E2F7 Can Regulate Proliferation, Differentiation, and Apoptotic Responses in Human Keratinocytes: Implications for Cutaneous Squamous Cell Carcinoma Formation

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Abstract

The E2F family of transcription factors plays a crucial role in the regulation of genes involved in cell proliferation, differentiation, and apoptosis. In keratinocytes, the inhibition of E2F is a key step in the control and initiation of squamous differentiation. Because the product of the recently identiﬁed E2F7a/E2F7b gene has been shown to repress E2F-regulated promoters, and to be abundant in skin, we examined its role in the epidermis. Our results indicate that E2F7b mRNA expression is selectively associated with proliferation-competent keratinocytes. Moreover, E2F7 was able to antagonize E2F1-induced proliferation and apoptosis. In contrast, although E2F7 was able to inhibit proliferation and initiate differentiation, it was unable to antagonize the differentiation suppression induced by E2F1. These data indicate that E2F7-mediated suppression of proliferation and apoptosis acts through E2F1-dependent pathways, whereas E2F7-induced differentiation acts through an E2F1-independent pathway. These data also suggest that proliferation, differentiation, and survival of primary human keratinocytes can be controlled by the relative ratio of E2F1 to E2F7. Because deregulated proliferation, differentiation, and apoptosis are hallmarks of cancer, we examined the expression levels of E2F1 and E2F7 in cutaneous squamous cell carcinomas (CSCC). We found that both genes were overexpressed in CSCCs compared with normal epidermis. Furthermore, inhibition of E2F7 in a SCC cell line sensitized the cells to UV-induced apoptosis and doxorubicin-induced apoptosis. Combined, these data suggest that the selected disruption of E2F1 and E2F7 in keratinocytes is likely to contribute to CSCC formation and may prove to be a viable therapeutic target. [Cancer Res 2009;69(5):1800–8]

Introduction

Squamous differentiation involves the migration of keratinocytes from the proliferation-competent undifferentiated basal layer of stratified squamous epithelia up through the nonproliferative differentiating suprabasal layers to the external surface of the skin or mucosa of the upper aerodigestive tract. Once at the external surface, the cells die and form part of the cross-linked envelope that contributes to the barrier function of the epithelium. Hence, stratified epithelial linings are subject to continuous cycles of proliferation, differentiation, and cell death. This program of squamous differentiation is complex and regulated primarily at the transcriptional level by several transcription factors, such as AP1, AP2, Sp1, and E2F (1–4).

Keratinocytes of the basal layer contain a mixture of rare slow-cycling stem cells, which are thought to give rise to rapidly cycling transit-amplifying (TA) cells responsible for replacing cells as they die or are sloughed off the external surface (5). The stimulus to initiate squamous differentiation is still unclear but it is thought that the TA cells undergo a vertical division (6, 7) in which the vertically displaced progeny undergo irreversible growth arrest. This withdrawal from the cell cycle is irreversible and is dependent on E2F inhibition. However, the proliferation arrest induced by E2F inhibition is not sufﬁcient to induce differentiation (8). Following growth arrest, the keratinocytes start to express various differentiation markers, such as transglutaminase type 1 or keratin 10, which are required for epithelial barrier function (1, 9). Paradoxically, although growth arrest is dependent on E2F inhibition, there is significant expression of E2F isoforms in differentiated keratinocytes where it can act as a negative regulator of the differentiated phenotype (8). Thus, the relative level of proliferation and differentiation in human keratinocytes is controlled, in part, by the relative abundance and activity of the E2F transcription factor complex.

