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Vitamin D receptor is required for proliferation, migration and differentiation of epidermal stem cells and progeny during cutaneous wound repair

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Short title: VDR in epidermal stem cells

*Abbreviation*s: VDR, Vitamin D receptor; *Krt* or KRT, keratin; FLG or *Flg*, filaggrin; bSC, hair follicle bulge stem cell; eSC, interfollicular epidermal stem cells; HF, hair follicle; IFE, interfollicular epidermis; SG, sebaceous gland; CON, control; cKO, conditional knockout; AJ, adherens junction; Tam, tamoxifen; BKO, β-catenin knockout; α6, integrin α6; mo, month

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ABSTRACT

Epidermal stem cells residing in the skin play an essential role in epidermal regeneration. When skin is injured, the stem cells are first activated to proliferate, and subsequently the progeny migrate and differentiate to regenerate the epidermis. Here, we demonstrate that the vitamin D receptor (VDR) is essential for these processes to occur. The requirement for VDR on epidermal stem cell function was revealed in conditional VDR knockout (cKO) mice, in which VDR was deleted from stem cells and progeny, and mice were maintained on a low calcium diet. First, self-renewal and niche formation of epidermal stem cells were impaired. Wound-induced activation of epidermal stem cells was blunted associated with a reduction of β-catenin signaling. Second, wound induced migration of stem cells and progeny was impaired as shown by lineage tracing and delayed migration of VDR silenced cells. Epidermal differentiation of progeny was impaired at the wounding site associated with reduced E-cadherin expression. Deletion of VDR also changed stem cell fate blunting hair development, increasing sebaceous glands, and altering expression and location of epidermal markers. These results suggest that VDR is required for self-renewal, migration and differentiation of epidermal stem cells and progeny during cutaneous wound healing.

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INTRODUCTION

The mammalian epidermis provides a protective barrier that retains essential body fluids. At the interface between the external environment and the body, skin is constantly subjected to physical trauma and must be primed to repair wounds in response to injury. Epidermal stem cells (SC) residing in the skin play essential roles for maintenance (Mascre *et al.*, 2012) and regeneration of epidermis during wound repair (Lim *et al.*, 2013; Plikus *et al.*, 2012). During embryonic development, the skin epithelia originate from ectoderm and differentiate into the interfollicular epidermis (IFE), sebaceous gland (SG) and hair follicle (HF). After birth, adult SC residing in different locations are responsible for the regeneration of IFE, SG, and HF. The putative epidermal SC, which reside in the basal layer of IFE (eSC), are at least in part regulated by β-catenin signaling to maintain IFE (Lim *et al.*, 2013). SC residing in the bulge region of HF (bSC) marked by CD34 expression, are responsible for hair follicles (Blanpain and Fuchs, 2006). When the skin is injured, eSC (Mascre *et al.*, 2012; Plikus *et al.*, 2012) and bSC (Ito *et al.*, 2005) are first to be activated to proliferate. Subsequently, the progeny migrate and differentiate to re-epithelized the wounds to regenerate the intact epidermis. A number of signaling pathways including β-catenin and calcium control the epidermal SC and progeny during regeneration processes in wound repair (Blanpain and Fuchs, 2006).

The vitamin D receptor (VDR) and its ligand of $1,25(OH)_2D_3$ are well known regulators of epidermal proliferation and differentiation (Bikle, 2011). The epidermis is the major source of vitamin D for the body, produced from 7-dehydrocholesterol

under the influence of UVB, and keratinocytes are capable of metabolizing vitamin D to its active ligand $1,25(OH)_2D_3$ (Bikle, 2011). However, the role of VDR and its ligand in epidermal SC has received less attention especially in the context of epidermal regeneration during wound repair. The role of VDR in epidermal SC is mainly studied in the context of hair regulation, as alopecia is a striking phenotype in mice lacking VDR (Li *et al.*, 1997). The global VDR knockout (KO) mice demonstrated no change of bSC (Palmer *et al.*, 2008). Gradual decrease in bSC was reported after hair defects, although $CD34^+$ niche formation was not affected (Cianferotti *et al.*, 2007). Moreover, it was reported by the same group that wound healing in the global VDR KO mouse was due to altered dermal fibroblast function but not epidermal repair (Luderer *et al.*, 2013).

However, we found a role for VDR in the keratinocyte during wound repair (Oda *et al.*, 2015). When we generated conditional VDR knockout (cKO) mice, in which VDR is deleted from Krt14 expressing epidermal SC and progeny, wound closure was delayed and expression of β-catenin target genes decreased. Our results indicated that VDR exerts its function through regulation of β-catenin signaling in the skin, which plays an important role in maintenance of epidermal SC such as $CD34^+$bSC (Choi *et al.*, 2013; Lien *et al.*, 2014) and eSC (Lim *et al.*, 2013). The VDR directly binds to β-catenin in the AF2 domain, and drive target gene transcription (Palmer *et al.*, 2008; Shah *et al.*, 2006). Delayed wound closure was significant in cKO mice compared to Cre negative control mice, when both mice were maintained on a low calcium diet. However, such a diet had no impact on control mice with intact VDR (Oda *et al.*,

2015). Here, we examined the role of VDR in epidermal wound repair under the experimental condition of lower dietary calcium. We tested the hypothesis that the defect in wound repair in mice lacking VDR in epidermal cells lay in the inability of 1) epidermal stem cells to be activated to proliferate, or 2) their progeny to migrate and differentiate to re-epithelialize the wound and regenerate epidermis. In particular, we explored the hypothesis that VDR with adequate dietary calcium regulates 1) epidermal stem cell activation through the interaction with β-catenin signaling and 2) epidermal differentiation via adherens junction signaling.

RESULT AND DISCUSSION

VDR is expressed in epidermal SC, and its deletion decreases gene expression of SC markers.

Previously, we generated VDR cKO mice, in which VDR is removed from KRT14 expressing epidermal SC and progeny by using the Cre-loxP strategy (Oda *et al.*, 2015). Homozygous floxed VDR mice with the KRT14 driven Cre transgene (VDR cKO) were compared to control littermates that have floxed VDR alleles but no Cre (CON). VDR was expressed in the bulge area of HF (bSC) and IFE that are marked by the stem marker KRT15, but efficiently removed in IFE and HF in cKO at 3 months (mo) (Fig. 1a). Deletion of VDR was further confirmed by mRNA analysis (Fig. 1b, 3 month (mo)), and western analysis (Fig. 1c, S1b), and PCR in both neonatal (P1-3) and adult stages (3 mo) (Fig. S1a). The expression of KRT15 was also substantially reduced in VDR cKO (Fig. 1a), as shown by mRNA analyses (Fig. 1b) and protein analysis (Fig. 1c, Fig. S1b) with statistical significance. Both cKO and

CON mice were maintained on a low calcium diet after weaning, in which serum calcium levels did not change, and 25(OH)D_3 levels slightly increased (Fig. S2a, b).

