p53 Is a Direct Transcriptional Repressor of Keratin 17: Lessons from a Rat Model of Radiation Dermatitis

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The intermediate filament protein keratin 17 (Krt17) shows highly dynamic and inducible expression in skin physiology and pathology. Because Krt17 exerts physiologically important functions beyond providing structural stability to keratinocytes whereas abnormal Krt17 expression is a key feature of dermatoses such as psoriasis and pachyonychia congenita, the currently unclear regulation of Krt17 expression needs to be better understood. Using a rat model of radiation dermatitis, we report here that Krt17 expression initially is down-regulated but later is strongly up-regulated by ionizing radiation. The early down-regulation correlates with the activation of p53 signaling. Deletion of p53 abolishes the initial down-regulation but not its subsequent up-regulation, suggesting that p53 represses Krt17 transcription. Because previous work reported up-regulation of Krt17 by ultraviolet irradiation, which also activates p53 signaling, the effect of ultraviolet radiation was reexamined. This revealed that the initial down-regulation of Krt17 is conserved, but the up-regulation comes much faster. Chromatin immunoprecipitation analysis in vivo and electromobility shift assay in vitro identified two p53-binding sites in the promoter region of Krt17. Thus, p53 operates as a direct Krt17 repressor, which invites therapeutic targeting in dermatoses characterized by excessive Krt17 expression.


INTRODUCTION

Keratins are a family of intermediate filaments that are critical for providing structural stability to epithelial cells. Recently, keratins have been found to exert a wide array of additional functions, including the control of cytokine expression, metabolic processes, and regulation of cellular growth, proliferation, migration, and apoptosis (reviewed in Kim and Coulombe, 2010; Pan et al., 2013; Ramot et al., 2009, 2014; also see Chung et al., 2015; Kim et al., 2006; Lu et al., 2007; Tong and Coulombe, 2006).

Keratin 17 (Krt17) is of special interest in this context. Krt17 shows highly selective intraepithelial expression in normal skin. In development, Krt17 expression coincides with hair follicle formation (McGowan and Coulombe, 1998), and prominent Krt17 expression is seen during postwounding hair follicle neogenensis (Fan et al., 2011). In adult mice, Krt17 is not expressed in the interfollicular skin but in the hair follicles in discrete regions of the outer root sheath, hair matrix, and shaft (McGowan and Coulombe, 2000). In hair cycle, Krt17 shows significant dynamic expression changes (Panteleyev et al., 1997). Krt17 is also expressed in glabrous skin and the nail matrix (McGowan and Coulombe, 2000; Swensson et al., 1998). Therefore, Krt17 gene expression is under strict and dynamic control, and better understanding of its regulation is critical for multiple aspects of skin biology.

Conceptually, Krt17 has been proposed as a “stress response keratin” that is up-regulated under pathological conditions such as wounding (McGowan and Coulombe, 1998; Paladini et al., 1996; Patel et al., 2006), oxidative or inflammatory stress of the epidermis (Arany et al., 1998; Komine et al., 1996; Proby et al., 1993; Schofield et al., 1995). Moreover, abnormal Krt17 expression is associated with several dermatoses, including pachyonychia congenita (McLean and Moore, 2011; McLean et al., 1995; Wilson et al., 2012, 2014), lichen planus (Schofield et al., 1995), viral warts (Arany et al., 1998; Proby et al., 1993; Schofield et al., 1995), and psoriasis (Bockelmann et al., 2005; Bracke et al., 2014; Jin and Wang, 2014, Wei et al., 1999). In psoriasis, abnormal Krt17 expression has even been invoked as an important pathogenic element and therapeutic target (Chang et al., 2011; Fu and Wang, 2012; Gudmundsdottir et al., 1999; Jin and Wang, 2014). Krt17 is also up-regulated by UVR (Bernerd and Asselineau, 1997; Bernerd et al., 2001; Del Bino et al., 2004; Hachiya et al., 2009).

