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The Antimicrobial Protein REG3A Regulates Keratinocyte Proliferation and Differentiation after Skin Injury

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Summary

Epithelial keratinocyte proliferation is an essential element of wound repair, and abnormal epithelial proliferation is an intrinsic element in the skin disorder psoriasis. The factors that trigger epithelial proliferation in these inflammatory processes are incompletely understood. Here we have shown that regenerating islet-derived protein 3-alpha (REG3A) is highly expressed in keratinocytes during psoriasis and wound repair and in imiquimod-induced psoriatic skin lesions. The expression of REG3A by kerati-nocytes is induced by interleukin-17 (IL-17) via activation of keratinocyte-encoded IL-17 receptor A (IL-17RA) and feeds back on keratinocytes to inhibit terminal differentiation and increase cell proliferation by binding to exostosin-like 3 (EXTL3) followed by activation of phosphatidylinositol 3 kinase (PI3K) and the kinase AKT. These findings reveal that REG3A, a secreted intestinal antimicrobial protein, can promote skin keratinocyte proliferation and can be induced by IL-17. This observation suggests that REG3A may mediate the epidermal hyperproliferation observed in normal wound repair and in psoriasis.

Introduction

A well-controlled and coordinated balance between immune defense and epithelial cell proliferation and differentiation is essential to normal wound repair. Keratinocytes are the predominant epithelial cell in skin and execute several complex processes that trigger

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inflammation as well as cell proliferation to maintain the structure and function of the epidermis (Eckert et al., 2002). After skin injury, the process of wound healing requires keratinocytes at the wound edge to resist microbial invasion; keratinocytes also migrate and proliferate to enable appropriate reepithelialization (Schauber et al., 2007; Gallo et al., 1994). However, in some inflammatory diseases such as psoriasis, an imbalance between proliferation and differentiation of keratinocytes results in epidermal hyperproliferation in excess of the extent of skin injury (Albanesi et al., 2007). Products of inflammatory cells present in both wound repair and psoriasis also contribute to the observed changes in keratinocyte proliferation and differentiation. Specifically, advances in understanding the pathophysiology of psoriasis have shown that increased interleukin-17 (IL-17) and IL-22 contribute to the cycle of inflammation and cellular proliferation that ultimately results in keratinocyte hyperproliferation and lesion formation in psoriasis (Lande et al., 2007; Zheng et al., 2007).

The cutaneous inflammatory response after injury includes the release of several proinflammatory cytokines such as IL-6, TNF-α, IL-17, and IL-22 (Finnerty et al., 2009; Lai et al., 2009). Many of these molecules induce inflammation to resist infection at the epithelial interface and stimulate wound repair. Other molecules released during injury, such as antimicrobial peptides, provide innate antimicrobial activity in wounds to resist infection and also stimulate cell growth and angiogenesis (Gallo et al., 1994). In skin, these pathways intersect at several levels. For example, IL-22 and IL-17 enhance the expression of antimicrobial peptides S100A7 and S100A8 in keratinocytes (Liang et al., 2006). Combined, these events provide a coordinated repair and defense strategy for the epidermis. However, in the skin disease psoriasis, the production of IL-17, IL-22, and antimicrobial peptides appears to also contribute to the pathophysiology of the disease (Lande et al., 2007; Zheng et al., 2007). Therefore, understanding the elements that participate in the cutaneous wound process is critical for both normal homeostasis and diagnosis or treatment of disease.

Other epithelial organs also employ strategies that combine antimicrobial defense and epithelial proliferation. For instance, IL-22 can augment the clonogenic potential of lung epithelial cells and accelerate repair after lung injury. In the intestine, regenerating islet-derived protein 3-alpha (REG3A), also named heptocarcinoma-intestine-pancreas (HIP) or pancreatic associated protein (PAP), functions as an antimicrobial protein in control of the bacterial proliferation (Brandl et al., 2008; Cash et al., 2006; Vaishnava et al., 2011). In addition to antimicrobial function, the expression of RegIIIγ, a mouse homolog of human REG3A, is increased after several tissue injuries, such as mucosal damage (Pull et al., 2005) and liver injury (Lieu et al., 2005), and has been suggested to influence tissue regeneration. Although REG3A or RegIIIγ in internal tissues has been extensively investigated, a role of REG3A or RegIIIγ in skin biology is less clear.

Given that the processes of both wound repair and psoriasis have a relative increase in IL-17 and a need for innate microbial defense, we investigated whether REG3A or RegIIIγ is expressed after skin injury and determine whether it has potential functional relevance in this system. Our findings uncover a vital component that appears to be inherent to cutaneous wound repair and of potential importance to the pathogenesis of psoriasis.

Results

REG3A or RegIIIγ is Abundantly Expressed in Epidermal Keratinocytes of Psoriasis Patients and Imiquimod-Induced Psoriatic Skin Lesions

Psoriasis is a skin disease characterized by excessive epithelial cell proliferation and enhanced antimicrobial defense (de Jongh et al., 2005; Wolk et al., 2006). Because of previous suggestions that REG3A influences tissue regeneration and immune defense in the

gut (Lieu et al., 2005; Okamoto, 1999; Vaishnava et al., 2011), we hypothesized that REG3A may also be expressed in hyperproliferative keratinocytes of psoriasis patients. To explore the expression of REG3A, we performed quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) on biopsies from the skin of 18 psoriasis patients. Compared to normal patients, *REG3A* mRNA was abundantly expressed in lesional skin of psoriasis patients, but not in normal skin or the lesional skin of patients with another form of skin inflammation (atopic dermatitis) or dysregulated keratinocyte proliferation (basal cell carcinomas) (Figure 1A). REG3A protein expression was detectable by immunohistochemistry localized to epidermal keratinocytes in psoriatic lesional skin, but not in the epidermis of normal skin (Figure 1B) or the epidermis of several skin carcinomas (Figure S1A available online). Interestingly, there is a large discrepancy of REG3A expression in nonlesional skin of psoriasis between Japanese and American patients in Figure 1A: *REG3A* was significantly increased in Japanese nonlesional psoriatic skin but was completely undetectable in American nonlesional samples (Figure S1B). To further confirm that REG3A is related to the psoriatic phenotype, we evaluated an imiquimod-induced psoriatic mouse model (Figure S1C) and found that both mRNA and protein of RegIIIγ were increased in imiquimod-induced psoriatic ear or back skin (Figures 1C–1E). These data demonstrate that REG3A or RegIIIγ is highly expressed in hyperproliferative keratinocytes of psoriasis but not universally expressed in normal skin or during other forms of skin inflammation or hyperproliferation.