We then investigated the impact of VDR deletion on gene expression profiles. Keratinocytes were isolated from VDR cKO and CON skin at 3 mo (n=3) as described (Nowak and Fuchs, 2009), and high purity RNA was isolated. The gene expression profiles were analyzed using an Illumina beads-based microarray including 25,600 annotated transcripts and 19,100 genes as described (Yoshizaki *et al.*, 2014) (data are available in GSE68727 under super-series GSE68729). These analyses revealed that the deletion of VDR significantly down-regulated the expression of epidermal stem cell markers, which were previously identified for bSC (Lien *et al.*, 2011) and eSC (Lim *et al.*, 2013) (Fig. S1c). The expression of Krt15 decreased in microarray analyses (fold change: -1.39 shown by heat map in Fig. S1c), that was consistent with decreases in mRNA and protein expression in cKO.

Deletion of VDR impairs self-renewal of epidermal stem cells in both the eSC and bSC niches.

We then conducted cell sorting to address whether the marked decreases in gene expression of stem cell markers is due to a decrease in the number of epidermal SC residing in the skin. Keratinocytes were isolated from the epidermis of VDR cKO and CON skin (n=3), in which both cKO and CON mice were maintained under low dietary calcium, in which the level of serum calcium did not change (Fig. S3b). They were incubated with antibodies against Sca1, CD34 and integrin α6 (α6) and separated by FACS analysis as described (Adam *et al.*, 2015). The IFE basal cells

containing eSC express Sca1, and HF bSC were marked by CD34 (Fig. 2a). The double peaks of $Sca1^+$ populations (arrow) decreased in cKO to generate a Sca1 weak population (Fig. 2b). The percent of basal cells ($Sca1^+\alpha6^+CD34^-$) per total cells was decreased in VDR cKO compared to CON by 51.8±2.8% (eg. from 33.7% to 16.6% in the sample shown) (n=3, P<0.05) (Fig. 2c, d). In addition, the $CD34^+$ peak (arrow) representing bSC was lower in cKO (Fig. 2e). The number of cells in the two subpopulations together of basal bSC ($Sca1^-\alpha6^{high}CD34^+$) and suprabasal bSC ($Sca1^-\alpha6^{low}CD34^+$) per total cells also decreased by 53.1±4.8% (eg. from 14.7% to 7.1% in sample shown) (n=3, P<0.05) with the greater loss in the suprabasal bSC (eg. from 7.15% to 2.86% in sample shown) (n=3, P<0.05) in cKO (Fig. 2f), as is well illustrated in the reduced number of $CD34^+$ cells in the cKO in the immunofluorescence results (Fig. 2g). These data demonstrated that VDR deletion impaired self-renewal for bSC and eSC, and niche formation for bSC.

Deletion of VDR blunts injury induced proliferation of epidermal SC and prevents activation of β-catenin signaling

We then determined the impact of deleting VDR on the ability of SC to respond to wounding to activate their proliferation. A full thickness 3mm skin biopsy was obtained from the back at 3 months (telogen skin) of cKO and CON mice. After 3 days, wounded skin and non-wounded control skin were harvested, and wound induced activation of SC was evaluated by measuring cell proliferation using PCNA. In CON skin, HFs were enlarged near the wound margin (shown by red bolt), where the number of PCNA positive proliferating cells markedly increased in both the HF

and IFE (Fig. 3a red triangles, Fig. S3a, b). However, cKO HFs remained abnormal, and PCNA positive cells were considerably fewer in both HF (dotted box) and IFE (Fig. 3a yellow triangles, Fig. S3a, b). in which results were quantitated by counting the number of PCNA positive cells in the epidermis and HF at the wound margin (Fig. 3b). Pathway analysis (IPA) on microarray data generated a mechanistic hypothesis that VDR deletion causes defects through inhibition of β-catenin signaling as shown by a heat map (Fig. S3c) and pathway model (Fig. S4). The β-catenin target gene of CD44 was specifically stimulated in epidermal cells in both IFE and HF at the wounding edge of CON (Fig. 3c), but was lower in both HF (dotted boxes) and IFE in cKO wounds (Fig. 3c. d). The decrease of Cd44 was restricted to epithelial tissues at the wounding edge but was not observed in non-wounded normal skin (qPCR) or in normal cells (microarray, Fig. S3c). qPCR demonstrated that wounding increased the expression of β-catenin target genes in CON but their increased expression was blunted in the VDR cKO (Fig. 3e) as shown previously (Oda *et al.*, 2015). However, in this case we compared the qPCR results with comparable wound biopsies from Krt14 specific β-catenin knockout mice (BKO) which as expected showed little expression of these genes at baseline or after wounding (Fig. 3e). These results demonstrate that VDR is essential for injury-induced activation of SCs and the stimulation of β-catenin signaling essential for SC activation.

Deletion of VDR impaired epidermal SC driven regeneration during wound re-epithelialization.

We then determined the role of VDR in regulating the ability of SC and progeny to re-epithelialize the wound. First, we conducted lineage tracing experiments to examine SC fate as described (Alcolea and Jones, 2014; Mascre *et al.*, 2012). Mice lacking VDR were generated by mating tamoxifen (Tam) inducible Krt14CreERT2 mice expressing Rosa TdTomato (TdT) (red fluorescence) with floxed VDR mice (cKO). They were compared to control mice (CON) with Krt14CreERT2 and RosaTdT but lacking floxed VDR. The scheme in Figure 4a demonstrates the strategy. A single injection of low dose Tam was given to label some of basal cells including eSC to facilitate the ability to follow individual eSC. After labeling, a full-thickness skin biopsy (2mm) was taken from the plantar epidermis, where only IFE eSCs contribute to wound re-epithelialization because HF is not present in plantar epidermis. As expected, only a few basal cells are labeled in both CON and VDR cKO one day after Tam injection in the biopsy samples as shown by strongly fluorescent cells (Fig. 4b white triangles). Weak fluorescence in all the basal cells are not considered as a labeling. After the wounds were completely healed (30 days), labeled eSC proliferate from the adjacent skin into the wound to regenerate the epidermis as strongly labeled cells are found in stable columns of basal and differentiated supra-basal cells. In contrast, cKO skin did not show clonal expansion (Fig. 4b cKO, Fig. S5), indicating an inability of SC to regenerate epidermis. However, cKO closed the wounds probably through unlabeled transient amplifying cells that may compensate SC defects. The results were quantitated by counting the number of red cells including weak background cells per epidermis on multiple

sections and normalized by CON (Fig. 4c, n=4, $P<0.05$). We also observed decreased ability of SC to differentiate the epidermis in cKO as shown by decreased Filaggrin (FLG) expression in supra-basal layer of cKO epidermis compared to CON epidermis (Fig. 4b right panels). Similar results were obtained in back skin wounding model (3 mm biopsy) (Fig. S5).