Although many transcription factors are thought to play a role in regulating the squamous differentiation program, the transcription factor E2F has emerged as a central regulator of many keratinocyte functions. The E2F family of transcription factors is an important class of cellular regulators that behave as both oncogenes and tumor suppressor genes in a context-speciﬁc manner (10, 11). The E2F family (E2F1 through E2F8 genes) shares signiﬁcant sequence identity in their DNA-binding domains and E2F1 to E2F6 share an ability to heterodimerize with the DP proteins (10, 11). E2Fs can be broadly classiﬁed as activators (E2F1, E2F2, and E2F3A) or suppressors/antagonists (E2F3B, E2F4, E2F5, E2F6, E2F7, and E2F8). This classiﬁcation refers primarily to the ability of the E2Fs to activate or inhibit transcription mediated through E2F response elements. However, this simple classiﬁcation does not reﬂect the pleiotropic nature of E2F activity. For example, E2Fs are involved in (a) the regulation of cell cycle traverse, (b) the...
regulation of apoptosis, (c) the regulation of differentiation, and (d) the sensing and response to DNA damage (2, 8, 12–16). The diverse nature of E2F action has significant implications for neoplasia because all the established functions of the E2F family are selectively targeted during neoplasia (17). Although the role of disrupted E2F activity in carcinogenesis may be obvious with regard to the regulation of proliferation and differentiation, other effects would seem paradoxical (e.g., E2F-induced apoptosis). For instance, disruption of the E2F-Rb pathway (e.g., p16 or Rb loss of function or cyclin D amplification), which controls passage through the G1 checkpoint of the cell cycle, is a common, and perhaps universal, defect in human cancers (11), which results in the overexpression/activation of E2Fs, such as E2F1 (2, 18). Because E2F1 is known to induce apoptosis, one would predict that transforming events that result in E2F1 overexpression should be self-defeating and cause E2F1-induced cell death (19). However, given the prevalence of cancer and the prevalence of E2F-Rb disruption in cancer, it is obvious that other events that inhibit apoptosis must occur during transformation. Hence, there must be a link(s) between E2F activity and the disruption of apoptosis in cancers. Indeed, it has been recently reported that the proapoptotic actions of E2F1 could be antagonized by the phosphatidylinositol 3-kinase/AKT pathway (20) and by E2F7 and E2F8 (21, 22).

An emerging theme in the E2F literature is the importance of the ratio of activating and suppressive E2Fs to the regulation of cellular homeostasis. In this regard, the role of E2F6, E2F7, and E2F8 has recently received considerable attention due to their antagonistic function with respect to E2F-induced proliferation and DNA damage responses (21–24).

We have previously shown that inhibition of E2F1, such as by an E2F dominant-negative construct, is able to induce growth arrest and sensitize cells to a differentiation stimulus but is not sufficient to induce differentiation (8). This suggested that inhibitors of endogenous E2F activity may exist that could initiate squamous differentiation in keratinocytes. Previously, we have excluded E2F6 as a possible candidate (15). However, a recent report from Gustavo Leone’s group showed that E2F7 could inhibit E2F2 activity and that E2F7 was preferentially expressed in skin (25). Considering the relative importance that E2F plays in regulating proliferation, differentiation, and apoptosis, we now examine the potential role that E2F7 may play in keratinocyte biology and how targeted dysregulation of E2F7 may contribute to keratinocyte transformation.

Materials and Methods

Keratinocyte isolation, stem cell enrichment, and keratinocyte culture. Human epidermal keratinocytes (HEK) were isolated from neonatal foreskins and cultured as described previously (18, 26). HEKs were cultured as proliferative keratinocytes or grown to confluence for 48 h to differentiate the cells. For transforming growth factor-β (TGF-β) experiments, subconfluent proliferative cultures of HEKs were treated with adjacent suprabasal keratinocytes were prepared by gentle trypsinization and stained with Cy5.5-Annexin V in binding buffer [0.01 mol/L HEPES (pH 7.4), 0.14 mol/L NaCl, 2.5 mmol/L CaCl2] for 15 min in the dark after gentle vortexing. For cell cycle analysis, propidium iodide was added to the cells (2 μg/mL final concentration) and cells were filtered through 60-μm mesh. In all instances, cell analysis was performed using the FACSx™ (BD Pharmingen) and E2F7b expression plasmids were a kind gift from Prof. Kristian Helin (European Institute of Oncology, Milano, Italy; refs. 35, 36). The CMV-Sp1 pocket protein-binding domain (2) was provided by Prof. Merlin Crossley (University of Sydney, Sydney, Australia). The E2F1 dominant-negative construct (E2F1d/n) codes for amino acids 116 to 235 of E2F1 and codes for the DNA-binding and heterodimerization domains but not the transactivation domain or pocket protein-binding domain (2). Plasmids coding for short hairpin RNA (shRNA) directed against E2F7 were purchased (SuperArray Bioscience Corp.) and transfected into HEKs and the human squamous cell carcinoma (SCC) cell line SCC25. In subsequent studies, the identification and enrichment for transfected cells was based on the concomitant expression of green fluorescent protein (GFP) by the transfected cells.

Confluent or proliferating HEKs were transfected in six-well plates and experiments were performed in triplicate following previously published protocols (2). Reporter activity was assayed 48 h after transfection using the CAT assay protocol (Roche) or the luciferase assay protocol, which has been described elsewhere (26, 38).

