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Identifying a Hyperkeratosis Signature in Autosomal Recessive Congenital Ichthyosis: Mdm2 Inhibition Prevents Hyperkeratosis in a Rat ARCI Model

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TO THE EDITOR

Autosomal recessive congenital ichthyosis (ARCI) is a Mendelian disorder of keratinization. LI patients are born as erythrodermic ''collodion'' babies but soon become covered with scales. The most commonly mutated genes in ARCI are Transglutaminase 1, Ichthyin, and the Arachidonate Lipoxygenases 12b and 3 (Russell *et al.*, 1995; Jobard *et al.*, 2002; Fischer, 2009). Hyperkeratosis (scaling) is a cardinal feature of disorders of keratinization, which are characterized by abnormal barrier function. For example, *Tgm1*-null mice die perinatally because of transepidermal water loss after birth, but when this skin is grafted onto nude mice, they form scales similar to ARCI patients (Kuramoto *et al.*, 2002). The epidermis is hyperproliferative, produces increased non-polar lipid, and the cornified layer is thickened. This scaling is the principle phenotype in ARCI patients, and the time taken to remove scale significantly affects their quality of life. Oral retinoids, the current treatment for ARCI, efficiently reduces scale formation but are also rarely associated with side effects, some serious (Rood *et al.*, 2007). Therefore, there is a need to develop new treatments for ARCI.

Current research on alternate ARCI treatments is focused on gene therapy (Choate *et al.*, 1996), or enzyme replacement therapy, adding recombinant Tgm1 to the skin (Aufenvenne *et al.*, 2012). A greater understanding of the common molecular mechanisms that lead to hyperkeratosis would facilitate the discovery of more directed therapies to reduce scaling in ARCI. Therefore, we investigated common gene expression changes in two different rat epidermal keratinocyte ARCI models, to identify the common networks of differentially expressed genes that are required for hyperkeratosis and, within these molecular networks, to identify new ''drug-able'' targets for the reduction of hyperkeratosis.

Two separate Arachidonate 12-lipoxygenase (*Alox12b*) short hairpin RNA (shRNA)–knockdown (kd) rat epidermal keratinocyte lines (Supplementary Figure S1a and S1b online) were compared with an existing Tgm1 shRNA–kd line (O'Shaughnessy *et al.*, 2010). Both *Tgm1*- and *Alox12b*-kd organotypic cultures were hyperkeratotic (Figure 1a). Comparison of *Tgm1*-kd with *Alox12b*-kd cultures revealed a common increase in keratin 1 expression, but keratin 10 reduction was observed only in *Tgm1*-kd cultures (Figure 1a and Supplementary Figure S1c online), suggesting both common and distinct programs of altered gene expression in the two models. Principal component analysis confirmed that, although phenotypically similar, overall gene expression differences were divergent (Figure 1b and Supplementary Figure S2 online). Despite these differences, we identified 155 genes that were commonly upregulated and 78 genes that were commonly downregulated in both Tgm1- and Alox12b-kd keratinocyte cultures (Supplementary Table S1 online). We identified upregulated genes related to the positive regulation of apoptosis and downregulated genes related to protein phosphorylation (Supplementary Figure S3 online).

The only significant network of interrelated genes was related to the genes involved in the positive regulation of apoptosis, and within this network we identified the p53 ubiquitin ligase *Mdm2* as an attractive candidate gene to target in order to reduce hyperkeratosis, as mice overexpressing Mdm2 display a hyperkeratotic epidermis in a p53-independent manner (Alkhalaf *et al.*, 1999). *Mdm2* upregulation in both Tgm1- and Alox12b-kd cultures was confirmed by quantitative reverse transcriptase in real-time PCR (Figure 1c and d). In organotypic culture, cytoplasmic

Abbreviations: ARCI, autosomal recessive congenital ichthyosis; kd, knockdown; pAkt, phosphorylated Akt