The migration of progeny also was examined in back skin wounding model. The migration of epithelial tongues was shown by epithelial specific proteins of E-cadherin (CDH1) and desmoglein 1 (DSG1). At early stage of wound healing (3 days after wounding on a 3 mm biopsy in back skin), epithelia migrated from the original wound site (Fig. 4d, red bolt) and extended (arrows) to reach to the middle of wounds (red triangles) in CON (Fig. 4d). In contrast, the length of the epithelial tongue was markedly shorter in the cKO (Fig. 4d stop signs with yellow triangles), as shown by quantitated data in Fig. 4e (n=3, $P<0.05$). The migration delay was observed only at early stage (3 days) but not at late remodeling stage (5 days).

Deletion of VDR impaired epidermal remodeling by reducing E-cadherin and epidermal differentiation.

IPA pathway analysis of microarray data also showed that VDR deletion decreased epithelial adherens junction (AJ) signaling as shown by a heat map (Fig. S6a) and pathway model (Fig. S6b). We have previously shown that E-cadherin mediated AJ signaling is essential for epidermal differentiation in a VDR dependent manner (Bikle *et al.*, 2012). Therefore, we determined the impact of deletion of VDR on E-cadherin and on the epidermal differentiation following re-epithelialization. Both epithelial

tongues were merged to restore the epidermal layers in both CON and cKO wounds by_5 days after wounding (Fig. 5a). The expression levels of E-cadherin (CDH1), a component of AJ to mediate epidermal differentiation, decreased in cKO wounds compared to CON (Fig. 5a). The epithelial specific desmosomal component of DSG1 was likewise decreased in cKO. The early IFE marker of keratin 1 (KRT1), was detected in the migrating epithelia covering the wound (arrow) through to the center of the wounds (red triangles) in CON (Fig. 5b), demonstrating that epithelia are differentiating while the cells are migrating to close the wounds. However, KRT1 expression did not extend across the wound and remained disorganized in a patchy pattern in the shortened epithelial tongues of cKO wound (Fig. 5b KRT1, cKO stop sign). Similarly, impaired differentiation was shown by the blunted extension of the intermediate marker of Involucrin (IVL) and the late marker of differentiation, Loricrin (LOR) (Fig. 5b). The decreased expression of CDH1 was observed only in newly regenerated epidermis covering the wounds (5 days) but not in surrounding non-wounded normal skin (Fig. 5a) or keratinocytes isolated from normal skin (heat map Fig. S6a).

To confirm whether the reduction in re-epithelialization in the cKO at 3 days is due to decreased migration and differentiation, we cultured human keratinocytes, in which VDR expression was blocked by transfection of siRNA (siVDR). The efficiency of VDR silencing in keratinocytes was confirmed by western blotting (Fig. 5c) and qPCR (Fig. 5d). After cells reached confluence, they were switched to supplement free media to block further proliferation and with low calcium medium (0.07mM) to

prevent differentiation. The monolayer culture was scratched with a pipette tip. After 16 hr, the cultures were photographed and the empty spaces remaining after cell migration were quantitated. Fewer siVDR cells migrated into the scratched area compared to control cells (siCON) as shown by representative images and quantitation (Fig. 5e, f). Cells were switched to high calcium media (1.2 mM) to induce epidermal differentiation. After 3 days, the expression levels of epidermal differentiation markers (*Krt1, Krt10, Lor, Flg*) were lower in siVDR cells compared to control cells (siCon), although basal keratins (*Krt14, Krt5*) did not change (Fig. 5g). These data demonstrate that VDR regulates E-cadherin mediated epidermal differentiation as well as keratinocyte migration, each essential for wound re-epithelialization.

VDR ablation alters epidermal stem cell fate for hair, epidermis and sebaceous glands.

As the keratinocyte culture system represents only interfollicular epidermis, we evaluated an impact of deletion of VDR on different epidermal lineages *in vivo*. Microarray data also demonstrated that VDR deletion *in vivo* down-regulated hair differentiation genes, but up-regulated genes involved with epidermal and sebocyte differentiation (Fig. S7c). The mRNA levels for representative differentiation markers for IFE (*Flg*) and SG (*Scd1*) were higher in the normal and wounded skin of VDR cKO and (Fig. 6a) and in β-catenin null skin (BKO) (same samples shown in Fig. 3e). The expression of HF markers of *Krt31* and *Dlx3* were low to nearly undetectable levels in VDR cKO and BKO skin (Fig. 6a). Consistent with mRNA data, the protein

expression levels of hair keratin KRT71 was lower and that of FLG was higher in VDR cKO in both whole skin and epidermis (Fig. 6b, Fig. S7a, b). The size of sebocytes and the SG was enlarged especially near the edge of the wound in VDR cKO (Fig. 6c) (260 + 55%, n=3, $P<0.05$) as observed in BKO skin (Lien *et al*., 2014). The expression of the FLG in interfollicular epidermis was similar in cKO. In contrast, FLG was specifically increased in the utricles in VDR cKO (Fig. 6d red arrows), indicating that hair follicle keratinocytes are converted to an epidermal phenotype. These results indicate that VDR regulates cell fate commitment of epidermal SC potentially through regulation of β-catenin signaling.

We propose that VDR regulates wound re-epithelialization in different locations and different stages of the healing processes. First, VDR may be essential for self-renewal of the epidermal SC residing in the bulge or epidermis, that are essential for injury induced activation through regulation of β-catenin signaling. VDR activated by its ligand, $1,25(OH)_2D_3$ is translocated to the nucleus where it facilitates the transcription of β-catenin target genes involved with the regulation of self-renewal and activation of epidermal SC through interaction with β-catenin and other transcription factors to regulate the genes involved in activation of epidermal SC. Second, VDR may play an important role in later stages of wound healing, regulating the migration of the progeny to the wounds, and their subsequent differentiation to regenerate the epidermis.

VDR may also direct a lineage commitment of epidermal stem cells. VDR ablation blunts hair fate by inducing SG fate and altering the regions of expression of

genes associate with IFE fate. The epidermal marker of FLG was detected in abnormal HF (utricles) of VDR cKO skin (Fig. 6e), suggesting that the bSC and outer root sheath epithelium are converted to an epidermal fate, an observation consistent to BKO mice and to an earlier study with the global VDR KO mice. These observations and the loss of β-catenin signaling in the VDR KO suggest that VDR may regulate stem cell fate_through β-catenin signaling. In contrast, VDR supports epidermal differentiation of the regenerating epidermis as IFE markers of KRT1/IVL/LOR and FLG were reduced in cKO wounds (Fig 5c, Fig. 4b). VDR likely regulates differentiation through calcium and E-cadherin mediated AJ signaling, in which adequate dietary calcium is required to maintain AJ formation, potentially accounting for the synergistic effects of a low calcium diet in the VDR cKO epidermis (Bikle, 2011).

