

# Ovol2 Suppresses Cell Cycling and Terminal Differentiation of Keratinocytes by Directly Repressing *c-Myc* and *Notch1*<sup>\*S</sup>

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Ovol2 belongs to the *Ovo* family of evolutionarily conserved zinc finger transcription factors that act downstream of key developmental signaling pathways including Wg/Wnt and BMP/TGF- $\beta$ . We previously reported Ovol2 expression in the basal layer of epidermis, where epidermal stem/progenitor cells reside. In this work, we use HaCaT human keratinocytes to investigate the cellular and molecular functions of Ovol2. We show that depletion of Ovol2 leads to transient cell expansion but a loss of cells with long term proliferation potential. Mathematical modeling and experimental findings suggest that both faster cycling and precocious withdrawal from the cell cycle underlie this phenotype. Ovol2 depletion also accelerates extracellular signal-induced terminal differentiation in two- and three-dimensional culture models. By chromatin immunoprecipitation, luciferase reporter, and functional rescue assays, we demonstrate that Ovol2 directly represses two critical downstream targets, *c-Myc* and *Notch1*, thereby suppressing keratinocyte transient proliferation and terminal differentiation, respectively. These findings shed light on how an epidermal cell maintains a proliferation-competent and differentiation-resistant state.

The *Ovo* gene family encodes evolutionarily conserved proteins including members from *Caenorhabditis elegans*, *Drosophila*, *Zebrafish*, chick, and mammals. *Ovo* proteins contain four DNA-binding C<sub>2</sub>H<sub>2</sub> zinc fingers at the C termini and possess transcriptional regulatory activities (1–5). *Drosophila ovo*, the founding member of the family, acts genetically downstream of Wg (fly Wnt homolog) and DER (fly epidermal growth factor receptor homolog) signaling pathways and is required for epidermal denticle formation and oogenesis (6–8). We and others have identified mammalian *Ovol* (*Ovo*-like) genes, including *Ovol1* (*movo1*), *Ovol2* (*movo2*), and *Ovol3* (*movo3*) in mice and *OVOL1*, *OVOL2*, and *OVOL3* in humans (9–12). Mammalian *Ovol/OVOL* (referred to as *Ovol* from here

on) genes also appear to reside downstream of key developmental signaling pathways. For instance, *Ovol1* is activated by the  $\beta$ -catenin-LEF1 complex, downstream effectors of Wnt signaling (13), and is a downstream target of TGF- $\beta$ /BMP7-Smad4 signaling (14, 15). A functional *Ovol1* gene is required for multiple developmental processes, including that of epidermis, hair follicles, kidney, and male germ cell differentiation (2, 4, 10). In both epidermis and testis, *Ovol1* restricts the boundary of late progenitor cells during development by promoting cell cycle exit (2, 4). Less is known about the biological function of *Ovol2* and *Ovol3*.

*Ovol2* is expressed in myriad embryonic and postnatal tissues (11, 16). Consistent with a widespread expression pattern, ablation of the *Ovol2* gene results in early embryonic lethality (16), which precludes a comprehensive analysis of its later developmental roles. Interestingly, *Ovol2* expression appears to correlate with a proliferative stem/progenitor cell state (16, 17). For instance, *Ovol2* protein is detected in the basal layer of skin epidermis (17), where proliferative epidermal stem/progenitor cells reside. Moreover, *Ovol2* is strongly expressed in inner cell mass as well as its *in vitro* equivalent, embryonic stem cells (16). The functional significance of *Ovol2* expression in proliferating stem/progenitor cells remains to be established.

Mammalian epidermis is an excellent model system to study the molecular circuits that control proliferation and differentiation. Using cultured keratinocytes and mouse models, important regulators of epidermal proliferation and differentiation have been uncovered (18). Among these, *c-Myc* is the most intriguing because of its multiple and seemingly opposing roles (19). Although *c-Myc* is expressed in basal cells and clearly important for keratinocyte proliferation, its constitutive overexpression in cultured keratinocytes causes progressively reduced growth, precocious terminal differentiation, and loss of cells that express a high level of  $\beta$ 1 integrin, a putative epidermal stem cell marker (20). The latter finding has been interpreted to indicate a *c-Myc*-stimulated premature exit from the stem cell compartment, a notion that is apparently supported by the observation of decreased  $\beta$ 1<sup>high</sup> or label-retaining cells in mice that overexpress *c-Myc* in the epidermal basal layer (20–22). Notch signaling, which is initiated by ligand binding to the Notch receptor followed by cleavage and nuclear translocation of the intracellular domain that in turn binds to RBP-J to generate a transactivation complex, is critical for terminal differentiation of keratinocytes (19, 23–26). Notch receptors (Notch1, 2, and 3) are not normally expressed in proliferating epidermal

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basal cells, and overexpression of the intracellular domain in these cells promotes a differentiating, spinous cell fate (23). Collectively, these findings suggest that fine-tuning c-Myc expression and suppressing Notch expression/signaling in the basal layer might be important for maintaining a differentiation-refractory progenitor cell state. To date, little is known about molecular mechanisms that directly repress c-Myc and Notch expression in epidermal progenitor cells.

In this work, we use HaCaT keratinocytes to explore the function of *Ovol2*. We show that *Ovol2* regulates independent yet related aspects of keratinocyte proliferation and differentiation, namely suppressing rapid amplification and terminal differentiation but maintaining long term proliferation potential in culture. We present evidence that *Ovol2* does so, at least in part, by regulating downstream targets *c-Myc* and *Notch1*.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Reagents**—293T cells (a human kidney epithelial cell line) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. UG1 mouse keratinocytes were cultured as previously described (13). HaCaT human keratinocytes were cultured in calcium-free Dulbecco's modified Eagle's medium/Ham's F-12 (3:1) supplemented with 15% fetal bovine serum that had the calcium chelated using Chelex beads (Bio-Rad). The following *Silencer* predesigned siRNAs<sup>2</sup> (Applied Biosystems/Ambion) were used at a concentration of 30–60 nM: *Ovol2* #1 (siRNA number 29207) 5'-GGCAUUCGUCCCUACAAAU-3', *Ovol2* #2 (siRNA number 29292) 5'-GGUAUUUCUUAGAGAG-AUC-3', and nontargeting negative control #1 siRNA. The following *Silencer Select* predesigned siRNA (Applied Biosystems/Ambion) was used at a concentration of 10 nM: *Ovol2* #3 (siRNA number s33860) 5'-AGAUCGAAAAUCAAGUUCA-3'; and the nontargeting negative control #1. c-Myc inhibitor, 10058-F4 (EMD Biosciences), was used at a concentration of 30  $\mu$ M and was added to cells 24 h after transfection. DAPT  $\gamma$ -secretase inhibitor (EMD Biosciences) was used at a concentration of 1  $\mu$ M and was also added 24 h after transfection. For differentiation assays, Ca<sup>2+</sup> was added at a final concentration of 2.8 mM 72 h after siRNA transfection, and treatment persisted for an additional 4 days.

**Nuclear Extracts and Western Blots**—Nuclear extracts were made as previously described (27). Protein concentrations were quantified (Bio-Rad protein assay reagent), and equal amounts of protein were run on 10% polyacrylamide gels, followed by transfer to nitrocellulose membranes and probing with antibodies. The following antibodies were used: rabbit anti-*Ovol2* (1:250) (16); mouse anti-Rad50 and mouse anti-p84 (1:2000; gift from P. L. Chen, University of California, Irvine); mouse anti-c-Myc (1:250; Santa Cruz Biotechnology); rabbit anti-HDAC1 (1:200; Santa Cruz Biotechnology); rabbit anti-K1, rabbit anti-loricrin, and rabbit anti-K14 (1:2000; gift from J. Segre, National

Institute of Health); and mouse anti- $\beta$ -actin (1:4000, Abcam). The proteins were detected using chemiluminescence (Pierce).

**Cell Counts and Clonal Assay**—For cell count experiments, HaCaT cells were seeded in triplicate in a 12-well plate and transfected at 20–30% confluence with 60 nM siRNA and Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. At 24-h intervals spanning 72 h post-transfection, the cells were trypsinized and counted using a hemacytometer. Clonal assays were performed as previously described (28). For first generation clonal assays, HaCaT cells were seeded at  $8 \times 10^4$  cells/well of a 6-well plate and transfected 24 h later with siRNA. The cells were trypsinized 24 h after transfection (day 2), replated at a density of 500 cells/well in 6-well plates, and retransfected with siRNA on day 8. After 14 days, three of the six wells/sample were fixed in 4% paraformaldehyde for 15 min, washed with  $1 \times$  PBS, and stained with a solution of 1% Rhodamine B, 1% Nile Blue before washing and air drying. The number and size of colonies were then scored. The remaining three wells were trypsinized, counted, and replated as second generation at a density of 500 cells/well in a 6-well plate.

