Oncogene Expression Cloning by Retroviral Transduction of Adenovirus E1A-immortalized Rat Kidney RK3E Cells: Transformation of a Host with Epithelial Features by c-MYC and the Zinc Finger Protein GKLF

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Abstract
The function of several known oncogenes is restricted to specific host cells in vitro, suggesting that new genes may be identified by using alternate hosts. RK3E cells exhibit characteristics of epithelia and are susceptible to transformation by the G protein RAS and the zinc finger protein GLI. Expression cloning identified the major transforming activities in squamous cell carcinoma cell lines as c-MYC and the zinc finger protein gut-enriched Krüppel-like factor (GKLF)/epithelial zinc finger. In oral squamous epithelium, GKLF expression was detected in the upper, differentiating cell layers. In dysplastic epithelium, expression was prominently increased and was detected diffusely throughout the entire epithelium, indicating that GKLF is misexpressed in the basal compartment early during tumor progression. The results demonstrate transformation of epithelioid cells to be a sensitive and specific assay for oncogenes activated during tumorigenesis in vivo, and identify GKLF as an oncogene that may function as a regulator of proliferation or differentiation in epithelia.

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Introduction
Cellular oncogenes have been isolated by characterization of transforming retroviruses from animal tumors, by examination of the breakpoints resulting from chromosomal translocation, by expression cloning of tumor DNA molecules using mesenchymal cells such as NIH3T3, and by other methods (1–5). Several human tumor-types exhibit loss-of-function mutations in a tumor suppressor gene that lead to activation of a specific oncogene in a large proportion of tumors. For example, c-MYC expression is regulated by the APC colorectal tumor suppressor; expression of GLI is activated by loss-of-function of PATCHED1 in human basal cell carcinoma and in animal models; E2F is activated by loss-of-function of the retinoblastoma susceptibility protein p105RB; and RAS GTPase activity is regulated by the familial neurofibromatosis gene NF1 (6–12). The comparative genomic hybridization assay and related methods have shown that numerous uncharacterized loci in tumors undergo gene amplification (13). These observations, and the infrequent genetic alteration of known oncogenes in certain tumor types, suggest that novel transforming oncogenes remain to be identified.

One limitation to the isolation of oncogenes has been the paucity of in vitro assays for functional expression cloning. Whereas most studies have used NIH3T3 or other mesenchymal cells as host for analysis of oncogenes relevant to carcinoma, the potential use of a host cell with epithelial characteristics has been discussed (2). In addition, several known oncogenes exhibit cell-type specificity. GLI, BCR-ABL, NOTCH1/TAN1, and the G protein GIP2 have been found to transform immortalized rat cells (14–18), but not NIH3T3 cells, demonstrating the potential use of alternate assays for oncogene expression cloning.

A consistent feature of human tumors is inactivation of the G1 phase cell cycle regulatory pathway that includes p105RB (19–22). Loss-of-function mutations affect p105RB or the cyclin-dependent kinase inhibitors, or gain-of-function mutations occur in cyclin-dependent kinases or associated cyclins. Such alterations are rate-limiting for tumor formation in vivo because inheritance of these defects predisposes to retinoblastoma, cutaneous malignant melanoma, and other tumors. During viral infection of normal cells, disruption of the same pathway is critical for successful induction of the cellular DNA replicative machinery to support viral replication. Therefore, viruses express proteins, such as adenovirus E1A, that affect cell cycle progression through direct interaction with cell cycle regulators including p105RB, p27kip1, and others (23–26).
Fig. 1. RK3E cells exhibit characteristics of epithelial cells. A, confluent RK3E cells in a culture dish were fixed and stained with uranyl acetate and lead citrate, and ultra-thin sections were examined using a Hitachi 7000 transmission electron microscope. The upper surface was exposed to growth media, and the lower surface was adherent. Electron dense aggregates typical of adherens junctions (arrows) and desmosomes (circled) are shown. Bars, 3.2 μm (top) or 1.3 μm (bottom). B, Northern blot analysis of RK3E cells (Lane 1) and REF52 fibroblasts (Lane 2). The filter was hybridized sequentially to a desmoplakin probe (top) and then to β-tubulin (bottom). C, vimentin expression by immunocytochemistry in RK3E (top) and REF52 (bottom) cells. Bars, 100 μm.
We previously developed and used RK3E cells, immortalized by E1A, to demonstrate the transforming activity of GLI (17). We now show that these cells exhibit multiple features of epithelia and detect known and novel transforming activities in tumor cell lines. The epithelial features of the cells and/or the mechanism of immortalization may explain the surprising sensitivity and specificity of the assay compared and/or the mechanism of immortalization may explain the surprising sensitivity and specificity of the assay compared with previous expression cloning approaches (27). Three of the four genes known to transform RK3E cells are activated by genetic alterations in carcinomas, and, of these genes, only RAS exhibits transforming activity in the commonly used host NIH3T3. We identify GKLF4 (3) as an oncogene that is expressed in the differentiating compartment of epithelium and misexpressed in dysplastic epithelium. We also suggest that GKLF may regulate the rate of differentiation and maturation and the overall cellular transit time through epithelium. The functional similarities shared with other oncogenes, including GLI or c-MYC, identify GKLF as an attractive candidate gene relevant to tumor pathogenesis.