Our studies support a clinical role for adequate dietary calcium and vitamin D in cutaneous wound healing. A significant percentage of the population is vitamin D deficient (Kojima *et al.*, 2013), and vitamin D deficiency is associated with poor wound healing (Burkiewicz *et al.*, 2012). Our study exploring the mechanisms by which VDR and calcium signaling regulate wound healing should further the development of methods to expedite wound healing.

MATERIALS AND METHODS

Animals

We used mice for floxed VDR, floxed β-catenin, Rosa tomato (TdT), Krt14-Cre, Krt14-CreERT2. Details for these mice are described in Supplemental information. All

experiments were approved by the Institutional Animal Care and Ethics Committee at the San Francisco VA Medical Center.

FACS analysis

A single cell suspension of freshly isolated primary keratinocytes was washed by FACS buffer (PBS containing 2% chelated FBS) and incubated with the following antibodies: integrin α6-PE (1:100 or 200, eBiosciences), CD34-eFluoro660 (1:100, eBiosciences) and Sca1-PerCP-Cy5.5 (1:200, eBiosciences) for 30 min on ice. The cells were washed with FACS buffer. Cell viability was assessed by 7-AAD labeling. FACS analysis was carried out using FACSAria sorters (BD Biosciences) running FACSDiva software (BD), and analyzed using the FlowJo program.

Lineage tracing

The red fluorescent reporter gene Rosa TdTomato (TdT) was introduced into mice expressing tamoxifen (Tam) inducible Krt14-CreERT2 with (cKO) or without (controls) the floxed VDR gene. First, the triple transgenic mice expressing floxed VDR and Krt14-CreERT2 and RosaTdT were prepared. The VDR gene was deleted and TdT expression was activated by intraperitoneal injection of tamoxifen (Sigma) dissolved in corn oil at postnatal day 21 (P21). Details for clonal fate mapping is described in supplemental information

Other methods are described in supplemental information.

CONFLICT OF INTERSTS

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The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1. Deletion of VDR decreases gene expression in epidermal stem cells.

(a) Immunostaining of VDR (green), Krt15 (red), and overlay of both (yellow with white triangles) of CON and VDR cKO skin. The schematic diagram (left) shows the location of bSC and eSC in the skin. Bar = 50 μm. (b, c) Expression of VDR and K15 in neonatal skin (P1-3) of VDR cKO and CON mice was detected by qPCR (b) and western blotting (c) (**P*<0.05; ****P*<0.001).

Figure 2. VDR deficiency decreased self-renewal potentials for epidermal SC.

FACS analyses for basal cells containing eSC ($Sca1^{+}\alpha6^{+}CD34^{-}$) and bSC ($Sca1^{-}\alpha6^{+}CD34^{+}$) in VDR cKO and CON are shown. (a) The scheme to show the location and expression markers for bSC and eSC. (b, e) Representative histograms for Sca1 (b) and (e) for CD34 to compare CON (blue) and cKO (red) are shown. (c, d) The sorting profiles and number of basal cells containing eSC ($Sca1^{+}\alpha6^{+}CD34^{-}$). (f) The profiles and number for suprabasal bSC ($Sca1^{-}\alpha6^{low}CD34^{+}$) and basal bSC ($Sca1^{+}\alpha6^{high}CD34^{-}$). (g) Immunostaining of CD34 (CD34 green, DAPI blue) on HFs (3-mo) with a diagram to show locations of basal and suprabasal bSC. These results were reproduced in two experiments and representative data are shown.

Figure 3. VDR deletion blunts injury induced proliferation of epidermal SC and activation of β-catenin signaling.

(a) Cell proliferation evaluated by PCNA staining in CON and VDR cKO mice 3 days after wounding (brown signal and blue counterstaining; Bar=50μm). (b) PCNA immunostaining quantification by Bioquant (**P* <0.05). (c)The β-catenin target gene CD44 expression in wounding edge after 3 days (CD44 red; DAPI blue; Bar=50μm). (d) CD44 immunostaining quantification by Bioquant (****P*<0.001). (e) The mRNA levels for β-catenin targets (Axin2, Wif1) and cell cycle regulators (Ccnd1, Ccnd2) in the wounded skin from VDR cKO and from β-catenin knockout wounded skin (BKO) evaluated by QPCR (**P*<0.05; n=3).

Figure 4. VDR ablation impairs SC driven epidermal regeneration during wound re-epithelialization.

(a) The strategy for lineage tracing using Krt14CreERT2RosaTdT. (b) Representative images of initial skin biopsy (0d) (left panels) and after 30 days of wound healing (middle panels), TdT labeled SC migrated to form clones in CON (white triangles, upper panels) but not in cKO (bottom panels). Differentiation was verified by FLG (right two panels) (TdT red; FLG green; DAPI blue). Bar = 25 μm (c) Quantification of the TdT labeled cells in the epidermis by Bioquant (***P*<0.05). (d) Migration of epithelial tongues at 3 days, in which wounding edge is shown by red bolt, and the

extent of the epithelial tongue is shown by arrows. Bar = 100 μm (e) Quantitated migration at 3 days (n=3, p<0.05).

Figure 5. VDR ablation prevents differentiation of progeny.

At late stage of wound healing (5 d after wounding), epithelial markers (a) and differentiation markers (b) are shown at the edge of CON and cKO wounds (brown with blue counterstaining). Arrows and red triangles show extension of differentiated cells in CON, and stop signs show their defects in cKO. Bar = 100 μm (c-g) VDR was silenced in cultured keratinocytes (siVDR). Blocking efficiency of VDR by western (c) and qPCR (d) (***P*<0.01) is shown. (e) Images for cell migration by the scratch assay (Bar = 25 μm) and (f) their quantitation (**P*<0.05, n=12). (g) mRNA expression of differentiation markers (qPCR, 1.2mM calcium, 3d culture) (n=3, P<0.05).

Figure 6. VDR ablation alters cell fate of epidermal SC for hair, epidermis and sebocytes.

(a) The mRNA levels for markers for hair (Krt31, Dlx3), IFE (Flg) and SG (Scd1) were evaluated in control and wounded skin (3 days after wound) from VDR cKO and BKO mice (3 mo) by qPCR (**P*<0.05). (b) Levels of hair keratin KRT71 and epidermal marker of FLG in epidermis of CON and VDR cKO mice by western blotting (***P*<0.01). (c) The enlarged sebocytes and SG at the wound edge (red bolt) are shown in HE stained skin of CON and VDR cKO. Bar = 100 μm (d) Increased

level of FLG in cKO skin compared to CON. FLG expression in utricles are shown by red arrows, Bar = 100 μm.

