C-type retroviruses has occurred frequently during mammalian evolution, but no human C-type retroviruses have to date been reported. The two C-type retroviruses, RD114 and MLV-A, can infect primary human lymphocytes in culture. Natural anti-Gal(α1–3)Gal antibodies in human serum may therefore have provided one barrier, probably in combination with induced cellular immunity, to the transmission of a number of carbohydrate-carrying enveloped viruses to humans, apes and Old World monkeys from other mammals.

Our data also show that human cells expressing Gal(α1–3)Gal carbohydrate modifications can be lysed by human serum, despite their expression of the complement inhibitory molecules DAF, CD46 and CD59 (determined by FACS analysis; data not shown). However, these transfected cells remain less sensitive to lysis by human serum than murine or canine cells (Fig. 2*a*). Therefore, lysis by human serum is probably controlled by a balance between the expression levels of complement inhibitory molecules and Gal(α1–3)Gal carbohydrate epitopes. This suggests that the expression of Gal(α1–3)Gal epitopes may need to be inhibited before animal organs (pig organs, for example) can be used efficiently in xenotransplantation to humans[26]. Indeed, although human DAF and CD59 expression in transgenic pigs allowed prolonged heart graft survival in baboons, the organs were eventually rejected[27]. □

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1. Welsh, R. M., Cooper, N. R., Jensen, F. C. & Oldstone, M. B. A. *Nature* **257** 612–614 (1975).
2. Welsh, R. M., Jensen, F. C., Cooper, N. R. & Oldstone, M. B. A. *Virology* **74,** 432–440 (1976).
3. Cooper, N. R., Jensen, F. C., Welsh, R. M. & Oldstone, M. B. A. *J. exp. Med.* **144,** 970–984 (1976).
4. Banapour, B., Sernatinger, J. & Levy, J. A. *Virology* **152,** 268–271 (1986).
5. Hoshino, H., Tanaka, H., Miwa, M. & Okada, H. *Nature* **310,** 324–325 (1984).
6. Bartholomew, R. M., Esser, A. F. & Muller-Eberhard, H. J. *J. exp. Med.* **147,** 844–853 (1978).
7. Takeuchi, Y. *et al. J. Virol.* **68,** 8001–8007 (1994).
8. Cosset, F. L., Takeuchi, Y., Battini, J. L., Weiss, R. A. & Collins, M. K. L. *J. Virol.* (in the press).
9. Barbacid, M., Bolognesi, D. & Aaronson, S. A. *Proc. natn. Acad. Sci. U.S.A.* **77,** 1617–1621 (1979).
10. Snyder, H. W. Jr & Fleissner, E. *Proc. natn. Acad. Sci. U.S.A.* **77,** 1622–1626 (1980).
11. Löwer, J., *et al. Virology* **109,** 409–417 (1981).
12. Larsen, R. D., Rivera-Marrero, C. A., Ernst, L. K., Cummings, R. D. & Lowe, J. B. *J. biol. Chem.* **265,** 7055–7061 (1990).
13. Galili, U. & Swanson, K. *Proc. natn. Acad. Sci. U.S.A.* **88,** 7401–7404 (1991).
14. Gustafsson, K., Strahan, K. & Preece, A. *Immun. Rev.* **141,** 59–70 (1994).
15. Strahan, K. M. *et al. Immunogenetics* **41,** 101–105 (1995).
16. Sandrin, M. S. & McKenzie, I. F. *Immun. Rev.* **141,** 169–190 (1994).
17. Hamadeh, R. M. *et al. J. clin. Invest.* **89,** 1223–1235 (1992).
18. Galili, U., Mandrell, R. E., Hamadeh, R. M., Shohet, S. B. & Griffiss, J. M. *Infect. Immun.* **56,** 1730–1737 (1988).
19. Galili, U., Anaraki, F., Thall, A., Hill-Black, C. & Radic, M. *Blood* **82,** 2485–2493 (1993).
20. Almeida, I. C., Krautz, G. M., Krettli, A. U. & Travassos, L. R. *J. clin. Lab. Anal.* **7,** 307–316 (1993).
21. Oriol, R., Ye, Y., Koren, E. & Cooper, D. K. *Transplantation* **56,** 1433–1442 (1993).
22. Sandrin, M. S., Vaughan, H. A., Dabkowski, P. L. & McKenzie, I. F. *Proc. natn. Acad. Sci. U.S.A.* **90,** 11391–11395 (1993).
23. Repik, P. M., Strizki, J. M. & Galili, U. *J. gen. Virol.* **75,** 1177–1181 (1994).
24. Welsh, R. M. *J. Immun.* **118,** 348–354 (1977).
25. Hirsch, R. L., Griffin, D. E. & Winkelstein, J. A. *J. Immun.* **127,** 1740–1743 (1981).
26. Fabre, J. W. *Nature Med.* **1,** 403–404 (1995).
27. McCurry, K. R. *et al. Nature Med.* **1,** 423–427 (1995).
28. Cosset, F. L. *et al. J. Virol.* **69,** 6314–6322 (1995).
29. McKenzie, I. F. C. *et al. Lancet* **ii,** 386–387 (1968).
30. Battini, J. L., Heard, J. M. & Danos, O. *J. Virol.* **66,** 1468–1475 (1992).

