles in signal transduction and embryonic development15. MAP3K1 deficient mice have a birth defect of the eye, due to abnormal epithelial morphogenesis in the embryonic eyelids16. While how MAP3K1 affects epithelial morphogenesis is still under investigation, the gene expression signatures in epithelial cells isolated from wild type and Map3k1 knockout mouse show that MAP3K1 may impede basal to suprabasal differentiation17. This role of MAP3K1, however, had neither been validated nor further explored.
The dioxin-like chemicals (DLCs) represent a large group of chemicals that are widespread environmental contaminants. The DLCs are generated either naturally through processes like forest fires and volcanic eruptions or by industrial activities, such as incomplete combustions. These chemicals are stable in the environment with a half-life of several years. Therefore, human exposure is inevitable. DLC exposure has been linked to many developmental defects. Using 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) as a model DLC congener, studies in laboratory rodents show that in utero dioxin exposure causes diverse developmental abnormalities, including, but not limited to, hydronephrosis, cleft palate, and vaginal thread formation. In the epidermis, dioxin is shown to accelerate terminal differentiation, leading to acanthosis and epidermal hyperkeratosis phenotypes in mice and potentiate terminal differentiation of human keratinocytes in vitro. Moreover, dioxin enhances differentiation of cells that have already committed to differentiation, insinuating that this environmental toxicant affects differentiation in a developmental stage-specific manner.

Mouse embryonic stem cells (mESCs) are the inner cell mass cells isolated from the pre-implantation blastocysts. When maintained under a well-defined culture condition, the mESCs have unlimited capacity of self-renewal, remain pluripotent with abundant expression of the pluripotency genes, such as Oct3/4 and Nanog. The mESCs also have potent potential of differentiation to generate all the cell types of the body. Under defined inducing conditions, they produce cells of the three primary germ layers, i.e. mesoderm, definitive endoderm and ectoderm, which in turn give rise to progenitor and mature cells of various lineages. Differentiation of mESC to generate epithelial cells for the purpose of tissue engineering and wound healing has been reported, but it has not been used to explore the genetic and/or environmental factors as the underlying etiology of epithelial disorders.

In this study, we differentiated mESCs to basal keratinocytes in vitro. Retinoic acid (RA) and bone-morphogenetic protein-4 (BMP4) were used to induce early stage commitment of the surface ectodermal lineages; Defined keratinocyte serum free medium (DKSFM) were used to drive further differentiation to and the expansion of basal keratinocytes. The resultant basal keratinocytes, with epidermal progenitor signatures, could be passaged at least 20 times with minimal terminal differentiation. Using this system, we investigated the effects of the environmental toxicant dioxin and Map3k1 gene mutations, either individually or in combination, on epidermal differentiation. The results highlight the utility of the in vitro system to investigate risk factors and multifactorial etiology in congenital skin disorders without extensive utilization of live animals.

Results
In vitro differentiation of mouse ESCs to keratinocyte. We differentiated the mESCs in vitro using a protocol adapted from Bilousova et. al. and Metallo et al. with modifications to increase efficiency at the initial phase of the procedure. Specifically, mESCs were re-suspended in EB media and the hanging drop methods were used for EB formation. Under these conditions, compact, sphere-shaped EBs formed in nearly 80% of the hanging drops. After plating on ColIV-coated plates and growing in DKSFM media, the EBs...
The role of MAP3K1 in keratinocyte differentiation. MAP3K1 is a signal transduction enzyme playing key roles in embryonic development and epithelial morphogenesis. Although the Map3k1−/− mice do not have overt skin defects, they display eye developmental defects due to abnormal epithelial morphogenesis and delayed healing of skin full-thickness wounds. To explore the role of MAP3K1 in skin biology, we re-analyzed the global gene expression in wild type and Map3k1−/− primary keratinocytes, using a more stringent cut-off criteria and performed GO analyses of the differentially expressed genes. We found that skin development was delayed in Map3k1−/− mice due to reduced expression of key genes involved in keratinocyte differentiation and cell-cell adhesion. These findings suggest that MAP3K1 is a critical regulator of keratinocyte differentiation and skin development, and may be a potential target for treating skin disorders.
a top biological process affected by MAP3K1 (Fig. 3A). Specifically, genes in epithelial terminal differentiation were significantly up-regulated in Map3k1-/- versus wild type cells (Supplementary Table s1).