ACCEPTED MANUSCRIPT

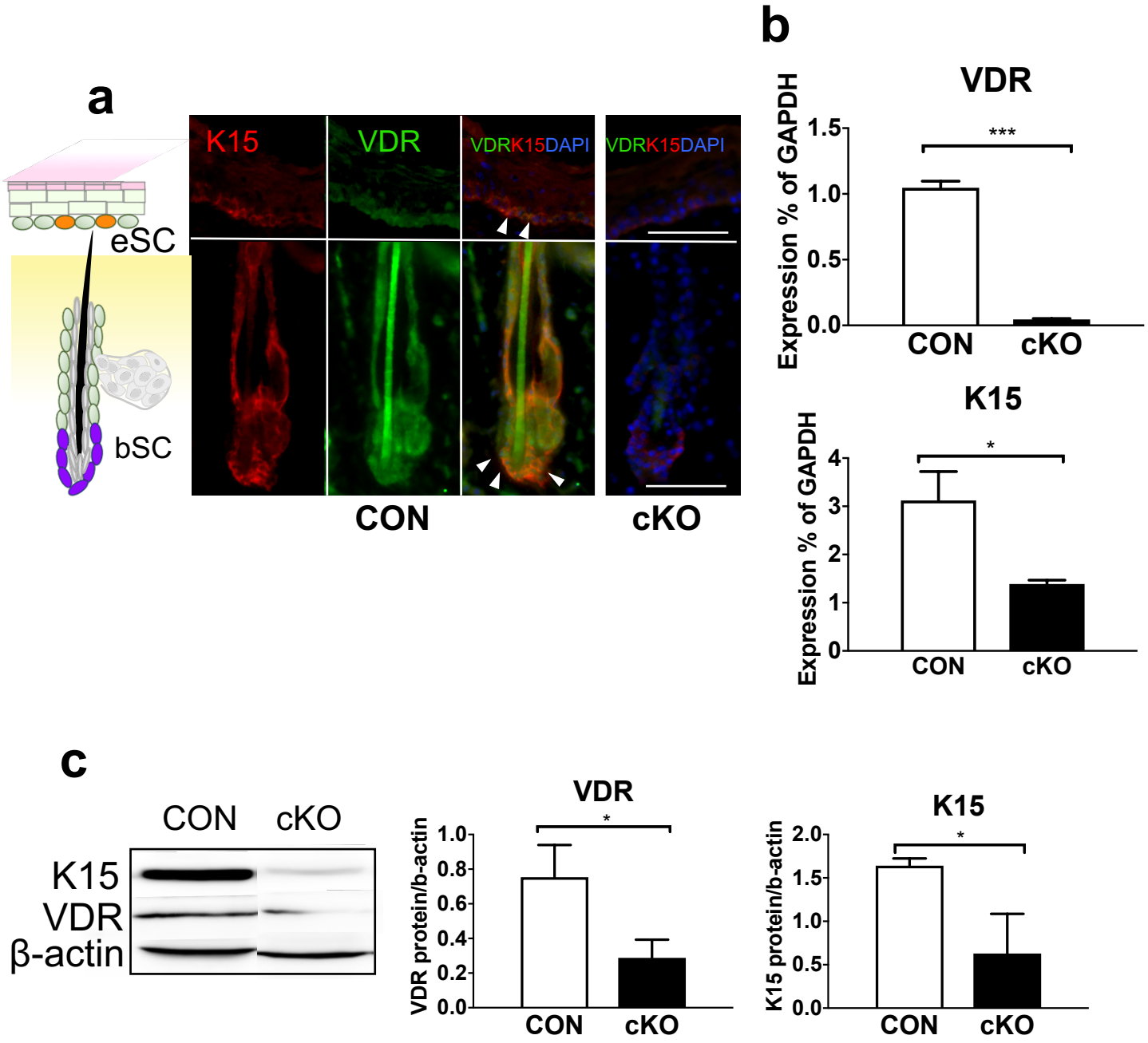
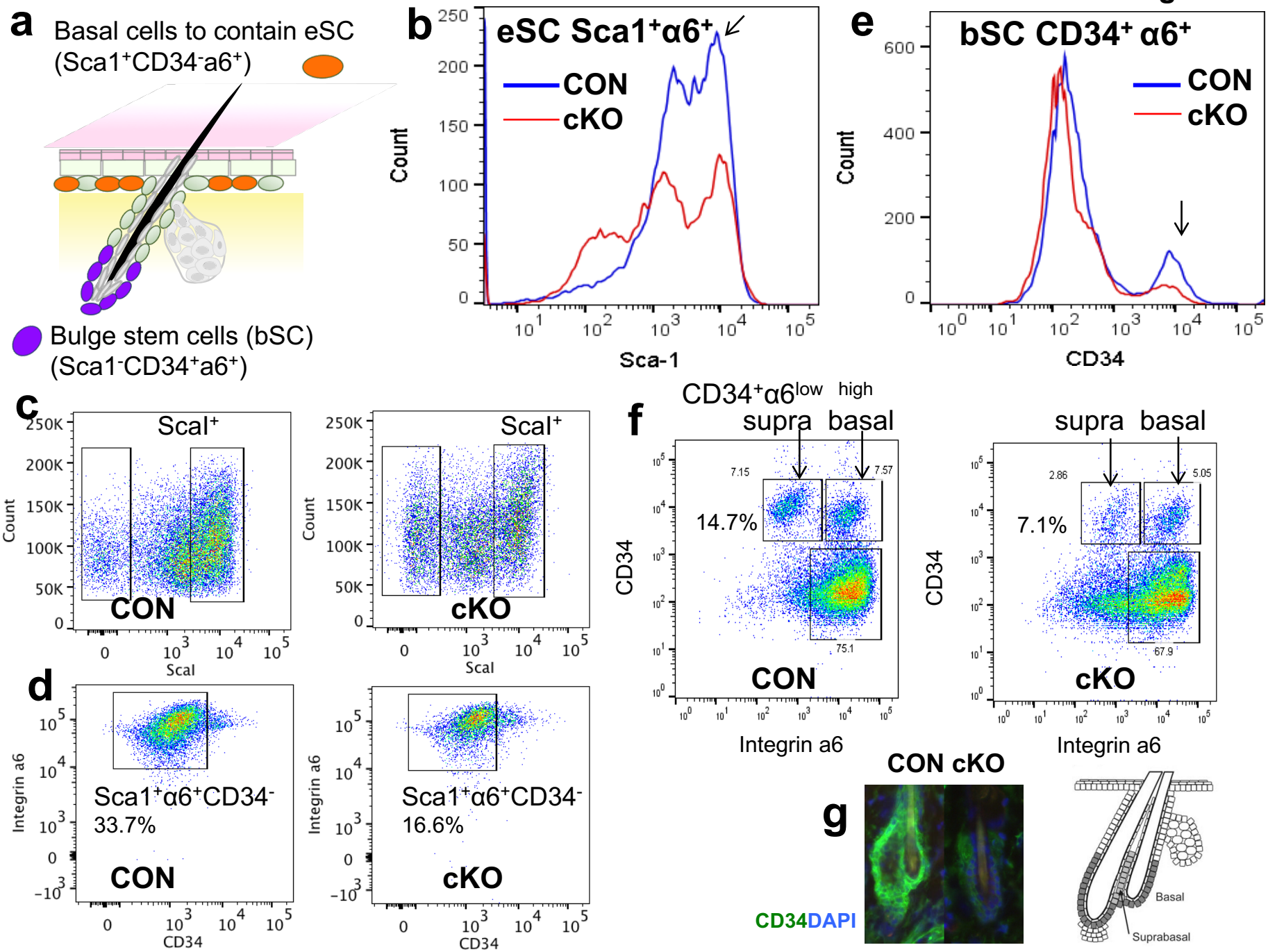