Results
RK3E Cells Have Characteristics of Epithelia. RK3E cells are a clone of primary rat kidney cells immortalized by transfection with adenovirus E1A in vitro (17). The cells exhibit morphological and molecular features that are epitheloid. They are contact-inhibited at confluence and are polarized with apical and basolateral surfaces and electron-dense intercellular junctions typical of adherens junctions and desmosomes (Fig. 1A). Northern blot analysis showed that RK3E cells, but not REF52 fibroblasts, expressed desmoplakin, a major component of desmosomes and an epithelial marker (Fig. 1B). By immunocytochemical staining, the mesenchymal marker vimentin was low or undetectable in RK3E cells, but was strongly positive in REF52 cells (Fig. 1C). Neither line reacted strongly with anticytokeratin or antidesmin antibodies. These results are consistent with the observation that E1A induces multiple epithelial characteristics without inducing cytokeratin expression (28).

Karyotype analysis revealed RK3E cells to be diploid with a slightly elongated chromosome 5q as the only apparent abnormality (17). Importantly, RK3E cells can be transformed by functionally diverse oncogenes such as RAS and GLI.

Four such transformed lines were each homogeneous for DNA content, as determined by fluorescence analysis of propidium iodide-stained cells derived from RAS- (one line) or GLI- (three lines) induced foci, indicative of a relatively stable genetic constitution (data not shown). These properties suggested that RK3E cells may serve as an in vitro model for identification and mechanistic analysis of gene products involved in the progression from normal epithelial tissue to malignancy.

cDNA Library Construction. To identify transforming genes, we used mRNA from human squamous cell carcinoma- or breast tumor-derived cell lines. These tumor types do not exhibit frequent alteration of RAS or GLI. After pooling mRNAs for each tumor type, oligo dT-primed cDNA libraries were constructed in bacteriophage λ (Table 1). The libraries were high-titer (assessed before amplification on agar plates) with a mean insert size of 1.6–1.7 kb. The amplified breast cDNA library was further assessed by plaque screening for the transcription factor hBRF, using a probe derived from the 5’ end of the protein coding region (bases 315–655, accession U75276). Each of the seven clones identified were derived from independent reverse transcripts, as determined by end sequencing, confirming that complexity of the library was maintained during amplification. The inserts ranged in size from 2.1–3.4 kb and contained the entire 3’ UTR and much or all of the protein coding region intact. Three of the seven clones extended through the predicted initiator methionine codon, whereas four others were truncated further downstream. These results suggested that the library is relatively free of COOH-terminally truncated clones and contains full-length cDNAs even for relatively long mRNAs. The overall abundance of hBRF mRNA has not been determined.

Isolation of c-MYC and GKLF by Expression Cloning. The libraries were cloned into the MMLV retroviral expression plasmid pCTV1B (27), packaged in BOSC23 cells (29), and high-titer virus supernatants were applied to RK3E cells. Fourteen foci, identified at 10–20 days after transduction, were individually expanded into cell lines. Thirteen of these foci contained a single stably integrated cDNA, as indicated by PCR (Fig. 2A). Eleven of these PCR products were identified as human c-MYC by end-sequencing and restriction enzyme analysis. The c-MYC cDNA in Lane 15 included the coding region and 193 bases of 5’ UTR sequence (accession V00568). As determined by sequencing or restriction mapping, the other c-MYC cDNAs extended further 5’ (Lanes 1, 3, 5–7, 9–13, 14), such that all of the clones contained the entire protein-coding region.

Table 1. Assesment of cDNA libraries

<table>
<thead>
<tr>
<th>Library</th>
<th>λ titer</th>
<th>cDNA size (N, R)</th>
<th>Probe</th>
<th>cDNA clones transduced</th>
<th>Transduced RK3E cells</th>
<th>Foci identified</th>
</tr>
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<tbody>
<tr>
<td>Squamous cell carcinoma</td>
<td>8.9 × 10^6</td>
<td>1.69 (10, 1.00–3.60)</td>
<td>NT</td>
<td>–4 × 10^6</td>
<td>–1.2 × 10^7</td>
<td>13</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>7.4 × 10^6</td>
<td>1.64 (18, 0.50–2.7)</td>
<td>hBRF</td>
<td>–4 × 10^6</td>
<td>–1.2 × 10^7</td>
<td>1</td>
</tr>
</tbody>
</table>

* The mean size of cDNAs in kb pairs; the number of clones sized by gel electrophoresis (N) and the size range (R) are indicated.

a Plaques (420,000) were analyzed by hybridization to the 5’ end of the RNA polymerase III transcription factor hBRF cDNA (see “Results”); NT, not tested.

b The mean size of cDNAs in kb pairs; the number of clones sized by gel electrophoresis (N) and the size range (R) are indicated.

c The mean size of cDNAs in kb pairs; the number of clones sized by gel electrophoresis (N) and the size range (R) are indicated.

d The number of RK3E cells transduced was estimated as the product of the transduction frequency (20%), the number of dishes screened (20), and the number of cells/dish (3 × 10^5).

