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E2F Modulates Keratinocyte Squamous Differentiation

IMPLICATIONS FOR E2F INHIBITION IN SQUAMOUS CELL CARCINOMA*

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E2F regulation is essential for normal cell cycle progression. Therefore, it is not surprising that squamous cell carcinoma cell lines (SCC) overexpress E2F1 and exhibit deregulated E2F activity when compared with normal keratinocytes. Indeed, deliberate E2F1 deregulation has been shown to induce hyperplasia and skin tumor formation. In this study, we report on a dual role for E2F as a mediator of keratinocyte proliferation and modulator of squamous differentiation. Overexpression of E2F isoforms in confluent primary keratinocyte cultures resulted in suppression of differentiation-associated markers. Moreover, we found that the DNA binding domain and the *trans*-activation domain of E2F1 are important in mediating suppression of differentiation. Use of a dominant/negative form of E2F1 (E2F d/n) found that E2F inhibition alone is sufficient to suppress the activity of proliferation-associated markers but is not capable of inducing differentiation markers. However, if the E2F d/n is expressed in differentiated keratinocytes, differentiation marker activity is further induced, suggesting that E2F may act as a modulator of squamous differentiation. We therefore examined the effects of E2F d/n in a differentiation-insensitive SCC cell line. We found that treatment with the differentiating agent, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), or expression of E2F d/n alone had no effect on differentiation markers. However, a combination of E2F d/n + TPA induced the expression of differentiation markers. Combined, these data indicate that E2F may play a key role in keratinocyte differentiation. These data also illustrate the unique potential of anti-E2F therapies in arresting proliferation and inducing differentiation of SCCs.

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The major function of the skin is to act as a barrier between the internal and external environment. The skin is divided into two layers, the dermis and the epidermis, of which the major cell type in the epidermis is the keratinocyte. During the strictly regulated process of differentiation, keratinocytes undergo morphological and biochemical changes, resulting in dead, enucleated, flat cells that are eventually sloughed from the skin surface. This process of differentiation is initiated by the irreversible growth arrest and suppression of proliferation-specific genes such as p53 (1), E2F1 (2, 3), cdk1 (4), and keratin 14 (5) in proliferative basal cells. Concomitant with the suppression of proliferation-specific genes, there is a corresponding induction of differentiation-specific genes, such as keratin 10 (6), cornifin (7), and transglutaminase type 1 (2). This process of growth suppression and induction of terminal differentiation is predominantly regulated at the transcriptional level by several transcription factor families such as AP1, Sp1, AP2, and E2F (8–10) and disruption of this process frequently accompanies the onset of neoplasia.

E2F was first identified as a nuclear factor capable of binding to the adenovirus E2 promoter (11). To date, six members of the E2F transcription factor family have been cloned, E2Fs 1–6 (12–24). E2F exists as a heterodimeric complex in association with a dimeric partner protein, DP1 or DP2 (25–27). This "free" E2F complex acts as a potent *trans*-activator of E2F-responsive genes. However, the activity of E2F is subject to regulation through inhibitory interactions with hypophosphorylated forms of the pocket proteins, pRb, p107, and p130 (13–18, 21, 28). Specifically, E2Fs 1–3 preferentially bind to pRb, while E2Fs 4–5 bind p107 and E2F5 binds p130. This direct association of E2F isoforms with their cognate pocket protein partner acts to repress E2F-mediated transcriptional activity. In some instances, this repression requires further interactions with specific histone deacetylases (29, 30). The presence of these various E2F-pocket protein complexes act to regulate passage through various phases of the cell cycle. In particular, certain complexes are associated with a specific phase of the cell cycle: the E2F5·p130 complex associates with G_0 (31), E2F1–3·pRb with G_1 (32) and E2F4·p107 with G_0/G_1 phase (18). Thus, the coordinated activation/inactivation of these complexes illustrate that cell cycle progression is controlled by complex transcriptional means. Despite clear evidence implicating E2F involvement in cell cycle regulation, there is also compelling evidence for other roles of E2F. For instance, E2Fs 1–3 have been implicated in the initiation of apoptosis (33–36). More recently, E2Fs have also been demonstrated to play a role in the regulation of myocyte, megakaryocyte, and adipocyte differentiation (37–39).