a
K15
VDR
VDRK15DAPI
VDRK15DAPI
eSC
bSC
CON
cKO
b
VDR
Expression % of GAPDH
1.5
1.0
0.5
0.0
***
CON
cKO
K15
Expression % of GAPDH
4
3
2
1
0
*
CON
cKO
c
CON
cKO
K15
VDR
β-actin
VDR
VDR protein/b-actin
1.0
0.8
0.6
0.4
0.2
0.0
*
CON
cKO
K15
K15 protein/b-actin
2.0
1.5
1.0
0.5
0.0
*
CON
cKO

Fig. 2


a
Basal cells to contain eSC
(Sca1+CD34-a6+)
Bulge stem cells (bSC)
(Sca1-CD34+a6+)
b
eSC Sca1+α6+
CON
cKO
Count
Sca-1
e
bSC CD34+ α6+
CON
cKO
Count
CD34
c
Scal+
CON
cKO
Count
Scal
d
Sca1+α6+CD34-
33.7%
16.6%
CON
cKO
Integrin a6
CD34
f
CD34+α6low high
supra basal
14.7%
7.1%
7.15
7.57
75.1
2.86
5.05
67.9
CD34
Integrin a6
CON cKO
g
CD34DAPI
Basal
Suprabasal

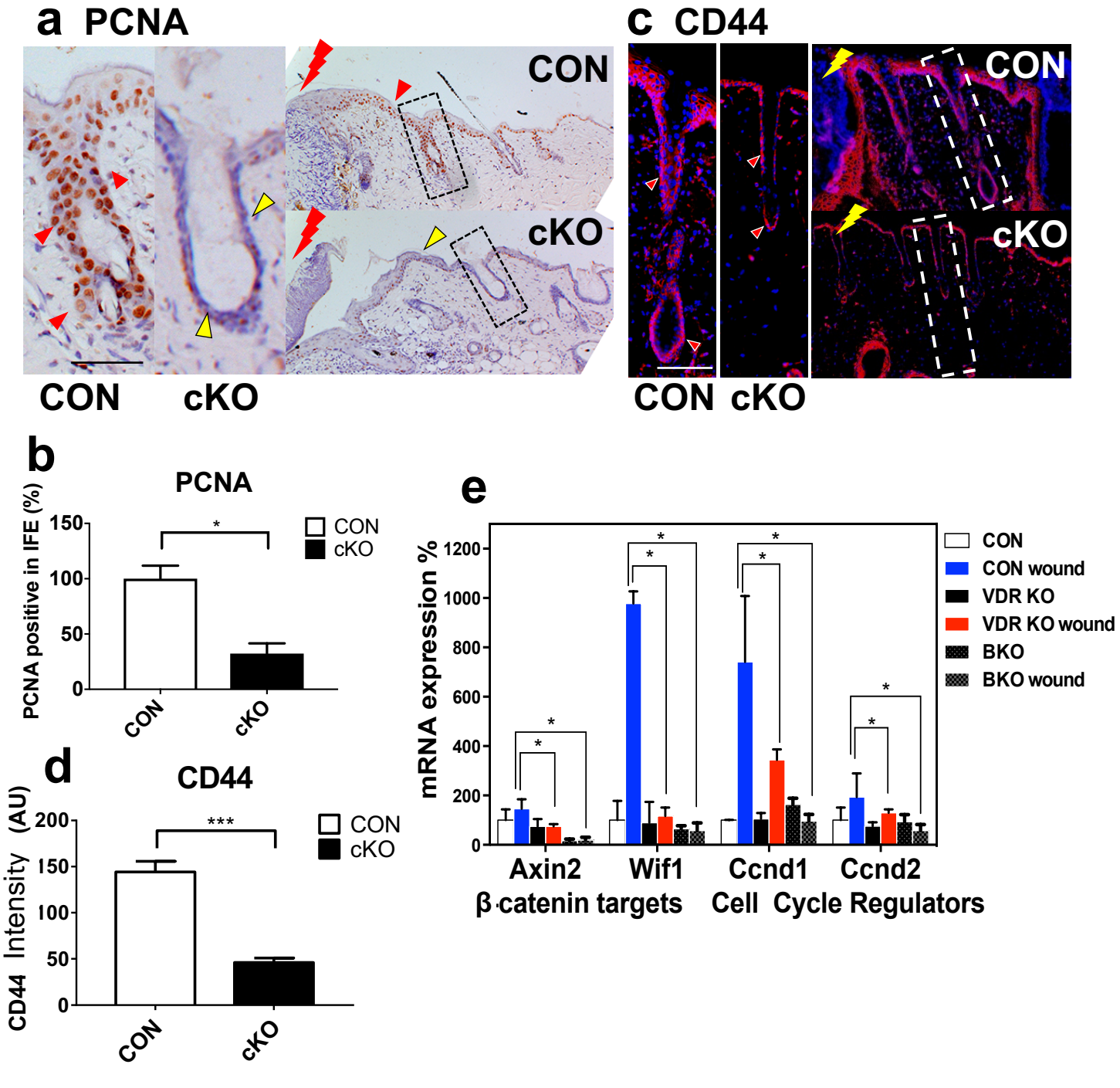

a PCNA
CON
cKO
CON
cKO
c CD44
CON
cKO
CON
cKO
b
PCNA
PCNA positive in IFE (%)
150
100
50
0
*
CON
cKO
CON
cKO
d
CD44
CD44 Intensity (AU)
200
150
100
50
0
***
CON
cKO
CON
cKO
e
mRNA expression %
1200
1000
800
600
400
200
0
CON
CON wound
VDR KO
VDR KO wound
BKO
BKO wound
Axin2
Wif1
Ccnd1
Ccnd2
β-catenin targets
Cell Cycle Regulators

