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Interferon Regulatory Factor 6 is necessary but not sufficient for keratinocyte differentiation

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Abstract

Regulation of epidermal proliferation and differentiation is critical for maintenance of cutaneous homeostasis. *Interferon Regulatory Factor 6* (*Irf6*) deficient mice die perinatally and exhibit ectopic proliferation and defective epidermal differentiation. We sought to determine if these disruptions of epidermal function were cell autonomous and used embryonic *Irf6*$^{-/-}$ keratinocytes to understand the specific role of Irf6 in keratinocyte proliferation and differentiation. In absence of Irf6, keratinocytes exhibited a heterogeneous phenotype with the presence of large cells. Irf6$^{-/-}$ keratinocytes displayed increased colony forming efficiency compared to wildtype cells, suggesting that Irf6 represses long-term proliferation. Irf6 was present at low levels in wildtype keratinocytes in culture and upregulated after induction of differentiation in vitro, along with upregulation of markers of early differentiation. However, *Irf6*$^{-/-}$ keratinocytes did not express markers of terminal differentiation. Overexpression of Irf6 in wildtype keratinocytes was insufficient to induce expression of markers of differentiation under growing conditions. Together, these results indicated that Irf6 is necessary, but not sufficient for keratinocyte differentiation. Finally, using a transgenic mouse expressing *Lac-Z* under the regulation of an enhancer element 9.7kb upstream of the *Irf6* start site, we demonstrated that this element contributes to the regulation of Irf6 in the epidermis and keratinocytes in culture.

Keywords

Interferon Regulatory Factor 6; keratinocyte; differentiation; mouse; enhancer

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Conflict of Interest

The authors declare no conflict of interest.

Introduction

Maintenance of the epidermis requires a delicate balance between restricted proliferation of keratinocytes in the basal layer, and differentiation of basal keratinocytes into the stratum corneum. The regulation of epidermal differentiation is a complex process that requires spatial and temporal regulation of many genes, including: Keratin (Krt) 14 and Krt5 which are expressed in the basal layer and shut down suprabasally when other genes, such as involucrin, loricrin, and filaggrin, are upregulated (Eckert, 1989). In addition, transcription factors, such as p63 (Mills *et al.*, 1999; Yang *et al.*, 1999), AP1 (Welter *et al.*, 1995) and AP2 (Leask *et al.*, 1991) family members, C/EBP (Maytin *et al.*, 1998), and SP1 (Byrne *et al.*, 1993) are involved in regulating epidermal differentiation. Recently, we identified Interferon Regulatory Factor 6 (Irf6) as a new critical transcriptional regulator of epidermal differentiation (Ingraham *et al.*, 2006; Richardson *et al.*, 2006).

Mutations in *IRF6* cause two allelic orofacial clefting syndromes, Van der Woude (VWS) and popliteal pterygium syndromes (PPS) (Kondo *et al.*, 2002). Removal of *Irf6* in mice results in severe epidermal malfunction, in addition to limb and craniofacial abnormalities (Ingraham *et al.*, 2006), and is a phenocopy of the *Sfn* (Fisher *et al.*, 1987), *Ikka* (Hu *et al.*, 1999; Takeda *et al.*, 1999), and *Rip4* (Holland *et al.*, 2002) knockout mice, genes necessary for proper epidermal morphogenesis. *Irf6*-deficient mice lack a functional barrier and are perinatal lethal.

More specifically, Irf6 is expressed suprabasally in the embryonic epidermis 17.5 days after conception (Ingraham *et al.*, 2006). Examination of the epidermis of *Irf6*-deficient mice revealed a lack of keratohyalin granules and stratum corneum that is accompanied by the absence of loricrin and filaggrin. Concomitantly, proliferative keratinocytes were found in the spinous layer, along with ectopic p63 and Krt14 (Ingraham *et al.*, 2006). These observations are indicative of a role for Irf6 as a repressor of proliferation and a promoter of differentiation. Recently, a feedback loop between Irf6 and p63 was identified (Moretti *et al.*, 2010; Thomason *et al.*, 2010). This occurs by ΔNp63 directly binding a conserved region 9.7-kb upstream of the *Irf6* transcriptional start site and inducing transcription of *Irf6*, which in turn down-regulates ΔNp63 post-translationally (Moretti *et al.*, 2010). Interestingly, the p63-binding site is located in a multispecies conserved sequence (MCS) genomic region that has *Irf6* enhancer activity (Rahimov *et al.*, 2008). However, whether this enhancer region also regulates *Irf6* in adult keratinocytes is unknown.

In this study, we provide evidence that Irf6 is necessary, but not sufficient, for keratinocyte terminal differentiation. These data, along with the requirement of Irf6 for epidermal development and maintenance in vivo, suggest that the effect of Irf6 is specific to keratinocytes. Furthermore, we demonstrate that a MCS enhancer 9.7kb upstream of the start site of Irf6 contributes to its regulation in adult epidermis and keratinocytes in culture.

Results

Irf6 deficient keratinocytes display an abnormal, but epithelial phenotype

We successfully cultured keratinocytes from e17.5 wildtype and Irf6$^{-/-}$ embryos. Microscopic observations revealed wildtype keratinocytes that appeared cobblestone-like, and largely uniform in size. In contrast, *Irf6*$^{-/-}$ keratinocytes exhibited a heterogeneous population with small, normal, cobblestone-like cells amongst cells that were much larger in size and irregularly shaped (Figure 1a). The overall cellular area of *Irf6*$^{-/-}$ keratinocytes was significantly increased compared to wildtype (Figure 1b), as well as the distribution of the cellular size, with an increase in the proportion of larger cells in the mutant cells (Figure 1b). However, cellular volume was unchanged between the two populations of cells (Figure 1b). An increase in cellular size has been observed with loss of the epithelial phenotype during epithelial-to-mesenchymal transition (EMT) (Lamouille *et al.*, 2007). To determine if the Irf6$^{-/-}$ keratinocytes had initiated EMT, we performed immunostaining to detect either the loss of basal epidermal marker, keratin (Krt) 14, or the gain of the mesenchymal marker vimentin (Steinert *et al.*, 1988) (Figure 1c top row). Both wildtype and mutant keratinocytes expressed Krt14 and lacked vimentin, demonstrating their epithelial phenotype. A few vimentin-positive cells were present in both cultures. However, they were Krt14-negative and dendritic-like. This is consistent with the only vimentin-positive cells being melanocytes and Langerhans cells in the epidermis (Franke *et al.*, 1979). Together, these data show that while there is a difference in cellular size distribution, *Irf6*$^{-/-}$ keratinocytes retain epithelial characteristics and do not express vimentin.

