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Intestinal-enriched Krüppel-like Factor (Krüppel-like Factor 5) Is a Positive Regulator of Cellular Proliferation*

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Abstract

Intestinal-enriched Krüppel-like factor (IKLF or KLF5) belongs to the family of mammalian Krüppel-like transcription factors. Previous studies indicate that expression of *IKLF* is enriched in the proliferating crypt epithelial cells of the intestinal tract. However, the biological function of IKLF is unknown. In the current study, we have shown that the level of *IKLF* mRNA was nearly undetectable in serum-deprived NIH3T3 fibroblasts but became acutely and significantly increased upon the addition of fetal bovine serum or the phorbol ester, PMA. This induction required protein synthesis because it was prevented by cycloheximide. Transfection of *IKLF* into NIH3T3 cells resulted in the formation of foci in a manner similar to that caused by the *activated Ha-ras* oncogene. Constitutive expression of *IKLF* in transfected NIH3T3 cells significantly increased the rate of proliferation when compared with cells transfected with an empty vector. The growth of *IKLF*-transfected cells was no longer inhibited by cell-cell contact or by low serum content. Moreover, these cells proliferated in an anchorage-independent fashion. We conclude that *IKLF* encodes a delayed early response gene product that positively regulates cellular proliferation and may give rise to a transformed phenotype when overexpressed.

Krüppel is a zinc finger-containing transcription factor that is responsible for segmentation of the *Drosophila melanogaster* embryo (1). In vertebrates, a large number of proteins have been identified that exhibit homology to Krüppel (2). One prominent example is Sp1 (3), a general transcription factor. Recently, a family of Krüppel-like factors (KLFs)[1] that are highly related to the mammalian Krüppel protein erythroid Krüppel-like factor (EKLF) (4) have been described (2,5,6). Many of these proteins were given a numerical designation by the Human Gene Nomenclature Committee (HGNC, Ref. 7), with EKLF designated as KLF1. The genes encoding many KLFs are expressed in a tissue-specific or -selective manner. In addition, evidence suggests that KLFs collectively exert important regulatory functions in diverse biological processes such as growth, development, differentiation, and apoptosis.

One tissue in which a number of KLFs appear to play an important regulatory role is the intestinal epithelium. This tissue is a dynamic system in which proliferation of stem cells located in the crypts is intimately coupled to their differentiation into mature daughter cells

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[1]The abbreviations used are: KLF, Krüppel-like factor; GKLF, gut-enriched KLF; BTEB2, basic transcription element binding protein 2; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; Egr-1, early growth response gene-1; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; IKLF, intestinal-enriched Krüppel-like factor; PMA, phorbol 12-myris-tate 13-acetate; SMC, smooth muscle cell.

once they exit the crypts (8,9). Expression of the genes encoding two KLFs, gut-enriched Krüppel-like factor (GKLF or KLF4, Ref. 10,11) and intestinal-enriched Krüppel-like factor (IKLF or KLF5, Refs. 12,13), is particularly active in the intestinal epithelium. However, their patterns of expression appear to be complementary rather than redundant. Whereas *GKLF* is primarily expressed in the differentiated epithelial cells, away from the proliferating zone, *IKLF* is found mainly in the proliferating crypt cell population. The *in vivo* pattern of *GKLF* expression is mirrored by its *in vitro* pattern; it is found mostly in cells that are growth-arrested (10,14). Moreover, constitutive expression of *GKLF* leads to the inhibition of DNA synthesis (10,15). In contrast, the physiological function of IKLF is less clear, although it has been proposed to have an opposing effect to GKLF in regulating epithelial cell differentiation (12).

The present study seeks to characterize the effect of IKLF on cellular proliferation. We demonstrate that expression of *IKLF* in cultured cells responds transiently and acutely to growth stimuli. In addition, forced expression of *IKLF* in transfected cells results in an accelerated rate of proliferation and a transformed phenotype as evidenced by formation of foci, loss of contact inhibition, as well as acquisition of serum- and anchorage-independent growth. Our results indicate that IKLF has a proproliferative effect, which lends support to the previous hypothesis that it may counteract the function of GKLF.