**Immunofluorescence**—HaCaT cells were seeded into a 96-well plate and 24 h later transfected with siRNA. Seventy-two hours after transfection, the cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS. Following permeabilization with cold 90% methanol for 5 min at room temperature and PBS washes, the cells were blocked in 10% normal goat serum for 1 h and then incubated with mouse anti-cleaved poly(ADP-ribose) polymerase (Asp<sup>214</sup>) (1:10; BD Pharmingen) and rat anti-phospho histone H3 (pS28) (1:20; BD Pharmingen) in 1% normal goat serum overnight at 4 °C. The cells were washed with PBS, and the nuclei were stained with 4',6-diamidino-2-phenylindole. The plates were run on an InCell Plate Reader, and the fluorescence was measured using InCell Developer Software.

**FACS Analysis**—HaCaT cells were seeded in duplicate at  $2.5 \times 10^5$  cells/10-cm dish and after 24 h were transfected with siRNA. The cells were collected 72 h after transfection. For propidium iodide staining, the cells were washed once with cold PBS, fixed with 70% ethanol for 24 h at  $-20$  °C, followed by two PBS washes. After being stained with a propidium iodide staining solution (50  $\mu$ g/ml propidium iodide, 0.1% sodium citrate, 0.03% Nonidet P-40, 50  $\mu$ g/ml RNase A, PBS) for 30 min at room temperature, the cells were then washed once with PBS. For  $\alpha 6$  integrin staining, the cells were washed once with 2% fetal bovine serum with PBS and stained on ice for 30 min with a phycoerythrin-conjugated rat anti-CD49f ( $\alpha 6$  integrin) antibody (1:40; BD Pharmingen) and the appropriate isotype control. The cells were washed two times with cold PBS, run on a FACS Calibur flow cytometer (BD), and analyzed with FlowJo software (Tree Star Inc.).

**Cyclic Amplification of Selected Targets**—Cyclic amplification of selected targets was carried out as previously described (3) using purified recombinant His<sub>6</sub>-*Ovol2* protein. Forty *Ovol2*-selected clones were sequenced to derive an *Ovol2* consensus sequence.

**Reporter Assays**—Assays were performed in 293T and HaCaT cells. The 293T cells were transfected using calcium phosphate as described (29) and HaCaT cells using Lipo-

<sup>2</sup> The abbreviations used are: siRNA, small interfering RNA; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; ChIP, chromatin immunoprecipitation; TA, transit amplifying; TGF, transforming growth factor; DAPT, *N*-(*N*-(3,5-difluorophenacetyl)-1-alanyl)-5-phenylglycine *t*-butyl ester.

fectamine 2000 (Invitrogen). Typically, transfection experiments were done in 12-well plates with each well transfected with a total of 1.6  $\mu\text{g}$  of plasmids including 0.1 or 0.8  $\mu\text{g}$  (for 293T or HaCaT cells, respectively) of pGL3-c-Myc (4), pGL3-Hes1 (where luciferase is under the control of the *Hes1* promoter; gift from C. C. W. Hughes, University of California, Irvine), or pGL4 Notch1 (where luciferase is under the control of the 2.4-kb *Notch1* promoter; gift from G. P. Dotto, Massachusetts General Hospital (30)), 0.08 or 0.3  $\mu\text{g}$  (for 293T or HaCaT cells, respectively) of  $\beta$ -actin- $\beta$ -galactosidase construct (transfection control), and varying amounts of pCB6-Ovol2A, an *Ovol2A* expression vector (as indicated in the figure legends). pCB6+ (empty vector containing the cytomegalovirus promoter) was used as filler DNA. The cells were harvested 24–36 h after transfection, and luciferase activity was measured in whole cell extracts using the luciferase assay system (Promega).  $\beta$ -Galactosidase activity was measured as previously described (31).

**Chromatin Immunoprecipitation (ChIP)**—HaCaT cells were seeded in 10-cm plates, and each plate was transfected at  $\sim 90\%$  confluency with 24  $\mu\text{g}$  of pCB6-Ovol2A using Lipofectamine 2000. The plates were cross-linked 24 h after transfection with 1% formaldehyde for 10 min at room temperature, and chromatin immunoprecipitates were isolated using the ChIP assay kit (Upstate Biotechnology Inc.) and anti-Ovol2 antibody (16) according to the manufacturer's instructions. PCR was performed using human c-Myc primers (4) and human Notch1 primers containing the following sequences: forward, 5'-ACCAGGAGCAGAGGACGTC-3'; and reverse, 5'-CTTT-CCTGGCACACCTCTTG-3'. The following PCR program was used: 94 °C for 5 min followed by 31–38 cycles (within the linear range of the primers) of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min followed by a final extension at 72 °C for 7 min.

**Real Time PCR**—Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Five  $\mu\text{g}$  of total RNA was reverse transcribed into cDNA using the Superscript III RNase H reverse transcriptase (Invitrogen) according to the manufacturer's instructions. All of the primers used for quantitative real time PCR were designed to span exon-exon borders to minimize the possibility of nonspecific amplification of genomic DNA. The following primers were used for quantitative real time PCR: glyceraldehyde-3-phosphate dehydrogenase forward, 5'-GGCATCCTGGGCTA-CACTGAG-3'; glyceraldehyde-3-phosphate dehydrogenase reverse, 5'-TGAGGTCCACCACCTGTTG-3'; c-Myc forward, 5'-TCTCTCCGTCCTCGGATTCTC-3'; c-Myc reverse, 5'-GGAGCCTGCTCTTTTCCAC-3'; Notch1 forward, 5'-AATGTGGATGCCGAGTTG-3'; and Notch1 reverse, 5'-CGGTCCATATGATCCGTGATG-3'. Quantitative real time PCRs were performed using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions on an iCycler real time quantitative PCR system (Bio-Rad). The thermal profile included 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 10 s and annealing at 63 °C for 1 min with the optics on for fluorescence monitoring. The reactions were run in triplicate and always included a standard curve and no template sample for each primer set as a control for the linear range and nonspecific PCR products, respectively. Glyceraldehyde-3-phosphate

dehydrogenase was used to normalize the data. The mean threshold cycle ( $C_t$ ) for individual reactions was identified using the iCycler IQ sequence analysis software (Bio-Rad).

**Three-dimensional Organotypic Culture**—Skin equivalents were prepared in 12-well tissue culture inserts (3- $\mu\text{m}$  pore Thinsert; Greiner Bio One) as previously described (32). Briefly, gels (400  $\mu\text{l}$ /insert) were prepared from collagen type I (BD Biosciences) at a final concentration of 2.5 mg/ml and seeded with 3T3 cells at  $2.5 \times 10^4$  cells/ml. The gels were submerged in Dulbecco's modified Eagle's medium, 10% fetal bovine serum and grown at 37 °C in a humidified, 95% air, 5% CO<sub>2</sub> atmosphere. After 48 h, the gels were released from the side of the insert using a pipette tip and allowed to contract for 5 days. The medium was replaced every 2 or 3 days.  $2 \times 10^5$  HaCaT cells (either negative control or *Ovol2*-depleted)/insert were added and allowed to grow for 2 days before being air-lifted and cultured in medium containing 2.8 mM calcium for 10 days. The medium was changed every other day.