Skin Injury Increases Epidermal RegIIIγ Expression

Psoriasis lesions can be triggered by physical injury to the skin of susceptible patients with this disorder (the Koebner response). We therefore next measured the expression of REG3A or RegIIIγ in keratinocytes during wound repair responses. To test this, we made aseptic, full-thickness incisions on the back of wild-type mice as previous reported (Lai et al., 2009). The expression of RegIIIγ mRNA was dramatically increased in the wound edge compared to normal skin (Figures 2A and 2B). Another mouse homolog of human REG3A, RegIIIα was also markedly increased in wounded skin (Figure 2C). Consistent with increased mRNA expression, the protein abundance of RegIIIγ was elevated in wounded skin compared to normal mouse skin (Figure 2D). Similar to human psoriatic epidermis, RegIIIγ was abundantly expressed by keratinocytes localized in hyperproliferative epidermis and hair follicle (Figure 2E). Taken together, these results demonstrate that skin injury increases RegIIIγ expression in epidermal and hair follicle keratinocytes.

IL-17 Induces the Expression of REG3A or RegIIIγ in Primary Keratinocytes and Mouse Skin Wounds

IL-17 and IL-22 are elevated in psoriatic skin and during wounding (Figures 1D, 1E, and S2A-S2C; Res et al., 2010; Rizzo et al., 2011). IL-22 has been shown to directly induce RegIIIγ in colonic epithelia cells (Zheng et al., 2008), so we next evaluated whether IL-17 or IL-22 could influence REG3A or RegIIIγ expression in keratinocytes. Neonatal human epidermal keratinocytes (NHEKs) treated with a panel of cytokines showed that IL-17A, but not several other factors elevated in psoriasis and wounded skin, induced REG3A expression in keratinocytes (Figure 3A). This induction was dose dependent with the maximum induction observed at 1,000 ng ml^{-1} of IL-17A (Figure 3B). Increased REG3A protein was also observed by immunohistochemical analysis of NHEKs treated with IL-17A (Figure 3C). Moreover, the induction of REG3A by IL-17 was dependent on the activation of IL-17 receptor A (IL-17RA). Treatment of NHEKs with short hairpin RNAs (shRNAs) targeted to IL-17RA was effective in inhibiting IL-17A-induced *REG3A* expression (Figure 3D). Moreover, murine IL-17a and the supernatant of wounded skin homoge-nate significantly increased RegIIIγ expression in primary murine epidermal keratinocytes, but this induction was completely abrogated by mouse IL-17a-neutralizing antibody (Figure 3E). In addition,

the administration of IL-17a-neutralizing antibody into mouse back skin decreased both mRNA and protein of RegIIIγ in wounds (Figures 3F and 3G). *Il17*$^{-/-}$ mice also showed significantly decreased RegIIIγ expression in skin wounds compared to control mice (Figure 3H). All these data suggest that IL-17 is an important molecule from wounded skin to induce REG3A or RegIIIγ.

Unlike the previously reported role of IL-22 in induction of RegIIIγ in colonic epithelia (Zheng et al., 2008), IL-22 by itself did not induce *REG3A* in keratinocytes (Figure 3A). Interestingly, the combination of various doses of IL-22 with 600 ng ml^{-1} of IL-17A induced a further increase in REG3A (Figure S3A). Although the receptor for advanced glycation endproducts (RAGE) is involved in S100A family protein expression induced by IL-17A and/or IL-22 (Heo et al., 2011), the cooperation of IL-17A and IL-22 in induction of REG3A was not dependent on this receptor, as shown by the fact that the treatment of NHEKs with shRNAs targeted to RAGE was effective in inhibiting the expression of IL-17A-induced or IL-17A+IL-22-induced *S100A7,* but not in IL-17A-induced or IL-17A +IL-22-induced *REG3A* expression (Figures S3B and S3C). Thus, these data show that IL-17A, rather than IL-22, activates its receptor IL-17RA to directly induce REG3A in skin keratinocytes, whereas IL-22 cooperatively increases REG3A in combination with IL-17A.

REG3A Increases Keratinocyte Proliferation via the Inhibition of Differentiation Genes

Because REG3A or RegIIIγ is increased in hyperproliferative keratinocytes in psoriasis (Figures 1B and 1C) and skin wounds (Figure 2E), we next investigated whether REG3A was involved in the regulation of keratinocyte proliferation and differentiation. When undifferentiated cultured NHEKs were exposed to recombinant REG3A, increased proliferation was observed as detected by an increase in cell number and percentage of cells observed in S phase (Figures 4A and 4B). Furthermore, we observed that REG3A dramatically inhibited the expression of the terminal differentiation marker genes keratin-10 *(KRT10),* filaggrin *(FLG),* and loricrin *(LOR)* but not differentiation gene keratin-5 *(KRT5)* (Candi et al., 2005) in cultured undifferentiated NHEKs (Figure 4C).

Furthermore, REG3A could inhibit keratinocyte differentiation triggered by elevated extracellular calcium, a potent signal of terminal differentiation that corresponds with increased Ca^{2+} present in the superficial epidermis. NHEKs were treated with 1.6 mM $CaCl_2$ in the presence or absence of recombinant REG3A. After 24 hr, a decreased cell number was observed in high calcium conditions (Figure 4D) and a decreased percentage of cells was observed in S phase (Figure 4E). In contrast, REG3A increased cell number even in high calcium (Figure 4D) and also rescued cells back to S phase (Figure 4E). Moreover, the calciuminduced increased expression of loricrin and filaggrin in keratinocytes was significantly inhibited by REG3A (Figures 4F and 4G). All these data suggest that REG3A can both increase keratinocyte proliferation and inhibit differentiation, elements characteristic of psoriasis and normal wound repair.