To evaluate whether the roles of MAP3K1 in epithelial differentiation could be recapitulated in the in vitro differentiation model, we differentiated the wild type and Map3k1-/- mESCs and examined the expression of marker genes at different time intervals. Wild type and Map3k1-/- cells had similar expression of Krt 18 at the early phase of differentiation (Fig. 3B). While these cells also had similar Krt 14 expression, they were strikingly different on Krt 1 expression at the later phase of differentiation (Fig. 3C). The expression levels of Krt 1 mRNA were tenfold more abundant in Map3k1-/- than in wild type cells. Similarly, there was a significant nearly twofold higher Krt 1 protein in Map3k1-/- than wild type cells (Fig. 3D and E). To validate the differentiation status of the D-KC cells, we examined additional markers. Compared to the wild type cells, the Map3k1 knock out cells had a slightly higher expression of Krt 10 and Involucrin, markers for spinous and granular cells, respectively; however, they had similar expression of Loricrin and Filaggrin, which are markers for the most outer layer stratum corneum (Fig. 3F). The in vitro data validate that MAP3K1 hampers epithelial differentiation, an idea originally insinuated from global gene expression studies.

Dioxin potentiate keratinocyte differentiation in vitro. The global environmental pollutant dioxin exhibits diverse developmental toxicities and is suggested to cause acanthosis and epidermal hyperkeratosis through derailing epithelial differentiation (Fig. 4A). As most dioxin effects are mediated by the Aryl Hydrocarbon Receptor (AHR), a ligand-activated transcription factor that regulates dioxin-responsive genes, we examined AHR expression during in vitro epithelial differentiation. The expression of Ahr was negligible in mESCs, but was significantly increased as soon as the cells started to differentiate in the EBs at day 2 of differentiation, consistent with previous observations (Fig. 4A). The Ahr expression continuously increased and remained at high levels after the cells committed to primary ectodermal lineages and became basal keratinocytes, suggesting that AHR signaling could be activated by dioxin as soon as the cells exit stemness.

To evaluate the effects of dioxin on differentiation, we examined cells differentiated in media with or without dioxin for 13 days. The presence of dioxin in the culture media did not alter the expression of Krt 18 and Krt 14, suggesting that dioxin did not change the course of differentiation from mESC to surface ectoderm and basal keratinocytes (Fig. 4B). After multiple passages (> 8), the steady-state D-KC exhibited a robust dioxin-induced AHR activation, reflected by the induction of Cyp1a1, the prototypical AHR target gene (Fig. 4C). While the presence of dioxin did not change Krt 14 expression, it increased Krt 1 expression by threefold, although such increase was not detected at the protein level likely due to insufficient sensitivity of the detection methods (data not shown).
not shown). In addition to Krt 1, Krt 10 and Involucrin mRNA were also slightly induced by dioxin treatment (Fig. 4D), supporting that dioxin treatment potentiates basal to spinous keratinocyte differentiation.

Dioxin plus Map3k1 loss-of-function further promote differentiation. In vivo, dioxin and Map3k1+/− have synergistic effects on impairing eye development. When neither dioxin nor Map3k1+/− alone are detrimental, their combination causes birth defects of the eye, a defect observed also in un-treated Map3k1−/− mice.44 Notwithstanding the intriguing phenotypic observations, how the environmental and genetic factors converge to disrupt the developmental programs has remained elusive. Given the similar effects of dioxin and Map3k1 gene mutation on promoting suprabasal differentiation, we postulated that the combination of these conditions exacerbated the differentiation abnormalities. In supporting of this idea, we noted that compared to the un-treated and dioxin-treated wild type cells, the dioxin-treated Map3k1−/− cells had also increased expression of Loricrin, another cornified envelop marker (Fig. 4D). We additionally tested the idea by treatment of D-KC derived from wild type and Map3k1+/− mESCs with 10 nM dioxin for 3 days and examination of Krt 1 expression. Compared to the wild type cells, the Map3k1+/− cells had higher Krt 1 expression (Fig. 5A). Dioxin treatment increased Krt 1 expression by threefold in wild type cells, and remarkably, it induced Krt1 expression further in Map3k1+/− cells. The Krt 1 expression in dioxin-treated Map3k1−/− cells reached to nearly eightfold of the levels in the untreated wild type cells and close to the levels in the untreated Map3k1−/− cells (Figs. 3C and 5A).

We also tested this idea in vivo by treatment of pregnant mice, carrying wild type and Map3k1+/− embryos, with 50 ug/kg dioxin on embryonic day (E)11.5. The embryos were collected on E15.5, as described previously44 and the embryonic skin was examined by immunohistochemistry. The E-cadherin staining labeled multiple layers of the epithelial cells in the embryonic skin, in which the Krt 1 positive cells were detectable at the most outer layer (Fig. 5B). Quantification of the signal intensities showed that neither dioxin exposure nor Map3k1−/− altered the level of Krt 1 expression; however, their combination significantly increased Krt 1 in more than 20 samples examined (Fig. 5C). The in vivo and in vitro data together raise an intriguing possibility that the gene-environment interactions significantly potentiate basal to suprabasal differentiation as a potential mechanism underlying the eye developmental abnormalities.