Several transcription factors are known to regulate Krt17 gene expression, including AP-1 (Kerns et al., 2010; Ma et al., 1997) and glioma-associated oncogene homolog 1/2 (Callahan et al., 2004; Sankar et al., 2013). Various cytokines,
including interferon-γ and interleukin-17A/interleukin-22 can also regulate Krt17 expression, possibly via the down-stream factors signal transducer and activator of transcription (STAT)1/3 and extracellular signal-regulated kinase (ERK)1/2 (Komine et al., 1996; Shi et al., 2011; Wei et al., 1999; Zhang et al., 2012). Recently, Rac1 activation was shown to up-regulate Krt17 expression (R. Chen et al., 2014). Whereas a 2-kb promoter of Krt17 can recapitulate its endogenous constitutive expression pattern (Bianchi et al., 2005), our knowledge on transcription factors that regulate Krt17 expression, and their complex interplay, remains sparse.

In this study, we report insights on the regulation of Krt17 expression, which had emanated from our recent investigations on radiation dermatitis (RD). RD is a common side effect in cancer radiotherapy, which is mediated by ionizing radiation (IR)-induced DNA damage and the release of inflammatory cytokines (reviewed in Hymes et al., 2006; Ryan, 2012; Schaue et al., 2012). Our results suggested that both IR and UVR down-regulate Krt17 gene expression by impacting on p53 activity.

RESULTS

Down-regulation of Krt17 expression correlates with activation of p53 in RD

We profiled gene expression in RD using a rat footpad model (Rezvani et al., 1998, 2002). An absolute quantification method for mRNA expression based on next-generation sequencing was used (Saha et al., 2002) as comparison to the more traditional microarray technology (Lee et al., 2006). To mimic the fractionated delivery regimen in clinical practice, a 10-Gy × 4 protocol with a 1-day interval was designed (Figure 1a and Supplementary Figure S1 online). This protocol produces a consistent phenotype, which manifests as dry desquamation at days 12–16 postirradiation (Figure 1b).

Although the full dataset of next-generation sequencing results has not yet been reported (unpublished data), we found that Krt17 showed a unique dual expression pattern: there was an early 2-fold reduction at T3/T6 and a 10-fold up-regulation at T9 (Figure 1c). p21, the classic downstream target of p53, was consistently elevated in the skin stressor that is known to activate p53 and up-regulate Krt17 (Bernerd and Asselineau, 1997; Bernerd et al., 2001; Komine et al., 1996). UVR is a common healing, infection, and inflammation (Paladini et al., 1996; Proby et al., 1993; Komine et al., 1996). UVR is a common skin stressor that is known to activate p53 and up-regulate Krt17 (Bernerd and Asselineau, 1997; Bernerd et al., 2001; Del Bino et al., 2004; Hachiya et al., 2009). In this study we examined the UVR response in greater detail.

Krt17 shows similar biphasic response after UVR

The discovery that p53 activation leads to down-regulation of Krt17 may be unexpected, as several previous reports showed that Krt17 is up-regulated in stress situations such as wound healing, infection, and inflammation (Paladini et al., 1996; Proby et al., 1993; Komine et al., 1996). UVR is a common skin stressor that is known to activate p53 and up-regulate Krt17 (Bernerd and Asselineau, 1997; Bernerd et al., 2001; Del Bino et al., 2004; Hachiya et al., 2009). In this study we examined the UVR response in greater detail.

The light source was calibrated, which emitted a mixture of UVB (290–320nm, 35%) and UVA (320–400nm, 65%; see Supplementary Figure S1b and c). We achieved a consistent desquamation phenotype at a single dose of 2.7 J/cm² (the skin remains normal at half of this dose). Under this treatment regimen, we found that the skin damage appeared much earlier, that is, erythema started at T2/T3, and desquamation at T5–T7. Therefore, we reasoned that a repressing effect of Krt17 may be found at earlier time points. Indeed, at 6–12 hours after UVR, p53 signaling was readily activated as shown by increased p21 expression (Figure 4a). Krt17 expression decreased at these time points, but such a
decrease was not observed in p53−/− mice (Figure 4b). At 48 hours after treatment, Krt17 was up-regulated in both mice (Figure 4b). The increased Krt6a expression was similar in both mice (see Supplementary Figure S7 online). Finally, the time frame and severity of the damage were also similar in both mice (n = 5 for each group; see Supplementary Figure S7). In summary, it appears that Krt17 up-regulation occurs during recovery from stress/damage and is p53 independent, but in the early phase of stress, Krt17 is suppressed by p53.