Previous work demonstrated a regulatory relationship between p63 and Irf6 (Moretti *et al.*, 2010; Thomason *et al.*, 2010). In order to localize Irf6 in murine embryonic keratinocytes, we performed immunostaining for Irf6 and p63 on wildtype and *Irf6*$^{-/-}$ keratinocytes under basal growing conditions (Figure 1c, bottom row). We observed Irf6 largely in the cytoplasm of the cells, while no signal was detected in Irf6$^{-/-}$ keratinocytes. p63 was localized in the nucleus of keratinocytes and was present in both wildtype and Irf6$^{-/-}$ keratinocytes.

Irf6 restricts the long-term proliferative potential of keratinocytes

Keratinocytes shut down proliferation in order to commit to terminal differentiation. To determine whether Irf6 represses proliferation in vitro, we compared the percentage of BrdU incorporation in wildtype and mutant keratinocytes (Figure 2, top left panel). We complemented the assay by evaluating the cell cycle profile of the same cells (Figure 2, top right panel). We did not observe statistically significant differences between the two groups. Data from our in vivo studies indicated the presence of ectopic proliferative keratinocytes in the absence of Irf6 while the proliferation of basal keratinocytes was not altered (Ingraham *et al.*, 2006). As these results may indicate a difference in long-term proliferative capacity, we performed a proliferation assay in which we counted the number of cells 6 and 10 days after plating (Figure 2 bottom left panel). Consistent with our BrdU incorporation assay and cell cycle analysis, we did not observe a difference six days after plating. However, Irf6$^{-/-}$ keratinocytes were more numerous than wildtype cells after 10 days. Additionally, we found that in the absence of Irf6, keratinocytes were more efficient at forming colonies than

wildtype cells (Figure 2 bottom right). Together, these data suggest that Irf6 restricts the long-term proliferative capacity of keratinocytes.

Irf6 is necessary for keratinocyte terminal differentiation in vitro

Loss of *Irf6* in the mouse resulted in abnormal epidermal morphogenesis. This prompted us to directly test whether Irf6 acts in a keratinocyte autonomous fashion to regulate keratinocyte differentiation. Murine keratinocytes can be induced to differentiate by increasing the calcium in the culture medium (Hager *et al.*, 1999). After reaching confluence in NMedium (0.06 mM $CaCl_2$), wildtype and $Irf6^{-/-}$ keratinocytes were purged from growth factors (LoCal medium, 0.06 mM $CaCl_2$) for 24h, then switched for KSFM with 0.15 mM $CaCl_2$ (K0.15) to induce differentiation. Both wildtype and $Irf6^{-/-}$ keratinocytes exhibited their characteristic cellular morphology (Figure 3a, d), similar to that previously observed in NMedium (Figure 1). After 72h in K0.15, wildtype keratinocytes appeared stratified in the middle of colonies, eventually forming peaks of elongated cells, as evidenced by birefringent scattering of light (Figure 3b). In contrast, the $Irf6^{-/-}$ cells remained mainly as a monolayer (Figure 3e).

Western blot analysis revealed low levels of Irf6 in wildtype cells under growing conditions (NMedium), and its upregulation upon differentiation (Figure 3f). No Irf6 protein was detected in $Irf6^{-/-}$ keratinocytes. Krt14 was detected at the same level in wildtype and mutant cells, regardless of culture conditions. However, p63 was upregulated in $Irf6^{-/-}$ keratinocytes under similar culture conditions. After induction of differentiation, p63 level decreased in wildtype keratinocytes, while it remained highly expressed in $Irf6^{-/-}$ keratinocytes. These data suggest that Irf6 is required to downregulate p63, but not Krt14, as keratinocytes are induced to differentiate. Krt10, Krt1 and involucrin, markers of early epidermal differentiation, were expressed in both wildtype and $Irf6^{-/-}$ epidermis (Figure S1) and cultures under differentiation conditions, which suggests that Irf6 is not necessary for early keratinocyte differentiation. In contrast, markers of terminal keratinocyte differentiation, loricrin and filaggrin, were expressed only in wildtype keratinocytes under differentiation conditions, indicating that Irf6 is necessary for keratinocyte terminal differentiation.

To determine if the differentiation defect was specific to the absence of Irf6, we infected $Irf6^{-/-}$ keratinocytes with an adenovirus that expressed Irf6 (Ad-Irf6) or green fluorescent protein (Ad-GFP) under the control of the CMV promoter (Bailey *et al.*, 2008a). All keratinocytes were successfully infected with the viral construct with no apparent toxicity (Figure 3c). Cellular size was similar between the groups, yet slightly increased compared to non-transfected keratinocytes (Ad-IRF6 = 1352 ± 66 μm^2, N = 4; Ad-GFP = 1429 ± 66 μm^2, N = 6; averages ± SEM). Under differentiation conditions, only a few Ad-Irf6 transfected $Irf6^{-/-}$ keratinocytes appeared stratified, suggesting only partial differentiation compared to wildtype cells (Figure 3b). Ad-Irf6 transfected $Irf6^{-/-}$ keratinocytes expressed Irf6 protein at a higher than endogenous level as shown by western blot (Figure 3f). The level of Krt14 was unchanged after transfection, while the level of p63 was reduced compared to $Irf6^{-/-}$ keratinocytes under differentiation conditions. In addition, Ad-Irf6 transfected $Irf6^{-/-}$ keratinocytes expressed Krt1 and involucrin at a higher level than in mutant cells while

Krt10 was expressed at a higher level than wildtype cells. However, despite abundant Irf6, these keratinocytes did not express filaggrin or loricrin (Figure 3f). Expression of all these proteins in *Irf6*$^{-/-}$ keratinocytes transfected with Ad-GFP were identical to *Irf6*$^{-/-}$ keratinocytes, indicating no impact of the transfection itself on these markers. These observations suggest that Irf6 regulates p63, but re-introduction of Irf6 was not sufficient to achieve terminal differentiation of keratinocytes.

Irf6 is not sufficient to promote keratinocyte differentiation

In order to explore the extent to which Irf6 is sufficient to induce keratinocyte differentiation, we used the Ad-Irf6 to overexpress Irf6 in wildtype embryonic keratinocytes (Figure 4). Keratinocytes transfected with a GFP-expressing vector (Ad-GFP) exhibited identical morphological features compared to Ad-Irf6 keratinocytes. Both groups showed cobblestone-like cells under growing conditions in NMedium (Figure 4c, d), and the presence of stratified cells consistent with differentiation in medium with calcium (Figure 4a, b, e, f). Under growing conditions, we did not observe stratification in cells overexpressing Irf6. Western blot analysis confirmed the overexpression of Irf6 in cells transfected with Ad-Irf6 compared to Ad-GFP control (Figure 4g). Both wildtype and overexpressing Irf6 keratinocytes exhibited a similar protein expression profile, indicating that Irf6 alone is not sufficient to induce keratinocyte differentiation. However, levels of p63, Krt10, Krt1, loricrin and filaggrin varied with culture conditions, with markers of differentiation being detected only in K0.15, and p63 only under low calcium concentration. NMedium supplemented with calcium was not sufficient to induce expression of differentiation markers, leading to the hypothesis that other components of these media influence keratinocyte differentiation in vitro.