Fluorescence-activated cell sorting analysis and cell sorting. Fluorescence-activated cell sorting (FACS) analysis was used to determine (a) apoptotic status using Cy5.5-Annexin V positivity (BD Pharmingen) and (b) cell cycle status using propidium iodide staining (39). Briefly, following transfection and treatment, HEKs or SCC25 cells were trypsinized and stained with Cy5.5-Annexin V in binding buffer [0.01 mol/L HEPES (pH 7.4), 0.14 mol/L NaCl, 2.5 mmol/L CaCl2] for 15 min in the dark after gentle vortexing. For cell cycle analysis, propidium iodide was added to the cells (2 μg/mL final concentration) and cells were filtered through 60-μm mesh. In all instances, cell analysis was performed using the FACSx™ (BD Pharmingen) and E2F7b expression plasmids were a kind gift from Prof. Kristian Helin (European Institute of Oncology, Milano, Italy; refs. 35, 36). The CMV-Sp1 pocket protein-binding domain (2) was provided by Prof. Merlin Crossley (University of Sydney, Sydney, Australia). The E2F1 dominant-negative construct (E2F1d/n) codes for amino acids 116 to 235 of E2F1 and codes for the DNA-binding and heterodimerization domains but not the transactivation domain or pocket protein-binding domain (2). Plasmids coding for short hairpin RNA (shRNA) directed against E2F7 were purchased (SuperArray Bioscience Corp.) and transfected into HEKs and the human squamous cell carcinoma (SCC) cell line SCC25. In subsequent studies, the identification and enrichment for transfected cells was based on the concomitant expression of green fluorescent protein (GFP) by the transfected cells.

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RNA extraction and quantitative reverse transcription-PCR and microarray analysis. Detailed procedures for total RNA extraction and reverse transcription-PCR (RT-PCR) have been previously described (40). Oligonucleotide primers for determining E2F7a, E2F7b (35), and E2F1 (27) expression have been described elsewhere. The involucrin primers used for amplification generated a 102-bp fragment and were as follows: 5′-GCAAGAATGTGAGCAACAGC-3′ (forward) and 5′-GTCGGTGGTTTCTGCTTT-3′ (reverse). The cdc2 primers used for amplification generated a 141-bp fragment and were as follows: 5′-AGTGGCAAAACGCTTCAAAGAAA-3′ (forward) and 5′-GTGGGGTGCACCTGTTACTC-5′ (reverse). A standard curve of β-actin expression was used to normalize expression of E2F7. The 20 μL quantitative RT-PCR contained 10 μL of Platinum SYBR Green qPCR SuperMix UDQ (Invitrogen), 0.6 μL of 20× bovine serum albumin (1 mg/mL; Invitrogen), 10 μmol/L of forward primers, 10 μmol/L of reverse primer, and 2 μL of cellular single-stranded cDNA. The reaction was performed in aRotor-Gene 3000 real-time rotary analyzer (Corbett Life Science) following the LightCytc® cycling program recommended by the manufacturer for the Platinum SYBR Green qPCR SuperMix.

Institutional ethics approval has been granted for the consensual collection of human tissue from patients. Normal epidermal sheets were prepared, following dispase digestion, from neonatal foreskins and...
Results

E2F7b can inhibit keratinocyte proliferation and is selectively expressed in proliferation-competent human keratinocytes in vitro and in vivo. Two isoforms of the E2F7 gene have been described (35): the shorter form, E2F7a, encodes a 728–amino acid protein, whereas the longer, more abundant form, E2F7b, encodes a 911–amino acid protein, which includes all the coding region of E2F7a plus an additional 183 amino acids. To estimate isoform-specific mRNA expression levels, we used previously published primers (35) that amplify the longer E2F7b-specific product or the combined E2F7a + E2F7b product. Figure 1 shows that proliferating HEKs expressed high levels of the proliferation-specific gene cdc2 and low levels of the differentiation-specific gene involucrin, whereas confluent/differentiated HEKs expressed relatively low levels of cdc2 and high levels of involucrin. E2F7b mRNA was more highly expressed in proliferating keratinocytes than in confluent differentiated keratinocytes (Fig. 1A). In addition, the level of E2F7a + E2F7b mRNA expression was almost identical to that of E2F7b alone, indicating that E2F7b is the predominant transcript found in keratinocytes and its expression is reduced when keratinocytes differentiate (Fig. 1A).