REG3A Promotes Wound Reepithelialization and Psoriatic Hyperproliferation

In response to epidermal injury, keratinocytes at the wound edge withdraw from terminal differentiation and undergo changes in gene expression followed by proliferation and migration (Epstein et al., 1983; Patel et al., 2006). We have observed that the amount of the terminal differentiation gene loricrin in wounded skin was significantly less than that in normal skin in vivo (Figure S4A) and REG3A inhibited the expression of loricrin and filaggerin in vitro (Figures 4C, 4F, and 4G). Therefore, the capacity of REG3A to inhibit keratinocyte terminal differentiation may promote cutaneous wound healing. To test this hypothesis, we first conducted an in vitro wound scratch assay. Recombinant REG3A increased the ability of both undifferentiated and differentiated keratinocytes to migrate and

reepithelialize a defined cell-free area (Figures 5A–5C). Furthermore, the overexpression of REG3A in undifferentiated keratinocytes accelerated wound reepithelialization (Figures S4B and S4C). In addition, IL-17A enhanced in vitro wound reepithelialization via the induction of REG3A as IL-17A lost its capacity to promote keratinocyte reepithelialization after *REG3A* was silenced (Figure S4D).

After in vitro wound scratch model, we next confirmed the role of RegIIIγ in promoting wound healing and psoriatic hyperproliferation in vivo. RegIIIγ significantly accelerated skin wound healing after 100 μg of RegIIIγ was intradermally injected into mouse back skin before wounding (Figure 5D), but the blockade of IL-17a or RegIIIγ by IL-17a- or RegIIIγ-neutralizing antibody, respectively, significantly delayed the healing process at day 3 (Figure 5E). However, the administration of recombinant RegIIIγ before IL-17a blockage could restore this event (Figure 5E). In addition to delaying wound healing, RegIIIγ blockage decreased ear thickness and epidermal hyperproliferation induced by imiquimod (Figures 5F and 5G). Thus, these findings confirmed that REG3A or RegIIIγ inhibited keratinocyte differentiation to promote wound reepithelialization or to induce psoriatic epidermal hyperproliferation.

REG3A Activates EXTL3-PI3K-AKT Signaling Pathway to Regulate Keratinocyte Differentiation

Having established the involvement of REG3A inthe regulation of keratinocyte differentiation and the role of REG3A in promoting wound healing, we next sought to explore the mechanism by which keratinocytes respond to REG3A. Exostosin-like 3 (EXTL3) has been implicated as a binding protein for REG1 in pancreatic β cell (Kobayashi et al., 2000) and as a potential receptor for HIP in pancreatic carcinoma cell line (Levetan et al., 2008). We thereby evaluated whether EXTL3 is required for the function of REG3A in keratinocytes. EXTL3 was observed to be expressed in keratinocytes (Figures S5A and S5B) and treatment with REG3A further increased its expression (Figure S5B). REG3A bound to EXTL3 on keratinocytes as demonstrated by immunoprecipitation of REG3A-treated keratinocytes with REG3A antibody and detection with antibody to EXTL3, or when EXTL3 was precipitated and REG3A antibody was used for detection (Figure 6A). Silencing of *EXTL3* significantly blocked the inhibitory effect of REG3A on differentiation as detected by the expression of loricrin in NHEKs (Figures 6B and S5C), and the administration of EXTL3 antibody into mouse back skin in vivo abrogated the inhibitory effects of RegIIIγ on both mRNA and protein of loricrin in wounds (Figures 6C and 6D). Silencing of *EXTL3* also inhibited the capacity of REG3A to increase cell proliferation (Figure 6E). However, although mannose receptor c-type 1 (MRC1) is a known receptor for C-type lectins and REG3A belongs to C-type lectin family, silencing of *MRC1* did not change the expression of loricrin (Figures S5D and S5E). Furthermore, although EXTL3 can influence heparan sulfate synthesis (Busse et al., 2007; Norton et al., 2005), the effect of silencing of *EXTL3* didn't appear to be due to the influence of this gene on proliferation induced by heparin binding growth factors (Radek et al., 2009; Trowbridge et al., 2002) because *EXTL3* silencing did not decrease in vitro wound reepithelialization induced by fibroblast growth factor 7 (FGF7) (Figure S5F) but did decrease REG3A-induced wound reepithelialization (Figure 6F). These data suggest that REG3A acts through EXTL3 expressed by keratinocytes and not through a dependence on the glycosyltransferase activity of EXTL3.

The finding of a functional REG3A receptor on keratinocytes compelled us to further explore EXTL3-activated downstream signaling pathways. Multiple pathways have been reported to regulate the proliferation and/or differentiation of keratinocytes, including mitogen-activated protein kinase (MAPK) and Wnt signaling pathways (Croyle et al., 2011; Yu et al., 2009). We thereby used inhibitors of those pathways to treat undifferentiated

NHEKs in the presence or absenceofREG3A. Among these inhibitors, phosphatidylinositol 3 kinases (PI3K) inhibitors wortmannin and Ly294002 significantly blocked the inhibitory effect of REG3A on loricrin expression (Figures 7A and 7B), but this was not blocked by MAPK inhibitor SB202190 and Wnt inhibitor DKK1 (data not shown). Wortmannin also inhibited keratinocyte proliferation induced by REG3A (Figure 7C). In contrast to RegIIIγ, wortmannin increased loricrin expression (Figure 7D) and delayed the healing of skin wounds in vivo (Figure 7E). It is known that the activation of PI3K induces the phosphorylation of AKT. To confirm that PI3K is the downstream signal molecule for EXTL3, we next checked whether REG3A activated PI3K to induce AKT phosphorylation after *EXTL3* was silenced. REG3A markedly increased the phosphorylation of AKT (Figures 7F and 7G) and this increase was inhibited after *EXTL3* was silenced (Figure 7F). Furthermore, the phophorylated AKT by REG3A was decreased after PI3K was inhibited by wortmannin (Figure 7G). All these data suggest that REG3A activates EXTL3-PI3K-AKT signaling pathway to regulate keratinocyte proliferation and/or differentiation.