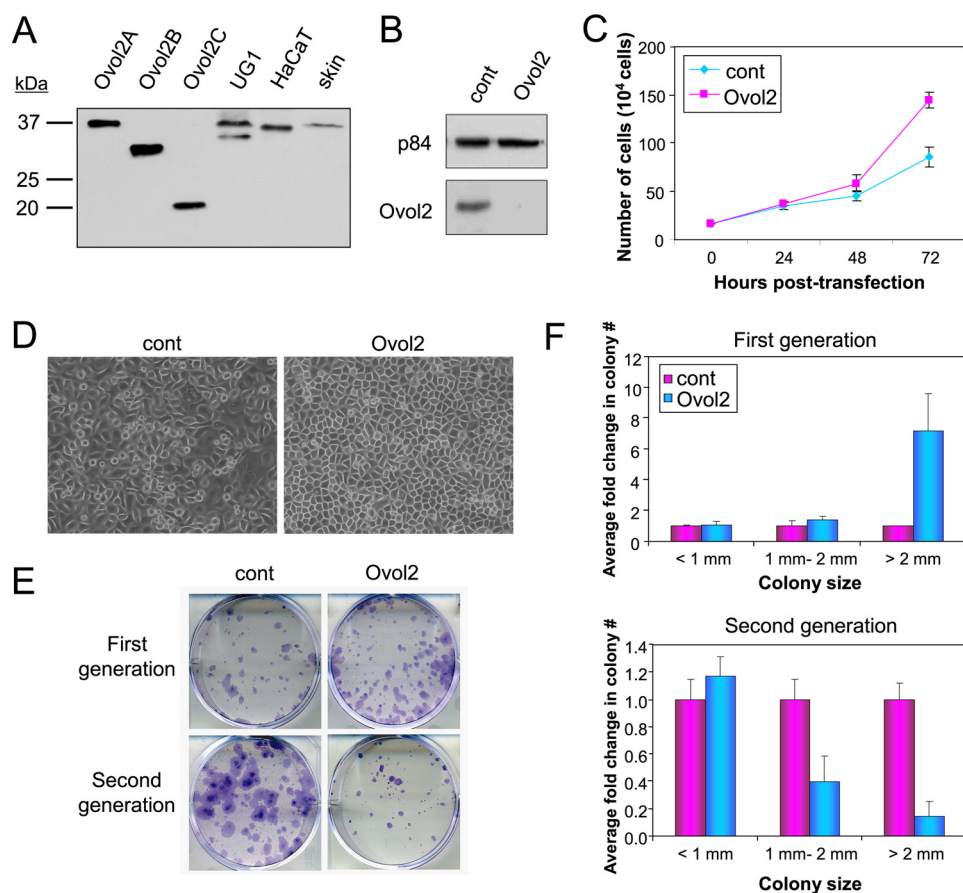
**Mathematical Modeling**—A cell population model consisting of a system of ordinary differential equations was considered (33, 34) (see supplemental text for equations and details of the model). In the system, after each stem cell ( $N_0$  in total) division, the daughter cells have the probability ( $p_0$ ) to remain a stem cell, or  $(1 - p_0)$  to become a progenitor ( $N_1$ ) cell that can undergo a maximum of  $M$  divisions before becoming growth arrested ( $N_{M+1}$ ) cells. For a smaller  $M$ , the solution of the system could be explicitly and analytically expressed. For a large  $M$ , the system was solved using NDSolve function in Mathematica. The parameter fitting was based on FindFit function of Mathematica.

## RESULTS

**Loss of *Ovol2* Leads to a Transient Increase in Growth and a Loss of Long Term Proliferation**—In both mice and humans, a single *Ovol2* gene encodes different protein isoforms with presumably opposing transcriptional regulatory activities (5, 11). We therefore first determined which isoform of *Ovol2* is expressed in epidermal keratinocytes. By Western blot analysis of nuclear extracts from mouse and human keratinocytes and mouse skin, we found that based on the sizes of the proteins seen in each of these samples, *Ovol2A* repressor is the dominant isoform expressed in epidermis *in vivo* and cultured keratinocytes *in vitro* (Fig. 1A). The other isoforms, namely *Ovol2B* and *Ovol2C*, were not detected under our experimental conditions.

Next we turned to use siRNAs to knockdown *Ovol2* in HaCaT human keratinocytes, an immortalized cell line that displays epidermal progenitor cell activity, *i.e.* forming a stratified epidermis under organotypic culture conditions (28, 35, 36). A number of different differentiation-specific markers are expressed in HaCaT cells under organotypic conditions, although stratification is incomplete and imperfect (37). These cells were chosen for our study because in culture they contain a small subset of quiescent stem/progenitor-like cells (28), mimicking epidermal homeostasis *in vivo*. When compared with cells transfected with a negative control siRNA that does not target any known gene, *Ovol2* proteins were effectively depleted with *Ovol2*-specific siRNAs, and knockdown persisted

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**FIGURE 1. Increased transient growth and decreased long term clonogenicity in *Ovol2*-depleted keratinocytes.** *A*, Western blot analysis of *Ovol2* isoform expression in keratinocytes and skin. Nuclear extracts were made from 293T cells transfected with *Ovol2A*, *Ovol2B*, or *Ovol2C* expression constructs, UG1 mouse keratinocytes, HaCaT cells, and mouse skin. Note that *OVOL2A* is, as expected, slightly smaller than *Ovol2A*. The second band in UG1 cells is likely a degradation product because its presence varies from experiment to experiment. *B*, Western blot showing efficient siRNA-directed depletion of *Ovol2* 72 h after transfection. Nuclear protein p84 was used as a loading control (*cont*). *C*, growth curve of short term, high density cultures of control and *Ovol2*-depleted cells ( $n = 3$ ). *D*, morphology of *Ovol2*-depleted keratinocytes 72 h after transfection. *E*, clonal analysis of control and *Ovol2*-depleted keratinocytes. *F*, quantitative analysis of results shown in *E*. Shown are the average values with standard deviations from two independent experiments each with triplicate samples.

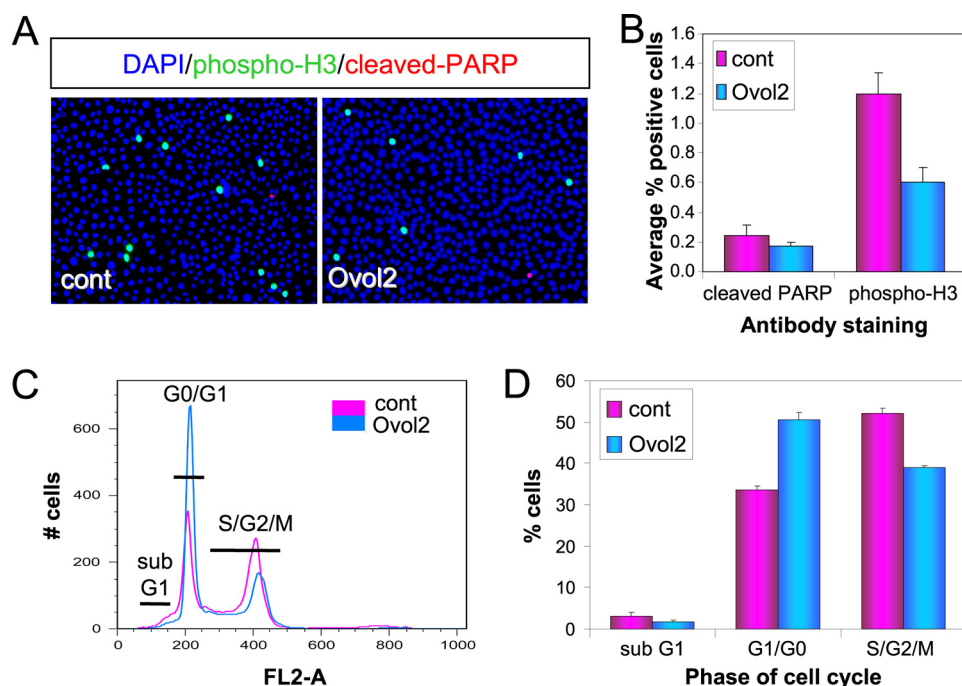
in culture for at least 6 days for the most effective siRNA (Fig. 1*B* and supplemental Fig. S1*A*). A growth curve analysis revealed a significantly higher number of cells in *Ovol2*-depleted high density culture, particularly at 72 h after siRNA transfection (1.7-fold  $\pm$  0.12) (Fig. 1*C*). Consistently, the *Ovol2* knockdown plates appeared more confluent, and the cells were more tightly packed on the plate than the negative control plates (Fig. 1*D*). Three different siRNA oligonucleotides (*Ovol2* #1 targets exons 3 and 4, *Ovol2* #2 targets the 3'-untranslated region, and *Ovol2* #3 targets exon 2) produced a similar trend, albeit to a lesser extent for the less effective siRNAs #2 and #3 (supplemental Fig. S1, *C* and *D*). This finding suggests that the increase in cell number is not an off target effect. Knockdown was also performed in mouse keratinocytes using adenoviral small hairpin RNA against *Ovol2*, and a similar increase in cell number was observed (data not shown). Subsequent experiments focused on the most effective siRNA (#1) and HaCaT cells.