EXPERIMENTAL PROCEDURES

Reagents

Cell culture media and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD) and Hyclone Laboratories (Logan, UT), respectively. Radioisotopes were purchased from PerkinElmer Life Sciences. Phorbol 12-myristate 13-acetate (PMA) and cycloheximide (CHX) were purchased from Sigma. The monoclonal antibody directed against the hemagglutinin A (HA) epitope (F-7, *sc*-7392) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Expression constructs containing full-length IKLF and HA-tagged IKLF (pBK-CMV-IKLF and pBK-CMV-IKLF·HA, respectively) were kindly provided by Dr. Jerry Lingrel (12). The expression construct containing activated Ha-Ras was a generous gift of Dr. Raul Urrutia (16). The expression construct containing full-length GKLF, PMT3-GKLF, was previously described (10).

Cell Culture

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a 5% CO_2 atmosphere. For experiments involving mitogenic stimulation, cells were first rendered quiescent by removal of serum from the medium for 24 h. Cells were then stimulated with fresh medium containing 15% FBS or 100 ng/ml PMA for various duration. In experiments in which CHX was included, quiescent cells were pretreated with 10 μg/ml CHX for 1 h before the addition of FBS or PMA. Treatments were then continued for another 1 h with the respective mitogen and CHX.

Northern and Western Blot Analyses

RNA was isolated using the Trizol method (Life Technologies) and resolved by denaturing agarose gel electrophoresis followed by transfer to nylon membranes. Complementary DNA probes encoding *IKLF* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were labeled using [α-^{32}P]dATP and the random-primed DNA labeling kit (Roche Molecular Biochemicals). Conditions of hybridization and washing were previously described (10). Western blot analysis was performed according to a previous protocol (10) using proteins extracted from stably transfected cells. The blots were probed with a monoclonal antibody directed against the HA epitope at a concentration of 200 ng/ml. Blots were then incubated

with a peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) at a dilution of 1:2,000. Signals were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Focus Formation Assay

Focus formation assays were performed according to a previously published protocol (17). Briefly, 5 μg of plasmid DNA containing the various effectors was transfected into NIH3T3 cells using LipofectAMINE (Life Technologies, Inc.). Cells were then maintained in DMEM and 10% FBS for 2–3 weeks at which time they were stained with methylene blue to demonstrate the foci. The number of foci in each dish was then manually counted.

Establishment of Stable IKLF-expressing Cell Lines

NIH3T3 cells were transfected with pBK-CMV-IKLF·HA or the pBK-CMV empty vector using LipofectAMINE. Two mg/ml G418 was added to the medium beginning 24 h after transfection to select for resistant clones, which were isolated 2 weeks later and expanded. The presence of HA-tagged IKLF was detected by Western blot analysis using the anti-HA monoclonal antibody.

Cell Proliferation and Serum and Anchorage Dependence Assays

Cell proliferation assays were performed by seeding the *IKLF·HA*- or empty vector-transfected cells at a density of 2×10^5 cells per well in 6-well plates. Cells were fed DMEM with 10% FBS every other day, and the number of cells in the wells were manually counted with a hemocytometer daily for up to 5 days following seeding. For the serum dependence assay, cells were seeded at a density 2×10^5 cells per well in 6-well plates and maintained in DMEM with 1% FBS. Cells were counted every other day up to 6 days after seeding. Anchorage dependence assay was performed according to a published protocol (17). *Ha-ras*-, *IKLF·HA*-, or empty vector-transfected cells were seeded at a density of 5×10^5 cells per 10-cm dish in a 0.33% top agar suspension, which was overlaid onto a 0.5% agar bottom layer. Cells were fed DMEM, 10% FBS, and 2 mg/ml G418 every other day. Colonies developed in the agar suspension were examined 3 weeks following seeding, and the number was tabulated under an inverted phase-contrast microscope. Photodocumentation was accomplished with a Nikon digital camera.

RESULTS

IKLF Encodes a Delayed Early Response Gene to Growth Stimulation

To investigate the responsiveness of *IKLF* expression to growth stimulation, we conducted Northern blot analyses in cultured fibroblasts. NIH3T3 cells were first rendered quiescent by removing the serum from the medium and then stimulated by 15% FBS or the phorbol ester, PMA. As seen in Fig. 1, *A* and *B*, the level of *IKLF* transcripts was barely detectable in serum-starved, quiescent cells (*time 0*). Upon addition of FBS (Fig. 1*A*) or PMA (Fig. 1*B*), the levels of *IKLF* transcripts increased acutely and transiently, reaching a maximum after 2–3 h of treatment before returning to baseline levels. The enhancing effect of both stimuli was prevented in cells pretreated with the protein synthesis inhibitor, cycloheximide (Fig. 1*C*, *CHX*). These results indicate that *IKLF* is an early response gene to growth stimulation although this responsiveness requires protein synthesis. *IKLF* would therefore fall into the category of delayed early response genes (18, 19).