Discussion

Proliferation and/or differentiation of keratinocytes is essential for the process of wound repair and is dysregulated in the skin disease psoriasis. REG3A or RegIIIγ has been proposed to regulate cell proliferation or differentiation after mucosal damage (Pull et al., 2005) and liver injury (Lieu et al., 2005) but was not known to be involved in skin homeostasis. Here we observed that REG3A or RegIIIγ may modulate epidermal repair through control of keratinocyte proliferation and differentiation. Our results revealed that REG3A or RegIIIγ is induced by IL-17, a prominent cytokine abundant in psoriasis, thus suggesting this as a link between IL-17-mediated inflammation and skin proliferation. The mechanism for REG3A-mediated proliferation involves inhibition of differentiation through the binding to its receptor EXTL3, followed by the activation of PI3K and AKT, events that we show have a major role in promoting wound re-epithelialization. Overexpression of REG3A in keratinocytes, possibly as a consequence of excess IL-17 and/or IL-22, may therefore lead to the epidermal hyperproliferation seen in psoriasis after skin trauma. Thus, the identification of REG3A as a stimulus for keratinocyte proliferation, and the elucidation of its mechanism of action, provides crucial information for understanding epidermal homeostasis, repair, and disease. These findings also offer potential targets for therapy.

Wounding breaches the intact physical and chemical cutaneous barrier against the outer environment. To reestablish an effective physical barrier, keratinocytes from the wound edge begin to migrate and reepithelialize the injury (Schauber et al., 2007). Previously, REG3A or RegIIIγ was undetectable in normal skin (Choi et al., 2007; Drognitz et al., 2006; Ogawa et al., 2003; Syder et al., 2003), but we show that acute wounding dramatically increased RegIIIγ expression in keratinocytes around the wound edge. However, despite lack of an acute injury, REG3A or RegIIIγ was also observed to be abundantly expressed in lesional skin of psoriasis patients or imiquimod-induced psoriatic mouse skin. The effects of REG3A or RegIIIγ on keratinocyte proliferation and its capacity to inhibit terminal differentiation link this system to essential processes involved in homeostasis of the skin and suggest that this could be a marker for skin injury and a potential prognostic indicator of psoriasis severity.

Multiple factors including cytokines, enzymes, and growth factors are involved in the initiation of the complex process of repair. Recently, IL-17A and IL-22 have been reported tomediate IL-23-induced hyperplasia of epidermis through the activation of signal transduction and activators of transcription 3 (STAT3) in vivo (Zheng et al., 2007) and IL-22 induces keratinocyte migration via downregulation of a set of differentiation-related genes in vitro (Boniface et al., 2005). However, whether IL-17 and/or IL-22 directly regulate

keratinocyte proliferation and differentiation is unknown. Here we show that IL-17 induced REG3A or RegIIIγ through the activation of IL-17RA to mediate cell proliferation. Furthermore, IL-22 cooperatively increased this to inhibit keratinocyte differentiation after skin injury, revealing that REG3A or RegIIIγ can be the link among IL-17, IL-22, and keratinocyte proliferation and/or differentiation. Because STAT3 is a downstream signal molecule for IL-22 to induce RegIIIγ in colonic epithelial cells, it remains to be deter-mined whether the activation of STAT3 by IL-17 is required for REG3A expression in keratinocytes. Furthermore, the mechanism of cooperation between IL-17 and IL-22 in regulation of REG3A needs further investigation. One speculation is that IL-22-induced STAT3 signaling converges with the NF-$_k$B pathway to cooperate with IL-17 in REG3A induction. However, it is possible that this cooperation occurs at the level of down-stream kinases that have been implicated in both IL-22R and IL-17R signaling, including MAPK and JUN N-terminal kinases (Kolls et al., 2008). REG3A might be an intermediate in this cyto-kine-regulated commitment to keratinocyte proliferation and/or differentiation as indicated by the paradigm of the crosstalk between epithelial cells and IL-17-expressing cells.

IL-17 is a potent proinflammatory mediator and is associated with several inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and asthma (Dong, 2006; Infante-Duarte et al., 2000; Langrish et al., 2005). Increasing evidence also shows that IL-17 participates in the pathogenesis of certain skin disorders, in particular psoriasis. IL-17 in synergy with IL-22 can induce critical events of psoriasis, including STAT3 activation and antimicrobial peptide elaboration (Tokura et al., 2010). Our results clarify the role of IL-17 in the context of IL-17-expressing-cell-dependent chronic inflammation in psoriasis and reveal that IL-17 induces REG3A to directly mediate keratinocyte differentiation in psoriasis, suggesting the important role of IL-17 and REG3A in hyperplasia of epidermis. Furthermore, this finding of REG3A as a downstream target of IL-17 has the potential to improve psoriasis therapy because current therapy in psoriasis that targets IL-17 results in systemic immunosuppression.

Although it was known that REG3A can mediate tissue regeneration, the underlying mechanism by which REG3A regulates cell proliferation and differentiation had been poorly explored. Here we show that REG3A depends on EXTL3 and activates PI3K and Akt to inhibit differentiation in keratinocytes. EXTL3 has been suggested tobe a receptor of REG proteins (Kobayashi et al., 2000; Levetan et al., 2008), but its downstream mediator had not been previously reported. We used shRNA interference and immunoprecipitation to confirm the functional association between REG3A and EXTL3 followed by screening of its down-stream mediators PI3K and AKT by using inhibitors for several pathways. Furthermore, even though EXTL3 is thought to be involved inheparan sulfate chain elongation, and heparan sulfate is required for FGF signaling (Radek et al., 2009; Trowbridge et al., 2002; Busse et al., 2007; Norton et al., 2005), silencing of *EXTL3* did not change the expression of syndecan-1 (data not shown) and did not inhibit the function of a heparan sulfate-depended growth factor, FGF7. These results suggest that EXTL3 silencing does not exert its effect on REG3A by an alteration of proteoglycan synthesis mediated by the glycosyltransferase activity of the EXT family of genes. However, further study is needed of the role of glycosaminoglycan synthesis and whether other signaling pathways as well as the EXTL3-PI3K-AKT pathway are involved in the REG3A response.

In conclusion, these findings support our discovery that REG3A or RegIIIγ is important for keratinocyte proliferation and/or differentiation after skin injury. Induction of REG3A or RegIIIγ is critical for wound reepithelialization and might be a previously unknown key element in the pathogenesis of psoriasis. Our findings implicate the potential of REG3A or RegIIIγ as a therapeutic target in psoriasis and wound healing. The identification of REG3A

function in skin provides insights into pathways contributing to antimicrobial defense, wound repair, and psoriasis and may ultimately lead to the development of different forms of treatment.