The expression of E2F7b in proliferation-competent keratinocytes suggested that it may act to inhibit E2F-dependent proliferation. Hence, we examined whether the transient overexpression of E2F7b in proliferating HEKs could inhibit proliferation. We found that overexpression of E2F7b in proliferating human keratinocytes resulted in a 2.7-fold accumulation of cells in the G0-G1 phase of the cell cycle compared with keratinocytes transfected with the empty vector (G0-G1/G2-M = 10.2 in control keratinocytes and 27 in E2F7b-transfected keratinocytes). This is consistent with a role, for E2F7b, as an inhibitor of E2F-dependent cell cycle traverse (25, 35, 42) in keratinocytes. Based on this, we speculated that if E2F7b were a physiologically relevant inhibitor of keratinocyte proliferation, then we would predict that exposure to growth-inhibitory agents such as TGFβ1, which produce a reversible growth inhibition, while suppressing differentiation (43, 44), might induce E2F7b expression. Keratinocyte proliferation is modulated in vivo by the opposing actions of endogenous...
mitogens, such as TGFα, and endogenous growth inhibitors, such as TGFβ1 (45, 46). Hence, TGFβ1 is a physiologically relevant suppressor of keratinocyte proliferation. TGFβ1 (20 ng/mL for 48 hours) caused a profound inhibition of BrdUrd incorporation and cdc2 mRNA expression while causing an approximate 8-fold increase in E2F7b mRNA expression (Fig. 1B).

Given the association of E2F7b with growth inhibition in human keratinocytes, we examined whether E2F7a or E2F7b was preferentially expressed in KSCs, which we have shown previously to be predominantly quiescent (29). We examined (a) whether the E2F7 mRNA expression profile in vivo was restricted to proliferation-competent cells and (b) whether E2F7 mRNA isoform expression was selectively restricted to the KSC or the proliferative populations of HEK. A, the basal layer of the epidermis comprises TA keratinocytes and KSCs from which PMD keratinocytes of the suprabasal layers are derived. Cell fractions enriched for KSC, TA, and PMD cells were isolated from human epidermal tissue (Materials and Methods) based on their staining intensity for α6 (FITC-A) and CD71 (PE-A) as shown in the representative FACS plot. Sorting based on the gates depicted in A enriched for cells from which RNA was isolated and subjected to quantitative RT-PCR to assess (B) E2F7a + E2F7b or E2F7b mRNA expression or (C) mRNA expression of the proliferation-specific gene cdc2 or the differentiation-specific gene involucrin. All data in B and C are normalized against the TA fraction. Columns, mean of duplicate determinations from two experiments; bars, SE.

Figure 2. E2F7 is expressed equally in stem cell–enriched and TA-enriched populations of HEK. A, the basal layer of the epidermis comprises TA keratinocytes and KSCs from which PMD keratinocytes of the suprabasal layers are derived. Cell fractions enriched for KSC, TA, and PMD cells were isolated from human epidermal tissue (Materials and Methods) based on their staining intensity for α6 (FITC-A) and CD71 (PE-A) as shown in the representative FACS plot. Sorting based on the gates depicted in A enriched for cells from which RNA was isolated and subjected to quantitative RT-PCR to assess (B) E2F7a + E2F7b or E2F7b mRNA expression or (C) mRNA expression of the proliferation-specific gene cdc2 or the differentiation-specific gene involucrin. All data in B and C are normalized against the TA fraction. Columns, mean of duplicate determinations from two experiments; bars, SE.

Figure 3. E2F7 can initiate squamous differentiation via E2F1-dependent and E2F1-independent pathways. A, proliferating HEKs were cotransfected with the proliferation-specific cdc2-CAT reporter gene and β-actin-Luc (internal standard) reporter gene with either E2F1, E2F7a, E2F7b, E2FΔd, or pcDNA3 control expression plasmid. B, proliferating HEKs were cotransfected with the differentiation-specific TG1-Luc reporter gene and β-actin-Luc (internal standard) reporter gene with either Sp1, E2F7a, E2F7b, or pcDNA3 control expression plasmid. C, confluent differentiated HEKs were cotransfected with either the TG1-Luc reporter gene or the K10-Luc reporter gene and β-actin-Luc (internal standard) reporter gene with either pcDNA3 control, E2F1, E2F7a, E2F7b, or combinations of these expression plasmids. In all instances, cells were left for 48 h after transfection before the reporter activities were measured. Data are normalized against the pcDNA3 control. Columns, mean of triplicate determinations from three experiments; bars, SE; *, P < 0.05, compared with pcDNA3 control.
E2F7 antagonizes E2F1-induced apoptosis in keratinocytes. Proliferating keratinocytes were cotransfected with (A) differing amounts of an E2F1 expression plasmid or (B) 1 μg of an E2F1 expression plasmid with differing amounts of an E2F7b expression plasmid plus a GFP expression plasmid. After 48 h, cells were assayed for Annexin V positivity in the GFP+ cell fraction by FACS. Columns, mean of triplicate determinations from two independent experiments; bars, SE. Data in A presented as the percentage of cells that were Annexin V positive. Data in B presented as the percentage of cells that were Annexin V positive in the control that only received 1 μg of an E2F1 expression plasmid. * P < 0.05, compared with 0 μg control.