Mapping the regulatory elements in the Krt17 promoter
To examine whether p53 directly regulates Krt17 expression, we cloned the 2-kb upstream promoter of the mouse gene, which accurately confers its constitutive expression pattern in vivo (Bianchi et al., 2005). Serial truncations were made to characterize this promoter (Figure 5a). In 293T cells treated with doxorubicin to activate p53 signaling, we found a 50% reduction of reporter activity (Figure 5c and e, and Supplementary Figure S8 online). This down-regulation is abolished when the −978 to −696 region was truncated. Two putative p53 binding sites were identified in this region (el-Deiry et al., 1992), suggesting direct regulation (Figure 5b). Similar p53 binding sites can be identified in rat and human promoters (see Supplementary Figure S9 online).

We further evaluated the regulation of Krt17 promoter by p53 via overexpression or RNAi knockdown. p53 overexpression repressed reporter activity, whereas knockdown of p53 significantly increased reporter activity (Figure 5d). The efficiency of RNAi knockdown was confirmed by western blot (Figure 5e and f), whereas a control RNAi did not change p53 expression (Figure 5g and h). To confirm a direct binding in vivo, we performed chromatin immunoprecipitation (CHIP) analysis. As expected, we found increased p53 binding in the promoter of p21 gene, which served as a positive control (Figure 6a and b). Similar p53 binding was found on Krt17 promoter (Figure 6a and c). Note that the CHIP data were performed with mice tissues that were irradiated only once at T0 (and is different from the rat experiments, therefore showing different p53 activation kinetics). The capability of p53 to bind the two putative regulatory sites was further confirmed by electromobility...
shift assay. Biotin-labeled synthetic oligos for both sites produced positive signals, which disappeared after nonlabeled competitive oligos were added but not when the binding sites were mutated (Figure 6d and e). Specific antibody to p53 produced the expected shifts of the electromobility shift assay signals, but not by a control IgG. Together, these results demonstrated a direct regulatory role of p53 on Krt17 gene expression.

**DISCUSSION**

In this study, we define Krt17 as a p53 target gene in the skin. The dynamic expression change of Krt17 in RD had escaped previous notice when whole-genome expression analysis was performed by microarray (Lee et al., 2006), possibly because of the more sensitive and accurate nature of next-generation sequencing. Krt17 shows a unique biphasic response, namely, down-regulation in the early stress response and up-regulation during the recovery stage. We found that p53 directly binds the promoter of Krt17 gene and down-regulates its expression, whereas the up-regulation is p53 independent. These results expand our view regarding the regulation of Krt17 expression.

p53 is a transcription factor that acts as a tumor suppressor and regulates cell stress response. Although p53 is known to activate target genes, the repressed genes can range from 15% to >80% after p53 activation (Böhlig and Rother, 2011; Menendez et al. 2009). In the skin, p53 has been shown to be important for normal epidermal differentiation, although some discrepancies have been reported in the literature. Some suggested that its expression is increased during epidermal differentiation (Mangiulli et al., 2009; Westfall et al., 2003), whereas other showed that p53 knockdown leads to enhanced squamous differentiation (Freije et al., 2014). The mechanisms by which p53 acts on keratinocytes are complex and involve both activation and repression of genes. p53 can indirectly repress Krt14 expression in basal keratinocytes, thereby promoting their differentiation (Cai et al., 2012). However, a more recent study showed that deletion of p53 enhanced differentiation, in part by regulating Krt6a expression (Cottle et al., 2016). Therefore, the impact of p53 is complex and multifaceted. Here, we provide evidence that p53 directly represses Krt17 expression. Our results might seem in contrast to the fact that p53 was increased in psoriatic plaques (Baran et al., 2005; Qin et al., 2002), which are characterized by Krt17 over-expression. It is possible that p53 is not active in this situation (Qin et al., 2002), and Krt17 is regulated by additional factors.

