Defects in transforming growth factor-β signaling cooperate with a *Ras* oncogene to cause rapid aneuploidy and malignant transformation of mouse keratinocytes

Adam Glick*†, Nicholas Popescu‡, Valarie Alexander*, Hikaru Ueno§, Erwin Bottinger¶, and Stuart H. Yuspa*

*Laboratory of Cellular Carcinogenesis and Tumor Promotion, and ‡Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD 20892; §Department of Cardiology, Kyushu University School of Medicine, Fukuoka 812-8582, Japan; and ¶Albert Einstein College of Medicine, Bronx, NY 10461

Edited by Michael Potter, National Institutes of Health, Bethesda, MD, and approved October 26, 1999 (received for review August 12, 1999)

Genetic inactivation of the transforming growth factor-β (TGF-β) signaling pathway can accelerate tumor progression in the mouse epidermal model of multistage carcinogenesis. By using an *in vitro* model of keratinocyte transformation that parallels *in vivo* malignant conversion to squamous cell carcinoma, we show that v-*ras*^{Ha} transduced primary TGF-β1−/− keratinocytes and keratinocytes expressing a TGF-β type II dominant-negative receptor transgene have significantly higher frequencies of spontaneous transformation than control genotypes. Malignant transformation in the TGF-β1−/− keratinocytes is preceded by aneuploidy and accumulation of chromosomal aberrations. Similarly, transient inactivation of TGF-β signaling with a type II dominant-negative receptor adenovirus causes rapid changes in ploidy. Exogenous TGF-β1 can suppress aneuploidy, chromosome breaks, and malignant transformation of the TGF-β1−/− keratinocytes at concentrations that do not significantly arrest cell proliferation. These results point to genomic instability as a mechanism by which defects in TGF-β signaling could accelerate tumor progression in mouse multistage carcinogenesis.

Defects in transforming growth factor-β (TGF-β) signaling are a frequent occurrence in cancer (1–4). In the mouse multistage skin-carcinogenesis model, loss of TGF-β1 expression is associated with accelerated progression *in vivo* (5, 6), and in the presence of a *ras* oncogene, genetic inactivation of autocrine TGF-β1 production or the TGF-β receptor yields squamous cell carcinoma (SCC) (7, 8). Nonneoplastic, immortal TGF-β1−/− keratinocyte cell lines exhibit an increased frequency of drug-induced gene amplification as compared with TGF-β1+/− controls (9), suggesting that defects in autocrine TGF-β1 secretion can cause genomic instability, which could be a mechanism for accelerated progression. Because expression of many genes involved in carcinogenesis, such as matrix proteins, integrins, metalloproteinases, and cytokines, is regulated by TGF-β1 (10), it is crucial to establish whether accelerated progression observed *in vivo* with v-*ras*^{Ha}-transduced TGF-β1−/− keratinocytes is due to TGF-β-regulated phenotypic changes or to genotypic changes resulting from decreased genomic stability. To address this question, we have assessed the effect of genetic inactivation of TGF-β signaling on the *in vitro* transformation frequency of v-*ras*^{Ha}-transduced keratinocytes, utilizing a well established *in vitro* assay that measures the acquisition of resistance to the growth arrest and differentiation signal induced by elevated medium calcium (11). Previous studies have shown that cells that become resistant to the calcium signal have *in vitro* and *in vivo* properties similar to cells derived from SCC, and, conversely, cells isolated from SCC are able to proliferate in medium with elevated calcium (11–13). We show that keratinocytes that are TGF-β1−/− (14) or express a TGF-β type II (TβRII) dominant negative receptor transgene (15) have a significantly higher frequency of transformation than control keratinocytes. In the TGF-β1−/− cells, transformation is preceded by the rapid development of aneuploidy and chromosomal aberrations. Transient inhibition of TGF-β signaling with a dominant negative TβRII adenovirus also provokes rapid changes in ploidy. Treatment with exogenous TGF-β1 suppresses *in vitro* conversion aneuploidy and chromosomal abnormalities. These results suggest that *in vivo* defects in TGF-β signaling may have a significant impact on the rate of genetic change that occurs during malignant progression.

Materials and Methods

Cell Culture. The TGF-β1−/− allele was bred onto a BALB/c background by mating TGF-β1+/− with BALB/c mice. Animals used were in the fourth- to sixth-generation cross. The AM3 transgenic line contains a truncated TβRII linked to a metallotheinen promoter and is on an FVB/n background (16). Epidermal keratinocytes were isolated from PCR-genotyped (5, 15) newborn mice by standard methods (17). Cells were cultured in Eagle's minimal essential medium/8% Chelex-treated FBS/0.05 mM $CaCl_2$ with antibiotics.

The v-*ras*^{Ha} replication-defective ecotropic retrovirus was prepared as described (18). The titer of virus was 1–2 $\times$ 10^7 virus/ml. The recombinant adenoviruses AdTGFβTR (19) and Adβgal (a gift of Luowei Li, National Cancer Institute) were prepared by using 293 producer cells and cesium chloride gradient purification (20). Keratinocytes were infected at a multiplicity of infection of 2–3 viruses per cell with the v-*ras*^{Ha} retrovirus and a multiplicity of infection of 40 viruses per cell with the recombinant adenoviruses. All virus infections were done in the presence of 4 μg/ml Polybrene (Sigma).

Assay for Malignant Conversion. Primary keratinocytes were seeded in 60-mm culture dishes, retrovirally infected on day 3, and then cultured for 15 days further in Eagle's minimal essential medium containing 0.05 mM calcium and 8% FBS. The cells were then switched to 0.5 mM calcium medium for 4–6 weeks, and colonies of calcium-resistant keratinocytes were identified by staining the dishes with 0.35% rhodamine/10% formalin and counted with a dissecting microscope. The number of colonies obtained was normalized to the cell number present before the calcium switch (11). Foci isolated from this assay produce

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: TGF-β, transforming growth factor-β; SCC, squamous cell carcinoma; TβRII, TGF-β type II receptor.