Figure 4. Dioxin potentiated suprabasal differentiation. Gene expression was examined using RT-PCR. (A) The Ahr expression was compared to levels in undifferentiated mESCs, set as 1. The expression of Krt 18, Krt 14, Krt 10, Inv, Lor, Flg and the AHR activation marker, Cyp1a1 were examined (B) in cells differentiated for 13 days and (C and D) in wild type and Map3k1−/− D-KC, in the presence and absence of 10 nM dioxin as indicated. Statistical analyses and relative expression are based on comparison to (A) undifferentiated mESCs and (B-D) control wild type samples, set as 1. *p < 0.05, **p < 0.01 and ***p < 0.001 are considered significant.
Discussion

In this paper, we describe an experimental system that differentiates mESCs to basal keratinocytes in vitro. The system enables convenient incorporation of genetic and environmental components, leading to the findings that Map3k1 loss-of-function and dioxin, while do not affect mESC differentiation to surface ectoderm and basal keratinocytes, jointly potentiate basal to suprabasal epidermal differentiation. Compelled by these in vitro findings, we examined the gene-environment interactions in vivo and found that in utero dioxin exposure indeed increased Krt 1 expression more abundantly in the Map3k1+/− than in the wild type embryos. These data suggest that the in vitro system described here can be used to explore complex conditions and etiology in the perturbation of epithelial differentiation.

Dioxin is a ubiquitous environmental agent that is stable and persistent in the environment and biological systems. Consistent with the notion that most toxic effects of dioxin are mediated through the AHR, we found a good correlation between Ahr expression and dioxin effects on differentiation. The minimal Ahr expression in mESCs and early phase of differentiation corresponded with unaltered differentiation from mESC to progenitor to basal keratinocytes in the presence of dioxin. The gradually increased Ahr and the steady-state high expression in the basal keratinocytes corresponded to potentiation of basal to spinous differentiation by dioxin. A similar observation has been made in the human cell culture models where dioxin is found to accelerate keratinocyte terminal differentiation, but does not change proliferation and apoptosis. It is worth noting that of the many clinical manifestations of dioxin exposure, chloracne, a hyperkeratotic skin disorder is the most consistent pathology observed in exposed humans. Thus, potentiation of basal to spinous differentiation observed here is likely relevant to dioxin-induced skin pathogenesis.

In Figure 5, we show that dioxin plus Map3k1 loss-of-function potentiated keratinocyte terminal differentiation. The Krt 1 expression was examined (A) by RT-PCR in wild type and Map3k1+/− D-KC with or without 10 nM dioxin treatment for 3 days, and (B) by immunohistochemistry in wild type and Map3k1+/− E15.5 embryos with or without 50 ug/kg dioxin treatment at E11.5; E-cadherin labeled all epithelial cells. (C) Quantification of Krt 1-positive signals in (B). *p < 0.05, **p < 0.01 are considered significantly different compared to wild type untreated samples, and ***p < 0.001 are considered significantly different compared to untreated samples of the same genotype. Scale bar represents 50 μm.
products have not been causally linked to Krt 1 expression and differentiation. The dioxin-AHR axis also regulates expression of genes that are not implicated in detoxification, such as transforming growth factor-α, epidermal growth factor²⁹, interleukin-1β and Plasminogen activator inhibitor-²⁵. Some of these gene products may mediate the toxicities of dioxin. For example, in a mouse model of embryonic palate fusion, Abbott et al. showed that dioxin induces epithelial differentiation abnormalities to cause cleft palate in wild type, but not EGF knockout mouse palates, implicating a role for EGF in dioxin toxicity²⁴.

We have previously shown that MAP3K1 is a signaling molecule crucial for eye development and that the Map3k1⁺/⁻ but not Map3k1⁻/⁻ embryos have eyelid closure defects due to epithelial morphogenetic abnormalities⁴⁶. More recently, we found that Map3k1 gene mutations sensitize the developmental programs to the toxicity of dioxin-like environmental chemicals⁴⁵. Specifically, in utero dioxin exposure induces eye defects in Map3k1⁻/⁻ but not wild type embryos. The in vitro mESC epithelial differentiation model, followed by in vivo validation, suggests that the gene (Map3k1)-environment (dioxin) interactions affect epithelial differentiation. The in vitro and in vivo data present a coherent narrative that Map3k1 mutation and dioxin have small effects on promoting basal to suprabasal differentiation, which is further potentiated by both agents together. The differentiation abnormalities induced by dioxin plus Map3k1⁺/⁻ resemble those in Map3k1⁻/⁻ cells, raising an intriguing possibility that the developmental defects occur when the differentiation abnormalities reaching beyond a threshold level.