4 The abbreviations used are: GLI, gut-enriched Krüppel-like factor; β-gal, β-galactosidase; UTR, untranslated region; MMLV, Moloney murine leukemia virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ISH, in situ hybridization.
In addition, two cell lines (Fig. 2A, Lanes 8 and 12) contained cDNAs coding for GKLF. Mouse and human GKLF cDNAs were previously isolated by hybridization with zinc finger consensus probes (30–32), but were not implicated as oncogenes or found to be induced during neoplastic progression. After cloning into plasmid, the sequences of these two cDNAs, termed SQC7 and SQC11, were obtained in total. As determined by comparison with multiple expressed sequence tags and two full-length coding sequence files in the database (accessions U70663 and AFO22184), each contained the predicted GKLF protein coding region bounded by 5' and 3' UTRs. An ATG in good context for translation initiation was located at base 330, with the predicted terminator codon at base 1740. Both isolates were artificially truncated at the Xhol site in the 5' UTR during library preparation. Because the transcripts had been processed using distinct AAUAAA polyadenylation signals, the cDNAs were slightly different in length and derived from independent mRNA molecules (Fig. 2A).

Sequencing revealed these two GKLF isolates to be identical within the residual 5' UTR and throughout the coding region. A single bp difference in the 3' UTR represents a PCR-induced error or a rare variant, as determined by comparison with ESTs. Comparison to a placenta-derived sequence (accession U70663) revealed three single bp differences in the coding region. These differences were resolved by alignment with other sequences in the database (accessions AFO22184 and AA382289) from normal tissues, indi-
Table 2  Retroviral transduction of reconstituted GKLF and c-MYC expression vectors

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Focus assay (no. foci/10 cm dish)</th>
<th>Colony morphology assay (no. transformed/total)</th>
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</thead>
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<tr>
<td>pCTV3K (vector)</td>
<td>0, 0</td>
<td>0/184</td>
</tr>
<tr>
<td>pCTV3K-SQC1 (c-MYC)</td>
<td>0, 0</td>
<td>0/232</td>
</tr>
<tr>
<td>pCTV3K-SQC5 (c-MYC)</td>
<td>&gt;1000, &gt;1000</td>
<td>ND²</td>
</tr>
<tr>
<td>pCTV3K-BR1 (c-MYC)</td>
<td>&gt;1000, &gt;1000</td>
<td>81/91 (89%)</td>
</tr>
<tr>
<td>pCTV3K-SQC7 (GKLF)</td>
<td>&gt;1000, &gt;1000</td>
<td>91/206 (44%)</td>
</tr>
<tr>
<td>pCTV3K-SQC11-2 (GKLF)</td>
<td>&gt;1000, &gt;1000</td>
<td>ND</td>
</tr>
<tr>
<td>pCTV3K-SQC11-3 (GKLF)</td>
<td>&gt;1000, &gt;1000</td>
<td>ND</td>
</tr>
</tbody>
</table>

a  RK3E cells were transduced with 4 ml of virus supernatant after calcium phosphate-mediated plasmid transfection of virus packaging cells.
b  RK3E cells were transduced with 0.4 ml of thawed viral supernatant. Cells were split 1:4 into selective media 30 h later. At 2 weeks, drug-resistant colonies were fixed, stained, and examined visually for morphological transformation. Numbers indicate colonies/10-cm dish. A duplicate transduction experiment yielded similar results (data not shown). No colonies formed in control dishes that were not exposed to virus.
c  pCTV3K-SQC1 is a c-MYC allele obtained by PCR that exhibited greatly reduced transforming activity compared with other alleles.
d  ND, not determined.
e  SQC11-2 and -3 are independent plasmid clones derived from the same PCR reaction (Fig 2A, Lane 12).

Reconstitution of Transforming Activity for c-MYC and GKLF. To demonstrate transforming activity, three independent PCR products each for the c-MYC and GKLF cDNAs were cloned into the retroviral expression vector pCTV3K (27), packaged into virions, and tested for transformation of RK3E cells in vitro (Fig. 2, B and C; Table 2). One of the c-MYC clones (pCTV3K-SQC1) possessed greatly reduced transforming activity in multiple experiments despite similar viral titers, as determined by induction of hygromycin resistance, suggesting that an error may have been introduced during PCR. Each of the other virus supernatants carrying GKLF and c-MYC transgenes induced >1000 foci/dish compared with no foci for virus controls.