Forced Expression of IKLF Causes Formation of Foci

As a means to measure the effect of IKLF on cell proliferation, we conducted the focus formation assay previously developed for testing the transforming activity of oncogenes such as *ras* (17,20). NIH3T3 fibroblasts were transfected with the various effector constructs and

foci scored 2–3 weeks after transfection. As shown in Fig. 2, an expression plasmid containing the HA-tagged IKLF produced ~30% of the number of foci caused by activated Ha-Ras. A second construct containing IKLF without the HA-tag gave rise to similar results (not shown). In contrast, an expression plasmid containing GKLF failed to produce any foci, as were mock-transfected cells (Fig. 2). These results suggest that forced expression of *IKLF* causes focus formation in a manner similar to activated Ha-Ras, an activity that was not paralleled by GKLF.

Constitutive Expression of IKLF Causes Accelerated Cell Growth

To further investigate the effect of IKLF on cell proliferation, we established several clonally derived NIH3T3 cell lines that had been transfected with pBK-CMV-IKLF·HA or the pBK-CMV empty vector and selected with the antibiotic, G418. Two independent clones from each construct were chosen and examined. Fig. 3*A* shows that the two pBK-CMV-IKLF·HA- but not the two pBK-CMV-transfected clones (*lanes 1* and *2 versus lanes 3* and *4*, respectively) produced a full-length HA-tagged IKLF as detected by Western blot using a monoclonal antibody against the HA epitope. When the growth characteristics of these cells were examined and compared over a course of 5 days following seeding at a low density, it became apparent that the *IKLF·HA*-expressing cells proliferated at a much faster rate compared with the control, empty vector-transfected cells (Fig. 3*B*). Moreover, whereas the control cells ceased to proliferate after reaching confluency, the *IKLF·HA*-transfected cells continued to grow to several layers (Fig. 3*C*). These findings suggest that the growth of *IKLF·HA*-transfected cells was no longer subject to contact inhibition, providing further evidence for a proproliferative effect of IKLF.

IKLF Causes Serum- and Anchorage-independent Growth

The stably transfected NIH3T3 cells were subjected to additional and more stringent tests of cellular proliferation and transformation. In one, cells were seeded and maintained in medium containing only 1% FBS. Under these conditions, empty vector-transfected cells failed to proliferate. In fact, many perished because of the low-serum content. In contrast, *IKLF·HA*-transfected cells continued to proliferate at a relatively brisk rate albeit slightly slower than that when maintained in 10% FBS (compare Figs. 4*A* and 3*B*). A second test involved growth in soft agar (17). As seen in Fig. 4*B*, *IKLF·HA*-transfected cells formed colonies in an agar suspension as were *Ha-ras*-transfected cells at a ~1:3 ratio. In contrast, empty vector-transfected cells remained as single cells in the agar suspension without ever forming any colonies (Fig. 4*C*). The morphology and size of the colonies produced by *IKLF·HA*- and *Ha-ras*-transfected cells were very similar to each other (Fig. 4*C*).

COMPLEMENTARY DISCUSSION

Complementary DNA clones encoding mouse IKLF were initially identified because of sequence homology to LKLF (12). A human homolog was subsequently isolated based on its binding to a specific *cis*-sequence in the promoter of the lactoferrin gene promoter (21). *In situ* hybridization studies of both adult (12) and fetal intestinal tissues (13) showed that expression of *IKLF* is concentrated in the base of intestinal crypts. The *in vivo* pattern of *IKLF* expression in the intestinal tract therefore correlates with a proliferative phenotype, although a direct effect of IKLF on cellular proliferation was not established by these studies. It should be noted that IKLF is identical to the previously isolated BTEB2 (22), which, because of a sequencing error, has a shorter open-reading frame than IKLF (13). In a rabbit model, expression of *BTEB2*/*IKLF* is induced in activated smooth muscle cells (SMCs) in the neointima of balloon-injured aorta (23). Similarly, increased *BTEB2* expression has been noted in proliferating SMCs at anastomotic vascular stricture (24) and this increased expression is a positive predictive factor for vascular restenosis in pathological conditions (25). Taken

together, these studies suggest that activation of *BTEB2/IKLF* expression is correlated with a proliferative state.