Figure 4. E2F7 antagonizes E2F1-induced apoptosis in keratinocytes. Proliferating TA cells were cotransfected with (A) differing amounts of an E2F1 expression plasmid or (B) 1 μg of an E2F1 expression plasmid with differing amounts of an E2F7b expression plasmid plus a GFP expression plasmid. After 48 h, cells were assayed for Annexin V positivity in the GFP+ cell fraction by FACS. Columns, mean of triplicate determinations from two independent experiments; bars, SE. Data in A presented as the percentage of cells that were Annexin V positive. Data in B presented as the percentage of cells that were Annexin V positive in the control that only received 1 μg of an E2F1 expression plasmid. * P < 0.05, compared with 0 μg control.

The increase in involucrin in the PMD cells is consistent with the differentiation-specific marker involucrin were higher in the PMD keratinocytes than in proliferating TA cells and quiescent PMD cells (data not shown). As expected, the mRNA levels for the proliferation-specific gene cdc2 was significantly higher in the quiescent KSCs compared with the proliferative TA cells. This suggests that the E2F7 mRNA levels may remain relatively stable during the KSC-TA transition, whereas pro-proliferative genes may be activated when KSCs progress to TA status.

E2F7a and E2F7b can sensitize keratinocytes to a differentiation stimulus. An essential prerequisite for the initiation of squamous differentiation is the inhibition of proliferation-associated E2F activity (2, 8, 47). To investigate whether E2F7 could function as an inhibitor of E2F activity in keratinocytes, proliferating HEKs were transfected with the cdc2-CAT reporter. This gene is a robust marker of proliferation and its activity is E2F dependent (18, 26, 43, 44). E2F-binding sites in the cdc2 promoter are critical for the optimal repression of its activity in cycling cells (26, 31). The cells were cotransfected with expression plasmids for E2F1, E2F7a, E2F7b, or E2F1d/n (2), alone or in combination. As expected, the activity of the cdc2 promoter was induced by the overexpression of E2F1, but not by the overexpression of E2F1d/n, E2F7a, or E2F7b (Fig. 3A). Coexpression of E2F1d/n, E2F7a, or E2F7b with E2F1 led to inhibition of E2F1-mediated induction of cdc2 promoter activity. These results indicate that all the expression plasmids are functional and that the E2F7 isoforms as well as E2F1d/n are potent suppressors of E2F activity in proliferating keratinocytes.

The inhibition of E2F activity is a prerequisite for the initiation of differentiation (2, 8, 47) and sensitizes keratinocytes to differentiation stimuli (8, 16). Because E2F7 is preferentially expressed in proliferation-competent keratinocytes and is an E2F antagonist, we investigated whether E2F7 could initiate squamous differentiation. Transient overexpression of E2F7a or E2F7b alone was able to induce the expression of TG1-Luc ~2-fold (Fig. 3B). We had previously shown that transient overexpression of the transcription factor Sp1 in keratinocytes was able to stimulate differentiation in E2F-inhibited keratinocytes (15). Therefore, we examined the effect of overexpressing a combination of an E2F inhibitor (E2F7a or E2F7b) and a differentiation stimulator (Sp1) on the activity of the differentiation-specific reporter TG1-Luc (Fig. 3B). We found that the simultaneous overexpression of E2F7a or E2F7b with Sp1 was able to induce TG1-Luc ~3.5-fold over the control and more than any of the constructs alone. These data show that E2F7a and E2F7b have the capacity to initiate differentiation and sensitize HEKs to differentiation stimuli.

We have previously shown that E2F2 to E2F5 are all able to suppress the expression of differentiation markers in keratinocytes (8). We now examined whether the E2F7 isoforms could antagonize the differentiation-suppressive effects of E2F. Using E2F1-mediated suppression as a reference in confluent HEKs, overexpression of E2F7a, E2F7b, or E2F1d/n significantly superinduced the activity of the differentiation-specific reporters TG1-Luc and K10-Luc (Fig. 3C). This contrasted to the suppression of these promoters by E2F1 (Fig. 3A). However, expression of E2F7a or E2F7b together with E2F1 failed to antagonize the E2F1-mediated suppression of differentiation marker activity. Combined, these results indicate that the E2F7 isoforms have the ability to suppress proliferation-associated E2F activity and initiate squamous differentiation.
However, these two important events are mediated via E2F1-dependent and E2F1-independent pathways, respectively.