Experimental Procedures

Patients

All skin samples were obtained from 18 psoriasis patients (7 American and 11 Japanese; mild to plaque-type psoriasis and not on current treatment), 7 atopic dermatitis patients, 26 basal cell carcinomas, or 2-3 actinic keratosis, bowen disease, seborrheic keratosis, and malignant melanoma with a 2-mm punch biopsy. 2-mm punch biopsy from 13 normal patients (5 American and 8 Japanese) was used as control. All these samples were used for RNA isolation or OCT section. The data of REG3A expression from lesional and nonlesional skin of psoriasis patients were analyzed as follows: 7 American psoriasis samples were normalized to 5 American normal patients and 11 Japanese psoriasis samples were normalized to 8 Japanese normal patients, and REG3A expression in lesional skin was the combination of 7 American and 11 Japanese psoriasis patients. All sample acquisitions, including skin biopsies, were approved by the Human Research Protection Program at the University of California, San Diego, and the ethical committee of the Catholic University of Korea, and performed in accordance with the declaration of Helsinki Principles. Informed consent was obtained for all procedures.

Mice

Mice were housed in VASan Diego Healthcare System Veterinary Medical Unit (VMU) and the animal facilities in Loyola University Chicago and East China Normal University. *Il17*$^{-/-}$ mice were purchased from Y. Iwakura's lab in University of Tokyo. All animal experiments were approved by VA San Diego Healthcare System, Loyola University Chicago IACUC committee, and East China Normal University Animal Care and Use Committee.

Cellular Proliferation

Neonatal human epidermal keratinocytes (NHEKs, Cascade Biologics) were cultured as previous reported (Lai et al., 2009). The indicated doses of recombinant REG3A purchased from Novus Biologicals or purified by our laboratory were used to stimulate NHEKs. After stimulation, cells were sent to the flow cytometry core in the Institute of Biomedical Sciences in East China Normal University to evaluate cell cycle. The cells' entry into S phase were counted as proliferative cells. In addition to FACS, MTT assay (Amresco) performed according to manufacturer's instruction was used to evaluate cell proliferation induced by REG3A.

shRNA Preparation and Targeting Gene Knockdown

Oligonucleotides encoding human REG3A, EXTL3, IL-17RA, and RAGE shRNA (Table S1) were designed. Blast search was performed with the National Center for Biotechnology Information (NCBI) database to ensure that the shRNA constructs were targeting only human REG3A, EXTL3, IL-17RA, or RAGE. The oligonucleotides were annealed and cloned into the pLL3.7 vector as the manufacturer described. 4 μg of pLL3.7 constructs containing shRNAs, 4 mg of packaging plasmid psPAX2 (Addgene), and 2 μg of envelope plasmid pMD2.G (Addgene) were used to transfected HEK293T cells by calcium phosphate precipitation method. 48 hr later, lentiviruses containing targeted gene shRNA were collected and used to transfect NHEK cells.

Real-Time Quantitative RT-PCR

Real-time RT-PCR-specific primers as shown in Table S2 were used to evaluate gene expression. RNA analysis was done as previously reported (Lai et al., 2009).

Cutaneous Injury In Vivo

The back skin of mice was wounded by biopsy punches as previously described (Nizet et al., 2001). 3 days later, RNA or protein from 2 mm of skin around wound edges was collected either for real-time RT-PCR or immunoblot. Or the wounded skin was stored in OCT (Leica) or paraffin (Shanghai Sanjing Gongmao Ltd. Co) for immunhistochemical analysis.

Immunoprecipitation, Immunoblotting, and Immunohistochemistry

2 mm mouse skin taken from mouse wound edge or cells treated with REG3A or EXTL3 shRNAs or wortmannin (Sigma) was lysed with RIPA buffer (pH 7.4) containing protease inhibitor cocktail (Roche). 15 μg of total protein was used for immunoprecipitation and 5 mg of total protein was used for immunoblot. RegIIIγ was detected by immunoblot with RegIIIγ antibody that was gifted by L.V. Hooper from the University of Texas Southwestern Medical Center at Dallas. AKT and EXTL3 were detected by immunoblot with AKT antibody (Cell Signaling) and monoclonal EXTL3 antibody (Santa Cruz).

4 μm of formalin-fixed, paraffin-embedded tissue sections was mounted on glass slides and used for immunohistochemistry and staining. 2% PFA was used to fixate the samples. After 10 min fixation and subsequent pretreated with antigen retrieval solution, the sections were stained with REG3A antibody that was developed byour group or loricrin-specific antibody (Santa Cruz). The sections were reprobed with rabbit IgG FITC conjugate antibody (Sigma) or goat IgG FITC conjugate antibody (Jackson Immunoresearch Laboratories) and then mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) and visualized by the microscope (Leica).

Wound Healing

In Vitro Keratinocyte Wound—The capacity of NHEKs to migrate, proliferate, and close a defined area of injury to a confluent monolayer in vitro was evaluated by modification of a previously reported assay (Cha et al., 1996; Radek et al., 2009). In brief, before wounding NHEKs were transfected with REG3A overexpression vectors by lipofectmin 2000 (Invitrogen) or *REG3A* shRNAs, *EXTL3* shRNAs by calcium phosphate precipitation. Cells were then scratched once per well vertically with a P1000 pipette tip to create an artificial wound. After being washed twice to remove cellular debris by PBS, cells were treated with 0.01 nM and 0.03 nM recombinant REG3A in the presence or absence of 1.6 mM $CaCl_2$ or 3 nM FGF7. PBS was used as a negative control (untreated cells). The same fields were photographed every 12 hr and the area migrated by keratinocytes was calculated with Image-pro Plus 6.0 (Media Cybernetics). The percent migration at indicated time point was calculated by subtracting the total area of the scratch at corresponding time point from the total area of the scratch at 0 hr, divided by the total area at 0 hr multiplied by 100.

In Vivo Wound Healing—100 μg of RegIIIγ or 12.5 μg of wortmannin (Calbiochem) was intradermally injected into mouse back skin or 100 mg of monoclonal mouse IL-17a or RegIIIγ antibody was i.p. injected 1 day before 100 mg of RegIIIγ injection. 4 hr later, wounds were made by 8 mm biopsy punches and photographed every day. The healing area was calculated by Image J. The percent of healing area at indicated time point was calculated by subtracting the total area of wounds at corresponding time point from the total area of wounds at 0 day, divided by the total area at 0 day multiplied by 100.