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Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours

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APOPTOSIS is a genetically encoded programme of cell death that can be activated under physiological conditions[1,2] and may be an important safeguard against tumour development[3–6]. Regions of low oxygen (hypoxia) and necrosis are common features of solid tumours[7,8]. Here we report that hypoxia induces apoptosis in oncogenically transformed cells and that further genetic alterations, such as loss of the *p53* tumour-suppressor gene or over-expression of the apoptosis-inhibitor protein Bcl-2, substantially reduce hypoxia-induced cell death. Hypoxia also selects for cells with defects in apoptosis, because small numbers of transformed cells lacking p53 overtake similar cells expressing wild-type p53 when treated with hypoxia. Furthermore, highly apoptotic regions strongly correlate with hypoxic regions in transplanted tumours expressing wild-type p53, whereas little apoptosis occurs in hypoxic regions of p53-deficient tumours. We propose that hypoxia provides a physiological selective pressure in tumours for the expansion of variants that have lost their apoptotic potential, and in particular for cells acquiring *p53* mutations.

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TABLE 1 p53-modulated hypoxic induction of apoptosis *in vivo*

	Number of apoptotic cells per 0.01 mm²		Fold
	Aerobic regions	Hypoxic regions	increase
p53$^{-/-}$	4.1 ± 1.8	7.9 ± 4.4	1.9
p53$^{+/+}$	3.7 ± 1.7	26.8 ± 9.4	7.2
Ratio +/+ to −/−	0.9	3.4 (6.1*)	

Quantification of apoptosis was performed on the TUNEL and EF5 stained sections described in Fig. 3. Fields containing both aerobic (EF5-negative) and hypoxic (EF5-positive) regions were randomly chosen while blinded to the apoptosis staining results (see, for example, Fig. 3*d*) and then photographed. The number of apoptotic cells per unit area was then calculated for each distinct region. Values stated are means ± s.d. ($n = 16$, 4 tumours each genotype).
* With aerobic values subtracted.

Solid tumours that possess low-oxygen regions have a poorer prognosis than well oxygenated tumours, independent of treatment[9]. To investigate how oncogenically transformed cells respond to hypoxia, Rat1 fibroblasts constitutively expressing a c-Myc–oestrogen receptor chimaera protein (MycER), which is activated by 4-hydroxytamoxifen (4hT)[10], were cultured either aerobically or anaerobically in the presence of 4hT and 10% serum for 48 h. Hypoxia induced substantial apoptosis (85 ± 2%; Fig. 1*b*) as compared to aerobic culture (10 ± 2; Fig. 1*a*). Bcl-2, which prevents c-Myc-mediated apoptosis following serum depletion[3], also blocked c-Myc-mediated apoptosis induced by hypoxia (Fig. 1*c–d,i*). These data indicate that oncogenic changes can modulate the susceptibility of cells to hypoxia-induced apoptosis.