Fig. 3

Fig. 4

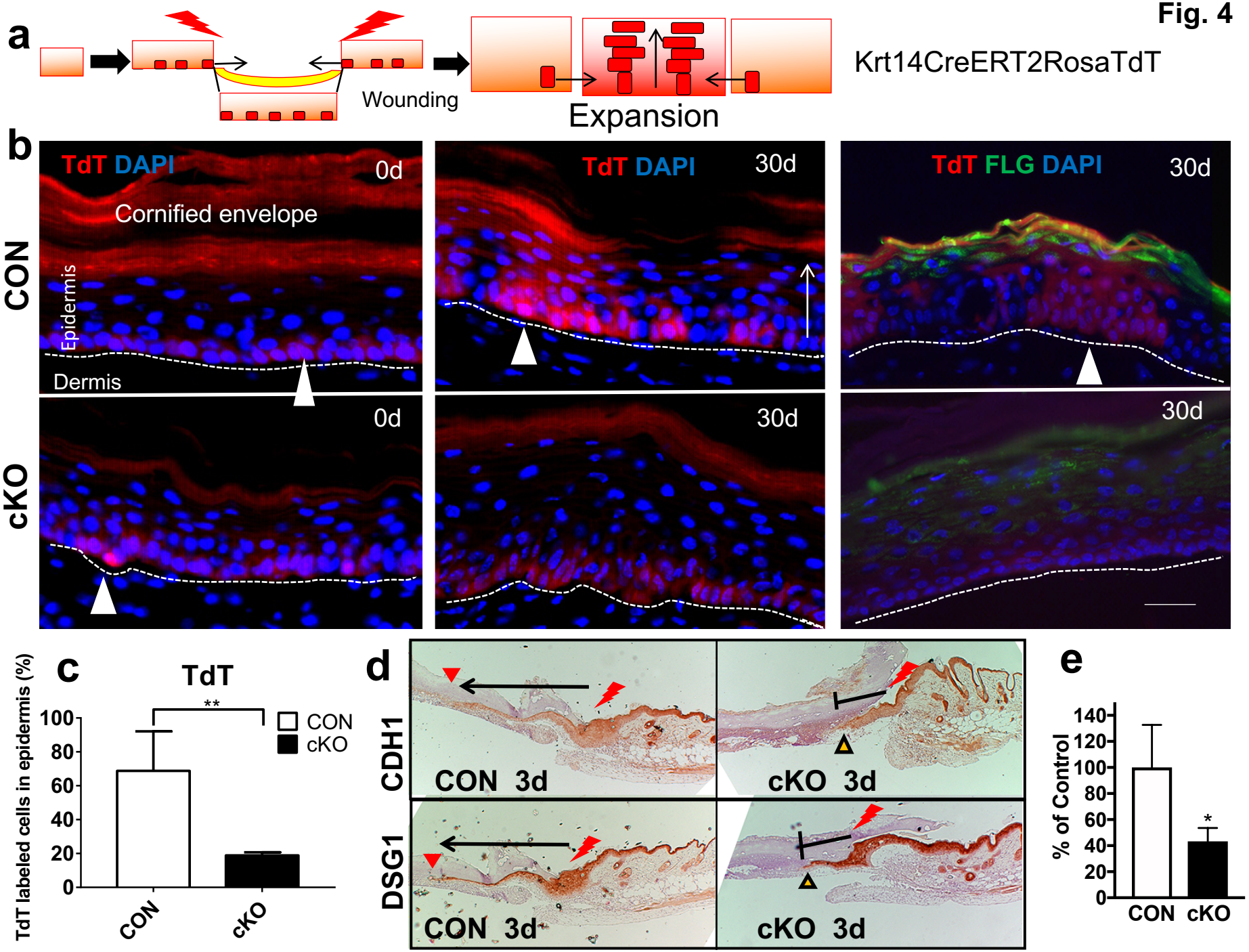

a
Wounding
Expansion
Krt14CreERT2RosaTdT
b
CON
cKO
TdT DAPI
0d
Cornified envelope
Epidermis
Dermis
TdT DAPI
30d
TdT FLG DAPI
30d
0d
30d
30d
c
TdT
**
CON
cKO
TdT labeled cells in epidermis (%)
100
80
60
40
20
0
CON
cKO
d
CDH1
DSG1
CON 3d
cKO 3d
CON 3d
cKO 3d
e
% of Control
140
120
100
80
60
40
20
0
*
CON
cKO