**E2F7b antagonizes E2F1-induced apoptosis in keratinocytes.** E2F1 is a potent inducer of apoptosis in a variety of cell types, including keratinocytes (48), and recent reports have shown that E2F6, E2F7, and E2F8 are capable of inhibiting E2F1-induced apoptosis in response to DNA damage (22–24). We therefore examined whether E2F7b was able to inhibit E2F1-induced keratinocyte apoptosis. We found that E2F1 induced a dose-dependent increase in keratinocyte apoptosis with ~35% of cells undergoing apoptosis in response to the transfection of 1 μg of E2F1 expression plasmid (Fig. 4 A). In contrast, the apoptosis induced by transfection of 1 μg E2F1 plasmid could be inhibited by cotransfection with E2F7b expression plasmid in a dose-dependent manner (Fig. 4B). These data show that E2F7b is able to antagonize E2F1-induced apoptosis in human keratinocytes. Thus, E2F7 seems to be able to antagonize E2F-dependent proliferation and apoptosis but not compete with E2F1-induced suppression of squamous differentiation.

**E2F1 and E2F7b are overexpressed in human CSCCs.** Because E2F1 is overexpressed in CSCCs (2) and E2F7b is able to antagonize E2F1-induced proliferation and apoptosis, we examined the expression patterns for both E2F1 and E2F7 in human CSCCs. Using a suite of five human CSCCs in situ, we found that E2F1 and E2F7 were overexpressed in all the SCC samples (Fig. 5). E2F7 was overexpressed >100-fold in four of five tumors compared with epidermis. To minimize the contribution of nonepithelial tissue components (e.g., stromal and inflammatory cells), we analyzed the epithelial-enriched component of the lesion obtained after dispase digestion of the tumor samples. The overexpression of E2F1 and E2F7 was not present in another primary human tumor type (i.e., osteosarcoma; Fig. 5). Hence, the elevation of E2F1 and E2F7 transcript levels is not common to all tumor types.

Because E2F7 is able to antagonize E2F1-induced apoptosis, we examined whether the inhibition of E2F7 in SCC cells would enhance their sensitivity to UV-induced DNA damage or doxorubicin-induced cytotoxicity (Fig. 6). Four different constructs of E2F7 shRNA (D1-D4) were tested for their ability to reduce E2F7 mRNA levels in SCC25 cells (Fig. 6A). The D1 construct inhibited E2F7 expression to ~10% that of the control (Fig. 6A). Transient overexpression of the D1 construct in SCC25 cells increased their sensitivity to UV-induced cytotoxicity and doxorubicin-induced cytotoxicity (Fig. 6B and C). These data suggest that E2F7 overexpression could reduce the ability of keratinocytes to respond to the major cutaneous carcinogen (i.e., UV radiation). Moreover, these data suggest that E2F7 inhibition can enhance the cytotoxic response to a chemotherapeutic agent in SCC cells.

**Discussion**

The E2F family of transcription factors is an important regulator of keratinocyte growth, survival, and differentiation. The biological activities of the E2F family can be broadly classified as pro-proliferative (E2F1-E2F3A), proliferation suppressive (E2F3B and E2F4-E2F8), differentiation modulating (E2F1-E2F8), or apoptotic modulating (E2F1 and E2F6-E2F8; refs. 8, 10, 11, 21, 22). Control of proliferation, differentiation, and survival are interdependent events and our data suggest that this interdependence results, in part, from the opposing actions of activating (e.g., E2F1) and inhibitory (e.g., E2F7) E2Fs. The inhibition of E2F activity is an obligate requirement for the initiation of squamous differentiation (8). We had previously speculated that an endogenous E2F inhibitor may exist to suppress E2F-dependent proliferation and initiate squamous differentiation (15) and the present study suggests that E2F7 may fulfill such a role. For instance, E2F7 (a) is expressed preferentially in proliferation-competent keratinocytes, (b) inhibits proliferation and apoptosis, and (c) is able to initiate differentiation, and E2F7 mRNA expression is induced in response to a physiologically relevant growth inhibitor, such as TGFβ1. Combined, these data indicate that E2F7 can regulate proliferation and survival in proliferation-competent keratinocytes (i.e., keratinocytes of the basal layer of the epithelium). Such a model predicts that proliferation and survival of proliferation-competent basal keratinocytes (comprising KSCs and TA cells) may be dictated by the opposing actions of activating (e.g., E2F1) and inhibitory (e.g., E2F7) E2Fs. Interestingly, our data suggest that the quiescent nature of KSCs is not due to the overexpression of E2F7 but may be due to reduced expression of pro-proliferative cell cycle genes, such as cdc2.