p53 is an integral part of DNA damage-induced apoptotic pathways[11]. Because cells exposed to hypoxia accumulate wild-type

p53 protein[12], we investigated the role of p53 in hypoxia-induced apoptosis using embryonic fibroblasts derived from wild-type and *p53*-deficient transgenic mice (Fig. 1*e–h, j*). Transformation of p53-expressing mouse embryonic fibroblasts (MEFs) by the adenovirus early region 1A (E1A) and activated Ha-*ras* oncogenes dramatically enhanced their susceptibility to hypoxia-induced apoptosis. Untransformed MEFs remained over 95% viable, whereas their transformed derivatives were only 11% viable after 48 h of hypoxia. p53 protein accumulated under hypoxia (not shown) and was required for efficient induction of apoptosis because oncogenically transformed cells lacking p53 were resistant to hypoxia-induced killing (72% viability after 48 h; Fig. 1*j*). Nonetheless, the $p53^{-/-}$ cells were also dying through apoptosis, indicating the existence of a less sensitive p53-independent apoptotic pathway[3]. The influence of p53 on apoptotic potential was not apparent until oxygen concentrations were reduced below 0.2% (Fig. 1*k*), corresponding to concentrations commonly found in solid tumours[8,9]. Thus, oncogenic transformation predisposes cells to hypoxia-induced killing through an apoptotic pathway modulated by p53.

The difference in viability of oncogenically transformed cells that differ in *p53* status suggests that p53-deficient cells would have a survival advantage over wild-type p53 cells in low-oxygen conditions. To test this hypothesis, we mixed transformed $p53^{-/-}$ (E1A, Ha-*ras*) cells stably expressing the *lacZ* gene product ($lacZ^+$) (Fig. 2*c*) with $p53^{+/+}$ (E1A, Ha-*ras*) $lacZ^-$ cells at a 1 to 1,000 ratio and treated them with multiple rounds of hypoxia and aerobic recovery. The percentage of $p53^{-/-}$ cells increased by approximately 2.4-fold following each treatment, and after 7 treatments the $p53^{-/-}$ ($lacZ^+$) cells had overtaken the $p53^{+/+}$ cells (Fig. 2*d–f*). Control cells (Fig. 2*a*) grown aerobically in parallel had no detectable increase in their percentage of $p53^{-/-}$ cells (Fig. 2*b*). These experiments demonstrate that hypoxia can select for apoptosis-resistant cells.

To investigate the relationship between oxygen concentration, *p53* status and apoptosis *in vivo*, we determined whether apoptotic regions correlated with hypoxic regions in tumours derived from the E1A- and Ha-*ras*-transformed $p53^{+/+}$ and $p53^{-/-}$ cells[13]. Apoptosis was assessed using the terminal deoxytransferase-mediated deoxyuridine nick end-labelling (TUNEL) assay. In TUNEL-stained sections, apoptotic regions were more prevalent in $p53^{+/+}$ tumours than in $p53^{-/-}$ tumours (see below), and apoptosis was most pronounced in regions distal to adjacent blood vessels (Fig. 3*a,b*).