The results of the current study indicate that expression of *IKLF* in cultured, quiescent NIH3T3 cells is acutely and transiently induced upon mitogenic stimulation by factors such as serum and phorbol ester. This induction is dependent on new protein synthesis as it is abolished in the presence of cycloheximide. A similar inductive response of *BTEB2/IKLF* was noted in cultured rabbit aorta-derived SMCs treated with PMA or basic fibroblast growth factor (26). The latter study also showed that expression of the immediate early response gene, *Egr-1*, is highly up-regulated by PMA and that Egr-1 binds to and activates the promoter of the *BTEB2/IKLF* gene (26). It is therefore possible that Egr-1 is an immediate mediator of induction of *IKLF* during proliferative responses.

Despite evidence from *in vitro* and *in vivo* studies demonstrating a correlation between *IKLF* expression and proliferation, it is not clear whether IKLF directly regulates cellular proliferation. The results of the current study are the first to show that constitutive expression of *IKLF* increases proliferation. We also show that IKLF alone is sufficient to cause a transformed phenotype as assessed by focus formation (Fig. 2), loss of contact inhibition (Fig. 3*C*), and gain of serum- (Fig. 4*A*) and anchorage-independent growth (Fig. 4, *B* and *C*). These observations therefore suggest that IKLF is potentially a mediator of cellular proliferation in the various *in vivo* and *in vitro* systems described above. Whether IKLF directly participates in regulating the cell cycle or whether expression of *IKLF* is increased in pathological conditions such as neoplasm remains to be investigated.

Based on the opposing patterns of expression of *GKLF* and *IKLF* in the intestine, Lingrel and co-workers (12) proposed that their gene products may have opposing effects in regulating proliferation and differentiation of the intestinal epithelium. Indeed, the antiproliferative effect of GKLF depicted by previous studies (10,14) and the proproliferative effect of IKLF demonstrated by this study support their hypothesis. Biochemical evidence also suggests that the two proteins may have opposing functions. For example, the promoter of the SMC differentiation marker gene, *SM22α*, is repressed by GKLF but activated by IKLF (27). Preliminary studies from our laboratory also indicate that GKLF and IKLF regulate the *GKLF* promoter in an opposing manner.[2] In view of the highly conserved sequence in the zinc finger region of the two proteins and the similar DNA sequences to which they bind (10,11, 12,14,21), it would not be surprising that the two KLFs may coordinately regulate the expression of a group of genes through similar if not identical *cis*-elements. Further studies will demonstrate the biochemical mechanisms by which GKLF and IKLF antagonize each other in the context of regulating complex biological processes such as cellular proliferation and differentiation.