The MCS9.7 enhancer region regulates Irf6 in adult keratinocytes

Recently, an enhancer of *Irf6* was identified (Rahimov *et al.*, 2008). This enhancer, termed *MCS9.7*, was sufficient to express a reporter gene in tissues that express endogenous *Irf6*, including the epithelial layers of developing limb buds and craniofacial structures at e11 (Rahimov *et al.*, 2008) and in the epidermis at e17.5 (Sperber, 2010). As *Irf6* is critical for epidermal morphogenesis, we asked whether this enhancer regulates Irf6 in adult skin and keratinocytes in culture. We performed X-gal staining on adult murine epidermis and detected strong beta-galactosidase activity throughout the suprabasal layers of the epidermis (Figure 5a). This expression pattern recapitulates that of Irf6 protein (Figure 5a).

We took advantage of our in vitro differentiation assay to test whether upregulation of Irf6 after differentiation was under the activity of the MCS9.7 enhancer. We extracted tail keratinocytes from adult mice positive and negative for the *MCS9.7-LacZ* transgene. Under growing conditions, we detected little or no beta-galactosidase activity (Figure 5b), even though Irf6 was expressed in these cells (Figure 5c). However, under differentiating conditions, we observed strong beta-galactosidase activity in stratified keratinocytes which correlated with stronger expression of Irf6 in these cells (Figure 5c). These data confirm that *MCS9.7* acts as an enhancer, but demonstrate that in vitro, *MCS9.7* is restricted to the most differentiated cells, while Irf6 has a broader profile of expression. Therefore, these data are further evidence that *MCS9.7* contributes to the regulation of Irf6 in keratinocytes.

Discussion

In this study, we showed that Irf6 was a critical regulator of proliferation and differentiation in keratinocytes in culture. In addition, we complemented, in murine keratinocytes, previous studies that demonstrated the regulation of steady-state levels of p63 by Irf6 (Moretti *et al.*, 2010; Thomason *et al.*, 2010). Finally, we better defined the enhancer activity of *MCS9.7*, a multispecies conserved sequence near *IRF6*.

Through culturing *Irf6*$^{-/-}$ keratinocytes for the first time, we tested the hypothesis that Irf6 acts in a keratinocyte autonomous fashion in regulating proliferation and differentiation. Phase contrast observations revealed the presence of large keratinocytes amongst smaller cobblestone-like keratinocytes in the absence of Irf6. This phenotypic difference appeared intrinsic to the cells as this phenotype was maintained after subculture. An increase in cellular size can be indicative of EMT (Lamouille *et al.*, 2007) or cellular stress such as wound repair (Martin, 1997). We detected the presence of Krt14 while vimentin was absent from *Irf6*$^{-/-}$ keratinocytes, indicating that *Irf6*$^{-/-}$ keratinocytes did not undergo EMT. Furthermore, cellular volume was unchanged, suggesting that the larger cellular size may be due to cellular spreading (Lamouille *et al.*, 2007). Thus, Irf6 is necessary to maintain keratinocyte size, but not epithelial phenotype.

Recent work demonstrated a regulatory relationship between Irf6 and p63. Our Western experiments showed an inverse relationship between the steady-state levels of Irf6 and p63, confirming that Irf6 represses p63 expression. In their studies, Moretti *et al.* (Moretti *et al.*, 2010) overexpressed Irf6 in human keratinocytes and found a reduction in CFE, indicating that Irf6 regulates the proliferative potential of keratinocytes. In our complementary studies, we observed that *Irf6*$^{-/-}$ murine keratinocytes were more efficient at forming colonies. These results were consistent with an increase in the number of mutant keratinocytes compared to wildtype cells long-term. Short-term, however, proliferation and cell cycle profile were similar between wildtype and mutant. Together, our results suggest that the initial proliferation of keratinocytes in culture is not Irf6-dependent. However, it appears that the absence of Irf6 leads to persistence of, or selection for, a population of keratinocytes with a high proliferation potential that after a lag-time of a few days initiate division. This translates into an increase in cellular proliferation and CFE. This cellular behavior observed in Irf6-deficient keratinocytes is reminiscent of keratinocytes with stem cell properties (Dunnwald *et al.*, 2003a). As epidermal stem cells have been described as more “sticky” than more differentiated cells (Bickenbach *et al.*, 1998; Jones *et al.*, 1993; Kaur *et al.*, 2000), it is tempting to propose that Irf6 plays a role in cellular adhesion. In this vein it is interesting to note that Irf6-deficient mice exhibit oral adhesion, fusion of the gastrointestinal track and syndactyly, all of which are forms of adhesion defects. Of note, we did not detect the presence of apoptotic cells under basal growth conditions regardless of the level of Irf6 in keratinocytes (data not shown). Furthermore, previous data in mammary epithelial cells showed that Irf6 promotes quiescence by favoring entry into the G(0) phase of the cell cycle (Bailey *et al.*, 2008b). And adenoviral re-expression of Irf6 in breast cancer cells that had lost Irf6 expression significantly reduced cellular proliferation (Bailey *et al.*, 2008a). Together, these data strongly support a role for Irf6 as a repressor of long-term keratinocyte proliferation.

A vital role for Irf6 in epidermal differentiation was first observed in vivo (Ingraham *et al.*, 2006), thus, we asked if *Irf6*$^{-/-}$ keratinocytes were competent to undergo differentiation in vitro. We observed that Irf6 was not necessary for early keratinocyte differentiation as we detected Krt10, Krt1, and involucrin in *Irf6*$^{-/-}$ keratinocytes after in vitro induction of differentiation. Conversely, Irf6 was required to achieve terminal keratinocyte differentiation, as loricrin and filaggrin were absent from calcium-induced differentiated Irf6$^{-/-}$ keratinocytes. These results confirm our previous in vivo data indicating the presence of early differentiation markers and the absence of later differentiation markers in Irf6$^{-/-}$ epidermis, and suggest that Irf6 might differentially regulate different aspects of keratinocyte differentiation. The re-introduction of Irf6 by adenovirus rescued stratification in mutant keratinocytes, although to a lesser extent than in wildtype. These phenotypic changes were accompanied by the expected increase in Krt10, Krt1 and involucrin and decrease in p63 expression, but lacked the expected expression of loricrin and profilaggrin. These data suggest that only partial rescue was achieved, despite Irf6 levels above that of endogenous levels. Other regulatory elements or cofactors may be required to fully rescue the *Irf6*$^{-/-}$ phenotype. Though Irf6 is expressed at low levels in basal conditions, it is upregulated upon differentiation. It is possible that the cellular environment of factors under growth conditions does not support Irf6-induced differentiation.