To determine the efficiency of transformation by GKLF and c-MYC, a colony morphology assay was used, as described previously (27). Virally transduced cells were selected in hygromycin at low confluence, and stable colonies were fixed, stained, and scored for morphological transformation by visual inspection as above for foci (Table 2). The c-MYC-transduced cells exhibited loss of contact inhibition and dense growth in 89% of colonies. The GKLF-transduced cells exhibited a transformed morphology in 44% of colonies. In comparison, a previous study showed that 70% and 40% of NIH3T3 colonies transduced by viruses carrying RAS and RAF exhibited a transformed morphology (27). We, likewise, tested virus supernatants for transformation of NIH3T3 cells. Neither c-MYC nor GKLF induced morphological transformation of NIH3T3 colonies, as previously described for GLI and others (data not shown; Ref. 17). These results identify the RK3E assay as not only highly specific, but also sensitive to the activity of a select group of oncogenes.

In lieu of sequencing the c-MYC alleles, we confirmed that wild-type c-MYC can transform RK3E cells. A human wild-type expression vector (pSRvMsv c-MYC tk-neo) induced foci using direct plasmid transfection of RK3E cells in multiple experiments. Foci were observed at a similar frequency using known wild-type or new c-MYC isolates when analyzed in parallel (data not shown). In addition, retrovirus encoding the estrogen receptor-c-MYC (wild-type) fusion protein induced morphological transformation of RK3E cells in the presence or absence of 4-hydroxy-tamoxifen (33). No effect was observed for controls (empty vector or a control containing a deletion in c-MYC residues 106–143).

Northern blot analysis of transformed RK3E cell lines demonstrated expression of the c-MYC and GKLF vector-derived transcripts (Fig. 3A). No endogenous transcripts were detected at the stringency used in this experiment. Compared with RK3E cells at subconfluence (Lane 1) or confluence (Lane 2), no consistent increase of E1A transcripts was detected in cells transformed by RAS, GLI, c-MYC, or GKLF, suggesting that these genes act upon cellular targets to induce transformation.

To detect the endogenous rat GKLF transcript, we used reduced-stringency wash conditions and a Smal fragment from the coding region exclusive of the COOH-terminal zinc fingers and with no sequence similarity to other genes in the database. By this approach, the apparent GKLF transcript was identified and migrated at 3.1 kb, similar to the human 3.0-kb transcript, in RK3E and all derivative-transformed cell lines (data not shown). A single transcript with the same mobility was detected by hybridization of the filter to full-length coding region probe. These studies revealed similar GKLF expression in RK3E and in derivatives transformed by RAS, GLI, or c-MYC. The results show that GKLF mRNA expression is not significantly altered by these other oncogenes and is consistent with function of GKLF in an independent pathway.

Cell lines derived from foci induced by c-MYC or GKLF were further tested for tumorigenicity in athymic mice by s.c. inoculation at four sites for each line (Table 3; Ref. 17). Tumors were >1 cm in diameter and were scored at 2–4 weeks after inoculation. Cells transformed by c-MYC induced tumors in 75% or 100% of sites injected (two lines tested). Three lines transformed by GKLF each induced tumors in 50–75% of sites injected. No tumors resulted from injection of RK3E cells, whereas a GLI-transformed cell line induced tumors in each of the four sites injected. In all, GKLF cell lines induced tumors in 8 of 12 injection sites, compared with 7 of 8 injection sites for c-MYC and 4 of 4 injection sites for GLI. GKLF-induced tumors also grew more slowly in vivo, reaching 1 cm in diameter by 3.4 weeks, on average, compared with 2.6 weeks for c-MYC and 3 weeks for GLI. The
moderately increased latency and decreased efficiency of tumor formation for \( \text{GKLF} \) cell lines may be attributable to the intrinsic rate of proliferation for these cells (Table 3). Although \( \text{c-MYC} \), \( \text{GLI} \), and \( \text{GKLF} \) cell lines all exhibited prolonged doubling times \text{in vitro} \) compared with \( \text{RK3E} \) cells, \( \text{GKLF} \) cells divided more slowly than the other transformed cell lines.