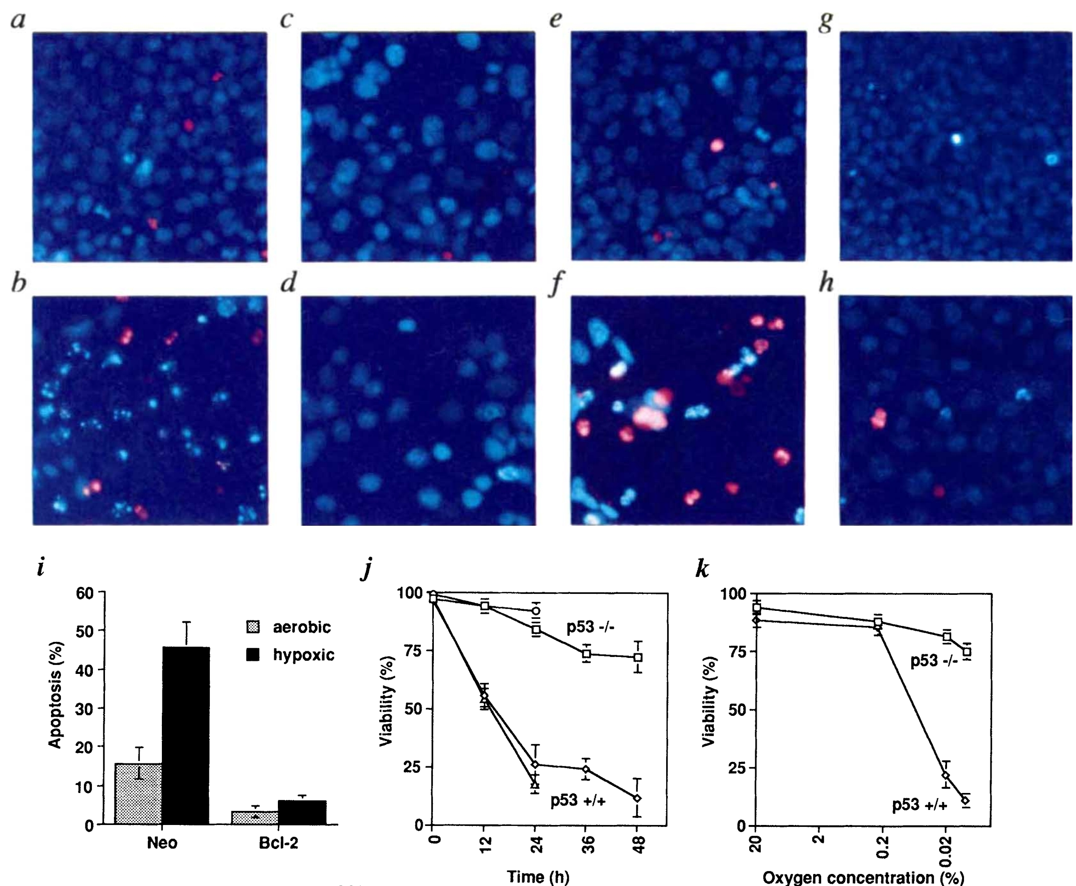

FIG. 1 Apoptotic response of oncogenically transformed cells to hypoxia. Following aerobic (*a, c, e, g*) or anaerobic (*b, d, f, h*) culture, nuclear morphology was visualized by staining with bisbenzimide (blue) and apoptotic cells were identified by their characteristic nuclear condensation and fragmentation[1]. Loss of membrane integrity was assessed by permeability to propidium iodide (PI; pink). Cell lines shown are MycER/Rat1 (*a, b*), MycER/Rat1 + Bcl-2 (*c, d*), $p53^{+/+}$ (E1A, Ha-*ras*) (*e, f*) and $p53^{-/-}$ (E1A, Ha-*ras*) (*g, h*) at ×270 magnification. Rat1 ($p53^{+/+}$) cells transformed by E1A and Ha-*ras* also underwent apoptosis when cultured under hypoxic conditions. *i*, Quantification of the percentage of apoptotic cells in control transfectants (Neo) or Bcl-2 transfectants of MycER/Rat1 cells cultured aerobically or under hypoxic conditions. Results shown in *i* are the means ± s.e.m. of two independently derived clones. *j, k*, Loss of viability due to induced apoptosis in $p53^{+/+}$ (E1A, Ha-*ras*) and $p53^{-/-}$ (E1A, Ha-*ras*) cells following culture at 0.02% oxygen (*j*), or following 24 h at various oxygen concentrations (*k*). Data shown in (*j, k*) are the means ± s.e.m. of two independent experiments. Each curve in (*j*) represents data from an independently derived clone. Untransformed $p53^{+/+}$ MEFs were 95% viable after 48 h of hypoxia (0.02% oxygen).

METHODS. MycER/Rat1 fibroblasts were generated by transfection of Rat1 fibroblasts with the plasmid pBpuro *c-myc*ER which encodes for a MycER chimaera protein that is activated by 4hT[10]. MycER/Rat1 cells expressing Bcl-2 protein (verified by immunoblot analysis) were generated by subsequent transfections with the plasmid pSFFV Bcl-2nl[27]. To activate Myc, 100 nM 4hT was added to the media of control and hypoxia-treated cells at the start of each experiment. MycER/Rat1 cells treated with hypoxia in the absence of 4hT showed similar low levels of apoptosis as hypoxia-treated parental Rat1 cells. Transformed $p53^{\pm/\pm}$ MEFs which express E1A and Ha-*ras* were generated as described[28]. Although p53 promotes apoptosis induced by hypoxia, p53 is not required for hypoxia-induced cell-cycle block[12]. Hypoxic incubations were performed in anaerobic incubators as described[12], with an oxygen concentration of 0.02% for 48 h unless otherwise noted. Before microscopy, cells were incubated with 5 μg ml^{-1} each of bisbenzimide (Hoechst No. 33342) and PI (*a–i*). Apoptosis/viability ratios were determined by scoring low-magnification photographs of randomly selected fields for cells with condensed and fragmented nuclear morphology (*i*), or by trypan-blue dye exclusion (*j, k*). All cells were cultured in media containing 10% fetal calf serum.