Although Irf6 is necessary for keratinocyte differentiation, we observed that it is not sufficient. Overexpression of Irf6 did not induce stratification in growth medium nor did it detectably alter stratification in differentiation medium. These results indicate that Irf6 alone cannot induce keratinocyte differentiation. Interestingly, KSFM with 0.15 mM $CaCl_2$ was the only medium permissive to keratinocyte differentiation. Addition of the same concentration of calcium to NMedium promoted stratification, but Krt10 or Krt1 were not detected. However, addition of calcium decreased p63 levels, suggesting a potential calcium-dependent regulation of p63.

Finally, we observed that *MCS9.7*, the recently identified enhancer of *IRF6* (Rahimov *et al.*, 2008) was active in some, but not all culture conditions. Specifically, we observed no beta-galactosidase activity in growing keratinocytes and a strong upregulation of activity in differentiated keratinocytes. However, we showed that Irf6 is present under both conditions. Possible explanations for these observations are the artificial nature of cell culture, potential lag time between the signal for transcription and the translation of Irf6, and differences between antibody staining for protein and colorimetric stain for a reporter enzyme activity. Furthermore, other regulatory elements may be required for Irf6 expression under basal conditions. We also demonstrated that the *MCS9.7* is present, with strong expression in the suprabasal layers of the adult murine epidermis, which recapitulates the localization of the Irf6 protein.

In summary, we have shown that Irf6 regulates long-term proliferation and is necessary but not sufficient to induce keratinocyte terminal differentiation. Cell cycle regulation and keratinocyte differentiation are critical to cutaneous wound healing and cancer, in which Irf6 could play a critical role (Bailey *et al.*, 2008a; Jones *et al.*, 2010).

Materials and Methods

Mice

All mice were cared for according to the ACURF at the University of Iowa. Two distinct *Irf6* mutant strains were used interchangeably to obtain *Irf6*-deficient embryos, $Irf6^{gt1/+}$ and $Irf6^{del1/+}$. The $Irf6^{gt1}$ allele was previously described (Ingraham *et al.*, 2006). The $Irf6^{del1}$ allele is a deletion of exons 3 and 4 that removed critical sequences in the DNA binding domain, created a frameshift and was the product of recombination of a conditional knockout allele for *Irf6* carrying *loxP* sites in introns 2 and 4 (Kinoshita and Schutte, unpublished results). The *MCS9.7-LacZ* is a stable transgenic mouse strain that carries the same *MCS9.7-LacZ* transgene used previously for transient transgenic analysis (Rahimov *et al.*, 2008). This transgene fuses *MCS9.7*, a multi-species conserved sequence with enhancer activity, to the reporter gene *LacZ*. Briefly, the 607 bp sequence element of *MCS9.7* was cloned into the XbaI site of the Hsp68 expression plasmid, upstream from the SV40 basal promoter, the *lacZ* reporter gene and the SV40 polyA site. Transgenic mice were generated with this vector by standard procedures at the University of Iowa transgenic facility using the C57BL/6 strain. Genotyping for the $Irf6^{gt1}$ allele was performed as described (Ingraham *et al.*, 2006). Conditions for genotyping of the $Irf6^{del1}$ allele were: f: gcagagtggagcacacttca, r: ttcactgagaaagcagcaatg; 2 min 95°C, 35 cycles of 15 sec at 95°C, 15 sec at 63°C, 45 sec at 72°C and a final 5 min at 72°C. Conditions for genotyping of the *MCS9.7-LacZ* transgene were previously described (Rahimov *et al.*, 2008); 5 min at 94° C, 35 cycles of 30 sec at 95° C, 45 sec at 58° C, 60 sec at 68° C and a final 3 min at 68° C. Homozygous and heterozygous animals were used interchangeably as the PCR conditions do not distinguish between the two genotypes.

Keratinocyte Culture, Colony Forming Efficiency, and Transfection

The presence of a copulatory plug is designated as embryonic (e) 0.5. Skin from e17.5 embryos or adult tail was incubated with 5 U/mL Dispase II (Roche Applied Science, Germany) at 4° C for 4h, and 2.5 U/mL Dispase II overnight, respectively. The epidermis was peeled from the dermis and incubated in 0.25% trypsin (Gibco Invitrogen, Carlsbad, CA) for 20 min at 37° C. Keratinocytes were grown in NMedium (Hager *et al.*, 1999) and used after their first passage. To induce differentiation, cells were grown in keratinocyte serum free medium (KSFM, Gibco Invitrogen) with 0.15 mM $CaCl_2$, or NMedium with 0.15 mM $CaCl_2$. To assay cellular proliferation, cells were trypsinized 6 and 10 days after plating. Cells were counted using a Coulter Counter® (Beckman Coulter, Brea, CA).

Transfection was performed with polybrene at 10 MOI with adenoviral vectors containing either Irf6 or GFP (Bailey *et al.*, 2008a).

Colony forming efficiency (CFE) was performed as originally described (Rheinwald *et al.*, 1975). Briefly, 5×10^3 keratinocytes were plated on collagen IV coated 60 mm dishes and grown for 12 days. Cells were stained with 25% Giemsa stain and fixed in Giordano buffer (37% formaldehyde, 0.66% w/v KH_2PO_4, 0.32% w/v Na_2HPO_4). Colonies were counted and percentage of CFE calculated.

Cellular area was measured using ImageJ (http://rsbweb.nih.gov/ij/). Seven images of wildtype and six images of *Irf6*$^{-/-}$ keratinocytes were used, and a minimum of 100 cells per image was measured.

Cellular volume was obtained after trypsinized keratinocytes were analyzed with a Coulter Counter® (Beckman Coulter). Samples were run in triplicate and averages of 6 to 7 samples per group calculated.

Antibodies

Rabbit polyclonal antibody against Irf6 was previously described (Bailey *et al.*, 2005). Rabbit anti-mouse vimentin (Muller *et al.*, 2009) was a generous gift from Dr. Kroeger and Dr. Magin (University of Bonn, Germany). Rabbit polyclonal antibodies against Krt14, Krt10, Krt1, involucrin, loricrin were from Covance (Emeryville, CA). Rabbit polyclonal antibody against filaggrin and monoclonal antibody against beta-actin were from Sigma (St. Louis, MO). Rat polyclonal antibody against BrdU was from Abcam (Cambridge, MA). Monoclonal antibody against p63 and anti-rabbit HRP secondary were from Santa-Cruz (Santa Cruz, CA). Anti-mouse HRP secondary was from GE Healthcare (Piscataway, NJ).