**Northern Blot Analysis of Tumors and Tumor-derived Cell Lines.** We then examined human tumors and cell lines by Northern blot analysis of total RNA (Fig. 3, B and C). \( \text{GKLF} \) expression in breast or squamous cell carcinoma cell lines was variable, with increased expression in the breast tumor line \( \text{ZR75-1} \) and the squamous tumors \( \text{SCC15} \) and \( \text{SCC25} \) (Fig. 3B). In human squamous cell carcinomas microdissected to enrich for tumor cells, \( \text{GKLF} \) expression was detected in each of 10 primary or metastatic tumors analyzed, with expression levels comparable with that for the cell line \( \text{SCC25} \) (Fig. 3C). The results suggest that \( \text{GKLF} \) represents a potent transforming activity that is consistently expressed in tumors as well as in tumor-derived cell lines. Because \( \text{GKLF} \) was isolated from cell lines that express the gene at a level found in tumors \text{in vivo} \), the results suggest that \( \text{GKLF} \) may represent a major transforming activity in tumors, as well as in cell lines.

**Gene Copy Number of \( \text{c-MYC} \) and \( \text{GKLF} \).** \( \text{c-MYC} \) was previously shown to be activated by gene amplification in \( \sim 10\% \) of oral squamous cancers and may be activated in these or other tumors by genetic alteration of \( \text{WNT-APC-\beta-catenin} \) pathway components (6, 34–37). To determine whether expression of \( \text{GKLF} \) in cell lines and tumors is, likewise, associated with gene amplification, we performed Southern blot analysis (Fig. 4, A and B). Filters were sequentially hybridized to \( \text{GKLF} \), \( \text{c-MYC} \), and \( \beta\)-tubulin. Increased copies of \( \text{c-MYC} \) were identified in two cell lines used for library construction, FaDu and MCF7. Increased hybridization to \( \text{c-MYC} \) was, likewise, observed for 1 of 11 oral squamous cell carcinomas (Fig. 4A, Lane 10) and for one of nine breast carcinomas (Fig. 4B, Lane 8). These results are consistent with the published frequencies of \( \text{c-MYC} \) amplification for these tumor types (34, 35, 38). No copy number gains of \( \text{GKLF} \) were observed, indicating that other mechanisms may contribute to expression of \( \text{GKLF} \) in tumors. The same may be true for \( \text{c-MYC} \) because gene amplification in FaDu cells was associated with reduced expression compared with other oral cancer cell lines (Fig. 3B).

**GKLF Expression Is Activated Early during Tumor Progression in Vivo.** Previously, expression of \( \text{c-MYC} \) was found to be up-regulated consistently in dysplastic oral mucosa and in squamous cell carcinomas, and tumors with the highest levels of \( \text{c-MYC} \) expression were associated with the
poorest clinical outcome (36, 39–41). To determine how 
GKLF mRNA expression is altered during tumor progression,
we analyzed squamous cell carcinoma of the larynx and 
adjacent uninvolved epithelium from the same tissue blocks 
using 35S-labeled riboprobes by ISH analysis. In apparently 
normal epithelium, 
GKLF expression was detected in the 
spinous layer above the basal and parabasal cells (nine 
specimens analyzed; Fig. 5, A–G, G–I; Table 4). No specific 
GKLF expression was detected in the basal or parabasal 
cells or in the underlying dermis. In contrast, a sense control 
probe produced grains at a much-reduced frequency in a 
uniform fashion across the epithelium. GAPDH expression 
served as a positive control and was detected diffusely 
throughout the entire epithelium (data not shown). The ob-

Fig. 4. Southern blot analysis of cell line- and tumor-derived genomic DNA. DNA (6 μg) was digested with EcoRI and separated by gel electrophoresis. The filters were hybridized sequentially to GKLF, c-MYC, and β-tubulin probes. *, samples with increased apparent copy number of c-MYC. Molecular weight markers are indicated on the right. NL, normal human lymphocyte DNA; A, oropharyngeal squamous cell carcinoma. Cell lines (Lanes 2–4) and tumors (Lanes 5–15) are shown. B, breast carcinoma. Cell lines (Lanes 2–5) and tumors (Lanes 6–14) are shown.
Fig. 5. ISH analysis of GKL.F. Paraffin-embedded (A-L) or fresh-frozen (M-O) tissues were analyzed using antisense (GKL.F-AS) or sense (GKL.F-S) \(^{35}\)S-labeled RNA probes. Each image (A-O) is 650 \(\mu\)m \(\times\) 530 \(\mu\)m. Sections were stained with H&E. Case 1, A-C, uninvolved epithelium in a patient with primary laryngeal squamous cell carcinoma; D-F, adjacent dysplastic epithelium within the same tissue block. Case 2, G-I, uninvolved epithelium; J-L, adjacent primary tumor nests within stroma in the same tissue block; *, a salivary gland and ducts. Case 3, M-O, metastatic laryngeal squamous cell carcinoma infiltrating a lymph node; +, lymphocytes.
Table 4  Expression of GKLF in oral epithelium and tumors

<table>
<thead>
<tr>
<th>Case</th>
<th>Histopathology (U,D,P,M)</th>
<th>Tissue source (PE/FF)</th>
<th>Method (N/ISH)</th>
<th>GKLF expression</th>
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<tbody>
<tr>
<td>1</td>
<td>U,D,P</td>
<td>PE</td>
<td>ISH</td>
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<tr>
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<td>M</td>
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<tr>
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<td>PE</td>
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<td>FF</td>
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* Each row corresponds to a tissue specimen. Levels of gene expression indicate changes identified within, rather than between, single tissue sections. For some cases, multiple specimens isolated during the same surgical procedure were analyzed. ISH results were confirmed by analysis of sections in duplicate.

erved pattern of GKLF expression is identical to the pattern in normal mouse skin (32).