We have previously reported that the induction of keratino-

cyte growth arrest, in response to growth inhibitors, is associated with decreased E2F1 mRNA expression (2, 3) and decreased E2F DNA binding activity (3). In contrast, squamous cell carcinoma (SCC)[1] cell lines showed no alterations in E2F DNA binding activity nor E2F1 mRNA expression in response to these same growth inhibitors (3). Given the role of E2F in proliferation, we proposed that the deregulation of E2F activity may contribute causally to the deregulated growth observed in SCC cell lines (3). Indeed, several lines of evidence support this contention. For example, deliberate deregulation of E2F activity *in vitro* using HPV 16 E7 protein resulted in growth inhibitor-insensitivity in normal keratinocytes (3). These data are further strengthened by more recent reports that E2F1 mRNA overexpression occurs in primary SCCs compared with normal epidermis (10). Furthermore, deliberate deregulation of E2F1 expression in the skin of transgenic mice induces hyperplasia and cooperates with *ras* in skin tumor development (40). Combined, these data suggested that the deregulation of E2F1 could contribute to skin cancer formation. While the overexpression and activation of E2F in human SCCs may contribute to their deregulated proliferation, we became interested in whether this overexpression would have other implications on the squamous differentiation program. To this end, we found some evidence that E2F1 may have a dual role as both a mediator of keratinocyte proliferation and a suppressor of squamous differentiation (10). If this were true, it would suggest that deregulated E2F in SCCs may serve to deregulate proliferation and repress terminal differentiation. Therefore, in the present study, we examined the possibility that E2F may act as a biologically relevant modulator of squamous differentiation and that E2F inhibition may provide the basis of a "differentiation therapy" for SCCs.

EXPERIMENTAL PROCEDURES

Cell Culture—Human epidermal keratinocytes (HEKs) were isolated and cultured from neonatal foreskins as described (3). The SCC cell line, KJD-1/SV40, were grown in culture as previously reported (10). Growth arrest and differentiation was induced by maintaining confluent cells in culture over 48 h or by treatment with the protein kinase C (PKC) activator, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, 50 ng/ml for 16–48 h). Both these treatment regimes induce robust and reproducible differentiation in HEKs but not in SCC cell lines (10, 41–43).

Transfection of Cells and Reporter Assays—The human *cdc2* promoter construct driving expression of a CAT reporter gene (cdc2-CAT) has been previously described (4). The 2.9-kb transglutaminase type 1 promoter linked to a firefly luciferase gene (TG-1 Luc), keratin 10 reporter (K10-Luc), β-actin-CAT reporter gene and β-actin-luciferase reporter gene have also been previously described (4, 6, 10, 41, 45). β-actin-CAT or β-actin-Luc reporters were used to normalize for transfection efficiency. The CMV-E2F1 construct was a kind gift from Dr. Kristian Helin (15). The CMV-E2F 2–5 constructs were generous gifts from Dr. David Livingstone (46). The mutant E2F1 constructs, Δ132E2F1 and Δ409E2F1, were kindly provided by Dr. Joseph Nevins (47). The E2F1 dominant-negative (E2Fd/n) construct codes for amino acids 116–235, spanning for the DNA binding domain and heterodimerization domain (10).

Transient transfections of cells were performed in a 10-cm^2 well, when cells were either 50 or 100% confluent. Reporter activity was assayed 48 h post-transfection. Transfection protocols for cultured HEKs using LipofectAMINE (Invitrogen, Australia) and KJD-1/SV40 cells using Effectene (Qiagen) have been described previously (10). Transfections were performed in triplicate and repeated at least three times.

Chloramphenicol acetyltransferase assays (CAT) were performed using a CAT ELISA kit (Roche Applied Science) as per manufacturer's instructions. The luciferase assay protocol has been previously reported (4, 41).

Protein Isolation and Western Blotting—Protein isolation and Western blotting protocols have been previously described (48). All antibodies were purchased from Santa Cruz Biotechnologies. Dilutions of rabbit polyclonal antibodies were as follows: E2F1 (sc-193) 1:200, E2F2 (sc-632) 1:1000, E2F3 (sc-878) 1:200, E2F4 (sc-866) 1:1000, and E2F5 (sc-999) 1:1000. All immunodetection was visualized after reaction with 1:3000 horseradish peroxidase-conjugated goat anti-rabbit and incubation with ECL reagent (48).

RNA Isolation and Detection by RT-PCR—KJD-1/SV40 cells were transfected as described above, but with the addition of a green fluorescent protein (GFP) expression plasmid (1:3, GFP:E2Fd/n). Forty-eight hours post-transfection, cells were harvested and the GFP-positive cells enriched by fluorescent analysis cell sorting (FACS; Ref. 49). Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) have been described (49, 50). Oligonucleotides for estimating the expression of TG-1 are as follows: 5′-TG-1 GCCGAGAGCACCACACAGACG, 3′-TG-1 CGTAGTAAATTCTCCCAGACTC; 5′-actin GAAATCGTGCGTGACATTAAG, 3′-actin CTAGAAGCATTTGCGGTGGACGATGGAGGG GCC. All amplifications were performed under linear conditions with respect to cycle number (50).