Cell cycle analysis and BrdU incorporation

Four days after plating, keratinocytes were trypsinized and incubated in hypotonic propidium iodide as described (Dunnwald *et al.*, 2003a). For 5-bromo-2'-deoxyuridine-5'-triphosphate (BrdU) incorporation, keratinocytes were plated on coverslips and incubated for 2h in 10 μM BrdU (Sigma, St. Louis, MO). After fixation in methanol-acetone (70% −30% v/v), the DNA was denatured with HCl according to manufacturer's instructions (Abcam, Cambridge, MA) and cells washed in PBS. Cells were incubated with an anti-BrdU antibody and counterstained with DAPI. Total number of cells and BrdU positive cells were counted and the percentage of BrdU-positive calculated. Over 1000 cells were analyzed per group.

Protein Analysis

RIPA and Urea-Tris (Dunnwald *et al.*, 2003b) extraction buffers were used for protein preparation. Equal amounts of protein were separated on 10% Bis-Tris (Invitrogen) SDS-Page gels under denaturing conditions. Proteins were transferred onto polyvinylidene fluoride membranes (BioRad Laboratories, Hercules, CA), blocked in 10% non-fat dry milk and incubated in primary antibodies. After incubation with HRP-conjugated secondary IgG antibodies, antigen detection was performed with the chemiluminescent detection system ECL (GE Healthcare, Piscataway, NJ).

Immunofluorescence

Keratinocytes in passage 1 were grown on collagen IV coated coverslips under different culture conditions and fixed as described (Michel *et al.*, 1996). After blocking with 3% goat serum (Vector Laboratories, Burlingame, CA), cells were incubated with primary antibodies, washed in PBS, and incubated in secondary antibodies. DAPI was used as a nuclear stain. Images were viewed with a Nikon Eclipse E800 (Melville, NY) and acquired with a SPOT RT Slider CCD camera using Spot Advanced software (Diagnostic

Instruments, Sterling Heights, MI). Black and white images were pseudocolorized and merged. For confocal microscopy, images were acquired using a Zeiss LSM 710 microscope (Thornwood, NY) and ZEN 2009 software (Thornwood, NY).

X-gal staining

X-gal staining was performed by fixing the cells in 2% formaldehyde, 0.5 M sodium phosphate buffer, 0.2% glutaraldehyde, 0.01% sodium deoxycholate, and 0.2% NP-40. Cultured keratinocytes and skin sections were stained with X-gal solution (0.3 M sodium phosphate buffer, 4 mM $MgCl_2$, 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 0.03% sodium deoxycholate, 0.06% NP-40, 8.4 μM X-gal). Tissue sections were counterstained with hematoxylin.

Statistics

Data are averages of at least three biological replicates. Statistical analysis was performed with appropriate tests for each study as indicated in the figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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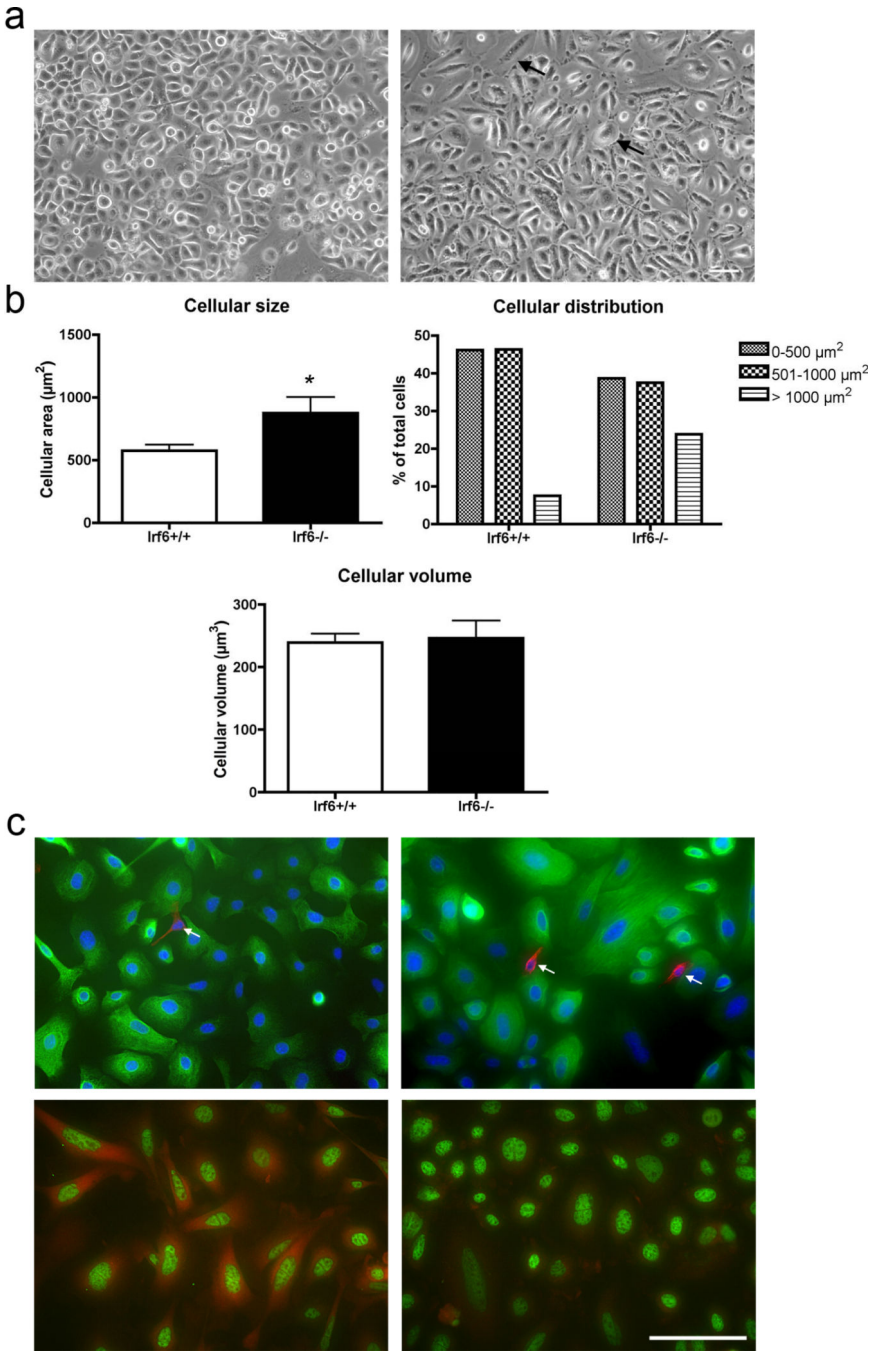