For each of 12 specimens analyzed, dysplastic epithelium exhibited increased GKLF expression throughout the epithelium (Fig. 5, D–F; Table 4, Cases 1, 2, 4, 9, 11, 12, and 15–17). In contrast to results obtained in normal-appearing epithelium, there was no reduction of expression in the basal and parabasal layers compared with superficial layers. For tissue sections that contained both uninvolved epithelium and adjacent dysplastic epithelium, the overall level of GKLF expression in dysplastic epithelium was prominently elevated compared with the GKLF-positive cell layers in uninvolved epithelium (Fig. 5, B, E, and H; Table 4, Cases 1, 2, 4, 11, 12, and 16). These results suggest that GKLF expression is qualitatively and quantitatively altered in dysplasia, that exclusion of GKLF from the basal and parabasal cell layers is lost early during neoplastic progression, and that GKLF exhibits properties of an oncogene not only in vitro, but also in vivo.

As shown by Northern blot analysis, GKLF transcripts are consistently present in tumor-derived mRNA (Fig. 3C; Table 4). To determine whether GKLF is expressed in tumor cells, we examined laryngeal squamous cell carcinomas by mRNA ISH. Expression was detected in each primary (13 cases) or metastatic (5 cases) tumor examined (Fig. 5, J–O; Table 4), with all or nearly all tumor cells associated with silver grains. The level of expression was somewhat heterogeneous, with higher levels found in the periphery and in nodules of tumor containing centrally necrotic cells or keratin pearls. As for dysplastic epithelium, expression in tumor cells was consistently elevated compared with uninvolved epithelium in the same sections (Fig. 5, H and K; Table 4, Cases 1, 2, 11, 12, and 16). However, expression in tumor cells was not higher than in dysplastic epithelium (Cases 1, 9, 11, 12, and 15–17). For several cases, expression in the most dysplastic epithelium was higher than in adjacent GKLF-positive tumor, suggesting that GKLF expression is specifically activated during the transition from normal epithelium to dysplasia, before invasion or metastasis.

Discussion

The results demonstrate that cells with an epithelial phenotype can be used for identification of transforming activities present in carcinoma-derived cell lines. The assay repeatedly identified two genes, and none of the isolated cDNAs were artificially truncated or rearranged within the protein coding region. This indicates that transformation of these cells is unusually specific to a few pathways or genes, including c-MYC, GKL, RAS, and GLI. c-MYC, RAS, and GLI are directly or indirectly activated by genetic alterations in diverse carcinoma types during tumor progression in vivo (9, 10, 42–44). For both breast and oral squamous carcinoma, the tumor types analyzed in this study, c-MYC gene amplification is one of the more frequent oncogene genetic alterations and is observed in 10–15% of cases. By analogy, novel oncogenes identified by the RK3E assay may be directly activated in neoplasms through gain-of-function mutations or indirectly activated by loss-of-function genetic alterations.

Whitehead et al. (27) developed the retroviral vectors that we used in this study for transduction of NIH3T3 cells, in which they isolated 19 different cDNAs encoding 14 different proteins. Known oncogenes were isolated, including raf-1, lck, and ect2. Other known genes included phospholipase C-γ2, β-catenin, and the thrombin receptor. In addition to the known genes, seven novel cDNAs were isolated, including several members of the CDC24 family of guanine nucleotide exchange factors. Only the thrombin receptor was isolated more than once, and many of the 14 different genes identified were truncated within the protein coding region. The diversity of cDNAs isolated in the NIH3T3 assay is in contrast to results obtained in the current study. The specificity of the RK3E assay may be attributable to the “tumor suppressor” activity of the E1A oncogene (28, 45). Although E1A antagonizes p105Rb and immortalizes primary cells, it also induces epithelial differentiation in diverse tumor types, including sarcoma, and suppresses the malignant behavior of tumor cells in vivo.

GKLF was previously isolated by hybridization to zinc finger probes (30–32). The human gene is located at chromo-
some 9q31 and is closely linked to the autosomal dominant syndrome of multiple self-healing squamous epitheliomata (31, 32, 46, 47). Affected individuals develop recurrent invasive, but well-differentiated, tumors morphologically similar to squamous carcinoma that spontaneously regress. Although GKLF has been proposed as a candidate tumor suppressor gene relevant to multiple self-healing squamous epitheliomata (32), our results suggest that activating mutations could account for the syndrome.