Because cultured keratinocytes are a heterogeneous population of cells with different proliferation potentials (28, 38, 39), we performed clonal assays in which cells are plated at a clonal

density to determine the proliferation potential of individual cells. Specifically, we transfected HaCaT cells with either control or *Ovol2* siRNA at days 1 and 8 to efficiently knockdown *Ovol2* during the first 14 days of clonal assay (this we term first generation). We then assessed the proliferation potential of the cells harvested from the first generation by replating a portion of them at a clonal density, but this time with no additional siRNA knockdown (this we term second generation). Consistent with an increased cell number in short term high density cultures described above, we observed a reproducible increase in the number of large (> 2 mm) colonies in *Ovol2*-depleted first generation cultures when compared with negative control (Fig. 1, *E*, top panels, and *F*, and supplemental Fig. S2, top panel). Trypan blue staining showed that *Ovol2* depletion did not affect the viability of the first generation cells (data not shown). Interestingly, after replating an equal number of first generation cells, we now observed a reduction in both colony size and number in the second generation culture derived from cells that had been previously depleted of *Ovol2* (Fig. 1, *E*, bottom panels, and *F*, and supplemental Fig. S2, bottom panel). This result suggests that depleting *Ovol2* leads to a reduction in the number

of keratinocytes that possess long term proliferation potential. Together, our results indicate that depletion of *Ovol2* results in a transient increase in growth but a decrease in long term proliferation.

**Reduced Active Cycling Accompanies the Growth Phenotype of *Ovol2*-depleted Cells—***A priori*, an increase in cell number can be caused by a decrease in cell death or an increase in cell proliferation. To distinguish between these possibilities, we stained negative control and *Ovol2* knockdown cells cultured under high density conditions for the presence of cleaved poly(ADP-ribose) polymerase, which symbolizes activated apoptosis (40), and phospho-H3 (pS28), which marks cells in the mitotic phase (specifically the prophase to anaphase transition) of the cell cycle (41). A slight decrease in the number of cleaved poly(ADP-ribose) polymerase positive cells was observed in *Ovol2* knockdown cells (0.17%  $\pm$  0.03) when compared with negative control cells (0.25%  $\pm$  0.07) (Fig. 2, *A* and *B*, left columns). This slight change is consistent with, but unlikely to completely account for the increase in cell number. Contradictory to our prediction, there was a significant decrease in the



**FIGURE 2. Decreased apoptosis and reduced active cycling accompany the growth phenotype of *Ovol2* knockdown cells.** HaCaT cells were transfected with either negative control (*cont*) or *Ovol2* siRNA and then used for analysis 72 h post-transfection. *A*, immunofluorescence of cells stained with anti-cleaved poly(ADP-ribose) polymerase (red) and anti-phospho-H3 (green). 4',6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei (blue). *B*, quantitative analysis of experiments in *A* reveals a slight reduction of apoptotic cells (*left*) and a significant reduction of mitotic cells (*right*) in *Ovol2*-depleted culture. The results are from a total of five samples from two independent experiments. *C*, representative histogram of cell cycle profiles. Note the increase in  $G_1$  population and concurrent decrease in  $S/G_2/M$  population in *Ovol2* knockdown samples. *D*, average percentage of sub- $G_1$ ,  $G_1/G_0$ , and  $S/G_2/M$  cells in control and *Ovol2*-depleted culture, as calculated from three samples each from two independent experiments.

number of phospho-H3-positive cells in *Ovol2* knockdown cells (average of 2-fold change  $\pm$  0.24) (Fig. 2, *A* and *B*, *right* columns). An overall reduction in the number of actively cycling cells was confirmed by FACS analysis of cell cycle profiles, revealing a 34% increase in  $G_1/G_0$  cells and a 25% decrease in  $S/G_2/M$  cells in the *Ovol2*-depleted high density culture (Fig. 2, *C* and *D*). Again, a slight reduction in apoptotic cells, as monitored by the sub- $G_1$  population, was observed.

**Mathematic Modeling Suggests That *Ovol2* Suppresses the Rate of Keratinocyte Cycling but Prolongs the Number of Divisions They Undergo**—To explore the cellular basis of these seemingly contradictory observations, we applied a simple cell lineage mathematic model (33, 34) to examine keratinocyte stem/progenitor cell evolution. We considered three distinct cell types (42): slow cycling stem cells that can proliferate indefinitely, faster cycling progenitor cells with a set proliferation potential (indicated by  $M$  number of cell divisions they are able to undergo), and growth-arrested cells (Fig. 3*A*). Using this model, we explored various parameters that would allow the recapitulation of our experimental observations. We found that a total of 36 rounds of progenitor cell divisions are necessary to mimic the observed growth of normal HaCaT keratinocytes (data not shown). Under this pre-condition, we found that decreasing  $t_1$  (cell cycle time for progenitor cells) but not  $t_0$  (cell cycle time for stem cells) was necessary to robustly capture the *Ovol2* depletion-induced transient increase in proliferation in high density culture (Fig.

for cultured keratinocytes to maintain their proliferation potential.

***Ovol2* Suppresses Active Keratinocyte Proliferation by Repressing *c-Myc* Expression**—How does *Ovol2* keep keratinocytes proliferation in check? We entertained the possibility that *Ovol2* might function by directly repressing a positive regulator of the cell cycle. *c-Myc* is an excellent candidate because 1) it is repressed by *Ovol1*, whose zinc finger domain is highly homologous to that of *Ovol2* (11, 13); and 2) its expression level in keratinocytes has been proposed to govern whether a stem cell should exit into the transit amplification (TA) stage, how well a TA cell proliferates, or whether it should terminally differentiate (20–22, 45, 46).

We first examined *c-Myc* expression in control and *Ovol2* knockdown cells. Loss of *Ovol2* indeed caused an up-regulation of both *c-Myc* mRNA and *c-Myc* protein levels (Fig. 4, *A* and *B*). Next we asked whether inhibiting *c-Myc* activity might rescue the growth phenotype of *Ovol2* knockdown cells. A small molecule inhibitor (10058-F4) that interferes with *c-Myc* binding to its partner in transcriptional activation (47) was added to keratinocytes cultured under clonal conditions with or without *Ovol2* knockdown. At an inhibitor concentration where minimal change of colony growth was observed in first generation control cultures, *Ovol2* knockdown no longer elicited a significant increase in transient proliferation (Fig. 4, *C* and *D*). In contrast, *Ovol2* knockdown still resulted in a dramatic reduction of colony