Figure 1. Characterization of Irf6$^{-/-}$ keratinocytes

(a) Phase contrast photomicrographs of *Irf6*$^{+/+}$ (left) and *Irf6*$^{-/-}$ keratinocytes (right). Black arrows indicate larger keratinocytes in the *Irf6*$^{-/-}$ population. (b) Cellular area of wildtype and *Irf6*$^{-/-}$ keratinocyte was traced on images as in (a) and averages (N = 6 or 7) plotted. *p < 0.05 after Student t-test. Distribution amongst three arbitrary categories of cellular sizes was calculated, and the difference determined by chi-square contingency test (*p < 0.05). Average of cellular volume (N = 6 or 7) after trypsinization was plotted. (c) Immunofluorescent staining of Krt14 (green, top row) and vimentin (red, top row), Irf6 (red,

bottom row) and p63 (green, bottom row). Nuclear DNA is labeled with DAPI (blue). White arrows indicate vimentin-positive, Krt14-negative melanocytes. Scale bar = 100 μm.

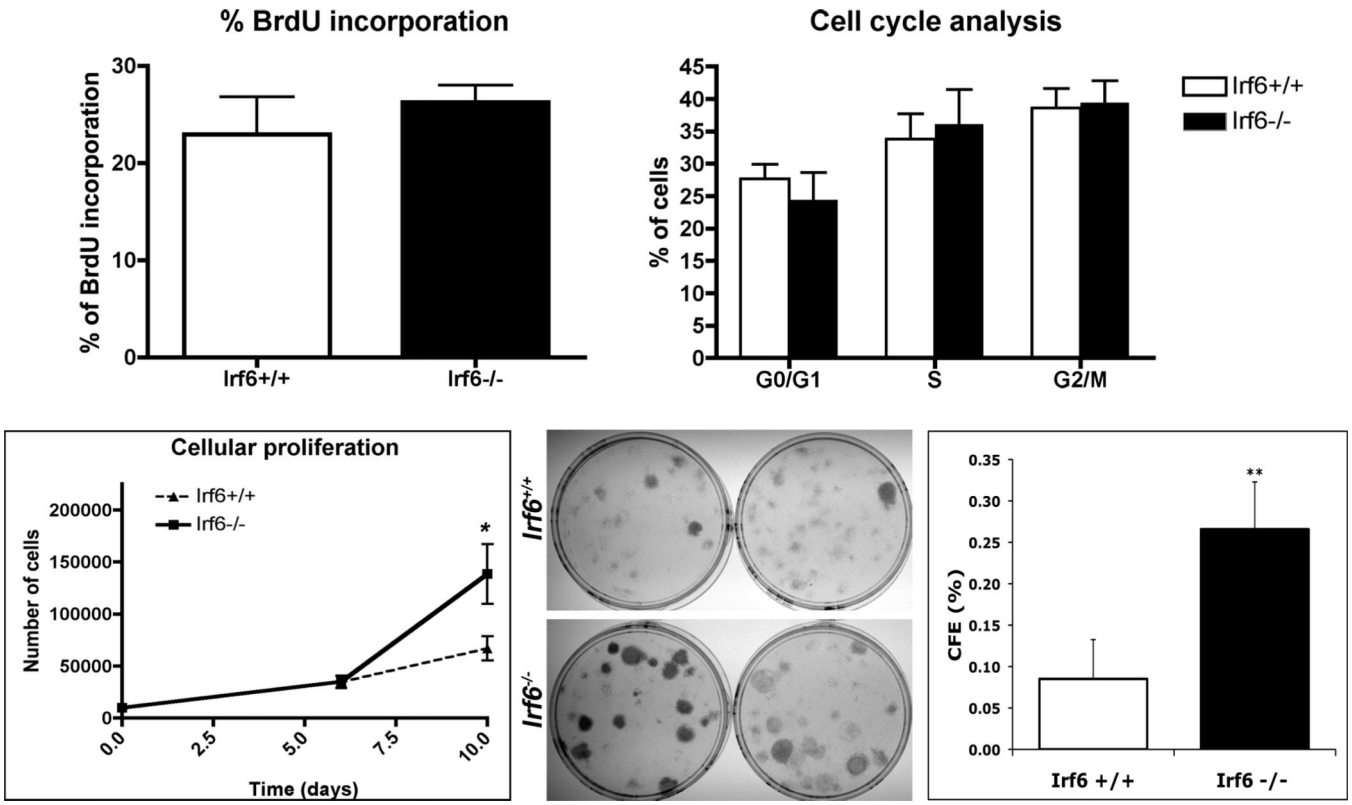

Figure 2. Irf6 restricts the long-term proliferative potential of keratinocytes

Percentage of BrdU incorporation is the ratio of BrdU positive cells over the total number of keratinocytes (top left). The distribution of keratinocytes in the cell cycle was determined from DNA content and expressed as percentage of total cells (top right). Number of cells 6 and 10 days after plating were plotted as a function of time (bottom let). Colony forming efficiency was performed and number of colonies (shown by the representative macroscopic view, bottom middle) was counted and percentage of colony forming efficiency calculated for *Irf6*$^{+/+}$ and *Irf6*$^{-/-}$ keratinocytes. Data are averages of three to five independent experiments (total N of 4 to 8 per group) ± SEM. *p<0.05; **p < 0.01 after Student t-test.