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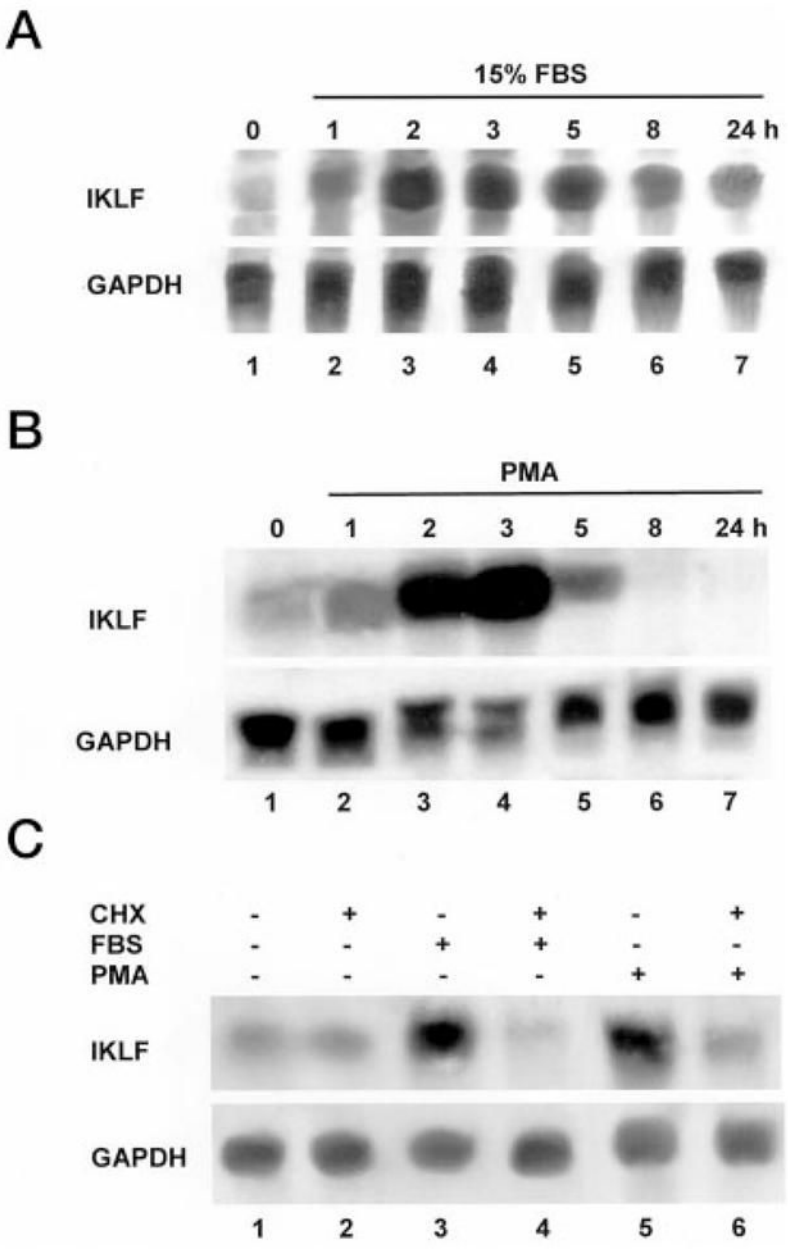

Fig. 1. Activation of IKLF expression in NIH3T3 cells upon mitogenic stimulation

Before addition of mitogens, cells were incubated in medium stripped of serum for 24 h. Medium containing fresh FBS (15% v/v, *A*) or PMA (100 ng/ml, *B*) were then added to the cells for the various times indicated. RNA was extracted from the cells and probed simultaneously for the *IKLF* and *GAPDH* transcript content. In *C*, serum-deprived cells were preincubated in the absence (*lanes 1*, *3*, and *5*) or presence (*lanes 2*, *4*, and *6*) of 10 μg/ml CHX for 1 h, followed by an additional 1 h of medium alone (*lanes 1* and *2*), medium containing FBS (*lanes 3* and *4*), or PMA (*lanes 5* and *6*) in the absence or presence of CHX. RNA was extracted and probed for both *IKLF* and *GAPDH*.

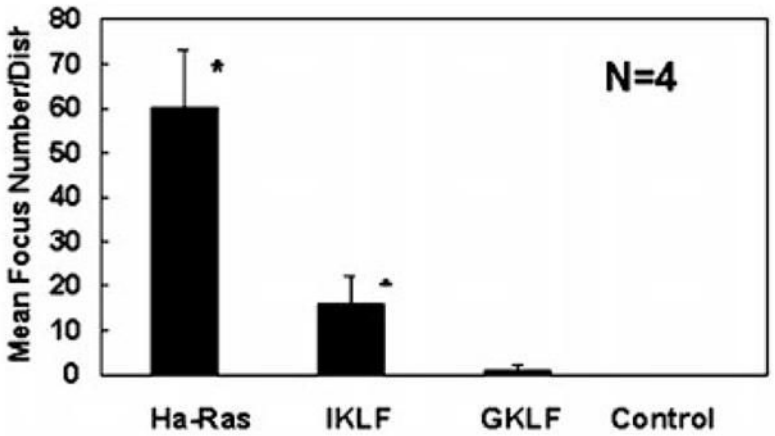

Fig. 2. Focus formation assay

Focus formation assay was conducted as described under "Experimental Procedures." NIH3T3 cells were transfected with 5 μg/10-cm dish of an expression plasmid containing Ha-Ras (16), IKLF·HA (12), or GKLF (10). The control was mock-transfected NIH3T3 cells. Foci were visualized by staining with methylene blue 2–3 weeks following transfection. Shown are the means of four independent experiments, each conducted in quadruplicate. *Bars* indicate S.E. *, $p < 0.01$ when compared with control, mock-transfected cells.