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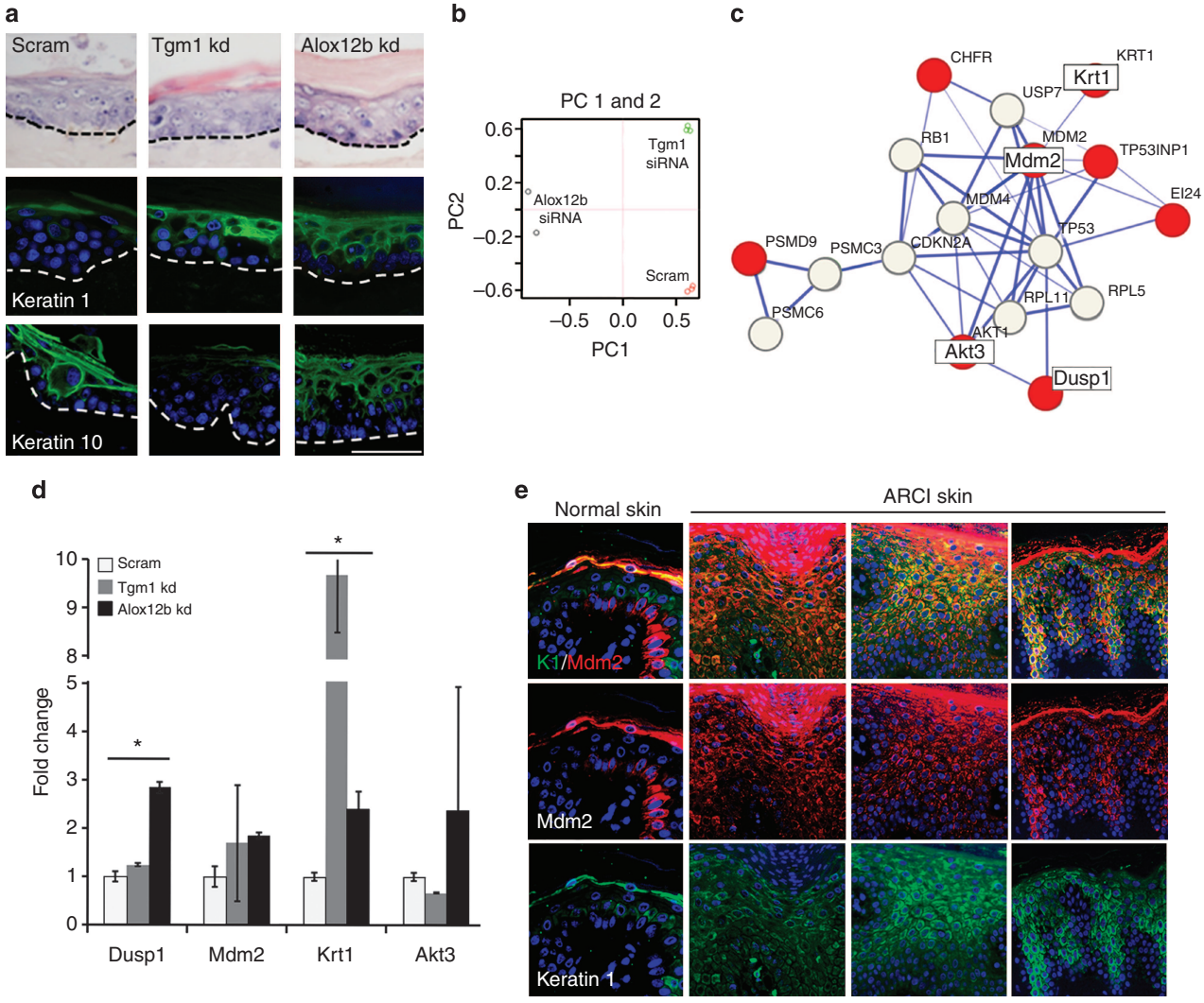