†To whom reprint requests should be addressed at: Laboratory of Cellular Carcinogenesis and Tumor Promotion, Building 37 3B-19, National Cancer Institute, Bethesda, MD 20892. E-mail: glicka@dc37.nci.nih.gov.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

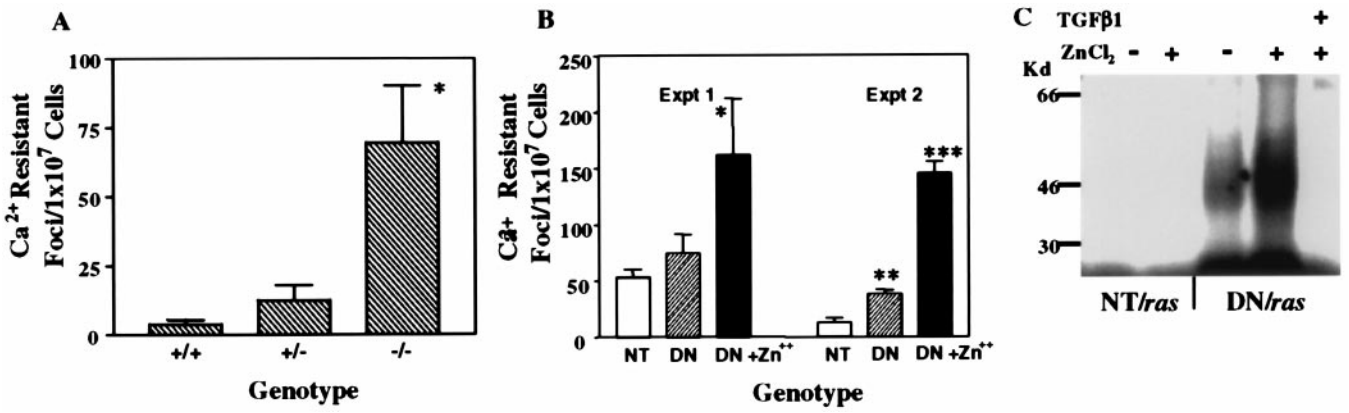

Fig. 1. v-*ras*^{Ha}-transduced TGF-$\beta-/-$ and AM3 dominant negative TβRII transgenic keratinocytes exhibit increased frequency of *in vitro* malignant conversion. The indicated cell types were infected with the v-*ras*^{Ha} retrovirus on day 3 and allowed to proliferate for 15 days before selection in 0.5 mM calcium as described in *Materials and Methods*. (*A*) Calcium-resistant foci per dish generated by TGF-$\beta1+/+$, TGF-$\beta1+/-$, and TGF-$\beta1-/-$ keratinocytes. Each bar represents the mean of nine independent experiments each with 7–10 dishes per genotype ±SEM. *, Significantly different from $+/+$ ($P = 0.0027$) and $+/-$ ($P = 0.0083$). (*B*) Calcium-resistant foci per dish generated by FVB/n control (NT), AM3 keratinocytes (DN), and AM3 keratinocytes cultured in the presence of $ZnCl_2$ (DN+Zn). Each bar represents the average of six dishes ±SEM. Two independent experiments are shown. $ZnCl_2$ did not alter the frequency of foci in the NT cultures. *, **, ***, Significantly different from NT ($P = 0.024$, 0.0009, 0.0001, respectively). (*C*) The truncated TβRII is induced by $ZnCl_2$ in the v-*ras*^{Ha}-transduced AM3 keratinocytes. ^{125}I-labeled TGF-$\beta1$ was crosslinked to monolayers of NT or AM3 cells cultured in the presence (+) or absence (−) of 25 mM $ZnCl_2$ for 24 hr. The crosslinked products were electrophoresed through a precast 4–12% polyacrylamide gel (NOVEX, San Diego). The truncated TβRII runs at a lower molecular mass than the native TβRII. The + in the TGF-$\beta1$ row indicates crosslinking in the presence of 100 nM unlabeled TGF-$\beta1$. Kd, kilodaltons.

carcinomas when tested *in vivo* in a skin graft (12). TGF-$\beta1$ was added 3 days after v-*ras*^{Ha} infection and maintained throughout the experiment, with media changes every 2 days. To induce expression of the dominant negative TβRII, AM3 keratinocytes were cultured in 25 μM $ZnCl_2$ 3 days after transduction with v-*ras*^{Ha}. ^{125}I-labeled TGF-$\beta1$ crosslinking was done as previously described (21). TGF-$\beta1$ secreted in 24-hr serum-free-conditioned medium was detected by using a sandwich ELISA (22). The amount of secreted TGF-$\beta1$ detected was normalized to the cell number for each genotype. Inhibition of [^{3}H]thymidine incorporation by TGF-$\beta1$ (21) was measured 8 days after v-*ras*^{Ha} transduction in complete medium with cells plated at 1×10^5 cells per well in 24-well culture trays.

Cell Cycle Analysis. Subconfluent cells were pulsed with 10 μM BrdUrd (Sigma) for 1hr, trypsinized, fixed in 70% ethanol, and then stained with either an FITC-labeled anti-BrdUrd monoclonal antibody and propidium iodide or propidium iodide alone, according to the manufacturer's specifications (Becton Dickinson). Stained cells were analyzed by flow cytometry. Asynchronous monolayers were irradiated in PBS by using a Mark I cesium source at a rate of 456 rad/min, or treated with 0.2 μg/ml nocodazole (Sigma) for 30 hr or 5 mM hydroxyurea (Sigma) for 18 hr before a 1-hr pulse with BrdUrd and harvest.