When comparing Krt17 expression in the skin of wild-type and p53−/− mice, it is evident that there is no difference in its expression in basal keratinocytes. Therefore, under physiological conditions, the constitutive expression of Krt17 probably is not under the control of p53. Indeed, it has been
shown that p53 levels in the epidermis are characterized by spontaneous pulses, which do not necessarily activate downstream signaling such as transcription of p21 (Loewer et al., 2010). Here, we demonstrate this regulation kicks in only after the p53 level increases after irradiation, leading to a reduction of Krt17 expression. We show that in the healing process after IR/UVR, up-regulation of Krt17 was seen in both mice. Bar = 100 μm. (e) Quantification of Ki67 staining in the skin. (f) Dry desquamation in mice 16 days after ionizing radiation. (T16). No difference in phenotypic outcome was noticed between WT and p53−/− mice (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001. Krt17, keratin 17.
as might be argued from preferential cell destruction by IR in basal keratinocytes. Consistent with this notion, in our previous work using avian feather as a model, we readily found that a cytokine response is of paramount importance, whereas activation of p53 does not correlate with IR-induced tissue damage (X Chen et al., 2014). To the best of our knowledge, such characterization of p53 response in RD has not been previously reported.

The functional role of Krt17 in RD remains unknown. We hypothesized that its down-regulation contributes to RD-associated epidermal atrophy by reducing cell proliferation (Kim et al., 2006) and/or adhesion (Sankar et al., 2013), whereas its up-regulation is related to desquamation (Kim et al., 2006) and/or adhesion (Sankar et al., 2013), and/or the healing process through as-yet unknown mechanisms. Further insight on the role of K17 in IR- and UVR-induced dermatitis might be learned from Krt17-null mice (McGowan et al., 2002; Tong and Coulombe, 2006). An inducible overexpression model may also help. Regardless, the current findings expand our understanding on the complex transcriptional regulation of keratin gene expression. This may be harnessed in the future to manage skin disorders characterized by abnormal Krt17 expression, such as psoriasis (Jin and Wang, 2014), pachyonychia congenita (Wilson et al., 2012), and cancers (Chung et al., 2015; Hobbs et al., 2015).

**MATERIALS AND METHODS**

**Experimental animals and irradiation**

C57BL/6 mice and Wistar rats were purchased from the Shanghai SLAC animal facility center. P53−/− mice were generously provided by Dr. Shengcai Lin (Xiamen University; originally from Jackson Lab #002899 and genotyped accordingly). All experimental protocols were approved by Fuzhou University Experimental Animal Ethics Board.

IR was performed at the Department of Radiation Oncology, Union Hospital of Fujian Medical University. The animals were anesthetized (pentobarbital 50 mg/kg) and loosely fasted on a foam board to expose the hind limbs. A lead cover was used to protect the rest of the body and expose the hind limbs to IR. A Varian Clinac 23ex linear accelerator (Varian Medical Systems, Inc., Palo Alto, CA) was used to provide the 9-MeV electron beam, at a dose rate of 500 cGy/min (X Chen et al., 2014). A 10-Gy × 4 regimen with a 1-day interval was applied to the rat skin. To induce dry desquamation in mice, a 40-Gy × 1 regimen was applied because the mice are more resistant to IR. For UVR, a light source from Liuyi Instrument Co (Beijing, China) was used, at a dose rate of 1.5 mW/s. The spectra were monitored using an RPS-900R calibrator (International Light Technologies, Peabody, MA).

**RNA extraction and profiling**

Skin samples were collected at designated times, and total RNAs were extracted using the Trizol reagent (Invitrogen, Guangzhou, China). After a quality check, RNAs were processed for gene expression profiling at Beijing Genome Institute (BGI, Shenzhen, China). The sequencing results were annotated according to the NCBI database (Chu et al. 2014; Saha et al., 2002).

**Quantitative polymerase chain reaction and western blot**

A PCR premix from CWBio (Beijing, China) was used, and each sample was repeated three times using a Roche LC480 Lightcycler (Roche Diagnostics, Shanghai, China). Primer sequences are available on request. Total protein was extracted using the Trizol reagent and dissolved in water containing 1% SDS. Protein concentration was determined by the bichinoninic acid method (Sangon, Shanghai, China). Antibodies used were Gapdh, β-actin (Beyotime, Shanghai, China), β-tubulin, and Krt17 (BBI, Shanghai, China).

**Immunohistology**

Skin samples were collected at designated times and fixed in 4% paraformaldehyde overnight. Paraffin sections 8 μm thick were collected for staining. For immunofluorescence, a Cy3-conjugated secondary antibody and counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole in phosphate buffered saline. For immunohistochemistry, a horseradish peroxidase–conjugated secondary antibody and 3-amin-9-ethylcarbazole substrate was used. Antibodies used were Krt17, Krt6a (BBI), Krt10 (Covance, Hong Kong, China), Ki67, and p53 (Santa Cruz, Dallas, TX).