Figure 1. Keratin 1 and Mdm2 upregulation in ARCI models and patient samples. (**a**) Immunofluorescence of keratins 1 and 10 in scrambled (Scram) control, Tgm1-kd organotypic cultures, and Alox12b-kd organotypic cultures. (**b**) Principal component analysis comparing gene expression patterns between scrambled controls, Tgm1-kd cells, and Alox12b-kd cells. Divergence between Alox12b- and Tgm1-kd cells indicates different gene expression profiles. (**c**) Network of gene–gene interactions of the commonly overexpressed genes involved in apoptosis; red genes are overexpressed in ARCI models. (**d**) Quantitative reverse transcriptase in real-time PCR analysis of apoptosis genes in the three scrambled controls, two Tgm1-kd keratinocyte cultures, and two Alox12b-kd keratinocyte cultures; error bars SD, $*P<0.05$ one-way analysis of variance. (**e**) Immunofluorescence of Mdm2 and keratin 1 in ARCI skin (3 of $n=7$) and a normal control. Bar = 50 μm (**a**, **e**). ARCI, autosomal recessive congenital ichthyosis; kd, knockdown; siRNA, small interfering RNA.

Mdm2 expression was increased and was more widespread (Supplementary Figure S4 online). This expression pattern was mirrored in ARCI patients in whom Mdm2 was strongly upregulated in the spinous layer in 7/7 samples, clearly colocalizing with upregulated keratin 1 (Figure 1e). p53 expression was undetectable in these cultures (not shown), and p63, which Mdm2 can also degrade, was not reduced in either kd culture (Supplementary Figure S4 online). Akt upregulation causes hyperkeratosis (Janes *et al.*, 2004), and Mdm2 activates Akt (Singh *et al.*, 2013). Consistent with this, serine-phosphorylated Akt (pAkt) was upregulated in both kd organotypic cultures (Supplementary Figure S4 online). Therefore, in ARCI, we hypothesized that Mdm2 functioned through a p53/p63-independent pathway involving increased Akt phosphorylation to cause hyperkeratosis.

Mdm2 overexpression alone in keratinocytes did not result in either keratin 1 or pAkt upregulation (Figure 2a). We therefore tested whether the inhibition of upregulated Mdm2 in the diseased state was sufficient to reduce hyperkeratosis. As Mdm2 is commonly upregulated in cancer (Berger *et al.*, 2004), a number of Mdm2 inhibitors are currently in phase I clinical trials (Secchiero *et al.*, 2011) and therefore could be rapidly adopted to treat ARCI. Nutlin-3 is a well-characterized and easily available Mdm2 inhibitor, which acts by preventing the interaction of Mdm2 with p53, as well as other protein targets (Nicholson *et al.*, 2012).

Treatment of *Tgm1*- and *Alox12b*-kd cells with nutlin-3 reduced both keratin 1 and pAkt expression levels (Figure 2b).