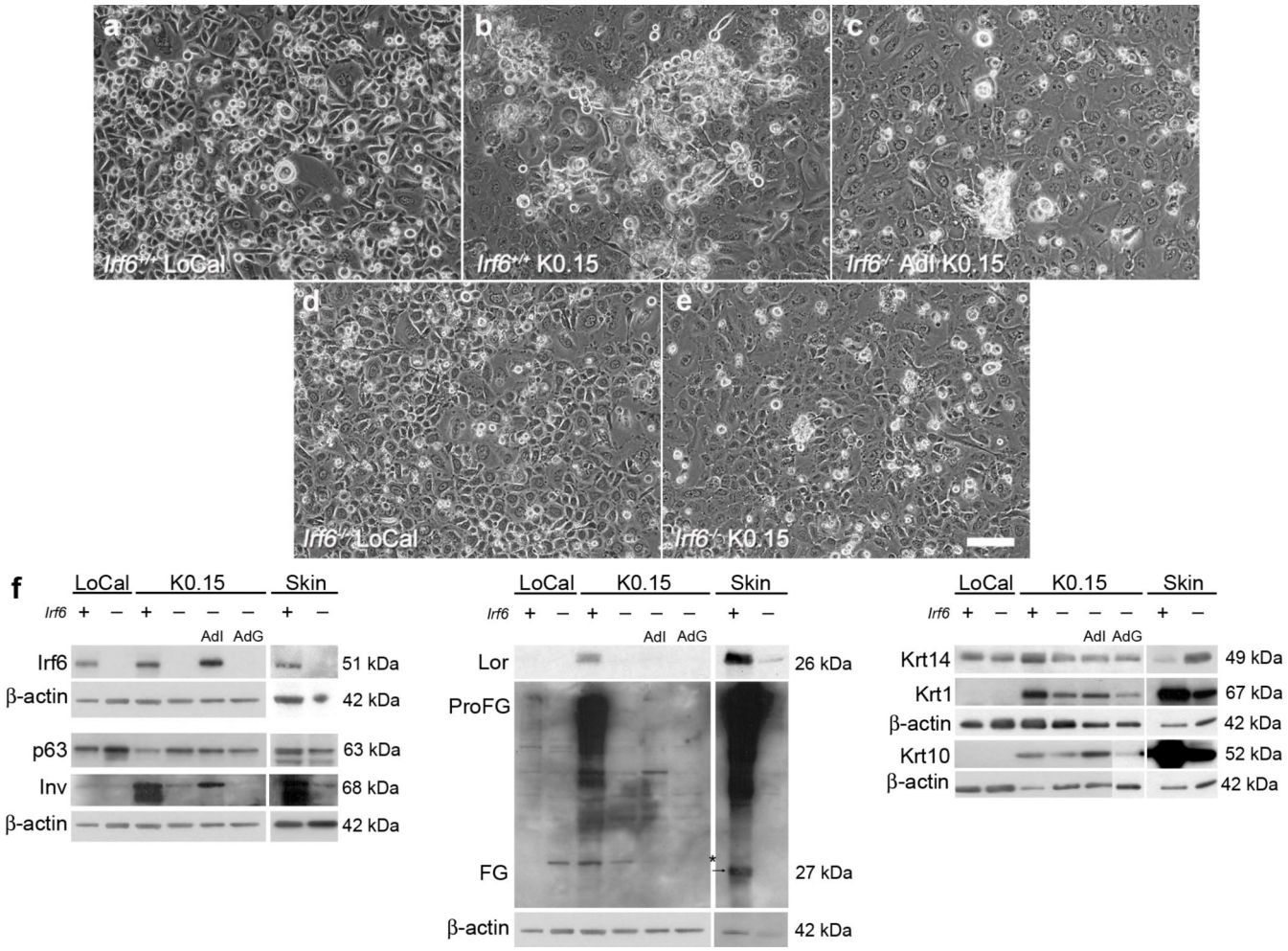

Figure 3. Irf6 is necessary for terminal differentiation of keratinocytes in vitro

Phase contrast images of *Irf6*$^{+/+}$ (a, b) and Irf6$^{-/-}$ keratinocytes (c–e) 24 hours in LoCal (a, d) or 72h in KSFM supplemented with 0.15 mM $CaCl_2$ (K0.15) to induce differentiation (b, c, e). *Irf6*$^{-/-}$ keratinocytes were transfected with an adenoviral construct containing Irf6 (AdI) or green fluorescent protein (AdG) cDNA. (f) Western blot analysis of protein extracts from cultured keratinocytes or embryonic skin for Irf6, Krt14, p63, Krt10, Krt1, Involucrin (Inv), loricrin (Lor) and filaggrin (FG; profilaggrin is also detected by this antibody and indicated as ProFG). Molecular weight for each protein is indicated. Black arrow in (f) indicates processed filaggrin in *Irf6*$^{+/+}$ skin; * indicates non-specific band. Scale bar = 100 μm.