Chromosome Analysis. Cells were treated for 3 hr with 0.1 μg/ml colchicine (Sigma), and chromosomes were prepared from metaphase cells by standard methods (23). Giemsa-stained metaphase spreads were identified, and the chromosome number per metaphase was counted by using an image captured with a video camera. Between 50 and 100 total metaphases were counted for each genotype at every time point from two or three independent experiments. Chromosomal aberrations were identified in a blinded manner on Giemsa-stained metaphase spreads according to standard cytogenetic nomenclature (24). For TGF-$\beta1$ treatment, TGF-$\beta1-/-$ keratinocytes were treated with 50 pg/ml porcine TGF-$\beta1$ (R & D Systems) in complete media, starting on day 3 after transduction with the v-*ras*^{Ha} retrovirus. Treatment was continued for 15 days, with the media being changed every 2 days.

Experimental Timeline. Primary mouse keratinocytes were infected with the v-*ras*^{Ha} retrovirus on day 3 of culture and, if indicated, with the recombinant adenoviruses on day 6 of culture (day 3 after retrovirus infection). Metaphase cells were harvested for chromosome analysis on days 4, 8, and 15 after infection with the v-*ras*^{Ha} retrovirus, and for flow cytometric analysis on days 4 and 8 after superinfection with the adenoviruses. Fifteen days after v-*ras*^{Ha} infection, cells were switched to 0.5 mM calcium medium to select for calcium-resistant foci.

Results

Defects in TGF-β Signaling Elevate Ras-Induced Malignant Conversion *in Vitro*. Primary keratinocytes from TGF-$\beta1-/-$ (14) and AM3 transgenic mice expressing a dominant negative TβRII (15, 16) were infected with the replication-defective retrovirus expressing v-*ras*^{Ha} and assayed for frequencies of malignant conversion *in vitro* (11). After retroviral infection on day 3, keratinocytes were allowed to proliferate for 15 days and then were switched to medium with elevated calcium (11). In this assay, the majority of v-*ras*^{Ha}-transduced keratinocytes undergo a growth arrest in response to elevated calcium, but rare clones of cells that are resistant to the growth arrest are able to proliferate and form colonies within the lawn of arrested cells (11). Cells resistant to elevated calcium are homologous to SCCs generated by chemical carcinogenesis protocols *in vivo* (11–13).

v-*ras*^{Ha}-transduced TGF-$\beta1-/-$ and AM3 keratinocytes generated spontaneous calcium-resistant colonies at frequencies significantly higher than the normal controls (Fig. 1). Fig. 1*A* shows that the TGF-$\beta1-/-$ keratinocytes generated foci at a frequency 17-fold higher than the TGF-$\beta1$ $+/+$ keratinocytes (6.9×10^{-6} foci per cell vs. 3.9×10^{-7} foci per cell) and 5-fold higher than the TGF-$\beta1$ $+/-$ keratinocytes (1.25×10^{-6} foci per cell). The difference between the TGF-$\beta1+/+$ and $+/-$ cells was about 3-fold, suggestive of a hemizygous dose effect, but did not reach statistical significance. When assayed with a TGF-$\beta1$ sandwich ELISA (22), TGF-$\beta1$ was undetectable in conditioned media from the TGF-$\beta1-/-$ cells, whereas the TGF-$\beta1+/+$ and TGF-$\beta1+/-$ keratinocytes secreted 80 ± 10 and 20 ± 3 pg/ml per 1×10^6 cells, respectively. DNA was isolated from several TGF-$\beta1+/-$ foci and tested for the presence of the

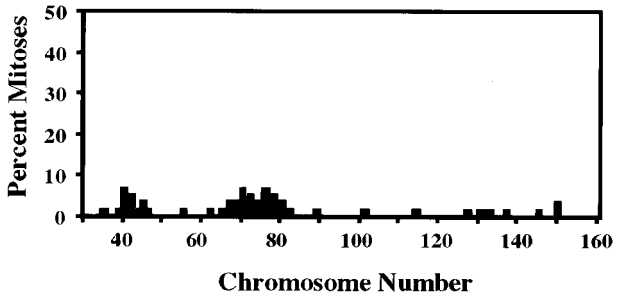

Fig. 2. TGF-β1$-/-$ calcium-resistant cells are aneuploid. Foci of calcium-resistant cells were pooled and expanded in 0.05 mM calcium medium for five passages before analysis of chromosome number as described in *Materials and Methods*. The histogram represents the percentage of the total metaphases analyzed (n = 60) with a specific chromosome number.

wild-type TGF-β1 allele by PCR. Six of six foci retained the wild-type allele, supporting the idea that reduced levels of autocrine TGF-β1 enhance spontaneous conversion (data not shown). Additionally, pooled calcium-resistant TGF-β1$-/-$ keratinocytes retained both the TGF-β type I and type II receptors and sensitivity to growth inhibition by exogenous TGF-β1$-/-$, as measured by inhibition of [^{3}H]thymidine incorporation (data not shown). These data indicate that other alterations in the TGF-β signaling pathway were not responsible for the conversion of these cells to calcium resistance.

Similar to the TGF-β1$-/-$ keratinocytes, the frequency of calcium-resistant foci generated by the AM3 transgenic keratinocytes was 2- to 3-fold higher than keratinocytes derived from the nontransgenic FVB/n littermates (Fig. 1*B*). Addition of $ZnCl_2$ to the culture medium increased expression of the dominant negative TβRII transgene (Fig. 1*C*), reduced the IC_{50} for growth inhibition to exogenous TGF-β1 from 100 pg/ml to 1 ng/ml (data not shown), and significantly increased the frequency of calcium-resistant foci. The higher level of foci between the two control keratinocyte cultures is likely due to genetic differences in the inbred strains of the two transgenic lines (BALB/c vs. FVB/n). Thus, the lack of autocrine TGF-β1 secretion and blocked TβRII signaling both increase the *in vitro* frequency of spontaneous malignant conversion.