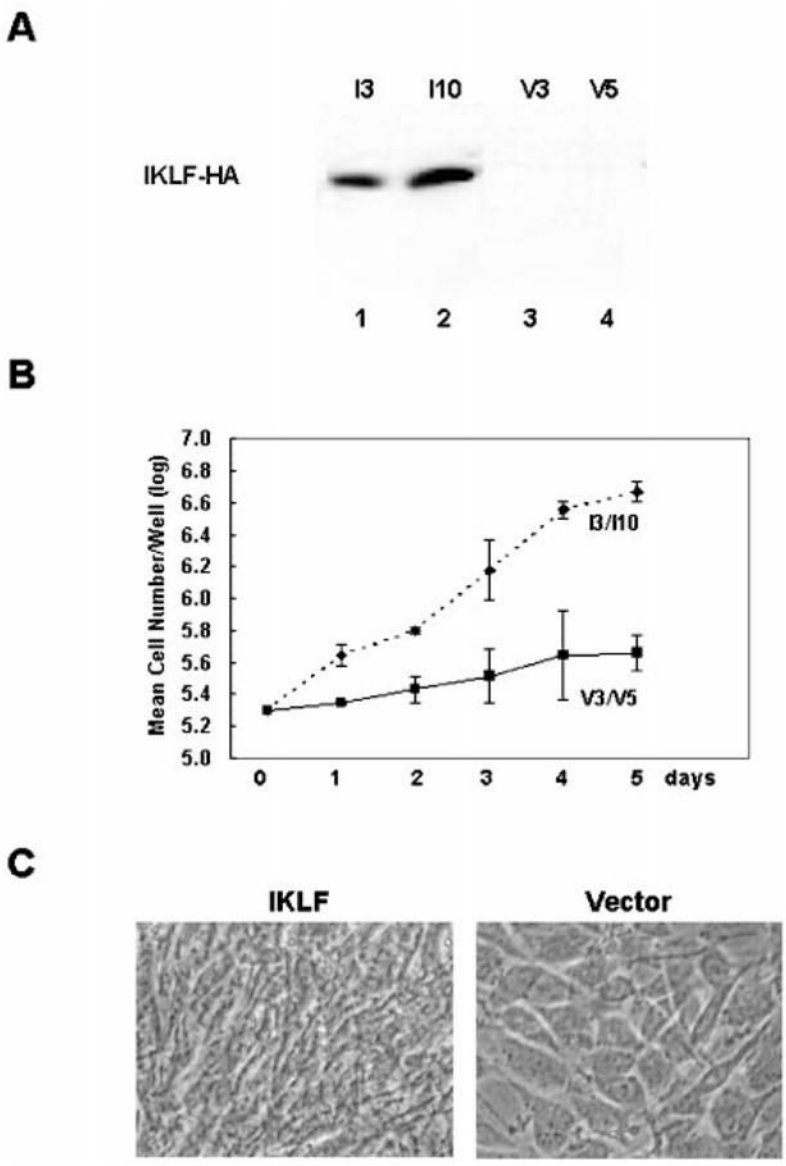

Fig. 3. Growth characteristics of cells stably transfected with

IKLF. Clonal derivatives of NIH3T3 cells stably transfected with pBK-CMV-IKLF·HA or pBK-CMV were selected with G418 and analy·ed for the content of IKLF·HA by Western blot analysis using an anti-HA monoclonal antibody (*A*). Shown are the results from two experiments each of *IKLF·HA*- and empty vector-transfected clones (*I3* and *I10 versus V3* and *V5*, respectively). In *B*, cells from each clone were seeded at a density of 2×10^5 cells/well in 6-well plates and fed every other day with DMEM containing 10% FBS. Three wells of cells from each clone were counted daily in triplicate, after seeding for up to 5 days. Shown are the mean cell numbers/well in log scale of *IKLF·HA*- and empty vector-transfected cells (*I3* and *I10 versus V3* and *V5*, respectively). *Bars* indicate S.E. *C*, typical morphology of *IKLF·HA*- and empty vector-transfected cells (*left versus right*, respectively) at 5 days after seeding. As shown, while the vector-transfected cells remained a monolayer, the *IKLF·HA*-transfected cells grew to multiple layers.