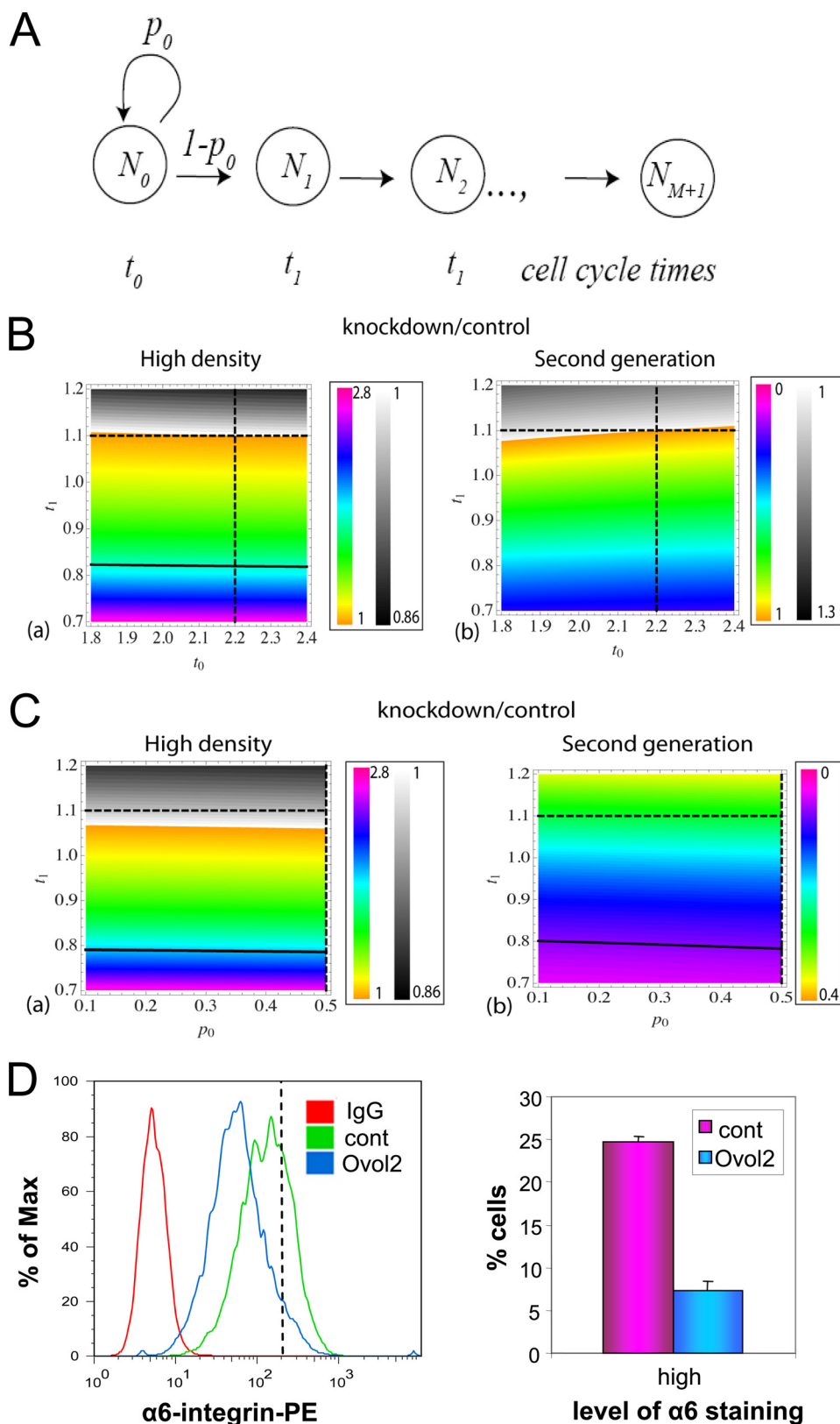
3*B*, *left* panel). However, this could not replicate the finding of decreased clonogenicity in second generation *Ovol2* knockdown culture (Fig. 3*B*, *right* panel). Importantly, using a smaller  $M$  in addition to decreasing  $t_1$  recapitulated both the increased growth in high density culture and the decreased clonogenicity in second generation culture of *Ovol2*-depleted cells (Fig. 3*C*). In contrast, decreasing  $p_0$  had minimal impact on the growth behavior of *Ovol2*-depleted cells regardless of whether  $M$  was reduced or not (Fig. 3*C* and data not shown). These results suggest that both decreased cell cycle time and compromised proliferation potential of progenitor cells are critical for the observed growth phenotype of *Ovol2*-deficient culture. Indeed, we observed a remarkable decrease in the expression of  $\alpha 6$  integrin, which when highly expressed, marks keratinocytes that possess long term proliferation potential and increased clonogenicity (43, 44), in *Ovol2* knockdown cells (Fig. 3*D*). This result provides further correlative support that *Ovol2* is required

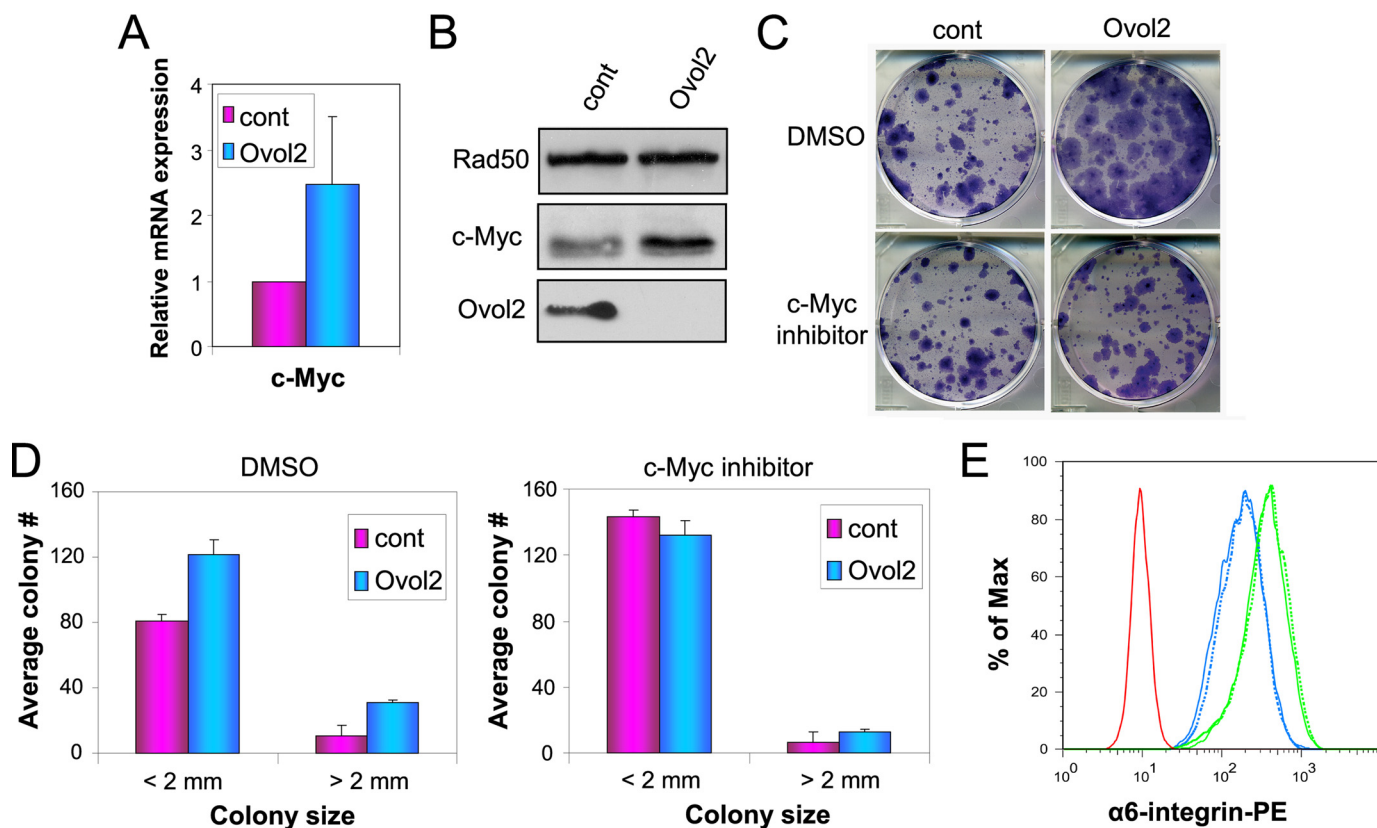
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formation in second generation cultures regardless of whether the *c-Myc* inhibitor was present or not (data not shown). Consistent with this finding, addition of the *c-Myc* inhibitor was unable to rescue the loss of  $\alpha 6^{\text{high}}$  cells caused by *Ovol2* depletion (Fig. 4E). Collectively, these results suggest that *c-Myc* up-regulation accounts at least in part for the *Ovol2* depletion-induced transient increase in proliferation but not for the loss of long term proliferation potential.

We next asked whether *c-Myc* is a direct target of *Ovol2*. Cyclic amplification of selected targets analysis revealed an *Ovol2* consensus DNA-binding sequence that is almost identical to the *Ovol1* cognate sequence (3) (Fig. 5A). Because a conserved *Ovol1* consensus was previously discovered in a 1.6-kb *c-Myc* promoter fragment to mediate *Ovol1* repression (4), we performed reporter assays to ask whether *Ovol2* also represses the *c-Myc* promoter. Transfection of an *Ovol2A*-expressing construct repressed the promoter-driven luciferase activity in a dose-dependent manner (Fig. 5B), whereas transfection of a construct expressing *Ovol2B* or *Ovol2C* that lacks the functional SNAG repressor domain had no effect (data not shown). When the *Ovol* consensus binding site was deleted or mutated, repression by *Ovol2A* was reduced but not abolished (Fig. 5B), suggesting the presence of additional, noncanonical *Ovol2A*-responsive element(s). Indeed, a minimal *c-Myc* promoter that contains only 100 bp upstream (proximal region) of the transcriptional start site (4) was repressed by *Ovol2A* in both 293T and HaCaT cells (Fig. 5C). This region does not contain any *Ovol2* consensus but contains recognition sequences for Smad3 and E2F that mediate transcriptional repression (48–50). Although mutation of the Smad3-binding sequence had no negative effect, mutation of the E2F-binding sequence significantly compromised repression by *Ovol2A* (Fig. 5D and data not shown). In ChIP assays, *Ovol2* was found to occupy both the distal consensus site and the proximal nonca-

nonical site of the endogenous *c-Myc* promoter, with binding to the noncanonical site being significantly more prominent (Fig. 5E). Taken together, our findings demonstrate that *Ovol2A* represses *c-Myc* expression via direct binding to the *c-Myc* promoter.