RESULTS

E2Fs 1–5 Can Suppress the Activity of Differentiation-specific Markers in Normal Human Keratinocytes—In order to determine whether the overexpression of E2F1 observed in SCCs (10) could affect the ability of the cells to undergo squamous differentiation, we examined the effects of E2F overexpression in normal keratinocytes.

Keratinocyte differentiation is characterized by irreversible growth arrest, the suppression of proliferation-specific markers (3, 51) and the induction of differentiation-specific markers (*e.g.* TG-1 Luc or K10-Luc). The implications of E2F1 overexpression in normal keratinocytes were therefore examined by measuring differentiation-specific marker activity in cells induced to differentiate by two independent pathways (confluence or PKC activation, Refs. 41, 52, and 53). Our data demonstrate that cultured HEKs have increased TG-1 Luc and K10-Luc activity when induced to differentiate either by prior treatment of cells with TPA or by growth to confluence for 48 h (Fig. 1). Transfection of E2F1 into these differentiated cells was accompanied by significant reduction in activity of these differentiation-specific markers. This observation indicates that E2F1 can suppress differentiation-specific reporters in keratinocytes induced to differentiate by two different stimuli.

In addition, co-transfection of E2Fs 1–5 and the TG-1 Luc reporter into differentiated keratinocytes showed that E2Fs 1–5 were all able to significantly suppress TG-1 Luc activity in confluent/differentiated HEKs (Fig. 2). This indicates that the ability to suppress differentiation markers is shared by E2Fs 1–5. Since E2Fs 4–5 do not contain a cyclin binding domain (18, 21) and are unable to induce apoptosis (33–36, 54–57), these data also suggest that the phenomenon observed is cell cycle phase-independent, cyclin binding domain-independent, and apoptosis-independent.

The DNA Binding Domain and trans-Activation Domain of E2F1 Is Essential for Suppression of Differentiation-specific Marker Activity—To examine the domain requirement of E2F1 to suppress squamous differentiation, we employed two E2F1 mutants (Fig. 3). The Δ132E2F1 mutant contains a point mutation in the DNA binding domain, which abolishes its activity (47). Similarly, the Δ409E2F1 mutant possesses a frameshift mutation that eliminates both the *trans*-activation domain and pocket protein binding domain of E2F1 (47). Both the DNA binding domain mutant (Δ132E2F1) and *trans*-activation domain mutant (Δ409E2F1) are unable to induce the proliferation-specific and E2F-responsive cdc2-CAT reporter (Fig. 3*A*). Furthermore, by measuring *TG-1* promoter activity, we show that both mutants are unable to suppress TG-1 Luc activity in

[1] The abbreviations used are: SCC, squamous cell carcinoma cell lines; HEK, human epidermal keratinocytes; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; ELISA, enzyme-linked immunosorbent assay.