Calcium-Resistant TGF-β1$-/-$ Keratinocytes Are Aneuploid. Aneuploidy is a hallmark of SCC induced in mouse epidermis with chemical carcinogens (25). To determine whether the calcium-resistant cells that developed from the TGF-β1$-/-$ keratinocytes had karyotypic changes associated with malignancy, chromosomes from metaphase cells were isolated and counted. Fig. 2 shows the percentage of the total metaphases analyzed with a specific chromosome number. Only 5–10% of the cells had a true diploid or tetraploid chromosome number, whereas the majority were aneuploid, having either a hyperdiploid (42 to 48) or hypotetraploid (65 to 75) chromosome number instead of an exact multiple of the diploid set (40).

Aneuploidy Is an Early Event in TGF-β1$-/-$ Keratinocytes After v-*ras*^{Ha} Transduction. To test whether aneuploidy developed before the selection for calcium resistance, chromosome numbers were determined in metaphase cells of TGF-β1$+/+$ and TGF-β1$-/-$ genotypes at different time points after v-*ras*^{Ha} transduction. Fig. 3 shows that 4 days after introduction of v-*ras*^{Ha}, nearly 50% of the metaphase spreads were diploid in both the TGF-β1$+/+$ (Fig. 3*A*) and TGF-β1$-/-$ (Fig. 3*D*) keratinocytes, with some tetraploid and aneuploid cells in both genotypes. By 8 days after v-*ras*^{Ha} infection, there was a significant increase in the percentage of tetraploid and octaploid metaphases in both genotypes and a decrease in the percentage of diploid cells (Fig. 3 *B* and *E*). These ploidy changes differ significantly from uninfected cells of both genotypes, which

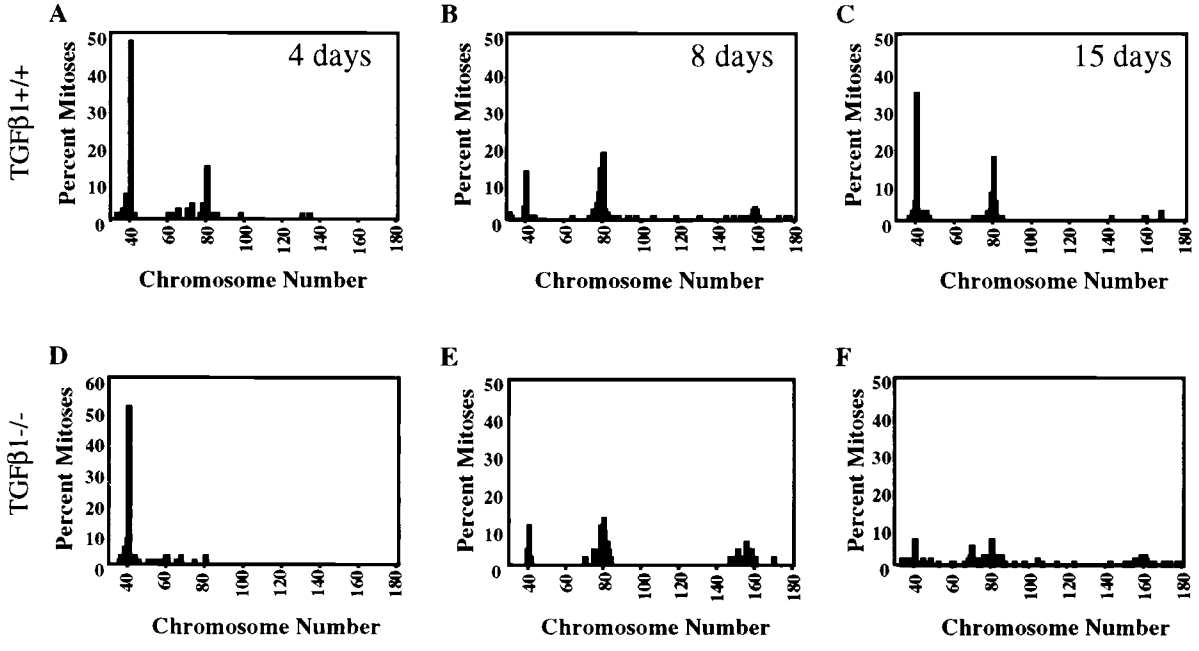

Fig. 3. Rapid aneuploidy in TGF-β1$-/-$ keratinocytes after transduction with v-*ras*^{Ha}. Percentages of metaphases with specific chromosome numbers in TGF-β1$+/+$ and TGF-β1$-/-$ keratinocytes at the indicated times after infection with the v-*ras*^{Ha} retrovirus. Each histogram represents the sum of three independent chromosome harvests. Between 50 and 100 metaphases were counted for each genotype at a specific time point, and the percentage of total mitoses with a specific chromosome number was plotted. Metaphase spreads with an extremely high chromosome number were excluded from the histogram for uniformity. (*A–C*) TGF-β1$+/+$ keratinocytes. (*D–F*) TGF-β1$-/-$ keratinocytes. Results are for 4 days (*A* and *D*), 8 days (*B* and *E*), and 15 days (*C* and *F*) after infection of TGF-β1$+/+$ and TGF-β1$-/-$ keratinocytes with v-*ras*^{Ha}.

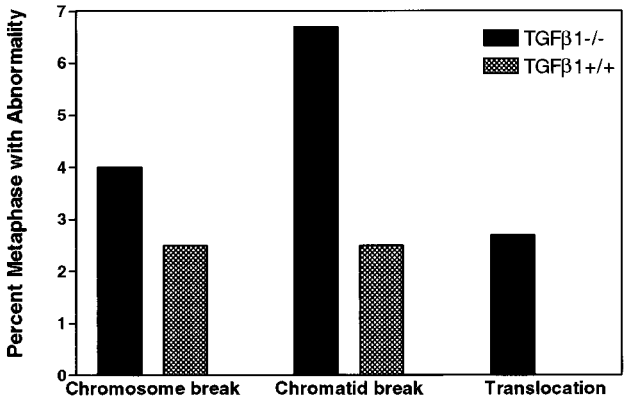

Fig. 4. v-*ras*^{Ha}-transduced TGF-β1$-/-$ keratinocytes have a higher frequency of chromosomal aberrations. Giemsa-stained chromosomes were isolated from keratinocyte cultures 15 days after v-*ras*^{Ha} transduction and analyzed for chromosomal abnormalities. $n = 70$ for TGF-β1$-/-$ cells; $n = 40$ for TGF-β1$+/+$ cells.