**FIGURE 4. Up-regulated *c-Myc* expression in *Ovol2*-depleted cells and rescue of their transient growth by a *c-Myc* inhibitor.** *A* and *B*, increased *c-Myc* mRNA (*A*) ( $n = 3$ ) and protein production (*B*) in the absence of *Ovol2* 72 h after transfection. cDNA in quantitative real time PCR was normalized using glyceraldehyde-3-phosphate dehydrogenase. For the Western blot, Rad50 was used as a loading control (*cont*) for the nuclear extracts. *C* and *D*, results of first generation clonal assays in the presence of dimethyl sulfoxide (*DMSO*) (vehicle control) or 30  $\mu\text{M}$  *c-Myc* inhibitor. The inhibitor was present for the entire duration of the first generation culture. Representative plate images are shown in *C*, and quantitative analysis is shown in *D*. The average values with standard deviations were calculated from triplicate samples in a single experiment, and the results are representative of two independent experiments. *E*, representative histogram of FACS analysis of  $\alpha 6$  integrin (CD49f)-stained cells 72 h after transfection (two independent experiments each with duplicate samples). The *solid* and *dotted* lines indicate samples that are treated with dimethyl sulfoxide and the *c-Myc* inhibitor, respectively. *Red*, isotype control; *green*, control siRNA; *blue*, *Ovol2* siRNA.

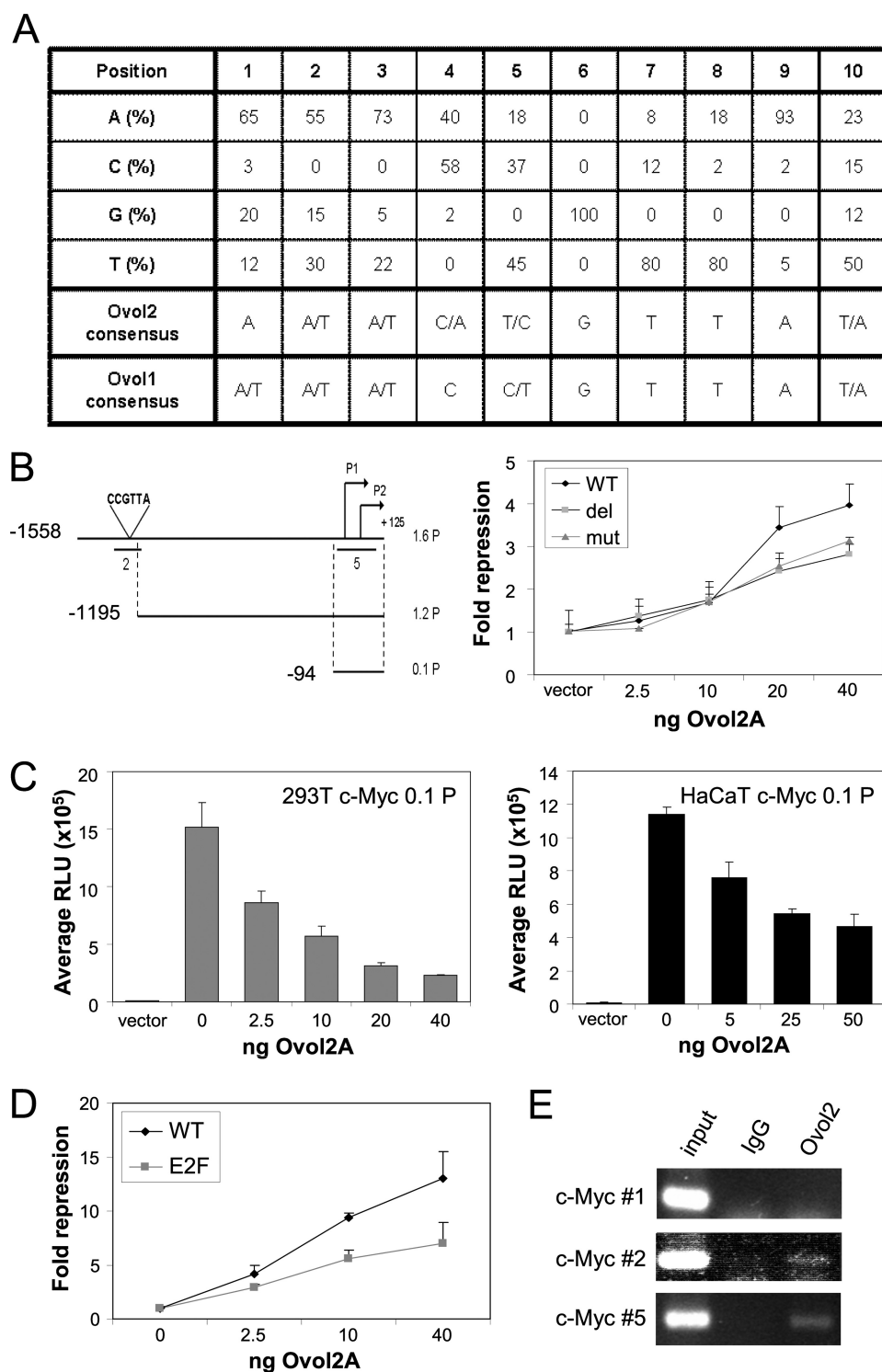
*Ovol2* Suppresses Keratinocyte Terminal Differentiation by Repressing *Notch1*—The growth and differentiation of keratinocytes are tightly linked. To test whether *Ovol2* depletion predisposes keratinocytes to terminal differentiation, we treated negative control and *Ovol2* knockdown cells with TGF- $\beta$  or calcium to induce growth arrest or terminal differentiation, respectively, and examined the expression of keratin 1 (K1) and loricrin, markers of terminal differentiation. Neither *Ovol2* knockdown or TGF- $\beta$  treatment alone was sufficient to induce K1 and loricrin expression (Fig. 6*A*). However, TGF- $\beta$  was able to induce loricrin expression when *Ovol2* was depleted. Moreover, induction of K1 and loricrin expression by calcium treatment was significantly more remarkable in *Ovol2*-depleted cells. The up-regulation of differentiation markers was also seen for *Ovol2* siRNAs #2 and #3 (supplemental Fig. S1*E*), indi-

cating that the premature differentiation is not an off target effect. To mimic conditions *in vivo*, we next turned to a three-dimensional organotypic culture system. Control or *Ovol2*-depleted HaCaT keratinocytes were grown on a fibroblast-collagen matrix under conditions that allowed the examination of early differentiation and stratification events. At a culturing time point when control samples showed expression of basal marker K14 but no detectable expression of differentiation marker K1 (51), *Ovol2*-depleted samples stained weakly for K14 but strongly for K1 (Fig. 6*B*). Collectively, our results suggest that *Ovol2* suppresses the differentiation tendency of keratinocytes; in its absence, keratinocytes are more prone to extracellular signal-induced terminal differentiation.