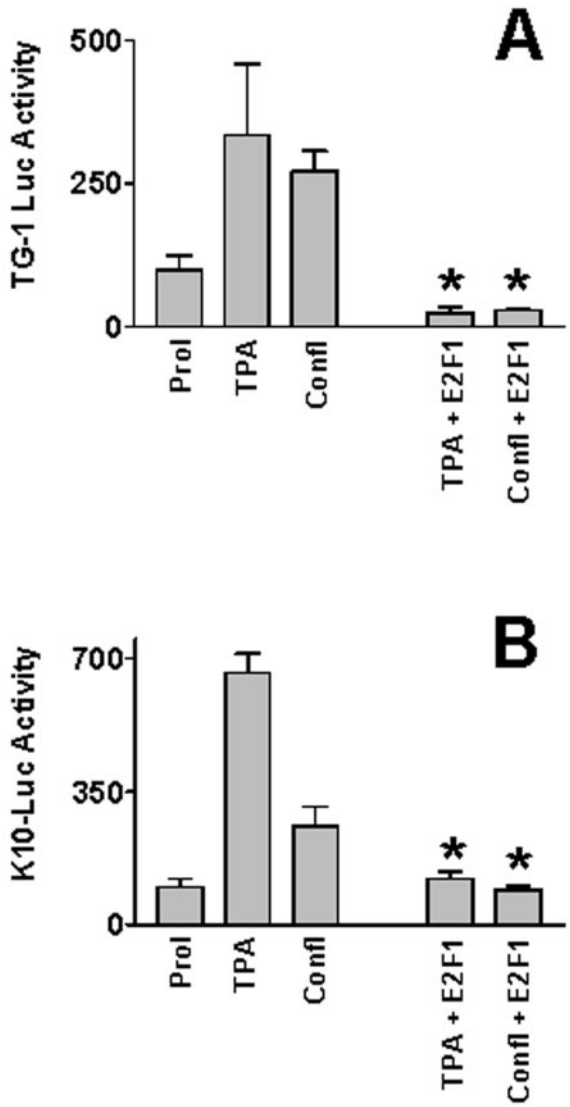


FIG. 1. **E2F1 suppresses differentiation markers in primary human keratinocyte cultures.** Human epidermal keratinocytes were transfected with a luciferase reporter linked to the transglutaminase-1 promoter (TG-1 Luc) (*A*) or keratin-10 promoter (K10 Luc) (*B*). Each construct was co-transfected with a β-actin-CAT reporter, to correct for transfection efficiency, as well as either an E2F1 expression plasmid or GFP expression plasmid. Cells were transfected when proliferating (*Prol*) or differentiated (*Conf*, TPA). Cells were induced to differentiate by growth to confluence or treatment of cells with medium supplemented with 50 ng/ml of TPA for 16 h prior to transfection. Data presented as mean ± S.E. of triplicate determinations of at least three experiments (*, $p < 0.05$ compared with *Prol*).

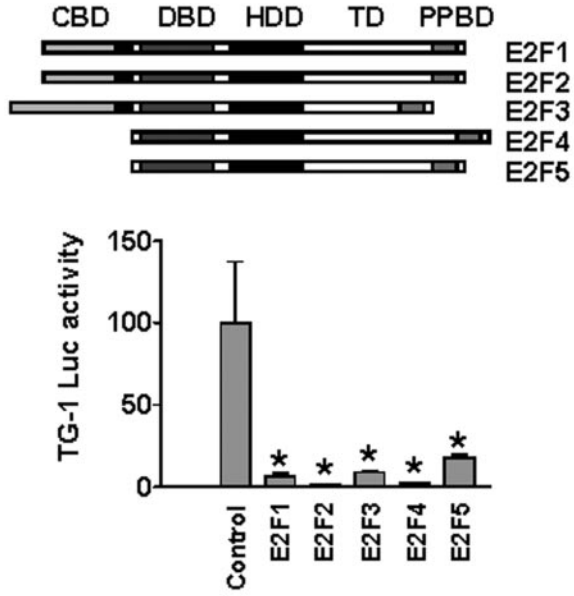


FIG. 2. **E2Fs 1–5 suppress TG-1 Luc in confluent cultures of primary keratinocytes.** Confluent cultures of human epidermal keratinocytes were transfected with a luciferase reporter linked to the transglutaminase-1 promoter (TG-1 Luc). Each construct was co-transfected with a β-actin-CAT reporter, to correct for transfection efficiency, as well as E2F expression plasmids (E2F1, E2F2, E2F3, E2F4, or E2F5) or GFP as control. Data presented as mean ± S.E. of triplicate determinations of at least three experiments and are normalized such that the value of the control is 100 (*, $p < 0.05$ compared with control). *CBD*, cyclin binding domain; *DBD*, DNA binding domain; *HDD*, heterodimerization domain; *TD*, *trans*-activation domain, and *PPBD*, pocket protein binding domain.

differentiated keratinocytes (Fig. 3*B*). These data demonstrate that both the DNA binding domain of E2F1 and the *trans*-activation domain of E2F1 are important for the suppression of differentiation-specific markers in HEKs. These data also suggest that suppression of differentiation is unlikely to be mediated by "squelching." It is interesting to note that E2Fd/n was able to superinduce/derepress TG-1 Luc activity to levels above that of differentiated HEKs. Thus, E2F1 overexpression suppresses TG-1 Luc and E2F inhibition induces TG-1 Luc. These data are consistent with a role for E2F as a modulator of squamous differentiation.

E2F Is Required For but Not Sufficient to Induce Squamous Differentiation—Given that the E2Fd/n could further induce TG-1 Luc activity in confluent cells, we examined the possibility that in proliferative cells the induction of differentiation was actively suppressed by E2F. If this were true, then inhibition of E2F in proliferative cells should suppress proliferation markers and induce differentiation markers. Indeed, inhibition of E2F causes suppression of the proliferation-specific marker activity, cdc2-CAT (Fig. 4*A*). However, inhibition of E2F is not sufficient to induce TG-1 Luc activity (Fig. 4*B*). Paradoxically, inhibition of E2F in confluent/differentiated HEKs superinduces/derepresses both TG-1 Luc and K10-Luc activity (Fig. 4*C*). These data indicate that (i) E2F suppression is not sufficient to induce differentiation, (ii) E2F inhibits the initiation of squamous differentiation (Fig. 1), and (iii) E2F inhibition is able to derepress/superinduce differentiation markers in differentiated HEKs. For these reasons, we believe E2F may be acting as a modulator of the differentiation phenotype.