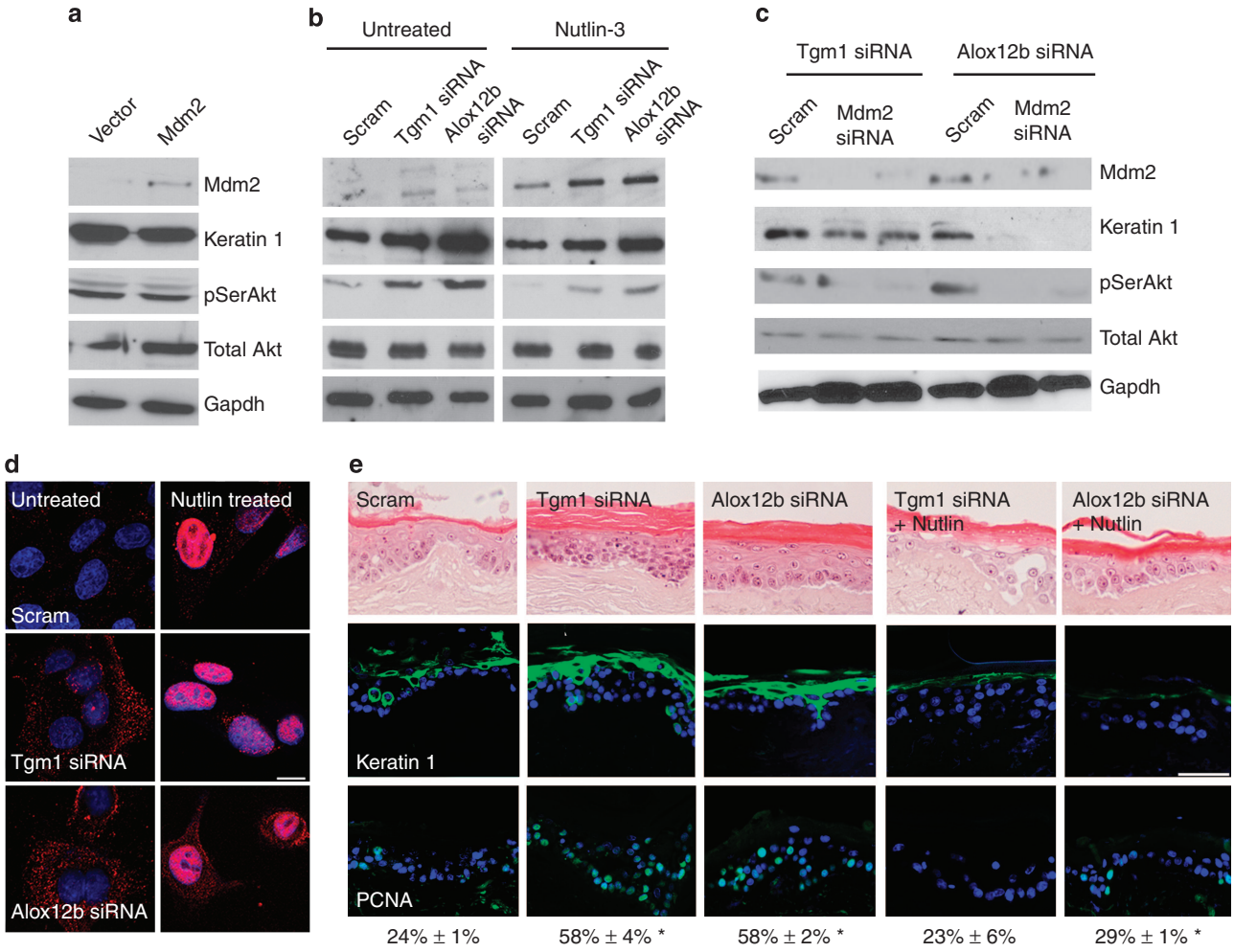

Figure 2. The Mdm2 inhibitor nutlin-3 reduces hyperkeratosis in ARCI models. (**a**–**c**) Western blot of Mdm2, keratin 1, pSerAkt, and total Akt in (**a**) rat epidermal keratinocytes overexpressing Mdm2, (**b**) Tgm1- and Alox12b-kd cells treated with nutlin-3, and (**c**) Tgm1- and Alox12b-kd cells expressing Mdm2 short hairpin RNA. Gapdh is the loading control. (**d**) Mdm2 immunofluoresence in Tgm1- and Alox12-kd cells with and without nutlin-3 treatment. (**e**) Histology of scrambled (Scram) control and kd cultures with or without nutlin-3 treatment. In addition, keratin 1 and proliferating cell nuclear antigen (PCNA) immunofluoresence with the mean percentage of PCNA-positive nuclei in the basal layer ± SEM (*$P<0.05$ for comparison either with Scram or with and without nutlin-3 treatment, $n=2$). Bars = 10 μm (**d**), 50 μm (**e**). ARCI, autosomal recessive congenital ichthyosis; kd, knockdown; siRNA, small interfering RNA.