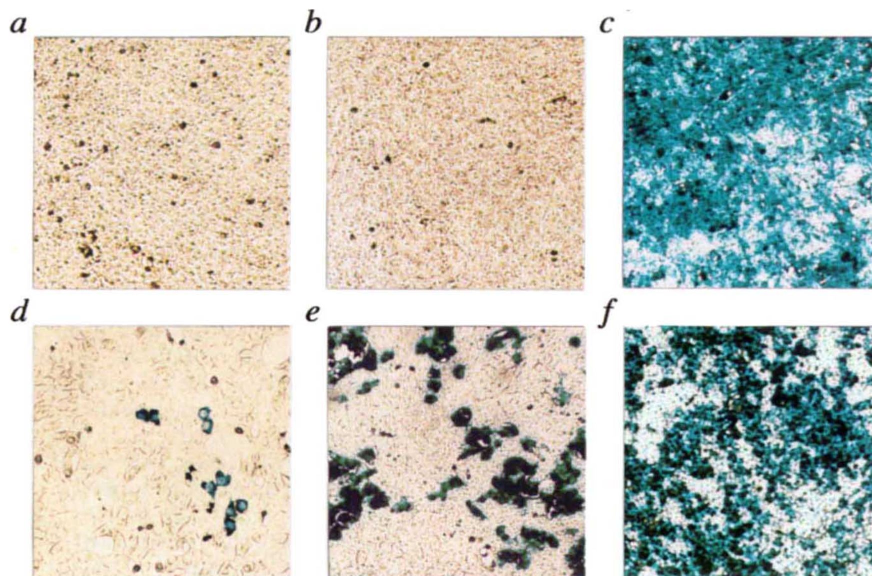

FIG. 2 Selection for $p53^{-/-}$ cells following hypoxia treatment *in vitro*. $p53^{-/-}$ (E1A, Ha-*ras*) cells stably expressing the *lacZ* gene product (lacZ$^+$; blue) were mixed with $p53^{+/+}$ (E1A, Ha-*ras*) cells (lacZ$^-$; clear) at a ratio of 1 to 1,000 and treated with multiple rounds of hypoxia and aerobic recovery. Representative photographs of β-galactoside-stained cells are shown at ×135 magnification: *a*, mixed cells at start of experiment; *b*, mixed cells cultured aerobically in parallel for the duration of the experiment; *c*, 100% $p53^{-/-}$ (lacZ$^+$) cells, and mixed cells after three (*d*), six (*e*) and seven (*f*) rounds of hypoxia treatment.

METHODS. $p53^{-/-}$ (lacZ$^+$) populations were generated by viral infection of $p53^{-/-}$ (E1A, Ha-*ras*) cells using the MFG-lacZ retrovirus[29], with subsequent enrichment for lacZ$^+$ cells by fluorescence-activated cell sorting[30]. Treated mixed cells were subjected to multiple rounds of hypoxia (2–3 days) followed by recovery (3–5 days) with sub-culturing when confluent. LacZ$^+$ cells were detected by staining for β-galactosidase activity[30].