Our differentiation protocol is modified from Metallo et al. and Bilousova et al. with improved efficiency²³,²⁴,²⁵. Using this protocol, we detected a gradual increase of Krt 18 expression, reaching the peak level in 10 days that are comparable to the time frame required for mouse stem cells to commit to ectodermal lineage in vivo²⁴. We further obtained Krt 14-expressing cells after culturing for approximately a month, though this time frame was much longer than that took in vivo for ectodermal to basal epithelial cell conversion. Given that the Krt 18-positive to Krt 14-positive conversion requires DNA methylation to turn off the Krt 14 promoter and multiple extracellular signals to activate the Krt 14 promoter, we speculate that these epigenetic and transcription machineries are less robust in vitro than in vivo²⁴,²⁵.

In vivo, the basal layer epidermis has low Ca²⁺ concentrations that support basal keratinocyte proliferation, whereas the suprabasal layers have high Ca²⁺ concentrations to promote terminal differentiation. Additionally, the three-dimensional (3D) in vivo microenvironment facilitate the intricate cell-matrix interactions and differentiation gene expression⁵⁷. The in vitro conditions, i.e. monolayer culture and the DKSFM media containing 0.15 mM calcium similar to the Ca levels in the basal layer epidermis, on the other hand, seem to favor basal keratinocyte proliferation but prevent differentiation⁵⁸,⁵⁹. Preliminary characterization shows that the D-KC, while lacking stem cell markers, are enriched with epidermal progenitors, extending the continuously growth and subculture capacities of the Krt 14-positive cells.

In summary, we have established an experimental model that differentiates mESCs to keratinocytes that recapitulates epithelial differentiation from E3.5 to E15.5. This system can be used as a convenient tool to trace epithelial differentiation; the resultant basal keratinocytes can be amplified and cultured for a long period of time, serving as resources for epithelial molecular biology research. Using this system, we show that Map3k1 loss-of-function mutation and dioxin act jointly to potentiate epithelial spurious differentiation, unveiling a potential mechanism through which gene-environment interactions, but not each agent separately, cause developmental abnormalities. Understanding the complex etiology of diseases will help the development of preventive strategies.

Materials and methods
Reagents, antibodies and chemicals. Soybean Trypsin Inhibitor (17,075–029), Tryple and Defined Keratinocyte SFM (DKSF) were from Gibco. Dulbecco's Modification of Eagle's Medium (DMEM; 10–017-CV) was purchased from Corning. Collagen (Col) IV (354,233) was from BD Biosciences. ESGRO Leukemia Inhibitory Factor (LIF) (10000x), ESG1107, retinoic acid (RA; 302–79-4) and Hoechst 33,342 (B2261) were from Sigma; recombinant mouse bone morphogenetic protein-4 (BMP4; 120-05ET) was from PEPROTECH. Dioxin, i.e. TCDD, was from AccuStandard and dissolved in dimethyl sulfoxide (DMSO; 67–68-5, Sigma). The CV) was purchased from Corning. Collagen (Col) IV (354,233) was from BD Biosciences. ESGRO Leukemia Inhibitory Factor (LIF) (10000x), ESG1107, retinoic acid (RA; 302–79-4) and Hoechst 33,342 (B2261) were from Sigma; recombinant mouse bone morphogenetic protein-4 (BMP4; 120-05ET) was from PEPROTECH. Dioxin, i.e. TCDD, was from AccuStandard and dissolved in dimethyl sulfoxide (DMSO; 67–68-5, Sigma). The rest cell culture media and reagents, and antibodies are listed in Supplementary Table s2 and s3.

Mice, mESC culture and differentiation. The wild type and Map3k1⁺/⁻ mice, in utero dioxin exposure and the collection and process of embryos for immunostaining were described before⁴⁴. The wild type, Map3k1⁺/⁻ and Map3k1⁻/⁻ mESCs were obtained from pregnant mice as described⁶⁰. The mESCs were expanded and maintained in DMEM supplemented with 15% Knockout™ Serum Replacement (Gibco), LIF (10000x), 2 mM glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 2-mercaptoethanol (Gibco, 1000x), 100 U/ml penicillin and 100 µg/ml streptomycin (Cytiva), in a humidified incubator with 5% CO₂ at 37 °C. Protocol describing mouse experiments and procedures was approved by IACUC of the University of Cincinnati, and all experiments were performed in compliance with the ARRIVE guidelines and UC guidelines and regulations.