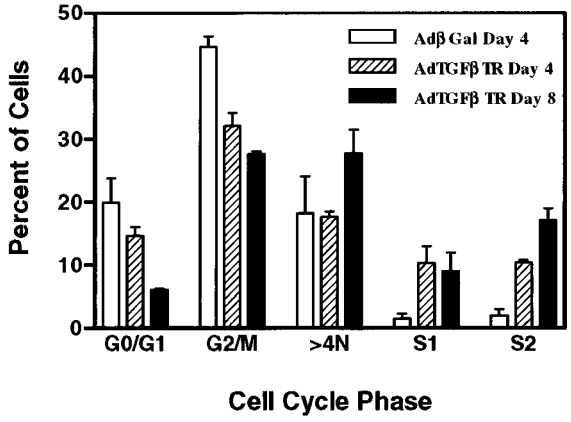

Fig. 5. Transient inactivation of TGF-β type II receptor causes rapid changes in ploidy. Percentage of cells in each phase of the cell cycle in normal and v-*ras*^{Ha}-infected BALB/c keratinocytes 4 and 8 days after superinfection with AdTGFβTR and 4 days after superinfection with Adβgal. The percentage of cells in each cell-cycle compartment was determined by two-color flow cytometric analysis as described in *Materials and Methods*. The S1 population represents the diploid S phase, and the S2 population represents the tetraploid S phase. Each bar is the average of two independent experiments.

remain predominantly diploid throughout the culture lifespan (data not shown). However, after 15 days, 70% of the TGF-β1$+/+$ v-*ras*^{Ha} cells capable of cycling had a diploid or tetraploid karyotype, whereas 80% of the TGF-β1$-/-$ v-*ras*^{Ha} cells were aneuploid, with chromosome numbers predominantly between 40 and 80 (Fig. 3 *C* and *F*). These results were confirmed by fluorescence-activated cell sorter analysis of anti-BrdUrd and propidium iodide-stained cells (data not shown). To determine whether the development of aneuploidy in the TGF-β1$-/-$ v-*ras*^{Ha} cells was associated with increased DNA damage, metaphase preparations from day 15 post-v-*ras*^{Ha} cultures were scored for karyotypic abnormalities. Of the TGF-β1$-/-$ cells, 13% contained translocations, chromosome breaks, or chromatid breaks, as compared with 5% of the TGF-β1$+/+$ cells. Fig. 4 shows the distribution of specific abnormalities for each genotype. Additionally, many of the TGF-β1$-/-$ metaphases contained multiple abnormalities, leading to 0.27 total abnormality per metaphase (20/74), whereas none of the TGF-β1$+/+$ v-*ras*^{Ha} cells contained multiple breaks or translocations, with 0.05 total abnormality per metaphase (2/40). Thus, the high frequency of aneuploidy is associated with a lower but significantly elevated frequency of chromosomal abnormalities.

To test for altered checkpoint function, v-*ras*^{Ha}-infected TGF-β1$-/-$ and TGF-β1$+/+$ keratinocytes were treated with γ irradiation (2 Gy), nocodazole (0.2 μg/ml), or hydroxyurea (5 mM). In both genotypes, γ irradiation and hydroxyurea caused a similar cell-cycle arrest, with a decrease in S phase from 26–27% to 1–2% and a corresponding increase in percentage of cells in cell-cycle stages G_0/G_1 and G_2/M. Similarly, treatment with nocodazole also caused a rapid inhibition of DNA synthesis and an increase in the percentage of cells in G_2/M from 32% to 60% in both genotypes. There was no evidence for escape of either genotype from the nocodazole block with prolonged treatment time or for differential sensitivity at reduced concentrations (data not shown).

Transient Interruption of TGF-β Signaling Causes Changes in Ploidy. To test whether inactivation of TGF-β signaling in a v-*ras*^{Ha}-transduced wild-type keratinocyte would provoke similar changes in ploidy, BALB/c keratinocytes were sequentially infected with the v-*ras*^{Ha} retrovirus and an adenovirus expressing a dominant negative TβRII (AdTGFβTR) (19), or a control adenovirus (Adβgal). Initial studies showed that resistance to growth inhibition by exogenous TGF-β1 was maximal 2 days after AdTGFβTR infection, but by 8 days postinfection, the cells had reacquired sensitivity to TGF-β (data not shown). v-*ras*^{Ha} induced a significant increase in the fraction of tetraploid cells in the BALB/c keratinocytes that was not altered significantly 4 days after inactivation of TGF-β signaling by superinfection with AdTGFβTR (Fig. 5). Tetraploidy is independent of the TGF-β signaling status because it is observed in v-*ras*^{Ha}-transduced cells of all genotypes. However, between 4 and 8 days after AdTGFβTR infection there was a progressive decrease in the percentage of diploid G_0/G_1 cells from 19% to 6%, and an increase in cycling cells with a DNA content of >4N (S2) to 19%. These changes in ploidy did not occur in the Adβgal-infected cells.