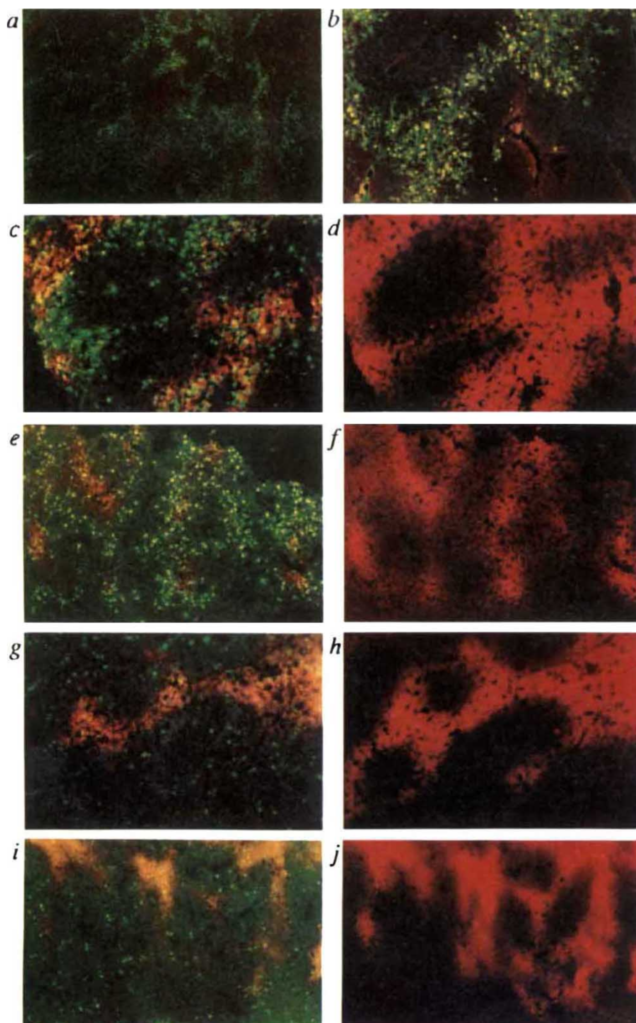

FIG. 3 Relationship between hypoxic and apoptotic regions in tumours differing in *p53* status. *a*, *b*, TUNEL staining (green) of a $p53^{+/+}$ paraffin tumour section showing apoptotic regions distal to blood vessels (counterstained with propidium iodide (red); *a*, magnification ×25; *b*, ×135). *c–j*, TUNEL staining (green) and EF5 staining (orange/red) of frozen tumour sections comparing the extent of apoptosis in similarly stained EF5-positive hypoxic regions of $p53^{+/+}$ tumours (*c–f*) and $p53^{-/-}$ tumours (*g–j*). Photographs *c*, *e*, *g*, *i* simultaneously show both TUNEL staining and the brightest EF5 staining using a dual-band pass fluorescence filter set, and photographs *d*, *f*, *h*, *j* show all EF5 staining of the same regions using a single-band pass filter set (*c*, *d*, *g*, *h*, magnification ×135; *e*, *f*, *i*, *j*, ×85). Representative photographs of multiple tumours are shown.

METHODS. $p53^{+/+}$ (E1A, Ha-*ras*) and $p53^{-/-}$ (E1A, Ha-*ras*) cells were transplanted subcutaneously into the backs of immunocompromised mice as described[13] (*a*, *b*, athymic nude mice; *c–j*, severe combined immunodeficient (SCID) mice). After tumours reached a volume of 0.2 to 1.2 cm^3, the tumours were either excised and fixed for paraffin section processing (*a*, *b*; 5 μm thick), or the mice were given an intraperitoneal injection of EF5 (2(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl)-acetamide) 1 h before tumour excision and freezing for frozen sectioning (*c–j*; 15 μm thick) as described[14]. In the absence of oxygen, EF5 is metabolically activated in viable cells and forms adducts with cellular macromolecules which can be detected with a monoclonal antibody[14]. TUNEL staining (fluorescein, green) was done according to the manufacturer's instructions (Oncor). Where applicable, EF5 staining (Cy3 fluorochrome, red) was done subsequently on the same section as described[14]. Hypoxic regions were present in all tumour sizes. Frozen sections of control tumours that were not exposed to EF5 had similar apoptotic patterning as tumours shown in *c–j*. 1-h exposures of aerobic or hypoxic cells to EF5 did not induce apoptosis *in vitro*.