The present study indicates that E2F7 has the capacity to initiate squamous differentiation and that this activity is mediated by unknown domains in the E2F7 molecule. This is most clearly shown by the observation that E2F7 could inhibit E2F1-induced...
activation of the cdc2 promoter but could not antagonize E2F1-mediated suppression of the differentiation-specific transglutaminase promoter. Moreover, E2F6 and E2F7 can both inhibit E2F1-dependent keratinocyte proliferation (ref. 15 and this study) and apoptosis (21–24); yet, E2F6 is unable to sensitize keratinocytes to subsequent differentiation stimuli, whereas E2F7 can. These data indicate that E2F7 mediates its effects in keratinocytes by two independent, yet complementary, pathways. The first is E2F1 dependent and regulates proliferation and survival, whereas the second controls the initiation of differentiation and is E2F1 independent. The presence of an E2F1-independent pathway suggests that E2F7-specific transcriptional gene targets exist that are involved in E2F7-mediated initiation of squamous differentiation. Significantly, these data also suggest that the control of keratinocyte proliferation, differentiation, and survival is governed by the combined effects of E2F7 operating through E2F1-dependent and E2F1-independent pathways.

The control of apoptosis, by E2Fs, is predominantly restricted to the proapoptotic actions of E2F1 (reviewed in ref. 10) and the antiapoptotic actions of E2F6 to E2F8 (21–24). Thus, unlike proliferation, which is controlled by E2F1 to E2F8, apoptosis regulation is restricted to a subset of E2Fs. For this reason, the control of apoptosis is likely to be more sensitive to changes in the E2F1/E2F7 ratio than would proliferation. This raises the issue of how transient spikes in E2F activity that are a requisite for passage through S phase of the cell cycle are prevented from inducing apoptosis. Our study would suggest that the transient activation of E2F that occurs during S phase may not induce apoptosis due to the antiapoptotic actions of E2F7. This is supported by data showing (a) that E2F7 is induced in the latter half of S phase (25, 35), (b) that E2F1 can induce E2F7 transcription/ expression, and (c) that E2F7 can suppress E2F1-dependent apoptosis (21, 22). These data indicate that an auto-feedback loop exists in which E2F1 and E2F7 are mutually antagonistic and control the expression and activities of each other (Supplementary Fig. S1). Based on the above evidence, we now propose that E2F7 plays a dual role in proliferating keratinocytes. Firstly, it acts as an antagonist to E2F-dependent proliferation. Secondly, it acts as an antagonist to E2F1-dependent apoptosis (i.e., E2F7 may be a survival factor), thus preserving the viability of the dividing cell. In such a model, the E2F1/E2F7 axis could play a similar role to that of the p53 tumor suppressor gene because the relative balance of E2F1 to E2F7 can regulate proliferation rates as well as responses to DNA damage. In this model, the major functions of E2F7 would be in proliferation-competent basal cells in which proliferation and apoptosis need to be constrained. On differentiation, the keratinocytes would lose the E2F7-suppressive effects on apoptosis. Because apoptosis is the final step in the formation of the cross-linked envelope, the loss of E2F7 in differentiated keratinocytes would remove a barrier to terminal differentiation-induced apoptosis.

The antiapoptotic action of E2F7 coupled with the ability of E2F1 to induce E2F7 expression may explain why E2F1 has been reported to protect against UV-induced apoptosis and carcinogenesis in mouse skin (13, 14). Although these reports would seem to contradict the prevailing concept that E2F1 is proapoptotic (10), our data may provide an explanation that accommodates the reported proapoptotic and antiapoptotic actions of E2F1 in keratinocytes. Berton and colleagues (14) and Wikonkal and colleagues (13) have recently shown that overexpressing E2F1 or ablating E2F1 expression in the context of genetically modified mouse skin inhibits or enhances UV-induced apoptosis and carcinogenesis, respectively (13, 14). We and others have shown (a) that E2F7 can suppress E2F1-induced apoptosis, (b) that E2F1 can induce E2F7 expression (25, 35), (c) that E2F1 and E2F7 are highly overexpressed in SCC (present study and ref. 2), (d) that E2F7 protects against genotoxicity (e.g., UV), and (e) that E2F7 depletion enhances sensitivity to cytotoxic/genotoxic stimuli (present study and refs. 21, 22). Hence, our interpretation would be that the antiapoptotic effects of E2F1, when overexpressed in mouse skin, and its ability to protect against genotoxic stimuli, such as UV radiation, may be due to E2F1-dependent induction of E2F7 (or E2F8) rather than a direct effect of E2F1.