Imiquimod Model of Skin Inflammation

10-week-old BALB/C mice were given 50 μg of imiquimod (InvivoGen) in the shaved back and 25 μg of imiquimod in right ear daily for 5 days as previous described (Ramirez-Carrozzi et al., 2011). For RegIIIγ blockage, 100 mg of RegIIIγ antibody was i.p. injected 1 day before 25 μg of imiquimod was used to induce psoriatic skin. Ear thickness was measured every day. On day 5, mice were euthanized and ear and back tissues were collected for immunohistochemical evaluation or RegIIIγ and IL-17a mRNA analysis.

IL-17a or EXTL3 Neutralization

In Vitro IL-17a Neutralization—Primary murine keratinocytes were isolated from 3-day-old neonates and cultured under standard culture conditions. 175 μg of total protein of 3-day wound homogenates was incubated with final concentration of monoclonal mouse IL-17a antibody (R&D) at 0.1 μg ml^{-1} for 30 min. Supernatants pretreated with or without IL-17a antibody were then used to stimulate primary murine keratinocytes for 24 hr. RNA was isolated for analyzing RegIIIy expression.

In Vivo IL-17a or EXTL3 Neutralization—100 μg of monoclonal mouse IL-17a antibody (R&D) or 10 μg of EXTL3 antibody (Santa Cruz) was intradermally injected into mouse back skin 24 hr and 4 hr before wounding. 3 days later, 2 mm skin around mouse wounds was taken for immunohistochemistry and analysis of RegIIIγ or loricrin expression.

Statistical Analysis

All data are present as mean ± SEM. We used two-tailed t tests to determine significances between two groups. We did analyses of multiple groups by one-way or two-way ANOVA with Bonferroni post test of GraphPad Prism Version 5. For all statistical tests, we considered p values <0.05 to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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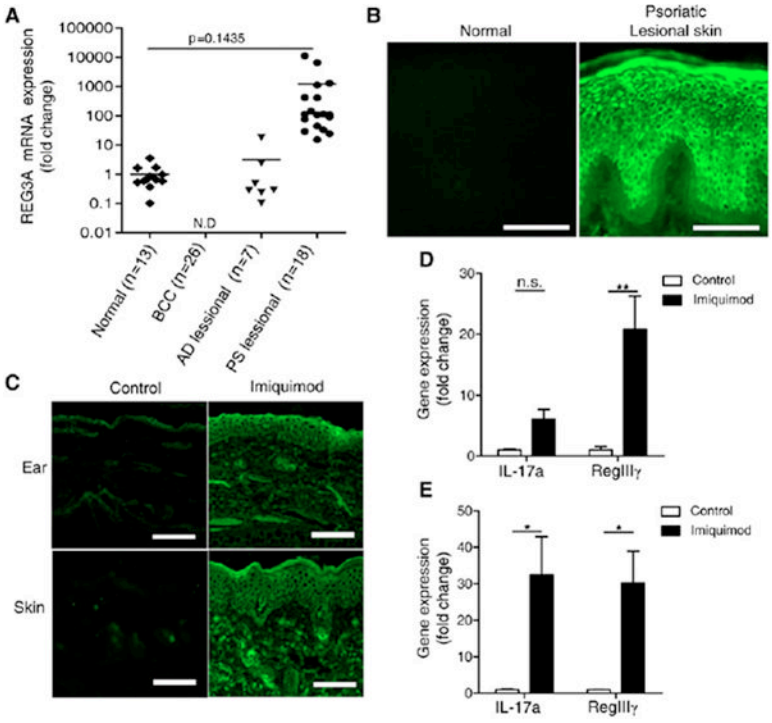

Figure 1. Psoriasis Patients and Imiquimod-Induced Psoriatic Mouse Skin Have High Abundance of REG3A or RegIIIγ in Lesional Skin

(A) Quantification of REG3A mRNA expression of skin from normal, basal cell carcinomas, atopic dermatitis, and psoriasis patients. N.D., not detectable.

(B) Immunofluorescent staining of skin in normal and psoriasis patients by REG3A antibody. Scale bars represent 200 μm.

(C) Immunofluorescent staining of normal and imiquimod-induced psoriatic mouse skin by RegIIIγ antibody. Scale bars represent 100 μm.

(D and E) Quantification of IL-17a and RegIIIγ mRNA expression of skin from normal and imiquimod-induced psoriatic mouse ears or backs. *$p < 0.05$, **$p < 0.01$; n.s., no significance. p values were determined by one-way or two-way ANOVA. Data are the means ± SEM. See also Figure S1.

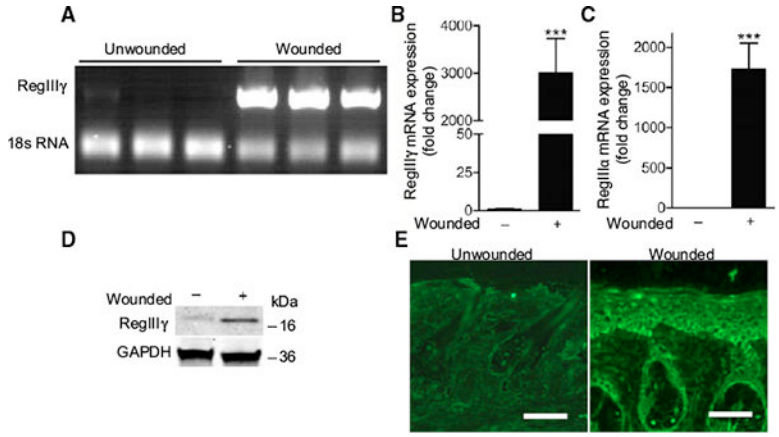

Figure 2. Skin Injury Increases Epidermal RegIIIγ Expression

(A) The expression of RegIIIγ mRNA of 2 mm skin surrounding the wound edge 3 days after aseptic injury.

(B and C) Quantification of RegIIIγ and RegIIIα expression of skin treated as in (A).

(D) Immunoblot of RegIIIγ in skin extracts from mouse wound edges.

(E) Immunofluorescent staining of skin in wild-type mouse treated as in (A) by RegIIIγ antibody. The scale bars represent 50 μm. ***$p < 0.001$. p values were determined by two-tailed t tests. Data are the means ± SEM of six mice for each. See also Figure S2.