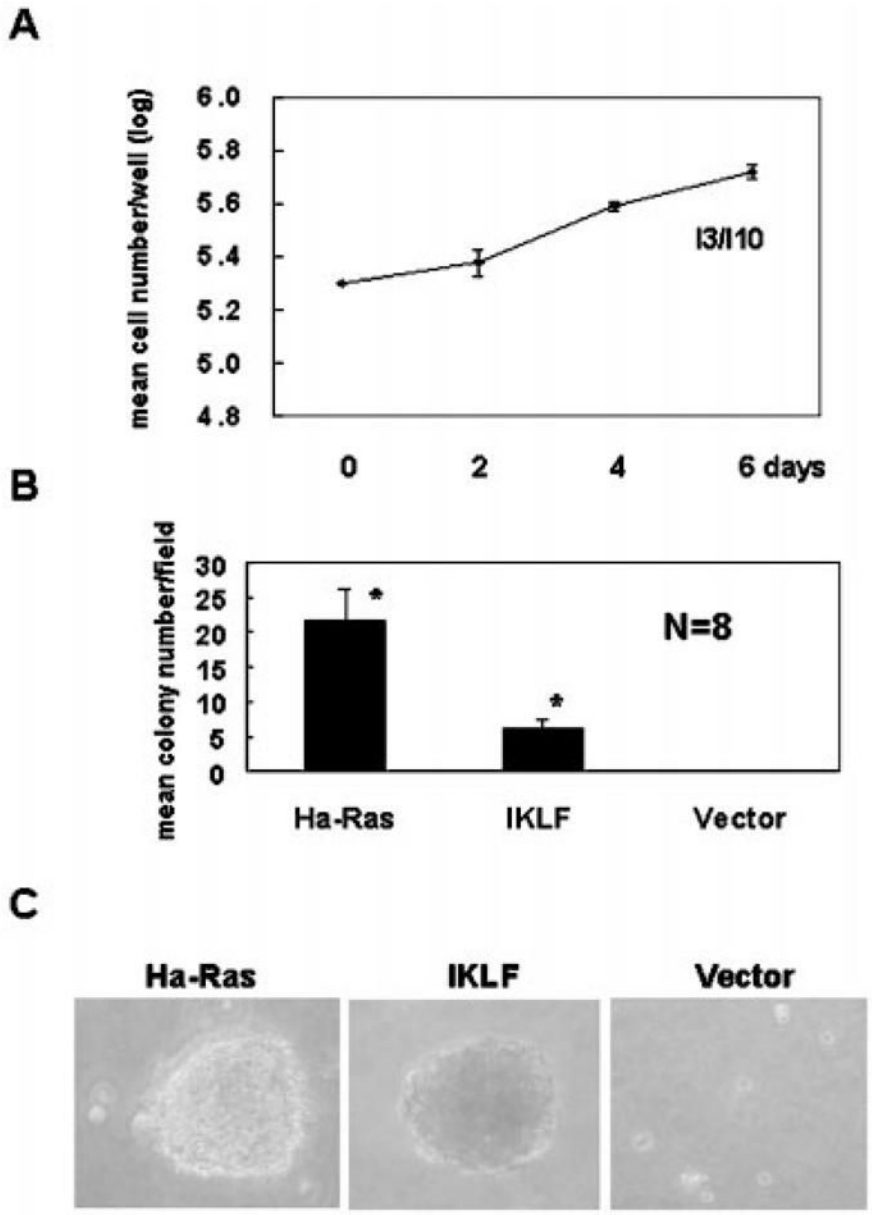

Fig. 4. Serum and anchorage dependence assays of *IKLF*-transfected cells

A, cells from the two *IKLF·HA*-transfected clones (*I3* and *I10*) were seeded at a density of 2 × 10^5 cells/well in 6-well plates and maintained in 1% FBS for up to 6 days after seeding. Cells were counted every other day as described in the legend to Fig. 3 and plotted over time. The two empty vector-transfected clones (V3 and V5) failed to grow (not shown). *B*, *Ha-ras*-, *IKLF·HA*-, and empty vector-transfected cells were seeded in agar suspension, which were fed DMEM with 10% FBS every other day for 3 weeks. Colonies of cells were scored by visual inspection using an inverted phase-contrast microscope. Shown are the mean colony numbers in 8 random fields under a × 100 magnification. *Bars* indicate S.E. *, $p < 0.005$ compared with empty vector-transfected cells (V3 and V5). *C*, photomicrograph of a typical colony of *Ha-ras*- and *IKLF·HA*-transfected cells (*left versus middle*, respectively). Empty vector-transfected cells (*right*) produced no such colonies.