The step-wise differentiation of mESCs to the epithelial lineages followed protocols in Bilousova et al. and Metallo, et al. with modifications. Briefly, on day 0, mESCs were trypsinized and resuspended in DMEM with 15% fetal bovine serum (FBS), known as embryoid body (EB) media; cells (5 × 10⁴ cells/25 µl) were placed as droplets on a Petri dish lid and incubated as “hanging drops” to enable the formation of EBs that contained cells of the three primitive germ layers⁶⁰. On day 2, the EBs (about 100 in number) were collected and transferred to a 100 mm CollI-V-coated tissue culture dish in EB medium plus 1 µM RA and 25 ng/ml BMP4, conditions that selectively induce surface ectoderm differentiation. On day 4, media were changed to DKSFM plus RA and BMP4 to promote epithelial lineage differentiation. On day 8, media were changed to DKSFM for keratinocyte amplification. On day 13, many cells with epithelial morphology were moving outward from the EB center. The clumps at the EB center was removed by vacuum aspiration; the remaining cells were detached with Tryple, resuspended in DKSFM containing 10 mg/ml trypsin inhibitor and passed to a new CollI-V-coated dish, as passage (P1). The
passaged cells can be continuously passaged for at least 20 generations and storage in and recover from liquid N₂. In some experiments, dioxin (10 nM) were included in the media at different phases of culture.

**RNA isolation, reverse transcription and quantitative polymerase chain reaction (qPCR).** Total RNA was isolated using PureLink RNA Mini Kit (12,183,025) and reverse transcription was performed using SuperScript IV reverse transcriptase (18,090,010; Invitrogen) following manufacturer's protocols. qPCR was carried out using PowerUp SYBR Green Master Mix (4,367,659, Applied Biosystems) and the signals were detected with an Agilent Technologies Stratagene Mx3000P PCR machine. The PCR reactions ran for 40 cycles under the appropriate parameters for each pair of primers and fluorescence values were used to construct the amplification curve. Specifically, at 95°C for 10 s and 60°C for 1 min. A dissociation curve was performed after amplification by gradual rise in temperature from 65 to 95 °C with fluorescence signal measurement every 0.5 °C. The results were normalized using Gapdh; ΔΔCt were used to calculate fold change. Data represent results of triplicates of 2 or more experiments. The sequences of PCR primers are listed in Supplementary Table s4.

**Immunofluorescence, microscopic image and quantification.** The embryonic tissue sections were processed and immunohistochemistry was done as described previously. Briefly, the embryonic/fetal heads were fixed in 4% paraformaldehyde at 4 °C overnight. The tissues were embedded in Optimal Cutting Temperature compound and frozen. The entire eye was processed for coronal sections at 12 μM. Cells grown on ColIV-coated coverslips were fixed with 4% paraformaldehyde at 4 °C for 10 min, permeabilized with PBS plus 0.2% Triton, and subjected to immunofluorescent staining. Primary antibodies were diluted at 1:100 and secondary antibodies and nucleus staining reagents were dilute at 1:400. Immunofluorescence and bright field images were captured using a Zeiss Axio microscope. For immunostaining of the embryonic tissues, the images were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The epithelial cell layers expressing distinctive E-cadherin were outlined and the mean intensity values of Krt1 staining were measured. Krt1 level in the images was determined after background subtraction.

**Global gene expression and pathway analyses.** The wild-type and Map3k1<sup>−/−</sup> primary mouse keratinocytes were subjected to high-density microarray hybridization; the differential gene expression was analyzed as reported before and are available at GSE201823. The data were re-analyzed through identifying the significantly differential expressed genes using the cut-off criteria: log2 fold change > 1 or < -1, False Discovery Rates (FDR) < 0.1, and intensity > 200, and the biological process enrichment using Metascape as previously described. The datasets generated and/or analyzed during the current study are available.

**Statistical analyses.** Means and standard deviations were calculated based on at least three independent experiments, and analyzed using student's two-tailed t-test. *p, †p < 0.05, **p < 0.01 and ***p, ###p < 0.001 were considered statistically significant.

**Data availability**
The datasets and cells generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
J.W., develop differentiation protocol, design experiments, data analyses and write manuscript; B.X., perform experiments, data analyses and write manuscript; E.K., perform experiment, data analyses; M.M., provide research materials; Y.X., design experiments, data analyses, write manuscript.

Competing interests
The authors declare no competing interests.

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