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To determine directly whether p53 influences the magnitude of apoptosis in tumour regions of similar oxygen deficiency, the specific hypoxia marker EF5[14] was injected 1 h before excision of $p53^{+/+}$ and $p53^{-/-}$ tumours. Frozen sections from these tumours were then stained by the TUNEL assay and with anti-EF5 antibody. Although no difference in hypoxic fraction or patterning was observed between tumours derived from the two *p53* genotypes, $p53^{+/+}$ tumours showed a more distinct spatial correlation between apoptotic and hypoxic regions compared to $p53^{-/-}$ tumours (Fig. 3*c–j*). The frequency of apoptosis was 3.4-fold greater in hypoxic regions of $p53^{+/+}$ tumours than in hypoxic regions of *p53*-deficient tumours, whereas no difference was seen in aerobic regions from the same tumours. Hypoxic regions in $p53^{+/+}$ tumours displayed 7.2 times more apoptosis than aerobic regions (Table 1). *In vivo*, other tumour-microenvironmental factors determined by vascularization and angiogenesis could act to induce apoptosis synergistically, although *in vitro* data indicate that hypoxia is sufficient (Fig. 1).

The data presented here have important implications for multistep carcinogenesis. Oncogenic changes occurring early in solid tumour development promote deregulated proliferation and can also increase cellular susceptibility to apoptosis[15–17]. We have shown that oncogenic transformation increases the sensitivity of cells to hypoxia and that hypoxia correlates spatially with apoptosis *in vivo*. The same genetic events that suppress hypoxia-induced apoptosis in oncogenically transformed cells also temporally coincide with reduced apoptosis in later stages of tumour development[5,18–21]. These observations suggest that hypoxia acts as a physiological selective agent against apoptosis-competent cells in tumours, thus promoting the clonal expansion of cells that acquire mutations in their apoptotic programmes.

p53 mutation and Bcl-2 overexpression also suppress apoptosis induced by radiation and chemotherapy[22–24]. Hence, hypoxia-mediated selection of cells with diminished apoptotic potential could also explain the resistance of many solid tumours to cancer therapy[24]. Although apoptosis induced by ultraviolet radiation has been implicated in the clonal selection of *p53* mutations in skin cancer[25], most solid tumours are not exposed to ultraviolet radiation during their development. In contrast, hypoxia is a physiological stress commonly found in developing solid tumours of diverse origins[8], and thus our results offer an explanation for why *p53* is one the most commonly mutated genes in human cancer[26]. □

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1. Bellamy, C. O. C., Malcomson, R. D. G., Harrison, D. J. & Wyllie, A. H. *Semin. Cancer Biol.* **6,** 3–16 (1995).
2. Thompson, C. B. *Science* **267,** 1456–1462 (1995).
3. Harrington, E. A., Fanidi, A. & Evan, G. I. *Curr. Opin. Genet. Dev.* **4,** 120–129 (1994).
4. Wyllie, A. H. *Curr. Opin. Genet. Dev.* **5,** 97–104 (1995).
5. Symonds, H. *et al. Cell* **78,** 703–711 (1994).
6. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. *Cell* **82,** 675–684 (1995).
7. Thomlinson, R. H. & Gray, L. H. *Br. J. Cancer* **9,** 539–549 (1955).
8. Vaupel, P. W. & Hockel, M. in *Tumor Oxygenation* (eds Vaupel, P. W., Kelleher, D. K. & Gunderoth, M.) 219–232 (Gustav Fischer, Stuttgart, 1995).
9. Hockel, M., Vorndran, B., Schlenger, K., Baussmann, E. & Knapstein, P. G. *Gynecol. Oncol.* **51,** 141–149 (1993).
10. Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G. & Evan, G. I. *Nucleic Acids Res.* **23,** 1686–1690 (1995).
11. Lane, D. P., Lu, X., Hupp, T. & Hall, P. A. *Phil. Trans. R. Soc.* B **345,** 277–280 (1994).
12. Graeber, T. G. *et al. Molec. cell. Biol.* **14,** 6264–6277 (1994).
13. Lowe, S. W. *et al. Science* **266,** 807–810 (1994).
14. Lord, E. M., Harwell, L. & Koch, C. J. *Cancer Res.* **53,** 5721–5726 (1993).
15. Bishop, J. M. *Science* **235,** 305–311 (1987).
16. Harrington, E. A., Bennett, M. R., Fanidi, A. & Evan, G. I. *EMBO J.* **13,** 3286–3295 (1994).
17. Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. *Cell* **74,** 957–967 (1993).
18. Bardeesy, N., Beckwith, J. B. & Pelletier, J. *Cancer Res.* **55,** 215–219 (1995).
19. Baker, S. J. *et al. Cancer Res.* **50,** 7717–7722 (1990).
20. Sinicrope, F. A. *et al. Cancer Res.* **55,** 237–241 (1995).
21. Bedi, A. *et al. Cancer Res.* **55,** 1811–1816 (1995).
22. Lotem, J. & Sachs, L. *Cell Growth. Differ.* **4,** 41–47 (1993).
23. Lowe, S. W. *Curr. Opin. Oncol.* (in the press).
24. Hickman, J. A., Potten, C. S., Merritt, A. J. & Fisher, T. C. *Phil. Trans. R. Soc.* B **345,** 319–325 (1994).
25. Ziegler, A. *et al. Nature* **372,** 773–776 (1994).
26. Greenblatt, M. S., Bennett, W. P., Hollstein, M. & Harris, C. C. *Cancer Res.* **54,** 4855–4878 (1994).
27. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. & Korsmeyer, S. J. *Nature* **348,** 334–336 (1990).
28. Lowe, S. W., Jacks, T., Housman, D. E. & Ruley, H. E. *Proc. natn. Acad. Sci. U.S.A.* **91,** 2026–2030 (1994).
29. Dranoff, G. *et al. Proc. natn. Acad. Sci. U.S.A.* **90,** 3539–3543 (1993).
30. Pear, W., Nolan, G. P., Scott, M. L. & Baltimore, D. *Proc. natn. Acad. Sci. U.S.A.* **90,** 8392–8396 (1993).