If E2F were to act as a modulator of squamous differentiation in keratinocytes, it would be of interest to determine which E2F isoforms are expressed in differentiated keratinocytes and hence which isoforms potentially contribute to the suppression. Whole cell extracts were blotted and probed with E2F 1–5 antibodies. Both proliferating and confluent HEKs were found to express E2Fs 1–5 protein (Fig. 5). These data indicate that any one of the E2F isoforms could have the potential to modulate squamous differentiation. E2F5 was the only isoform whose expression was increased while E2F2 was the only isoform whose expression was decreased in differentiated cells.

Inhibition of E2F in the Presence of a Differentiation-inducing Agent Reinstates Differentiation in a Squamous Cell Carcinoma Cell Line—If E2F acts as a modulator of differentiation, this may explain the differentiation-resistance observed in cancer cells in which E2F is overexpressed. The KJD-1/SV40 cells represent a SCC cell line that was produced by transforming normal keratinocytes with the SV40 virus. These cells were used in transfection studies in order to determine whether the inhibition of E2F was able to reinstate TG-1 Luc activity. When

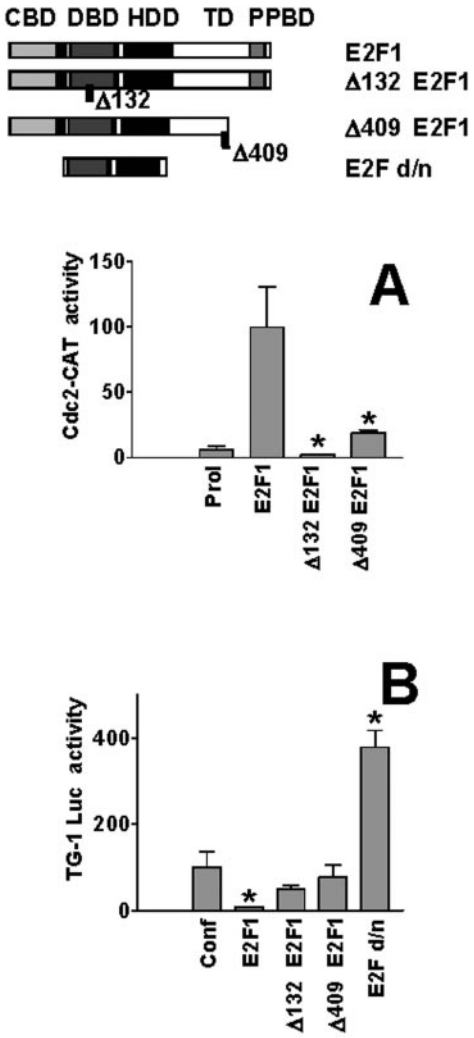


FIG. 3. **E2F1 suppression of TG-1 Luc requires the DNA binding domain, trans-activation domain and pocket protein binding domain.** *A*, proliferating (*Prol*) and *B*, differentiated (*Conf*) keratinocytes were transfected with a reporter linked to either the (*A*) *cdc2* promoter (cdc2-CAT) or (*B*) transglutaminase-1 promoter (TG-1 Luc). Each plasmid was co-transfected with a β-actin-CAT or β-actin-Luc reporter, to normalize for transfection efficiency, as well as an expression plasmid for one of the following: E2Fd/n, E2F1, Δ132E2F1, Δ409 E2F1, or GFP as control. Data presented as mean ± S.E. of triplicate determinations of at least three experiments (*, $p < 0.05$ compared with prol (*A*) or conf (*B*)). *CBD*, cyclin binding domain; *DBD*, DNA binding domain; *HDD*, heterodimerization domain; *TD*, *trans*-activation domain; and *PPBD*, pocket protein binding domain.