Exogenous TGF-β1 Suppresses Malignant Conversion and Aneuploidy. To further support a link between inactivation of TGF-β signaling, aneuploidy, and malignant conversion, v-*ras*^{Ha}-transduced TGF-β1$-/-$ keratinocytes were treated with exogenous TGF-β1 and assayed for frequency of calcium resistance and aneuploidy. Treatment of the TGF-β1$-/-$ cells with exogenous TGF-β1 in the conversion assay caused a dose-dependent reduction in the number of calcium-resistant foci generated by the v-*ras*^{Ha}-transduced cells, with as little as 25 pg/ml causing a 3- to 4-fold reduction in malignant foci (Fig. 6*A*). It is unlikely that suppression simply results from a TGF-β1-induced growth arrest, because in short-term assays under similar conditions of cell density the IC_{50} for growth inhibition of v-*ras*^{Ha}-transduced keratinocytes is $\approx$300 pg/ml (Fig. 6*C*). When TGF-β1$-/-$ v-*ras*^{Ha} cells were treated with 50 pg/ml TGF-β1 for 18 days, the percentage of aneuploid metaphases was reduced from 80% to 47%, although there was no effect on the frequency of tetraploid cells (Fig. 6*B*). Flow cytometric analysis of parallel dishes showed that in both TGF-β1-treated and -untreated cultures, the percentage of cells in S phase was 20% and 21%, respectively. In addition, the number of detectable chromosome breaks in the v-*ras*^{Ha}-transduced TGF-β1$-/-$ cells was reduced from 0.27 break per metaphase to 0.058 break per metaphase. These results indicate that the development of aneuploidy and chromosome breaks in the TGF-β1$-/-$ keratinocytes is directly related to the lack of autocrine TGF-β signaling. Reactivation of this signaling pathway by the addition of exogenous TGF-β1 can suppress malignant conversion *in vitro* as well as associated genetic change.

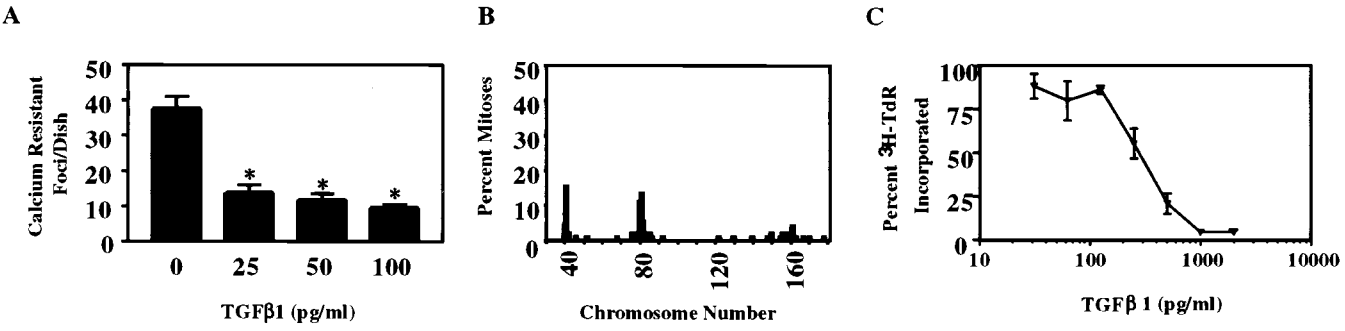

Fig. 6. Suppression of malignant conversion and aneuploidy by exogenous TGF-β1. (*A*) TGF-β1 suppresses the frequency of calcium-resistant foci in TGF-β1$-/-$ keratinocytes. The indicated doses of TGF-β1 were added to keratinocytes 3 days after infection with v-*ras*^{Ha} and maintained through the course of the experiment. Each histogram represents the average of five to seven dishes. *, Significantly different from untreated keratinocytes ($P < 0.0001$). (*B*) The development of aneuploidy in TGF-β1$-/-$ v-*ras*^{Ha} keratinocytes is suppressed by treatment with TGF-β1. Histogram shows the percentage of total mitoses with specific chromosome numbers. Treatment with TGF-β1 (50 pg/ml) was begun 3 days after infection with v-*ras*^{Ha} and continued for 15 days. A total of 90 metaphases from 2 independent experiments were counted. (*C*) Dose response for inhibition of DNA synthesis by exogenous TGF-β1 in v-*ras*^{Ha}-transduced TGF-β1$-/-$ keratinocytes. DNA synthesis was measured by the incorporation of [^{3}H]thymidine (^{3}H-TdR).

Discussion

Previous studies in the mouse multistage carcinogenesis model have shown that disruption of the TGF-β1 signaling pathway leads to accelerated malignant progression *in vivo*. TGF-β1 is not detectable in chemically induced papillomas with a high frequency of premalignant progression (5, 6), and v-*ras*^{Ha}-transduced TGF-β1$-/-$ primary keratinocytes rapidly progress to SCC in a reconstituted skin graft (7). Furthermore, transgenic mice expressing a skin-targeted dominant negative TβRII produce carcinomas with shorter latency and higher frequency than nontransgenic controls (8). Our results show that disruption of the autocrine TGF-β signaling, either through genetic ablation of the TGF-β1 locus or with a dominant negative TβRII, causes malignant conversion *in vitro* of a primary epithelial cell whose only other genetic alteration is the presence of an activated v-*ras*^{Ha} oncogene. It is likely that the assay used to measure conversion, selection for the calcium-resistant phenotype, is a true measure of malignancy, because resistant foci express markers of progression such as keratin 13 and form SCCs in a reconstituted skin graft (11). In the TGF-β1$-/-$ strain, the frequency of malignant conversion *in vitro* of each genotype correlates with the levels of secreted TGF-β1, whereas in the AM3DN strain, the frequency correlates with the level of expression of the TGF-β type II DN receptor. Thus, elevated malignant conversion *in vitro* is directly related to inhibition of TGF-β secretion or response.