Mdm2 expression was increased—a known effect of Nutlin-3 treatment (Kranz and Dobbelstein, 2006)—suggesting that Mdm2 function is being inhibited in our ARCI models. Reduction of keratin 1 and pAkt was also seen in both *Tgm1*- and *Alox12b*-kd cells transfected with an *Mdm2* shRNA plasmid, suggesting that the effect of nutlin-3 was Mdm2 specific (Figure 2c). Nutlin-3 treatment changed Mdm2 localization from cytoplasm to nucleus (Figure 2d) and reduced hyperkeratosis and proliferation in the kd organotypic culture (Figure 2e).

Mdm2 is cytoplasmic in ARCI, not nuclear, as is observed in skin cancer (Berger *et al.*, 2004), and therefore it is likely that cytoplasmic Mdm2 distribution is functionally significant in hyperkeratosis. It is possible that Mdm2 directly interacts with keratin 1, and therefore it would be interesting to determine the interacting partners of Mdm2 in a barrier-disrupted situation such as ARCI. Evidence for a potential interaction between Mdm2 and keratin 1 comes from a proteomic screening for Mdm2-interacting proteins in the presence and absence of nutlin-3, which reveals an interaction between Mdm2, keratins, and keratin-associated proteins that is lost with nutlin-3 treatment (Nicholson *et al.*, 2012). Therefore, we present a pathway critical to hyperkeratosis in ARCI, and potentially other hyperkeratotic diseases, which is modulated by Mdm2-inhibiting drugs.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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In Vivo siRNA Targeting of CD28 Reduces UV-Induced DNA Damage and Inflammation

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TO THE EDITOR

Exposure to UV radiation is the major factor for the development of non-melanoma skin cancer, including squamous cell and basal cell carcinoma. The initial event in UV carcinogenesis upon photon absorption by DNA is the formation of cyclobutane pyrimidine dimers (CPDs), and to a lesser extent, pyrimidine-pyrimidone 6,4-photoproducts. When these photoproducts are left unrepaired or misrepaired, they can lead to mutations in critical genes such as the tumor suppressor gene p53 (Benjamin *et al.*, 2008). However, depending on the dose of UVB exposure and the amount of DNA photoproducts, damaged cells undergo apoptosis or survive through cellular repair mechanisms (Clydesdale *et al.*, 2001; Schwarz and Schwarz, 2009), preventing or allowing mutations to be formed from unrepaired photoproducts. UVB-induced DNA damage also serves as a trigger for immunosuppression via the secretion of IL-10 and tumor necrosis factor-α, inhibition of dendritic cell function and induction of regulatory T cells (Tregs; Schwarz and Schwarz, 2009, 2011). Certain cytokines may increase the repair of DNA damage by stimulating nucleotide excision repair (NER). For instance, exogenous administration of IL-12 and IL-23 was able to abrogate the immunosuppressive effect of UVR through induction of NER (Cline and Hanawalt, 2003; Schwarz and Schwarz, 2009). It has been demonstrated that CD80/CD86–CD28/CD152 signaling has an important role in the prevention of UVB-induced immunosuppression and tumor formation (Beissert *et al.*, 1999; Loser *et al.*, 2005). However, the individual role of CD28 has not been investigated in short-term UV effects, including DNA damage. Here, we have targeted CD28 with small interfering RNA (siRNA) to determine the role of this signaling pathway in the preservation of UVB-induced skin alterations.

Mice were exposed to 80 mJ cm^{-2} UVB (a moderate dose, as previously determined (Pflegerl *et al.*, 2009) immediately after CD28 siRNA or control siRNA injection (Figure 1a), and skin samples were collected at 1, 6, and 24 hours after UVB exposure for hematoxylin and eosin and immunohistochemical thymine dimer staining. We found similar number of thymine dimers (a specific form of CPDs), positive cells and sunburn cells (SBC), in the epidermis of CD28 siRNA- and control siRNA-injected mice 1 and 6 hours after UVB

Abbreviations: CPD, cyclobutane pyrimidine dimer; NER, nucleotide excision repair; Treg, regulatory T cell

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