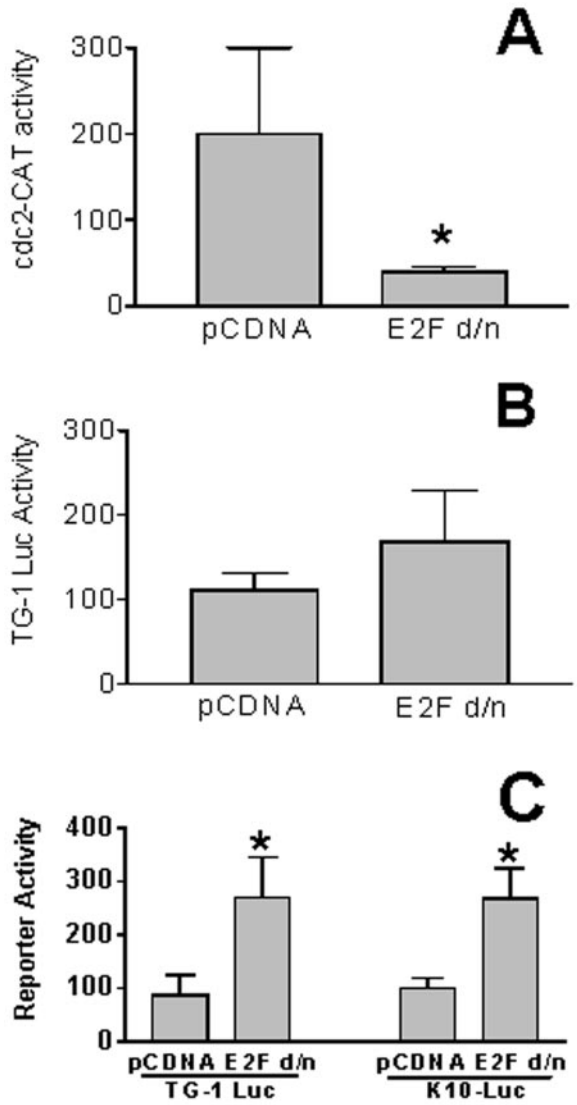


FIG. 4. **E2F may modulate squamous differentiation.** *A* and *B*, proliferating and *C*, differentiated keratinocytes were transfected with a reporter linked to the *cdc2* promoter (cdc2-CAT) (*A*), transglutaminase-1 promoter (TG-1 Luc) (*B*), or keratin 10 promoter (K10-Luc) (*C*). Each condition was transfected with a β-actin-CAT or β-actin-Luc reporter, to normalize for transfection efficiency, as well as E2Fd/n or pCDNA-GFP as control. Data presented as mean ± S.E. of triplicate determinations of at least three experiments (*, $p < 0.05$ compared with control).

proliferating KJD-1/SV40 cells were transfected with E2Fd/n, TG-1 Luc activity was not altered (Fig. 6*A*). In contrast, our earlier studies had shown that expression of E2Fd/n in KJD-1/SV40 cells could inhibit the proliferation-specific marker, cdc2-CAT (10). When proliferating KJD-1/SV40 cells were treated with TPA, there was also no induction of TG-1 Luc activity. However, treatment of KJD-1/SV40 cells with both a differentiation-inducing agent (TPA), and E2Fd/n resulted in a significant increase in TG-1 Luc activity (Fig. 6*A*). To demonstrate that the reinstatement of differentiation-specific activities were not restricted to our reporter-based assay, we repeated the experiment using the induction of TG-1 mRNA as marker of differentiation (Fig. 6*B*). Using this independent strategy we were able to confirm that a combination of TPA and E2Fd/n was able to induce differentiation markers in the previously differentiation-resistant KJD-1/SV40 cell line.

DISCUSSION

This study presents evidence implicating the E2F family as potent and biologically relevant modulators of squamous differentiation. This extends the known functions of E2F as key regulators of proliferation (58, 59) and apoptosis (58, 60, 61) to include regulation of keratinocyte terminal differentiation. Such a role may have significant implications to our understanding of squamous neoplasia and to the development of differentiation therapies for SCC. A role for the E2F family as differentiation modulators is based on the following observations: (i) E2F1 suppresses differentiation-specific markers regardless of stimuli used to induce squamous differentiation, (ii) the ability to suppress squamous differentiation is shared by E2Fs 1–5, (iii) inhibition of E2F is not sufficient to induce keratinocyte differentiation but can superinduce/derepress differentiation markers in differentiated cells, (iv) the superinduction/derepression mediated by E2Fd/n was observed for both TG-1 Luc and K10-Luc activity, (v) E2F isoforms are

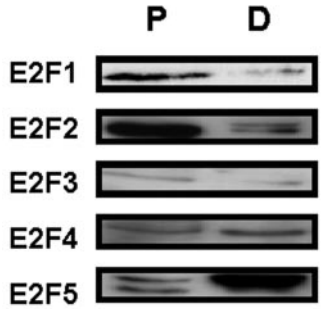
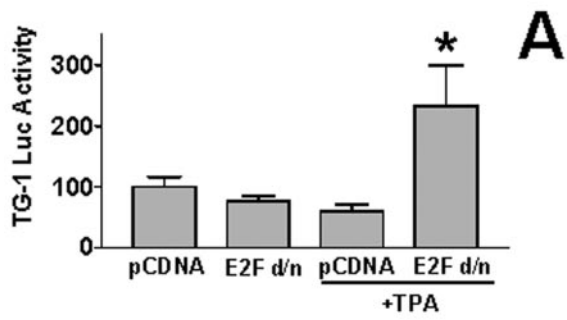


FIG. 5. **E2Fs 1–5 are expressed in primary human keratinocytes.** Proliferative (*P*) and differentiated (*D*) keratinocytes were harvested, total cellular protein isolated, and 5 μg of protein subjected to Western blot analysis for the expression of E2Fs 1–5.