Notch signaling plays a pivotal role in promoting keratinocyte differentiation. We therefore wondered whether this sig-

**FIGURE 3. Increased proliferation rate and decreased proliferation potential of *Ovol2*-depleted keratinocytes.** *A*, a schematic diagram of keratinocyte stem cell evolution in culture.  $N_0$ , number of stem cells;  $N_1$  to  $N_M$ , number of progenitor cells;  $N_{M+1}$ , number of growth arrested cells;  $p_0$ , probability of the daughter cells of a stem cell to remain a stem cell;  $M$ , number of progenitor cell divisions;  $t_0$  and  $t_1$ , cell cycle times of a stem and a progenitor cell, respectively. *B*, the effects of varying  $t_0$  and  $t_1$  on cell number (ratio between knockdown and control cells) when  $p_0 = 0.5$  and  $M = 36$ . The *solid black line* in high density cultures (*panel a*) shows the expected ratio based on experimental data; note the absence of black line in second generation culture (*panel b*). The values set for control keratinocytes are marked by *dotted black lines*. *C*, the effects of varying  $t_1$  and  $p_0$  on cell number when  $M = 20$ . Note the presence of *black lines* (fitting to experimental data) in both high density (*panel a*) and second generation (*panel b*) cultures irrespective of varying  $p_0$ . *D*, FACS analysis using anti- $\alpha 6$  integrin-phycoerythrin (CD49f) antibody 72 h after transfection. Shown on the *left* are representative profiles from a single experiment, and the average values of three samples each from two independent experiments are shown on the *right*. The cut-off value used for quantitative analysis is indicated by the *dotted line*, with cells to the *right* of the line being scored as  $\alpha 6^{\text{high}}$  and those to the *left* being scored as  $\alpha 6^{\text{low}}$ . *cont*, control.

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**FIGURE 5. *Ovol2* directly represses *c-Myc*.** *A*, result of cyclic amplification of selected targets analysis revealing an *Ovol2* binding consensus, which is compared with the known *Ovol1* binding consensus (4). *B*, *left panel*, diagram (adapted from Ref. 4) of the human *c-Myc* promoter in plasmid 1.6 P (wild type pGL3-*c-Myc*) and in the deletion constructs 1.2 P-del and 0.1 P. Transcription start sites are indicated as P1 and P2. *Right panel*, repression of 1.6 P wild type and mutant promoters containing deletion (1.2 P-del) and point mutations (1.6 P-mut) of the CCGTTA *Ovol2* consensus binding site in 293T cells. *C*, *Ovol2* represses the *c-Myc* minimal promoter (0.1 P) lacking any *Ovol2* consensus. Shown are reporter assays in 293T (*left panel*) and HaCaT (*right panel*) cells. *RLU*, relative luciferase unit. *D*, *Ovol2* repression of the minimum promoter requires the presence of an E2F-binding site. Each *bar* represents the average of triplicate samples in a single experiment, and the results are representative of multiple experiments ( $n = 3$ ). The *error bars* are the standard deviations of triplicate samples. Luciferase activities were normalized for transfection efficiency by using a  $\beta$ -*actin* promoter driving *lacZ* as an internal control. *E*, ChIP assays showing *Ovol2* binding to both the distal consensus (site 2) and proximal nonconsensus regions (site 5) in the *c-Myc* promoter. See *B* for ChIP primer positions. An upstream region of the *c-Myc* promoter (outside of 1.6 P) was used as a control for nonspecific binding of *Ovol2* (site 1). *WT*, wild type.

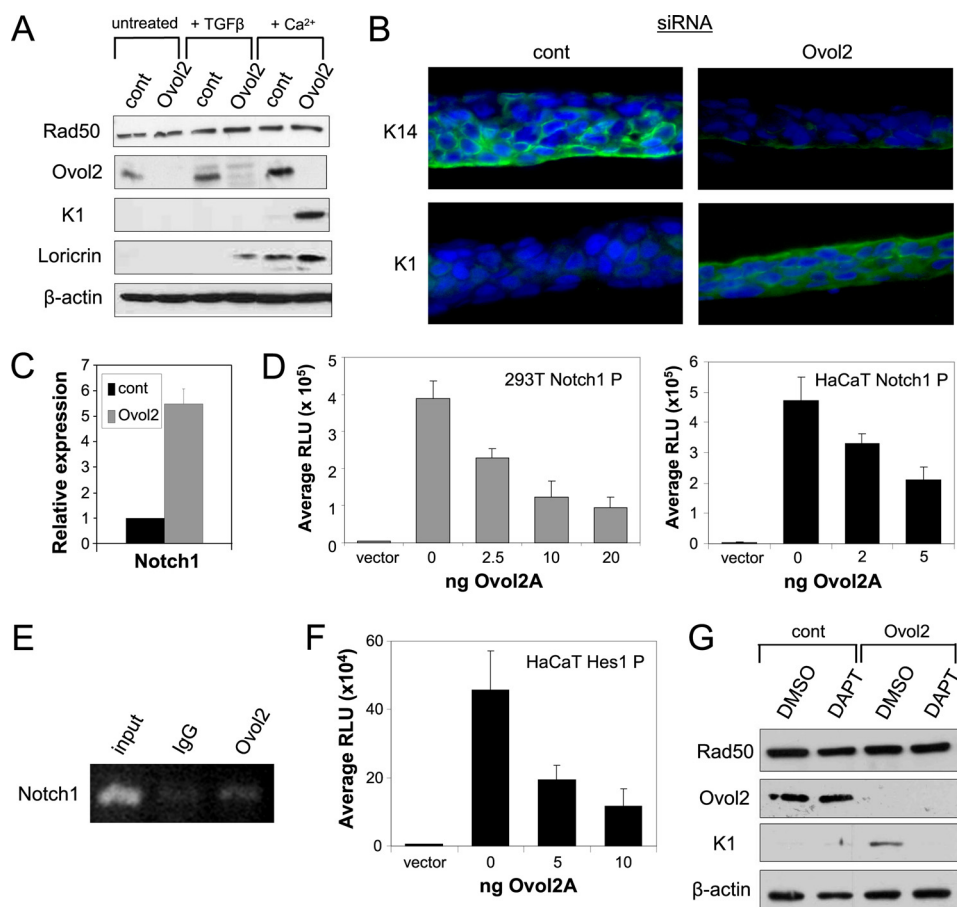
naling pathway is affected by the loss of *Ovol2*. Examination of our microarray data (to be published else where) revealed a 1.9-fold increase in *Notch1* transcript levels in *Ovol2*-depleted keratinocytes, whereas no change was seen for other *Notch* genes. Real time PCR confirmed *Notch1* up-regulation at 24, 48, and 72 h after transfection of *Ovol2* siRNA (Fig. 6C and data not shown). Consistent with this finding, we found that *Ovol2A* was able to repress the *Notch1* promoter in a dose-dependent manner in both 293T and HaCaT cells (Fig. 6D). ChIP analysis showed that *Ovol2* bound to the endogenous *Notch1* promoter (Fig. 6E), suggesting that *Notch1* is a direct target of *Ovol2*. Moreover, the promoter activity of *Hes1*, a known target of Notch signaling (52), was repressed by *Ovol2A* in a dose-dependent manner (Fig. 6F). These results demonstrate that *Ovol2* modulates Notch1 expression and downstream signaling.

To address the importance of Notch 1 in the role of *Ovol2* in regulating differentiation competence, we repeated the calcium induction experiment, this time including DAPT, a  $\gamma$ -secretase inhibitor that blocks Notch signaling (53, 54). The induction in K1 expression by the loss of *Ovol2* was completely abolished when DAPT was added (Fig. 6G). Different concentrations of DAPT were used and yielded similar findings (data not shown). In contrast, blocking Notch signaling did not rescue *Ovol2* depletion-induced loss of long term colony formation (data not shown). Therefore, we conclude that *Ovol2* normally suppresses Notch signaling to prevent premature terminal differentiation of keratinocyte progenitor cells.

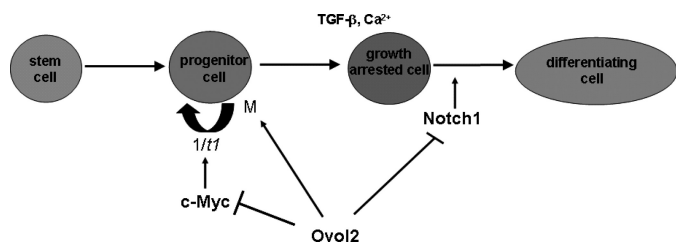
## DISCUSSION

Our study has elucidated an important function for *Ovol2* in human epidermal keratinocyte growth and differentiation. We have shown that *Ovol2* regulates three related cellular aspects: proliferation rate, proliferation potential, and differentia-





**FIGURE 6. Up-regulated *Notch1* expression in *Ovol2*-depleted cells and rescue of their accelerated differentiation by Notch signaling inhibitor DAPT.** *A*, HaCaT cells were transfected with either negative control (*cont*) or *Ovol2* siRNA and 72 h post-transfection were treated with either TGF- $\beta$  or Ca<sup>2+</sup> to induce growth arrest or terminal differentiation, respectively. Western blots were performed to examine the expression of K1 and Loricrin. *B*, representative results of three-dimensional culture to show that *Ovol2* depletion leads to precocious K1 expression. *C*, *Ovol2* knockdown cells show elevated *Notch1* mRNA expression 72 h after transfection ( $n = 3$ ). *D*, *Ovol2* represses the *Notch1* promoter in both 293T cells (*left panel*) and HaCaT cells (*right panel*). See legend to Fig. 5 for more information. *E*, ChIP assay showing that *Ovol2* binds to the *Notch1* promoter. The upstream region of the *c-Myc* promoter (Fig. 5*D*, site 1) was used as a negative control. *F*, *Ovol2* represses the *Hes1* promoter in HaCaT cells. *G*, differentiation assay was performed as in *A*, except that dimethyl sulfoxide (DMSO) or DAPT was added to the cells before differentiation; shown are Ca<sup>2+</sup>-treated samples.