**Figure 6.** E2F7 inhibition sensitizes SCC cells to UV-induced apoptosis and doxorubicin-induced apoptosis. A, proliferating SCC25 cells were cotransfected with expression constructs coding for a control shRNA and shRNAs directed against E2F7 (D1-D4) + a GFP expression plasmid. After 48 h, GFP+ve cells were enriched by MoFlo and E2F7 mRNA was measured by quantitative PCR. B, mean of triplicate determinations from two experiments; bars, SE. Columns, mean of triplicate determinations from two experiments; bars, SE. Proliferating SCC25 cells were cotransfected with the D1 shRNA construct + a GFP expression plasmid. After 48 h, GFP+ve cells were enriched by MoFlo and the cells were plated. After 24 h, cells were exposed to varying doses of UV radiation and then left for 24 h or (C) treated with varying doses of doxorubicin for 48 h. Cell viability was then determined. Points, mean of triplicate determinations from two independent experiments; bars, SE. Solid line, line of best fit.
The prosurvival actions of E2F7 observed in this and other studies (21, 22) suggest that, under certain circumstances, E2F7 may be oncogenic. These circumstances may arise in CSCCs where E2F7 and E2F1 are overexpressed due to the dysregulation of the E2F-Rb axis. Disruption of the Rb-E2F axis is common in SCC and is associated with deregulated E2F activity, hyperproliferation (2, 18, 49), and an induction of E2F7 expression. This results in the establishment of a new and highly elevated steady-state level of E2F1 and E2F7 (Supplementary Fig. S1), resulting in insensitivity to the relatively small fluctuations in E2F activity that would normally discriminate between growth or apoptotic responses. Given that apoptosis is also sensitive to changes in the E2F1/E2F7 ratio, we would predict that the overexpression of E2F1 and E2F7 in CSCC would be associated with an elevation in the basal level of apoptosis but that the ratio of proliferation to apoptosis would remain relatively similar. This is supported by earlier published data (49, 50). However, the consequence of having very high basal levels of E2F1 would be that the loss of E2F7 would leave the cells vulnerable to E2F1-mediated apoptosis. This is supported by our observation that inhibition of E2F7 expression in SCC cells makes the cells sensitive to the apoptotic effects of the common skin carcinogen, UV. These data imply that the overexpression of E2F7 observed in the patient SCCCs would render them resistant to the apoptotic effects of UV radiation and hence more susceptible to acquiring UV-induced mutations.

The above model predicts that the overexpression of E2F7 in SCC contributes to tumorigenesis and that E2F7 may be an attractive “druggable” target. Targeting E2F activity as a potential therapeutic strategy is not without precedent. For example, a decoy E2F response element has undergone clinical trial for the treatment of restenosis injury in vascular grafts (51). In addition, it has also been proposed that E2F inhibition could be used as the basis for a differentiation therapy for SCC (8, 16). Of relevance to the present report is the proposition that E2F1-induced cytotoxicity could form the basis for an anticancer strategy (52). Although it may seem counterintuitive to activate an oncogene (e.g., E2F1) in a patient as an anticancer strategy, we suggest that a more effective strategy may be to combine an E2F inhibitor with a traditional chemotherapeutic (present study and refs. 25, 35). In this strategy, the inhibition of E2F7 would remove a survival signal and would sensitize cells to E2F1-dependent and E2F1-independent cytotoxic stimuli.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 7/16/2008; revised 11/27/2008; accepted 12/18/2008; published OnlineFirst 02/17/2009.

Grant support: Australian National Health and Medical Research Council (#453929) and Queensland Cancer Fund (#014512). N. Saunders is supported by a Principal Research Fellowship awarded by the Garnett Passe and Rodney Williams Memorial Foundation. P. Kaur is supported by Senior Research Fellowship from the Australian National Health and Medical Research Council.

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We thank all those individuals who donated their tissue for these studies.

References


www.aacrjournals.org 1807 Cancer Res 2009; 69: (5). March 1, 2009

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Correction: Article on E2F7 in SCC

In the article on E2F7 in SCC in the March 1, 2009 issue of Cancer Research (1), data from murine keratinocytes was inadvertently shown instead of human epidermal keratinocytes. The corrected Fig. 2A appears below. The legend for Fig. 2A should read as follows: "A, the basal layer of the epidermis comprises TA keratinocytes and KSCs from which PMD keratinocytes of the suprabasal layers are derived. Cell fractions enriched for KSC, TA, and PMD cells were isolated from human epidermal tissue (Materials and Methods) based on their staining intensity for α6 (FITC-A) and CD71 (PE-A). Enriched fractions were reanalyzed and a representative merged image demonstrating the purity of the fractions is shown." The conclusions and interpretation of the data in the article are unaltered by this correction.


Published OnlineFirst 8/25/09.
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doi:10.1158/0008-5472.CAN-09-17-COR2

Figure 2.
E2F7 Can Regulate Proliferation, Differentiation, and Apoptotic Responses in Human Keratinocytes: Implications for Cutaneous Squamous Cell Carcinoma Formation

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