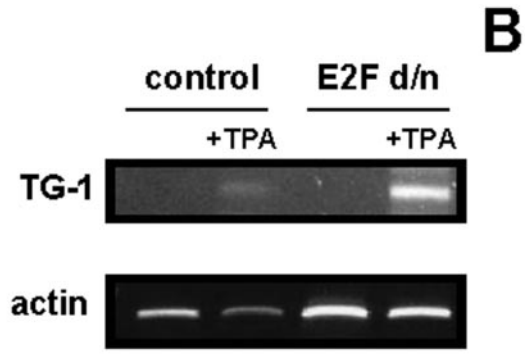


FIG. 6. **E2F inhibition makes KJD-1/SV40 cells permissive to TPA-mediated differentiation.** Proliferating KJD-1/SV40 cells were transfected with a luciferase reporter linked to the transglutaminase-1 promoter (TG-1 Luc). Cells were co-transfected with β-actin-CAT reporter (*A*), to adjust for transfection efficiency, GFP (*B*), and either the E2Fd/n plasmid or pCDNA-GFP control (pCDNA). After transfection, cells remained in growth media or were treated with 50 ng/ml of TPA for 48 h. *A*, data are presented as mean ± S.E. of triplicate determinations of at least three experiments and normalized such that the value of the control is 100 (*, $p < 0.05$ compared with control). *B*, transfected cells (GFP-positive) were selected for by FACS. mRNA levels for actin or TG-1 were then estimated in the transfected cells by RT-PCR.

FIG. 7. **Model illustrating the requirement for independent stimuli to induce growth inhibition or differentiation in keratinocytes.**

expressed in both proliferating and differentiated keratinocytes, (vi) differentiation-insensitive SCCs overexpress E2F1 (10, 43), and (vii) E2F inhibition makes SCC cell lines permissive to differentiation stimuli. Together, these data lend support to the notion that E2F possesses biological properties expected of a modulator of squamous differentiation.

Our data indicate that E2F may play a dual role in promoting keratinocyte proliferation and modulating squamous differentiation. In this capacity, E2F members would participate in proliferation control of undifferentiated keratinocytes and would actively prevent them from undergoing differentiation. However, once a cell receives a signal to withdraw from the cell cycle and commits to differentiation (associated with loss of proliferation-competence), E2Fs would assume an active role as negative modulators of differentiation. In this model, E2Fs would be predicted to regulate the extent of the differentiation response and in concert with other differentiation-specific activators/repressors, the level of differentiation. In this way, the function of E2F may be considered analogous to the inhibitory function of the cyclin-dependent kinase inhibitor, $p21^{Cip1/WAF1}$, in regulating primary mouse keratinocyte differentiation (62). However, studies with p21 suggest that it is actively involved in repression of differentiation. In contrast, our data indicate that E2F inhibition is required for the initiation of differentiation but is not sufficient to initiate differentiation. Of interest is the observation that E2Fs, in the present study, and p21, in an earlier report (62), mediate their effects via a cell cycle-independent process. In addition, our data with the E2Fd/n suggests that the process of differentiation is not a direct result of growth arrest but an independent process arising after the cessation of proliferation. If differentiation was a result of growth arrest, inhibition of E2F should suffice to cause both growth arrest and entry into terminal differentiation. However, our results demonstrate that although E2F inhibition was sufficient to suppress the activity of proliferation-specific markers, it was not able to induce differentiation-specific marker activity. This indicates that terminal differentiation is not merely a result of growth arrest, but that differentiation is initiated and modulated by mechanisms yet to be understood that lie downstream of growth arrest and require an independent stimulus (Fig. 7). This observation is strengthened by previous reports that growth arrest of epidermal keratinocytes is not sufficient to induce differentiation (2, 4, 49).