GKLF encodes a nuclear protein that functions as a transcription factor when bound to a minimal essential binding site of 5'-CAGG/GGG/GG/GAGGG-3' (48). The 470 residue polypeptide exhibits modular domains that mediate nuclear localization, DNA binding, and transcriptional activation or repression (31, 32, 49, 50). In mice, GKLF expression is found predominately in barrier epithelia, including mucosa of the mouth, pharynx, lung, esophagus, and small and large intestine (30, 32). A role for GKLF in differentiation or growth arrest was suggested by the onset of expression at the time of epithelial differentiation (approximately embryonic day 13; Refs. 32 and 51) and by similarity within the zinc finger domain to family members erythroid Krüppel-like factor and lung Krüppel-like factor that were previously associated with growth-arrest or differentiation-specific gene expression (52, 53). Similarity to these other genes is limited to the DNA-binding zinc finger region.

Our results show that GKLF can induce proliferation when overexpressed in vitro. Analysis of expression in dysplastic cells and tumor cells in vivo provides independent evidence that GKLF exhibits properties expected of an oncogene. Genetic progression of carcinoma seems to involve genes and pathways important for homeostasis of normal epithelium (6, 7, 9, 54). For example, the zinc finger protein GLI is expressed in normal hair shaft keratinocytes, whereas c-MYC is expressed in normal epithelium of the colonic mucosa. In tumors derived from these tissues, GLI and c-MYC are more frequently activated by recessive genetic changes in upstream components of their respective biochemical pathways than by gain-of-function alterations such as gene amplification. Up-regulation of GKLF expression in dysplastic epithelium and tumor cells in vivo is particularly interesting as expression seems not to be increased by proliferation in vitro. Expression of the endogenous GKLF mRNA in RK3E cells was similar in cycling versus contact-inhibited cells (data not shown). In contrast, GKLF is significantly induced in NIH3T3 cells during growth arrest (30). These different results suggest that cell type-specific mechanisms can regulate GKLF expression, and that GKLF may play different roles in epithelial versus mesenchymal cells.

Squamous epithelium is divided into compartments (55, 56). In the basal cell layer, proliferative reserve or stem cells possess long-term or unlimited self-renewal capacity, whereas the parabasal transit amplifying cells undergo several rounds of mitosis and then withdraw from the cell cycle to differentiate into spinous cells that form the mid strata of the epithelium. These cells then undergo terminal differentiation and programmed cell death at the surface. Proliferation and differentiation are normally balanced such that overall cell number remains constant. In contrast to GLI and c-MYC, GKLF expression in skin seems limited to the differentiating compartment (32). A simple model is that GKLF normally regulates the rate of maturation and shedding and the overall transit time for individual cells. The thickness of epithelium, which varies greatly in development and in different adult tissues, may be regulated not only by alterations in the rate of cell division in the basal layer, but also in response to GKLF or similarly acting molecules in the suprabasal layers. This model is consistent with the relatively late induction of GKLF during mouse development, and is testable by modulating expression of GKLF in transgenic animals or using raft epithelial cultures in vitro. Activation of GKLF in the basal layer of dysplastic epithelium suggests that dysplasia and progression to invasion and metastasis could result from loss of normal compartment-specific patterns of gene expression.

In summary, GKLF, c-MYC, and GLI are potent oncogenes in epithelioid RK3E cells in vitro, are analogous with respect to their expression in normal epithelium, and have potentially complex roles in the regulation of epithelial cell proliferation, differentiation, or apoptosis (6, 7, 9, 44, 56–58). How GKLF contributes to these processes will require a better understanding of its function and of the pathways that regulate GKLF activity in epithelia.

Materials and Methods

Immunocytochemistry. Immunocytochemical assays were performed in the Immunopathology Laboratory at The University of Alabama at Birmingham. Antibodies to vimentin and desmin were from Dako (Carpenteria, CA). A mixture of anticytokeratin included AE1/AE3 (Biogenics, San Ramon, CA), CAM5.2 (Becton Dickinson, San Jose, CA), and MAK-6 (Zymed, South San Francisco, CA). Human tissue served as a positive control for each antibody. No signal was obtained in the absence of primary antibody.