**Luciferase assay**

A 2-kb promoter of the mouse Krt17 gene was cloned into the pGL3 basic reporter, and serial truncation was performed. Full-length human p53 open reading frame was cloned into pEGFP-N1 for overexpression. shRNA for p53 target sequence was GACTC CAGTGGTAATCTAC, and scramble control was AGATACGACA GAGGACACT. For luciferase assay, 293T cells were plated in 24-well plates 24 hours before transfection. Cells were cultured with 10% fetal bovine serum in DMEM (Gibco, Guangzhou, China). For
each well, 1 μg DNA was transfected, including 250 ng reporter/CMV-LacZ, and the desired amount of p53 expression or RNAi plasmids. Cells were lysed 48 hours posttransfection, and luciferase activities were measured (Beyotimes). LacZ activity was used to normalize the results. For doxorubicin (Sigma, Shanghai, China) treatment, cells were cultured at 1 μM for 24 hours.

CHIP analysis

CHIP analysis was performed using a commercial kit (Beyotimes). Mice footpad skin samples were collected after a single IR exposure. Freshly isolated samples were cross-linked in 4% paraformaldehyde on ice for 6 hours, homogenized, and centrifuged to remove the insoluble debris. The supernatants were sonicated to break the genomic DNA. Primary antibody used was p53 (Santa Cruz), and normal rabbit IgG was used as control (BBI). PCR was performed with the following primers: p21 forward: 5’-CCTTTCTATCAGCCCAGAGGATACC-3’; reverse: 5’-GGGACGTCCTTAATTATCTGGGGTC3’; Krt17 forward: 5’TGGAACTTTAGCTGAGGCTCTGCTGCTG-3’; reverse: 5’-GGGAAGATGTGCTATGACAGCAGAG-3’. Quantification of the CHIP signal was performed according to Xue et al., 2014.

Electromobility shift assay

Electromobility shift assay was performed using a commercial kit (Beyotimes). Briefly, 293T cells were lysed with hypotonic buffer and

Figure 5. p53 regulates Krt17 promoter. (a) Scheme of truncation of a 2-kb mouse Krt17 promoter. Arrows indicate the position of chromatin immunoprecipitation (CHIP) primers. (b) Sequence and annotation of a segment of the promoter. (c) Doxorubicin (Dox) treatment down-regulates Krt17-luc, which is dependent on the −978 to −696 region. (d) p53 overexpression down-regulates whereas RNAi knockdown of p53 up-regulates Krt17-luc. Numbers on the horizontal axis indicate the amount of plasmids transfected (ng). (e, f) Western blot showing Dox treatment increases whereas RNAi decreases p53 protein levels. (g, h) Control RNAi induces no change in p53 protein level. *P < 0.05; **P < 0.01; ***P < 0.001. Krt17, keratin 17.
nuclear protein extracted. For probe, double-strand DNAs were synthesized with 5'-biotin label on each oligo (Sangon). p53 binding site 1: 5’CAGAAAAACTGGGAGAAATGGGCGCTATCACCTGGG3’, mutation: 5’TGTAAATAATGGGAATTATCC3’; p53 binding site #2: 5’GGGCCTGTTTGGACATCACA3’, mutation: 5’TTGACTAATTGTAAATCACA3’. Competing DNA (no label): 5’TACAGAAACATGTCAAGCATCAGGACATCACA3’. For supershift, 2 µl of specific antibody was used in a 10-µl reaction, incubated for 20 minutes at room temperature, separated on nondenatured 4% polyacrylamide gel, and transferred to a nylon membrane. The membrane was cross-linked by UV for 10 minutes, incubated with horseradish peroxidase-conjugated streptavidin, and developed using BeyoECL Plus (Beyotimes).

Statistics
All experiments were repeated at least three times, and results are shown as mean ± standard error of the mean. For gene expression analysis, at least five mice were used for each time point. Two-tailed
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Student t test was used to calculate the P-value (Lu et al., 2007; Naruse et al., 2014), where *P < 0.05, **P < 0.01, and ***P < 0.001.

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 www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.12.021.

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