Fig. 5

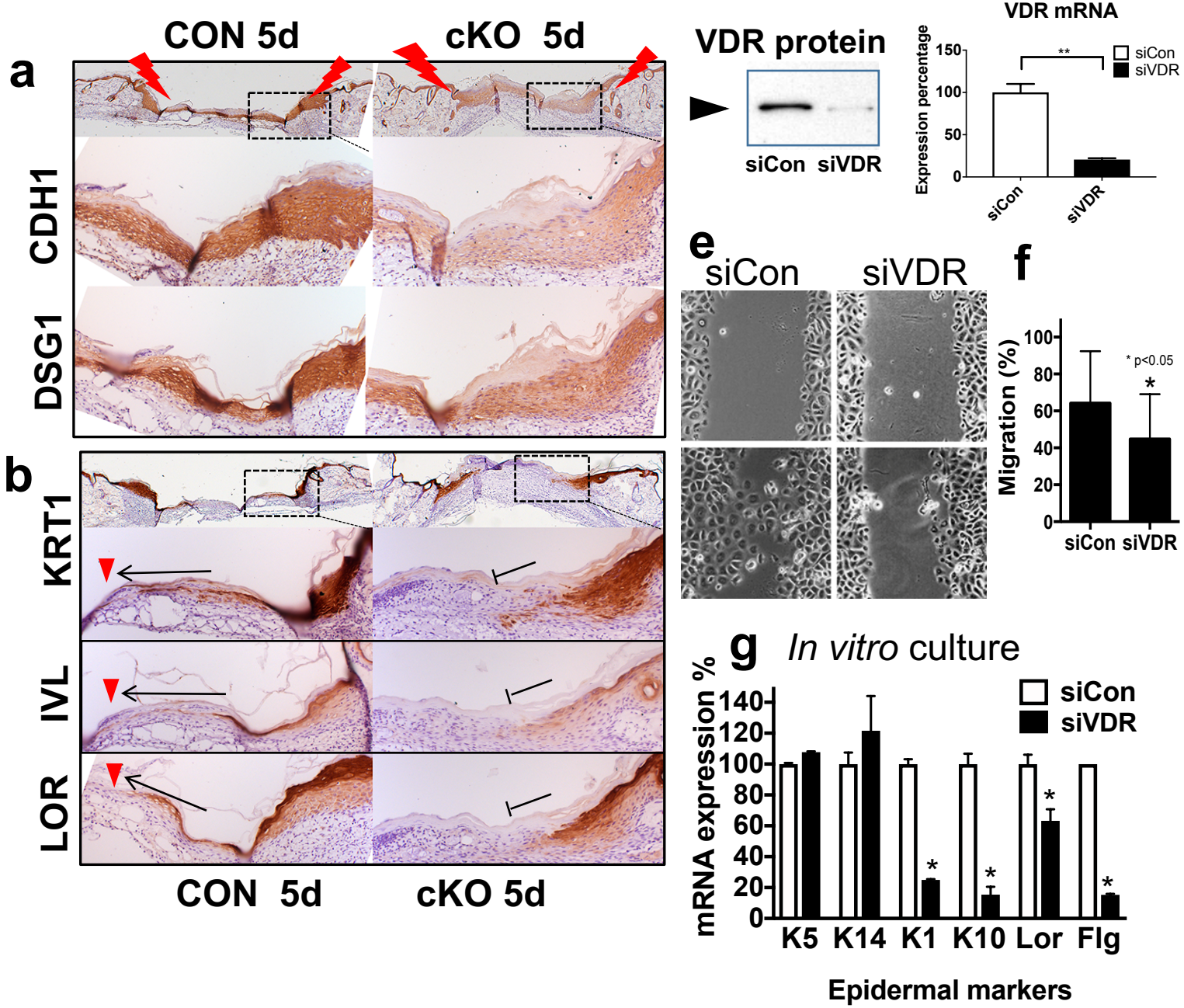
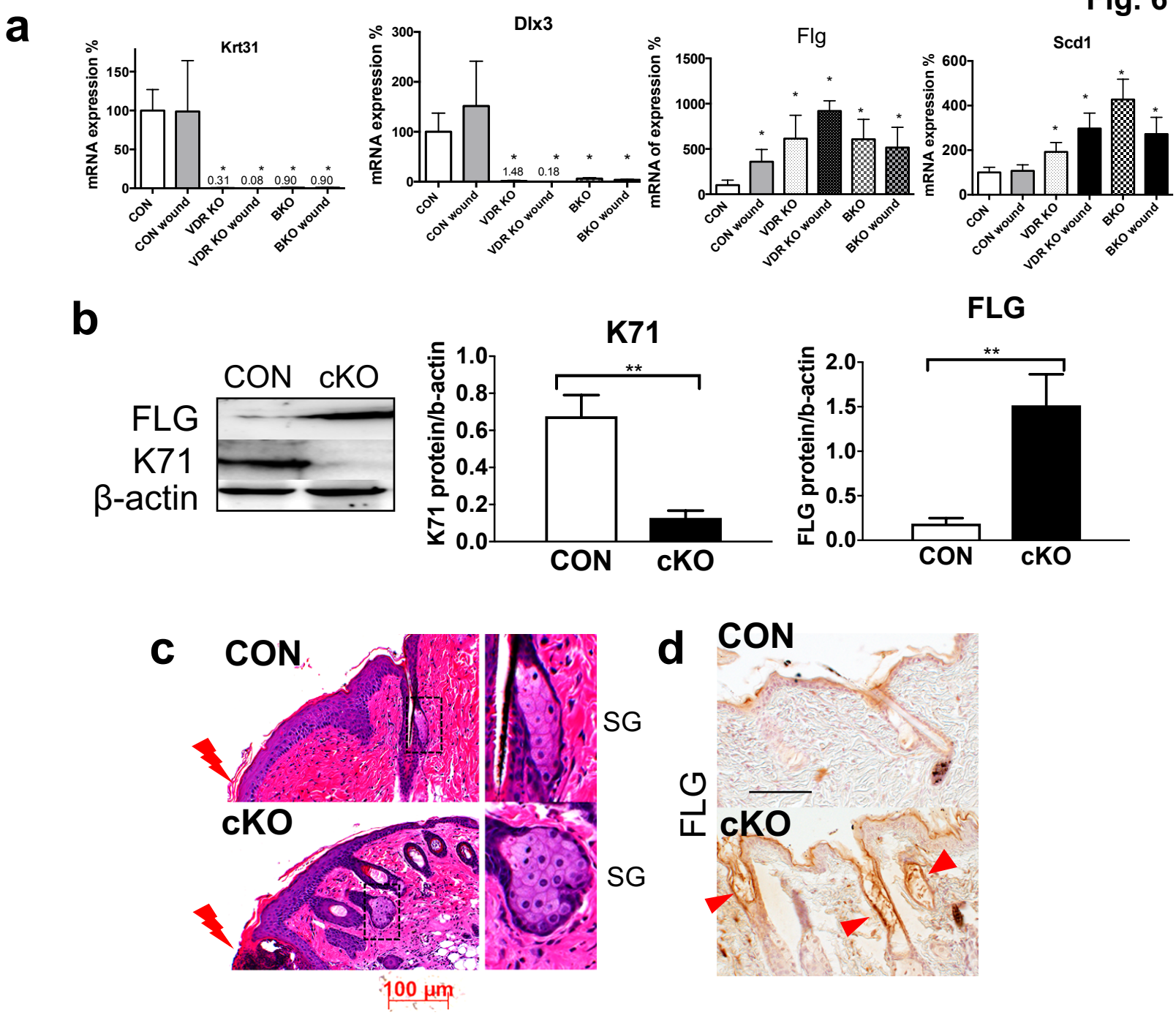

a
CON 5d
cKO 5d
CDH1
DSG1
b
KRT1
IVL
LOR
CON 5d
cKO 5d
c
VDR protein
siCon siVDR
d
VDR mRNA
Expression percentage
150
100
50
0
**
siCon
siVDR
siCon
siVDR
e
siCon
siVDR
f
Migration (%)
100
80
60
40
20
0
* p<0.05
*
siCon siVDR
g
In vitro culture
mRNA expression %
140
120
100
80
60
40
20
0
siCon
siVDR
*
*
*
*
K5 K14 K1 K10 Lor Flg
Epidermal markers

Fig. 6


a
Krt31
mRNA expression %
0.31
0.08
0.90
0.90
Dlx3
1.48
0.18
Flg
mRNA of expression %
Scd1
CON
CON wound
VDR KO
VDR KO wound
BKO
BKO wound
b
CON
cKO
FLG
K71
β-actin
K71
K71 protein/b-actin
FLG
FLG protein/b-actin
**
c
CON
cKO
SG
100 μm
d
CON
cKO
FLG