Construction of cDNA Libraries. Two cDNA libraries were constructed using the ZAP-Express cDNA synthesis kit (Stratagene, La Jolla, CA). A library was prepared from human squamous cell carcinoma cells derived from tumors of the oro-pharynx. Equal quantities of total mRNA from cell lines SCC15, SCC25, and FaDu (American Type Culture Collection, Manassas, VA) were pooled. Similarly, equal quantities of mRNA from the breast cancer cell lines MCF-7, ZR75-1, MDAMB-453, and T47D (American Type Culture Collection) were pooled. For each pool, poly(A)+ mRNA was selected by two cycles of oligo-dT cellulose affinity chromatography, and 5 μg were reverse transcribed using an oligo-dT linker primer and MMLV reverse transcriptase. Double-stranded cDNA was synthesized using Escherichia coli RNase H and DNA polymerase I. cDNA was ligated to λZAP EXPRESS bacteriophage arms and packaged into virions. The λ size and the frequency of nonrecombinants were determined before amplification of the library on bacterial plates (Table 1). The frequency of nonrecombinant clones was estimated to be <2% by complementation of β-gal activity (blue/white assay). Phage were converted to pBKCMV plasmids by autoexcision in bacteria. Insert sizes in randomly selected clones were determined at this step by gel electrophoresis of plasmid DNA digested with Sall and Nofl (Table 1). The pBKCMV plasmid libraries were amplified in soft agar at 4 × 10^7 colony forming units/ml (27). After incubation at 37°C for 15 h, bacterial cells within the agar bed were isolated by centrifugation, amplified for 3–4 doublings in culture, and plasmid DNA was purified using a Qiagen column (Qiagen, Inc., Chatsworth, CA).

To generate libraries in a retroviral expression vector, cDNA inserts were excised from 10 μg of plasmid using Sall and XhoI. After treatment with Klenow and dNTPs and extraction with phenol, the DNA was ligated to 5′ phosphorylated BstXI adapters (5′-TCAGTTACTAGG-3′ and 5′-CCTGAGTAACTGACACA-3′), as described (27). After treatment with Nofl, excess adapters were removed by gel filtration, and the residual vector was converted to a 9.0-kb dimer using the Not site and T4 DNA
ligase. The cDNA was size-fractionated by electrophoresis in Sea Plaque agarose (FMC BioProducts, Rockland, ME) and fragments 0.6–8.5 kb were isolated and ligated to the BstXI- and alkaline phosphatase-treated MMLV retroviral vector pCTV1B (27). E. coli MC1061/p3 were transformed by electroporation and selected in soft agar as above.

**Retroviral Transduction.** The libraries were analyzed in two transfection experiments performed on consecutive days. For each library, ten 10-cm dishes of BOSC23 ecotropic packaging cells at 80%–90% confluence were transfected using 10 µg of plasmid DNA/dish (29). The transfection efficiency for these cells was ~60%, as determined using a β-gal control plasmid. Viruses were collected in a volume of 9.0 ml/dish at 36–72 h after transfection, filtered, and the 9.0 ml was expressed into a 10 cm dish containing RK3E cells at ~30% confluence. Polybrene was added to a final concentration of 10 µg/ml. After 15 h, and every 3 days thereafter, the cells were fed with growth media (17). A total of 20 RK3E dishes were transduced for each library. A β-gal retroviral plasmid transduced at least 20–30% of RK3E cells in control colonies. For colony assays, glyceromycin was used at 100 µg/ml. Cell proliferation rates for transformed cells lines was measured by plating 2 × 104 cells in duplicate and counting cells 96 h later using a hemacytometer (Table 3).

**PGR Recovery of Proviral Inserts.** PGR reactions used 200 ng of cell line genomic DNA, 20 µm Tris-HCl (pH 8.8), 87 µm potassium acetate, 1.0 µm MgCl2, 8% glycerol, 2% DMSO, 0.2 m M of each dNTP, 32 pmol of each primer (5'-CCTACTCTCTCTACTGCTC-3'; 5'-AACAAATTGGAC-TAATCGATACG-3'; Ref. 27), 5 units of Taq polymerase (Life Technologies, Inc., Gaithersburg, MD), and 0.3 units of Pfu polymerase (Stratagene, La Jolla, CA) in a volume of 0.05 ml. Cycling profiles were: 95°C for 1 min; then 95°C for 10 s, 59°C for 40 s, 68°C for 8 min (35 cycles).

**DNA.** genic DNA libraries were analyzed in two transfections, using 30 µg/dish of BOSC23 ecotropic packaging cells at 80%–90% confluence. Viruses were collected at 9.0 ml/dish at 36–72 h after transfection, filtered, and the 9.0 ml was expressed into a 10 cm dish containing RK3E cells at ~30% confluence. Polybrene was added to a final concentration of 10 µg/ml. After 15 h, and every 3 days thereafter, the cells were fed with growth media (17). A total of 20 RK3E dishes were transduced for each library. A β-gal retroviral plasmid transduced at least 20–30% of RK3E cells in control colonies. For colony assays, glyceromycin was used at 100 µg/ml. Cell proliferation rates for transformed cell lines was measured by plating 2 × 104 cells in duplicate and counting cells 96 h later using a hemacytometer (Table 3).

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**References**