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Regulation of cell adhesion and anchorage-dependent growth by a new β_1-integrin-linked protein kinase

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THE interaction of cells with the extracellular matrix regulates cell shape, motility, growth, survival, differentiation and gene expression, through integrin-mediated signal transduction[1–3]. We used a two-hybrid screen to isolate genes encoding proteins that interact with the β_1-integrin cytoplasmic domain. The most frequently isolated complementary DNA encoded a new, 59K serine/threonine protein kinase, containing four ankyrin-like repeats. We report here that this integrin-linked kinase (ILK) phosphorylated a β_1-integrin cytoplasmic domain peptide *in vitro* and coimmunoprecipitated with β_1 in lysates of mammalian cells. Endogenous ILK kinase activity was reduced in response to fibronectin. Overexpression of $p59^{ILK}$ disrupted epithelial cell architecture and inhibited adhesion to integrin substrates, while inducing anchorage-independent growth. We propose that ILK is a receptor-proximal protein kinase regulating integrin-mediated signal transduction.

A partial cDNA, BIT-9, was isolated in a two-hybrid screen[4] using a bait plasmid expressing the cytoplasmic domain of the β_1 integrin subunit. The BIT-9 insert was used to isolate clones from a human placental cDNA library. A 1.8 kilobase (kb) clone, Plac5, was found to contain a high degree of similarity to cDNAs encoding protein kinases (Fig. 1*a–c*), and recognized a widely expressed transcript of 1.8 kb in northern blots (Fig. 1*d*). Deduced amino-acid residues 186–451 from Plac5 comprise a domain which is highly homologous with the catalytic domains of a large number of protein tyrosine and serine/threonine kinases (Fig. 1*b*). Residues 33–164 comprise four repeats of a motif originally identified in erythrocyte ankyrin[5] (Fig. 1*c*), probably defining a domain involved in mediating additional protein–protein interactions[6,7]. Affinity-purified anti-ILK antibodies (see methods, Fig. 3) were used in western blot analyses of mammalian cell extracts, and detected a conserved protein of apparent M_r of 59K ($p59^{ILK}$, Fig. 1*e*).

For analysis of kinase activity *in vitro*, a bacterially expressed fusion protein, $GST\text{-}ILK^{132}$, was band-purified after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and incubated with [γ-^{32}P]ATP in the presence or absence of the exogenous substrate myelin basic protein (Fig. 2). $GST\text{-}ILK^{132}$

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