**FIGURE 7. Working model of *Ovol2* function in keratinocyte growth and differentiation.** The arrows indicate positive regulation, whereas blunted lines indicate negative regulation.

tion tendency (Fig. 7). In doing so, *Ovol2* contributes to the overall genetic program that maintains keratinocytes in a proliferation-competent and differentiation-resistant state, that of a stem/progenitor cell.

*Balancing Cell Cycling with Long Term Proliferation Potential in a Single Progenitor Cell Population: Role of Ovol2*—Loss of *Ovol2* leads to an initial transient increase in growth but decreased long term clonogenicity. Similar cellular changes have been reported for keratinocytes deficient for Rac1 or over-

expressing *c-Myc* (20–22, 55–57). In a widely accepted model of epidermal homeostasis, it is posited that stem cells that reside in the epidermal basal layers divide infrequently to give rise to TA cells that cycle faster but are destined to terminally differentiate after a few rounds of cell divisions (42). In the framework of this model, the growth phenomenon of *c-Myc*- and Rac1-altered cells has been explained by a differential effect of these genes on the different cell type populations present in a heterogeneous keratinocyte culture. One might envision that exit of the so-called stem cells from a slow cycling state will increase the percentage of actively cycling TA cells, thereby resulting in a transient burst in growth. However, because the number of cell divisions that a TA cell can undergo is limited (58), this will ultimately cause a depletion of cells with long term proliferation potential. In an alternative model, misregulation of these genes might affect a single population of progenitor cells, making them cycle faster, thereby exhausting the number of cell divisions that they are programmed to undergo. Our mathematical modeling of the behavior of control and *Ovol2*-depleted keratinocytes presents a scenario that is more consistent with the second model. Reducing the cell cycle time

( $t_1$ ) and proliferation potential ( $M$ ) of the progenitor cell population indeed can recreate the observed increase in transient growth and decrease in clonogenicity, whereas changing the cell cycle time ( $t_0$ ) and/or exit probability ( $p_0$ ) of a slow cycling stem cell population cannot. From this analysis, we infer that although there clearly is heterogeneity in terms of proliferation rate (slow cycling *versus* fast cycling) in cultured keratinocytes, such heterogeneity may not be functionally relevant to the growth of these cells. This said, it is important to note that our modeling was done using previously described experimental parameters, namely that  $\sim 10\%$  of cultured HaCaT keratinocytes are slow cycling stem cells and that these stem cells cycle at a rate that is half of the TA cells (28). These constraints dictate that the rare presence of slow cycling stem cells generates a minimal impact on overall culture growth.

In principle, our findings are consistent with the recently proposed model that a single population of proliferating progenitor cells is sufficient to maintain normal homeostasis of mouse tail epidermis (42, 59, 60) and suggest that the same might be true for cultured human keratinocytes. More impor-

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tantly, our studies uncover an important novel player, transcription factor *Ovol2*, that regulates the cellular activities of keratinocytes. If a normal progenitor cell has an intrinsically set number of cell cycles that they can undergo (*i.e.* a set proliferation potential), then when cycling faster (decreasing  $t_1$ ), they will exit the cell cycle faster. This calls for means that keep proliferation rate in check to achieve populational longevity. Moreover, any molecular alterations that minimize this proliferation potential (*i.e.* decreasing  $M$ ) will cause premature growth arrest. It is likely that both mechanisms (proliferation rate and potential) account for the observed reduction in the number of total cycling cells as well as long term clonogenicity in *Ovol2*-depleted culture. As such, our combinatory experimental and modeling approach sets a useful paradigm to study the function of other critical regulators of stem/progenitor cell homeostasis in epidermis as well as in other tissues.

**Molecular Mechanism of *Ovol2* Function in Controlling Keratinocyte Proliferation**—Consistent with the finding of similar phenotypes caused by *Ovol2* depletion and *c-Myc* overexpression, our results demonstrate that *c-Myc* up-regulation accounts for the transiently increased proliferation in *Ovol2* knockdown cells. However, inhibiting *c-Myc* function in these cells is not sufficient to rescue the loss of long term proliferation potential. These data corroborates our modeling finding that *Ovol2* independently regulates proliferation rate and proliferation potential. Although its role in the proliferation rate control is in part mediated by repressing *c-Myc* expression, *Ovol2* must regulate additional molecular events to control proliferation potential.

The finding that *Ovol2*, like *Ovol1*, also represses *c-Myc* is intriguing. *Ovol1* is predominantly expressed in suprabasal cells, whereas *Ovol2* is predominantly expressed in basal cells. However, our previous analysis of *Ovol1/Ovol2* compound mutants has provided evidence for possible functional redundancy and compensation between the two *Ovol* genes in an *in vivo* setting (17). Sharing a common molecular target offers a partial explanation for this observation. Importantly, despite regulating a common target, depletion of the two *Ovol* proteins leads to apparently opposite cell biological outcomes because of their different sites of expression: loss of *Ovol1* results in expansion of late epidermal progenitor cells, whereas loss of *Ovol2* results in depletion of long term proliferating keratinocytes. This further highlights the importance of intricate *c-Myc* regulation at multiple stages of keratinocyte progenitor cell evolution: down-regulation of *c-Myc* is important for both keeping a long-lived progenitor cell population and initiating postmitotic differentiation (19). Further studies are now necessary to examine whether *Ovol2* is also required for maintaining epidermal progenitor cells *in vivo*.

Another interesting finding of our study is that *Ovol2* is able to repress transcription via an E2F-binding sequence. We have previously shown that *Ovol1* also represses the *c-Myc* minimal promoter, where this E2F site resides (4). It is possible that DNA binding via an E2F protein underlies both *Ovol1* and *Ovol2* repression of *c-Myc*. A likely candidate is E2F4/5, which is known to form a repressor complex with Smad3 and p107 to repress *c-Myc* expression (48–50). Additional studies are needed to test this notion.

***Ovol2*, *Notch1*, and Keratinocyte Differentiation**—Our work highlights a role for *Ovol2* in negatively modulating terminal differentiation of keratinocytes even when differentiation-inducing signals are present. However, when such signals are absent, the loss of *Ovol2* alone is insufficient to trigger differentiation, suggesting that *Ovol2* is not a master switch of differentiation but instead regulates differentiation competence. We have shown that *Ovol2* performs this function by repressing *Notch1* expression as well as downstream signaling. Previous studies have underscored the importance of down-regulating Notch signaling in basal progenitor cells to prevent premature spinous cell differentiation (23) and shown that epidermal growth factor receptor signaling negatively regulate *Notch1* transcription. While epidermal growth factor receptor repression of *Notch1* is via an indirect mechanism that involves tumor suppressor p53 (30), we have found *Notch1* to be a direct transcriptional target of *Ovol2*. Together, these findings indicate that multiple mechanisms are in place to repress *Notch1* expression and activity in the proliferating epidermal progenitor cells.

The ability to suppress differentiation is conceivably very important for normal tissue progenitor cells to maintain a differentiation-resistant state, but this ability, when hijacked by malignant cells, can lead to differentiation refractory tumor growth. In fact aberrant epidermal growth factor receptor signaling is frequently linked to tumorigenesis. Importantly, *Ovol2* not only suppresses terminal differentiation but also poses a limit on the rate of proliferation in keratinocyte progenitor cells. This may be significant for developmental progenitor cells to maintain an undifferentiated state while restricting unwanted growth. Studies of tissue-specific knock-out mouse models are ongoing to examine whether the same holds true *in vivo*.

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