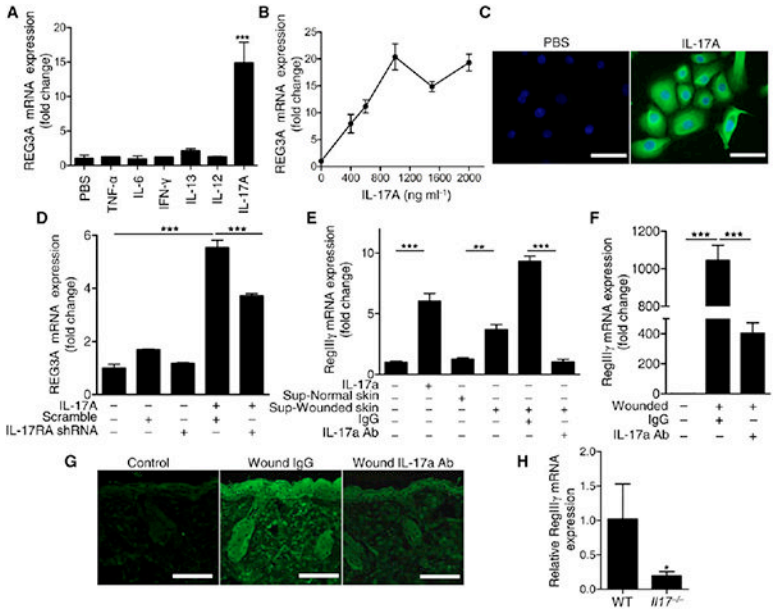

Figure 3. IL-17 Activates IL-17RA to Induce REG3A or RegIIIγ Expression in Primary Keratinocytes and Skin Adjacent to Wound Edge

(A) Quantification of REG3A mRNA expression in undifferentiated NHEKs stimulated by different cytokines.

(B) The expression of REG3A mRNA induced by the indicated doses of IL-17A.

(C) Immunofluorescent staining of undifferentiated NHEKs treated with 1,000 ng ml^{-1} IL-17A by REG3A antibody. Scale bars represent 100 μm.

(D) The expression of REG3A after IL-17RA was silenced.

(E) RegIIIγ expression in undifferentiated primary murine keratinocytes stimulated by the supernatant of skin wound homogenate pretreated with control IgG or IL-17a-neutralizing antibody.

(F) RegIIIγ expression in mouse skin wounds after IL-17a was neutralized by IL-17a-neutralizing antibody.

(G) Immunofluorescent staining of skin in wild-type mouse treated as in (F) by RegIIIγ antibody. The scale bars represent 100 μm.

(H) RegIIIγ expression in skin wounds of Il17–/– and wild-type mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. p values were analyzed by two-tailed t test or one-way ANOVA. Data are the means ± SEM of n = 3. See also Figure S3.

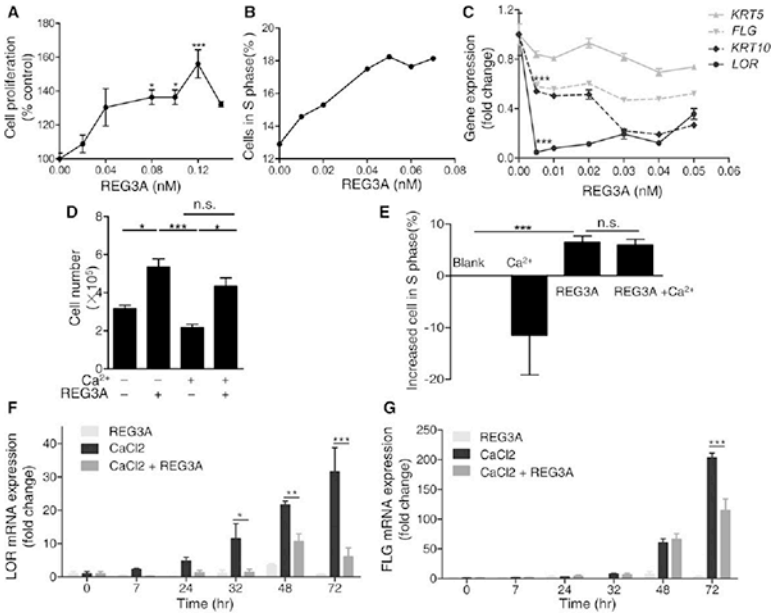

Figure 4. REG3A Increases Keratinocyte Proliferation via the Inhibition of Differentiation Gene Expression

(A) REG3A induced undifferentiated NHEK proliferation analyzed by MTT.

(B) Undifferentiated keratinocytes in S phase by flow cytometry. The percentage of cells in S phase increased by REG3A was calculated by the amount of cells in S phase at each indicated concentration divided by the amount of total cells.

(C) Quantification of differentiated gene expression in undifferentiated NHEKs.

(D) Cell number after NHEKs were treated with 0.1 nM REG3A and/or 1.6 mM $CaCl_2$.

(E) Differentiated keratinocytes back into S phase induced by REG3A.

(F and G) $CaCl_2$-induced loricrin *(LOR)* and filaggrin *(FLG)* expression in the presence or absence of REG3A. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., no significance. p values were analyzed by one-way or two-way ANOVA. Data are the means ± SEM of n = 3.