The frequency of focus formation in the TGF-β1$-/-$ cells is approximately 6.5×10^{-6}, significantly higher than expected for a spontaneous mutagenic event (26), and suggests that the absence of autocrine TGF-β signaling produces or amplifies genomic instability. In the initiation-promotion model of tumor induction in the skin of mice, the majority of early papillomas are diploid (25). Malignant progression is accompanied by significant genetic change at the chromosomal level, including gross aneuploidy (25), trisomy of chromosomes 6 and 7 (27), as well as amplification of the mutated c-*ras*^{Ha} allele (28, 29), and allelic loss (30–32). As found previously with BALB/c keratinocytes (33), v-*ras*^{Ha}-transduced TGF-β1$+/+$ and$-/-$ cells rapidly become tetraploid, most likely due to endoreduplication. However, 15 days after transduction, the majority of TGF-β1$-/-$ keratinocytes, as well as the pooled calcium-resistant foci, are aneuploid, either hyperdiploid (41 to 45) or near tetraploid (70 to 79), similar to the SCC that develops from chemically initiated mouse skin (34) or v-*ras*^{Ha}-transduced primary BALB/c keratinocytes (33). These results suggest that genetic instability precedes the acquisition of resistance to calcium. More detailed studies are needed to determine whether specific chromosomes are gained or lost in the calcium-resistant cells. In addition, sequential infection of v-*ras*^{Ha}-transduced BALB/c keratinocytes with AdTGFβTR causes a significant reduction in the percentage of cells with diploid DNA content and an increase in cells with a DNA content >4N. Based on BrdUrd and propidium iodide fluorescence-activated cell sorter analysis, the cells with >4N DNA content were actively cycling. Thus, even a transient inactivation of autocrine TGF-β1 signaling is sufficient to provoke significant changes in DNA ploidy.

Aneuploidy can result from abnormalities in genes regulating chromosome segregation at mitosis as well as from defects in cell-cycle checkpoints that couple replication and mitosis (35). Human analogues to yeast mitotic-checkpoint genes have been isolated and shown to be mutated and to be a potential cause of aneuploidy in some colon carcinomas (36). In addition, mouse embryo fibroblasts with defects in p53 become polyploid and exhibit an altered response to microtubule-disrupting agents (37, 38). However, the responses to DNA damage and nocodazole, both of which activate p53-regulated checkpoint(s) (38–41), are unaltered in the primary v-*ras*^{Ha}-transduced TGF-$\beta-/-$ keratinocytes. This result is in accord with a functionally wild-type p53 in TGF-$\beta-/-$ keratinocyte cell lines (9). Because the v-*ras*^{Ha}-transduced TGF-β1$-/-$ keratinocytes arrested in the presence of nocodazole, it is unlikely that they harbor mutations in the mouse *bub* genes (36). Thus, intrinsic mitotic segregation defects or p53-related DNA-repair checkpoint defects are unlikely to account for the high frequency of aneuploidy in the TGF-β1$-/-$ v-*ras*^{Ha} keratinocytes.

Accelerated entry into S phase can also be a significant source of genetic damage, which can result in gene amplification and karyotypic instability (35, 42). The increased frequency of chromosome and chromatid breaks detected in the v-*ras*^{Ha}-transduced TGF-β1$-/-$ keratinocytes points, in part, to a mechanism involving aberrant cell-cycle regulation and subsequent errors in replication. Aneuploidy could result from chromosome gains via fusion of unrepaired breaks and subsequent nondisjunction, or loss of defective chromosomes at mitosis. These defects must arise from a cooperative interaction between the constitutive activation of the Ras pathway and the absence of TGF-β signaling, because a TGF-β1$-/-$ genotype or AdTGFβTR infection did not effect ploidy in the absence of v-*ras*^{Ha}. Several targets of TGF-β1, including cdc25a, c-myc, p21^{waf1}, and p15^{ink4b} (43–46), are potential candidates for this type of cooperative interaction. Both c-myc and cdc25a are down-regulated by TGF-β1 and can cooperate with *ras* to

transform fibroblasts (47, 48), and transient overexpression of c-myc can cause chromosomal instability in rodent fibroblasts (49). Alternatively, altered regulation of cyclin-dependent kinase inhibitors in TGF-β signaling-defective cells could cause significant disturbance of normal G_1/S-phase progression to yield DNA damage and subsequent aneuploidy.

Finally, our results show that exogenous TGF-β1 can suppress malignant conversion *in vitro* of an epithelial cell as well as the genetic hallmarks of premalignant progression, chromosomal damage and aneuploidy. These data provide support for the notion that activation of this signaling pathway in preneoplastic human epithelial cells could suppress malignant progression. Although treatment of the v-*ras*^{Ha}-transduced TGF-β1$-/-$ keratinocytes with TGF-β1 significantly reduced the percentage of aneuploid metaphases, there was no effect on tetraploidy, suggesting that pathways leading to tetraploidy are independent of TGF-β signaling. It is also not clear whether TGF-β1 treatment inhibits aneuploidy and other genetic changes or whether cells with these changes are more sensitive to growth-inhibitory or apoptotic effects of TGF-β1. However, because suppression occurs at concentrations that do not cause significant growth arrest of v-*ras*^{Ha}-transduced keratinocytes, restoration of cell cycle balance rather than simple growth suppression is likely to underlie the action of TGF-β1 in this system.

We thank Dr. Luowei Li for Adβgal and Dr. Ester Fernandez-Salas for advice on purification of recombinant adenoviruses. We also thank members of the Yuspa laboratory for helpful discussions and for critical reading of the manuscript.