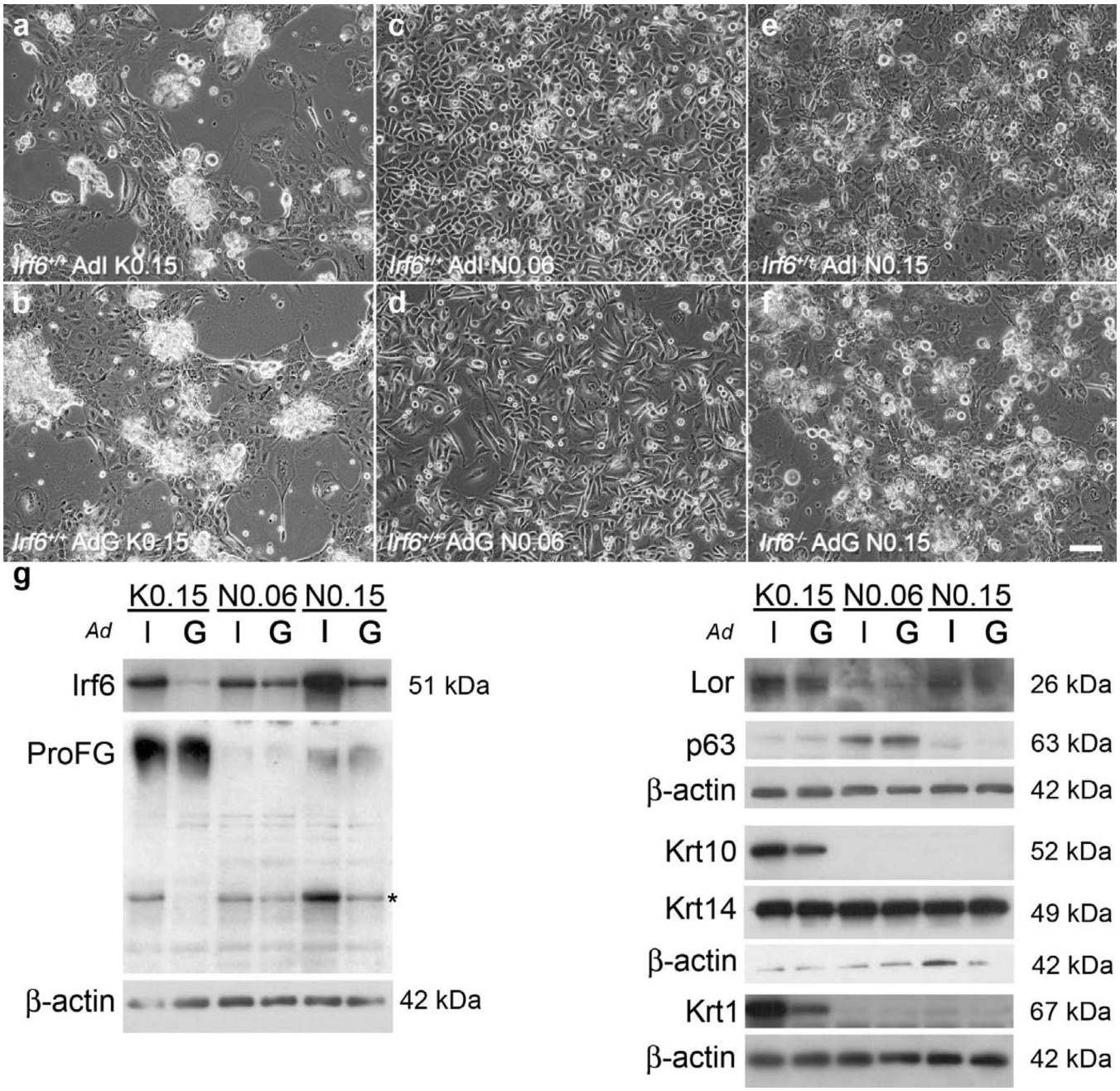

Figure 4. Overexpression of *Irf6* does not induce keratinocyte differentiation

(a) Microscopic phase contrast images of *Irf6*$^{+/+}$ keratinocytes were transfected with an adenoviral construct (Ad) containing either Irf6 (I) or GFP (G) cDNA. Cultures were then purged of growth factors and grown in KSFM with 0.15 mM $CaCl_2$ (K0.15), NMedium (N), or NMedium with 0.15 mM $CaCl_2$ (N0.15) for 72 hours. (b) Proteins were extracted for Western blot analysis and probed for Irf6, Krt14, p63, Krt10, Krt1, loricrin (Lor) and filaggrin (profilaggrin is also detected by this antibody and indicated as ProFG; * indicate non-specific band). Molecular weight for each protein is indicated. Scale bar = 100 μm.

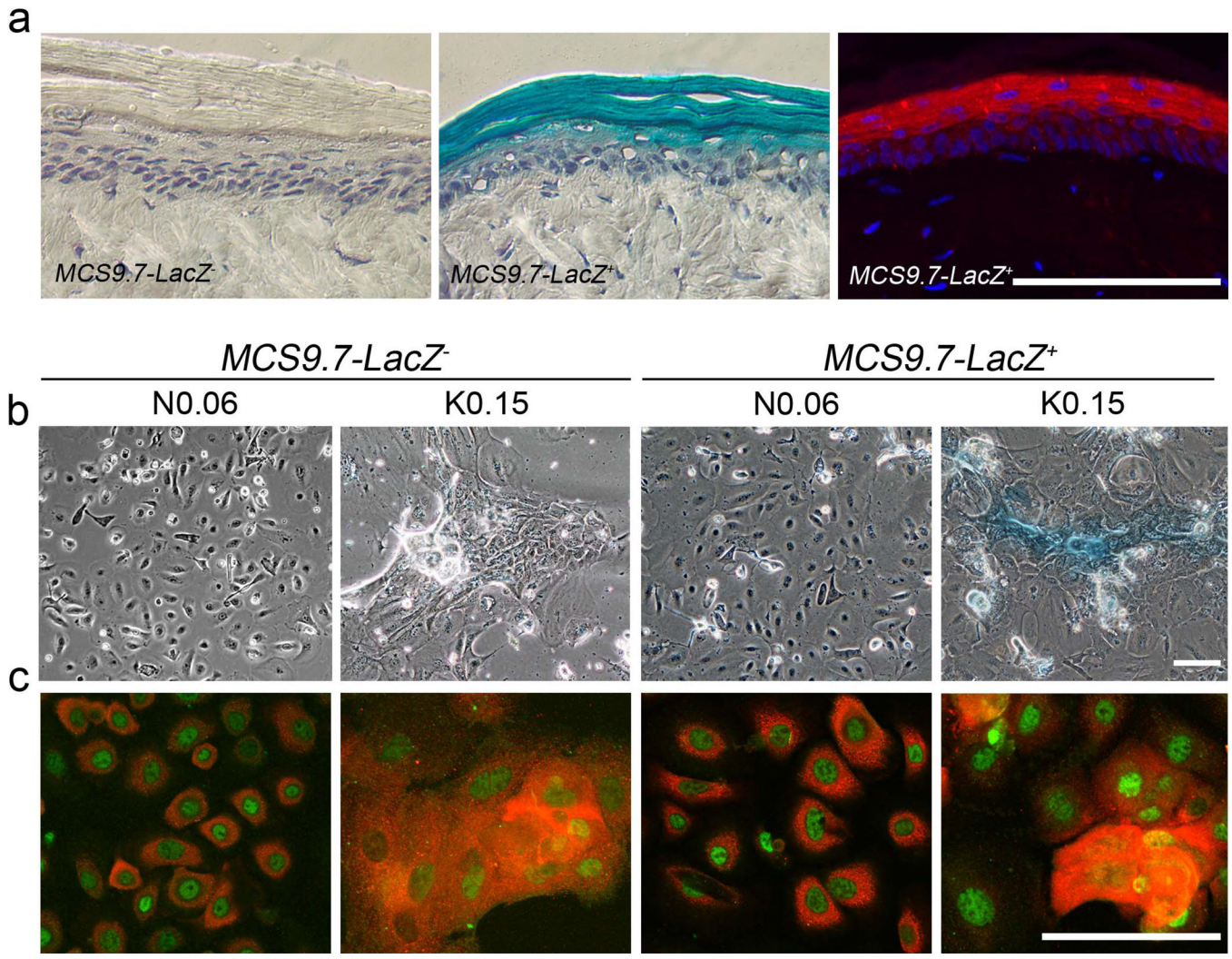

Figure 5. The MCS9.7 enhancer regulates *Irf6* in adult epidermis and keratinocytes

a) X-gal staining of *MCS9.7-LacZ* negative (*MCS9.7-LacZ*$^{-}$, left) and *MCS9.7-LacZ* positive (*MCS9.7-LacZ*$^{+}$) adult tail skin (middle), both counterstained with hematoxylin. Immunofluorescent staining of Irf6 (red) on *MCS9.7-LacZ* positive adult tail skin (right). Nuclear DNA is labeled with DAPI (blue). (b) X-gal staining of *MCS9.7-LacZ* negative and *MCS9.7-LacZ* positive keratinocytes under growing (N0.06) and differentiation conditions (K0.15). (c) Immunofluorescent staining of Irf6 (red) and p63 (green) on *MCS9.7-LacZ* negative and *MCS9.7-LacZ* positive keratinocytes under growing and differentiation conditions. Scale bar = 100 μm.