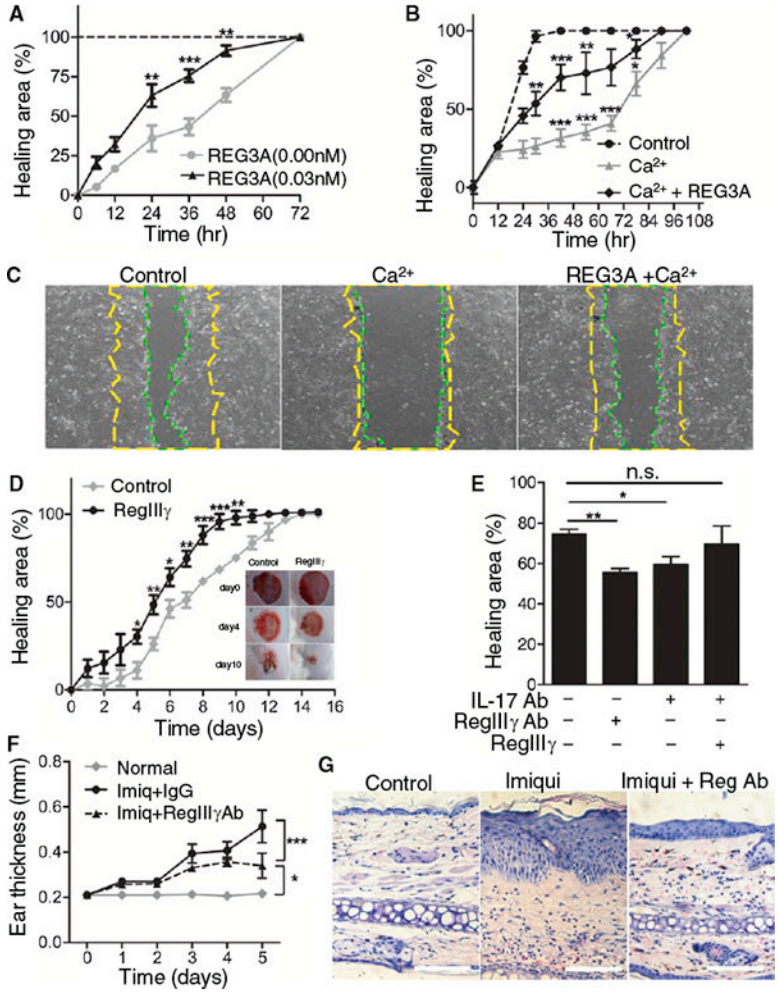

Figure 5. REG3A or RegIIIγ Promotes Wound Reepithelialization and Psoriatic Hyperproliferation

(A) Percentage of scratch wound healing of undifferentiated NHEKs.

(B) Percentage of scratch wound healing of differentiated NHEKs.

(C) Identical fields treated as in (B) at 0 and 24 hr scratch. The yellow dotted line represents the area of the scratch at $t = 0$ hr. The green dotted line represents the area of the scratch at $t = 24$ hr.

(D) RegIIIγ promoted wound healing in vivo.

(E) Wound healing at day 3 after IL-17a or RegIIIγ blockage.

(F) Imiquimod-induced ear thickness with or without RegIIIγ blockage.

(G) Imiquimod-induced epidermal hyperpro-liferation with or without RegIIIγ blockage. Scale bars represent 100 μm.

*p < 0.05, **p < 0.01, ***p < 0.001; n.s., no significance. p values were determined by one-way or two-way ANOVA. Data are represented as the mean ± SEM of n = 3–6. See also Figure S4.

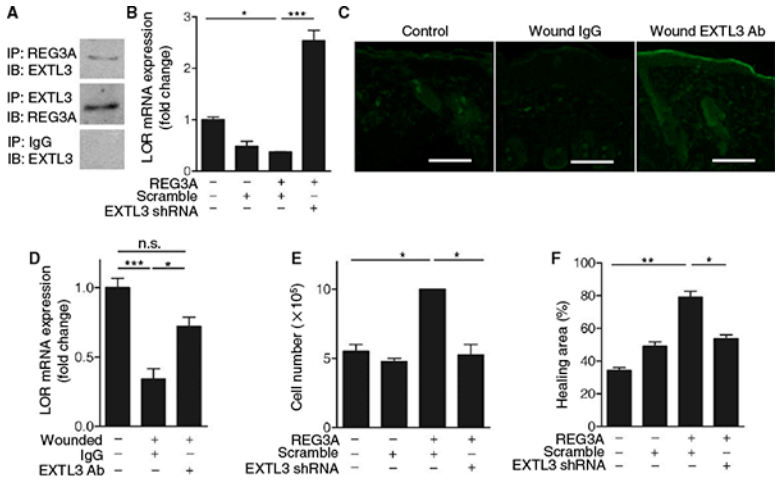

Figure 6. EXTL3 Is Required for REG3A Activation in Keratinocytes

(A) REG3A bound to EXTL3 in human keratinocytes.

(B) Loricrin expression induced by REG3A after *EXTL3* was silenced.

(C) Immunofluorescent staining of skin by loricrin antibody after EXTL3 blockage. The scale bars represent 100 μm.

(D) Loricrin expression in mouse wounded skin treated as in (C).

(E and F) Cell proliferation and wound healing induced by REG3A after *EXTL3* was silenced.

*p < 0.05, **p < 0.01, ***p < 0.001; n.s., no significance. p values were determined by one-way ANOVA. Data are means ± SEM of n = 3. See also Figure S5.

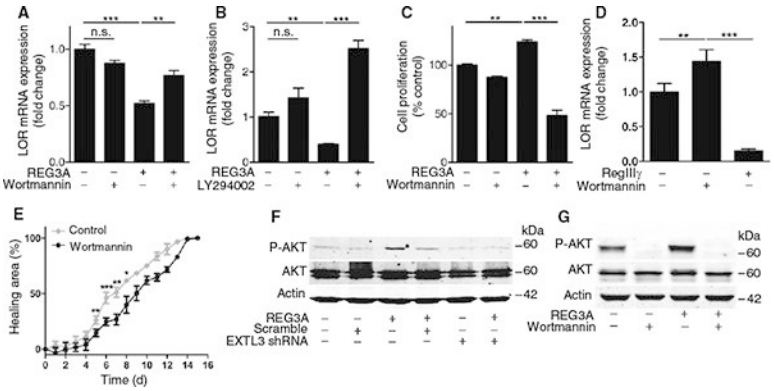

Figure 7. REG3A or RegIIIγ Activates PI3K-AKT Signaling Pathway to Regulate Keratinocyte Differentiation

(A and B) Loricrin expression of NHEKs treated with REG3A and/or inhibitors wortmannin and Ly294002.

(C) The proliferation of NHEKs treated as in (A).

(D) Loricrin in wounds of mice treated with RegIIIγ or wortmannin.

(E) Wound healing of mice treated with wortmannin.

(F) Phosphorylation of AKT after NHEKs were treated REG3A and/or *EXTL3* shRNA.

(G) Phosphorylation of AKT of NHEKs treated with REG3A and/or wortmannin.

*p < 0.05, **p < 0.01, ***p < 0.001; n.s., no significance. p values were determined by one-way ANOVA or two-way ANOVA. Data are the means ± SEM of n = 3.