1. Park, K., Kim, S. J., Bang, Y. J., Park, J. G., Kim, N. K., Roberts, A. B. & Sporn, M. B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8772–8776.
2. Kadin, M. E., Cavaille-Coll, M. W., Gertz, R., Massague, J., Cheifetz, S. & George, D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6002–6006.
3. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B., *et al.* (1995) *Science* **268,** 1336–1338.
4. Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., *et al.* (1996) *Science* **271,** 350–353.
5. Glick, A. B., Kulkarni, A. B., Tennenbaum, T., Hennings, H., Flanders, K. C., O'Reilly, M., Sporn, M. B., Karlsson, S. & Yuspa, S. H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6076–6080.
6. Cui, W., Kemp, C. J., Duffie, E., Balmain, A. & Akhurst, R. J. (1994) *Cancer Res.* **54,** 5831–5836.
7. Glick, A. B., Lee, M. M., Darwiche, N., Kulkarni, A. B., Karlsson, S. & Yuspa, S. H. (1994) *Genes Dev.* **8,** 2429–2440.
8. Amendt, C., Schirmacher, P., Weber, H. & Blessing, M. (1998) *Oncogene* **17,** 25–34.
9. Glick, A. B., Weinberg, W. C., Wu, I. H., Quan, W. & Yuspa, S. H. (1996) *Cancer Res.* **56,** 3645–3650.
10. Roberts, A. B. & Sporn, M. B., (1990) *Handb. Exp. Pharmacol.* **95,** 419–472.
11. Morgan, D., Welty, D., Glick, A. B., Greenhalgh, D., Hennings, H. & Yuspa, S. H. (1992) *Cancer Res.* **52,** 3145–3156.
12. Yuspa, S. H. & Morgan, D. L. (1981) *Nature (London)* **293,** 72–74.
13. Strickland, J. E., Greenhalgh, D. A., Koceva-Chyla, A., Hennings, H., Restrepo, C., Balaschak, M. & Yuspa, S. H. (1988) *Cancer Res.* **48,** 165–169.
14. Kulkarni, A. B., Huh, G. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M. & Karlsson, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 770–774.
15. Bottinger, E. P., Jakubczak, J. L., Haines, D. C., Bagnall, K. & Wakefield, L. M. (1997) *Cancer Res.* **57,** 5564–5570.
16. Bottinger, E. P., Jakubczak, J. L., Roberts, I. S., Mumy, M., Hemmati, P., Bagnall, K., Merlino, G. & Wakefield, L. M. (1997) *EMBO J.* **16,** 2621–2633.
17. Dlugosz, A. A., Glick, A. B., Tennenbaum, T., Weinberg, W. C. & Yuspa, S. H. (1995) *Methods Enzymol.* **254,** 3–20.
18. Roop, D. R., Lowy, D. R., Tambourin, P. E., Strickland, J., Harper, J. R., Balaschak, M., Spangler, E. F. & Yuspa, S. H. (1986) *Nature (London)* **323,** 822–824.
19. Yamamoto, H., Ueno, H., Ooshima, A. & Takeshita, A. (1996) *J. Biol. Chem.* **271,** 16253–16259.
20. Seth, P., Rosenfeld, M., Higginbotham, J. & Crystal, R. G. (1994) *J. Virol.* **68,** 933–940.
21. Glick, A. B., Danielpour, D., Morgan, D., Sporn, M. B. & Yuspa, S. H. (1990) *Mol. Endocrinol.* **4,** 46–52.
22. Danielpour, D. (1993) *J. Immunol. Methods* **158,** 17–25.
23. Zimonjic, D. B. & Popescu, N. C. (1994) *Cancer Genet. Cytogenet.* **72,** 161.
24. Harnden, D. G. & Klinger, H. P., eds. (1985) *An International System for Human Cytogenetic Nomenclature* (Karger, New York; in collaboration with *Cytogenet. Cell Genet.*), pp. 66–76.
25. Aldaz, C. M., Conti, C. J., Klein-Szanto, A. J. P. & Slaga, T. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2029–2032.
26. Lee, J., Acs, G., Blumberg, P. M. & Marquez, V. E. (1995) *Bioorg. Med. Chem. Lett.* **5,** 1331–1334.
27. Aldaz, C. M., Trono, D., Larcher, F., Slaga, T. J. & Conti, C. J. (1989) *Mol. Carcinog.* **2,** 22–26.
28. Bianchi, A. B., Aldaz, C. M. & Conti, C. J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6902–6906.
29. Quintanilla, M., Brown, K., Ramsden, M. & Balmain, A. (1986) *Nature (London)* **322,** 78–80.
30. Bremner, R. & Balmain, A. (1990) *Cell* **61,** 407–417.
31. Buchmann, A., Ruggeri, B., Klein-Szanto, A. J. P. & Balmain, A. (1991) *Cancer Res.* **51,** 4097–4101.
32. Burns, P. A., Kemp, C. J., Gannon, J. V., Lane, D. P., Bremner, R. & Balmain, A. (1991) *Oncogene* **6,** 2363–2369.
33. Aldaz, C. M., Conti, C. J., Yuspa, S. H. & Slaga, T. J. (1988) *Carcinogenesis* **9,** 1503–1505.
34. Conti, C. J., Aldaz, C. M., O'Connell, J., Klein-Szanto, A. J. P. & Slaga, T. J. (1986) *Carcinogenesis* **7,** 1845–1848.
35. Hartwell, L. H. & Kastan, M. B. (1994) *Science* **266,** 1821–1828.
36. Cahill, D. P., Lengauer, C., Yu, J., Riggins, G. J., Willson, J. K., Markowitz, S. D., Kinzler, K. W. & Vogelstein, B. (1998) *Nature (London)* **392,** 300–303.
37. Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S. & Vande Woude, G. F. (1996) *Science* **271,** 1744–1747.
38. Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H. & Reid, B. J. (1995) *Science* **267,** 1353–1356.
39. Lanni, J. S. & Jacks, T. (1998) *Mol. Cell Biol.* **18,** 1055–1064.
40. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W. & Vogelstein, B. (1998) *Science* **282,** 1497–1501.
41. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B. & Fornace, A. J., Jr. (1992) *Cell* **71,** 587–597.
42. Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C. & Wahl, G. M. (1992) *Cell* **70,** 937–948.
43. Iavarone, A. & Massague, J. (1997) *Nature (London)* **387,** 417–422.
44. Munger, K., Pietenpol, J. A., Pittelkow, M. R., Holt, J. T. & Moses, H. L. (1992) *Cell Growth Differ.* **3,** 291–298.
45. Hannon, G. J. & Beach, D. (1994) *Nature (London)* **371,** 257–261.
46. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y. & Wang, X. F. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5545–5549.
47. Land, H., Chen, A. C., Morgenstern, J. P., Parada, L. F. & Weinberg, R. A. (1986) *Mol. Cell Biol.* **6,** 1917–1925.
48. Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M. & Beach, D. (1995) *Science* **269,** 1575–1577.
49. Felsher, D. W. & Bishop, J. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3940–3944.