A role for E2F in differentiation is not entirely unprecedented since E2Fs are known to modify myocyte, megakaryocyte, and adipocyte differentiation (37–39). In myocytes and megakaryocytes, E2F1 suppression is required in order for differentiation initiation to occur (37, 38). This situation is similar to keratinocytes although studies with myocytes and megakaryocytes did not extend to examining effects in differentiated cells (37, 38). More recently, it was reported that E2F1 and E2F4 act antagonistically in regulating terminal adipocyte differentiation (39). In this model, E2F plays an active role in adipogenesis in which E2F1 predominates in proliferating adipocytes and serves to initiate differentiation (39). Subsequently, E2F4 is activated and displaces E2F1, thereby inhibiting E2F1 activity (39). Thus, the mechanism by which E2F controls adipocyte differentiation is through E2F1 promoting differentiation whereas in keratinocytes, we propose that E2Fs 1–5 suppress the initiation of differentiation. Data from the adipocyte study compared with results from the present study suggest that the role of E2F in differentiation may be cell type-specific, tissue-specific, and transformation-specific. This latter point would also explain why an earlier report with the HaCaT cell line (63) differs to that of primary keratinocyte

cultures. HaCaT cells are an immortalized keratinocyte cell line known to lack p53 (64), undergo spontaneous transformations (65), demonstrate mixed responses to differentiation stimuli (43) and exhibit different patterns of E2F isoform expression when compared with human primary keratinocytes (43).

The mechanism by which E2F suppresses differentiation-specific markers is unknown. However, our data allow us to exclude a number of mechanisms. Our data clearly indicate that E2Fs 1–5 suppress the activity of differentiation-specific markers. Since E2Fs 1–3 can induce apoptosis and E2F4 cannot (33–36, 54–57), it is unlikely that the loss of differentiation-specific marker activity is due to E2F-induced death of the cell. Moreover, since E2F4 and E2F5 suppress squamous differentiation, yet lack a cyclin binding domain, we can conclude that E2F suppresses squamous differentiation through a cyclin binding domain-independent pathway. Similarly, we can exclude the nuclear export sequence of E2F 4 and 5 since E2Fs 1–3 lack this sequence (66, 67) yet are still able to suppress differentiation. While it could be argued that E2F overexpression may cause a squelching phenomenon, and hence suppression of differentiation-specific markers, this would also seem unlikely. For instance, single mutations within different domains of E2F1 (Δ132 and Δ409) render them unable to suppress differentiation markers thereby arguing against a squelching phenomenon. In contrast to the above findings, our data consistently indicate that the *trans*-activation domain, pocket protein binding domain and DNA binding domain of E2Fs are critical for suppressing squamous differentiation. Similarly, the antagonistic effects of E2Fd/n, which lacks the pocket protein binding domain and *trans*-activation domain, would suggest that these domains are central to the ability of E2F to suppress differentiation. This critical need for the *trans*-activation domain implies that the recruitment of other cofactors, such as pocket proteins and histone deacetylases, may be required. This would be consistent with an earlier report implicating pocket proteins in myocyte differentiation (68). It is also noteworthy that although both the E2F1Δ409 mutant and E2Fd/n lack the *trans*-activation domain and pocket protein binding domain, only the E2Fd/n is able to superinduce/derepress differentiation marker activity. This suggests that there may be an additional "repressor" domain between amino acid 235 and 409 of E2F1 involved in differentiation suppression. Furthermore, the absence of an E2F consensus sequence in the *TG-1* promoter (69) would imply that an indirect mechanism may be involved. Thus, our data are consistent with a model in which E2Fs alter the transcription of genes that in turn regulate squamous differentiation.

Previous data indicate that E2F contributes causally to SCC formation. For example, keratinocyte growth arrest and differentiation are characterized by decreased E2F DNA binding activity and E2F1 mRNA expression (3). This decrease in expression and activity does not occur in growth inhibitor-insensitive SCC cell lines (3) and finally, deregulation of E2F contributes to SCC formation in transgenic mice (40). Combined with the present study, we can propose a model of squamous neoplasia in which E2F deregulation promotes aberrant proliferation and differentiation suppression in keratinocytes. It is these two properties that present E2F as a unique target for SCC therapy. For instance, E2F inhibition would serve the dual purpose of inhibiting E2F-induced aberrant proliferation of cancer cells and render these cells permissive to entering a differentiation program in the presence of an appropriate differentiating stimulus. Therefore, these data suggest that the inhibition of E2F in SCC cells may provide the foundation for a differentiation therapy. In this regard anti-E2F therapies are already in trial in non-neoplastic proliferative disorders (44). Our data suggest that it may be timely to extend these trials to neoplastic disease such as SCCs.

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E2F Modulates Keratinocyte Squamous Differentiation: IMPLICATIONS FOR E2F INHIBITION IN SQUAMOUS CELL CARCINOMA

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