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Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival

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The paired-box (*PAX*) genes encode a family of nine well-characterized paired-box transcription factors, with important roles in development and disease. Although *PAX* genes are primarily expressed in the embryo, constitutive expression promotes tissue hyperplasia. Rare tumor-specific mutations of *PAX* genes implicate an oncogenic role, and persistent *PAX* expression characterizes several tumors. Yet, a cancer-wide analysis of *PAX* gene expression to investigate a general role for *PAX* genes has not been performed. We analysed the pattern and requirement for *PAX* gene expression in a panel of common cancer cell lines. Very frequent *PAX* gene expression was identified in tumor cell lines, including lymphoma, breast, ovarian, lung, and colon cancer. In addition, the *PAX2* gene was frequently expressed in a panel of 406 common primary tumor tissues. Apoptosis was rapidly induced in ovarian and bladder cancer cell lines following RNA interference to silence *PAX2* expression, despite concomitant *TP53* and/or *HRAS* mutations. These data suggest that *PAX* genes are frequently expressed in cancer, and that endogenous *PAX* gene expression is required for the growth and survival of cancer cells.
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Introduction

The PAired-boX (PAX) gene family has been well characterized in both human (*PAX*) and mouse (*Pax*), and consists of nine genes (Dahl *et al*., 1997; Chi and Epstein, 2002). Each of the *PAX* genes encodes a transcription factor containing a paired-box DNA-binding domain, with or without octapeptide and/or homeodomains (Dahl *et al*., 1997). Subclassification of the *PAX* genes into subgroups (*PAX1*/*PAX9*, *PAX2*/*PAX5*/*PAX8*, *PAX3*/*PAX7*, *PAX4*/*PAX6*) on the basis of sequence correlates with expression patterns in developing embryonic tissues (Chi and Epstein, 2002). In each of these tissues, the *PAX* genes specify positional information in the developing embryo. The *PAX* genes are tightly regulated in both temporal and spatial expression patterns, and expression is observed primarily during fetal development, with the genes being switched off during latter phases of terminal differentiation of most structures. This pattern is maintained in the mature organism. However, in a few adult tissues, *PAX* genes are expressed in a restricted fashion (Kozmik *et al*., 1993; St-Onge *et al*., 1997; Eccles, 1998). In contrast, abnormal cell growth and proliferation is associated with high levels of *PAX* gene expression in several diseases occurring in otherwise terminally differentiated tissues (Bernasconi *et al*., 1996; Winyard *et al*., 1996; Murer *et al*., 2002).

Haploinsufficiency of *PAX2*, *PAX3*, and *PAX6* genes cause dysmorphic syndromes with developmental abnormalities (Glaser *et al*., 1992; Jordan *et al*., 1992; Tassabehji *et al*., 1992; Sanyanusin *et al*., 1995). In affected mutant tissues, dysmorphogenesis is associated with increased apoptosis in cells that normally express the *PAX* gene (Borycki *et al*., 1999; Ostrom *et al*., 2000; Porteous *et al*., 2000; Torban *et al*., 2000; van Raamsdonk and Tilghman, 2000). Conversely, deregulated expression of *PAX2* blocks caspase 2-induced apoptosis in HEK293 cells, suggesting a role for *PAX2* in cell survival. Yet, the continuous expression of *PAX* genes is not required at late stages of differentiation or following terminal differentiation of cells. For example, *PAX2* expression must be switched off for completion of epithelial differentiation in the kidney (Dressler *et al*., 1993). Persistent expression of *PAX* genes in terminally differentiated tissues is associated with a blockage in tissue differentiation and hyperplasia (Dressler *et al*., 1993; Morrison *et al*., 1998b).

Chromosomal translocations involving *PAX3*, *PAX5*, *PAX7* or *PAX8* genes in alveolar rhabdomyosarcoma, B-lymphoid malignancies, or thyroid cancer, respectively, suggest that *PAX* genes have an oncogenic capacity when constitutively expressed, either as part of a fusion gene, or as a whole gene (Barr *et al*., 1993; Davis *et al*., 1994; Morrison *et al*., 1998a; Kroll *et al*., 2000; Cazzaniga *et al*., 2001; Marques *et al*., 2002). In addition, *PAX* genes are persistently expressed in several embryonal tumors (Dressler and Douglass, 1992; Eccles *et al*., 1992, 1995; Bernasconi *et al*., 1996) and adult

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tumors (Fabbro *et al*., 1994; Gnarra and Dressler, 1995; Stuart *et al*., 1995b; Scholl *et al*., 2001; Silberstein *et al*., 2002), and reduced survival has been observed in rhabdomyosarcoma or melanoma cell lines following treatment with *PAX3* antisense oligonucleotides (Bernasconi *et al*., 1996; Scholl *et al*., 2001). Yet, constitutive expression of *PAX5* is not sufficient to induce transformation of the mouse neuroectoderm (Steinbach *et al*., 2001). It is therefore unclear whether *PAX* gene expression plays a significant role in cancer in general, and it is not known to what extent *PAX* genes are expressed in cancer.

To investigate the frequency and significance of *PAX* gene expression in cancer, we characterized *PAX* gene mRNA levels in 54 common cancer cell lines, including 51 cell lines of the NCI-60 cell line panel. Approximately 90% of the cancer cell lines expressed *PAX* genes, while a high proportion of primary tumors, including breast, ovarian, lung, colon, prostate, and lymphoma, expressed *PAX2*. The role of *PAX* gene expression in several different cancer types was investigated in melanoma (*PAX3*), and in ovarian and bladder cancer cell lines (*PAX2*). Both *PAX2* and *PAX3* were functionally important in the cancer cell lines, as rapid induction of apoptosis was observed following treatment of cancer cells with either siRNA, or antisense oligonucleotides designed to inhibit *PAX2* or *PAX3* expression. These data suggest that endogenous *PAX* gene expression in cancer is common, and is required for the growth and survival of many cancer types.

Results and discussion

Melanoma cell lines depend on PAX3 *for survival*

Scholl *et al*. (2001) previously analysed five melanoma cell lines, and showed that treatment with a *PAX3* antisense oligonucleotide resulted in induction of apoptosis and reduced cell survival in the melanoma cell lines specifically expressing *PAX3*. To confirm the requirement for *PAX3* in melanoma cell survival, we transfected two melanoma cell lines from the NCI-60 cell line panel, M14 (*TP53* mutant) and UACC-62 (*TP53* wildtype), with *PAX3* antisense oligonucleotide, as previously described (Scholl *et al*., 2001). Treatment of the cell lines with the *PAX3* antisense oligonucleotides led to significantly decreased cell growth, and increased annexin V staining in flow cytometric analysis, suggesting that treated cells underwent apoptosis when exposed to the antisense oligonucleotide. Treatment with sense *PAX3* oligonucleotide resulted in no significant change in the cell survival (Figure 1). Annexin V positive staining was detected in 45% of UACC-62 cells and 18% of M14 cells 24 h after antisense treatment, but in only 23 and 13% of sense *PAX3* oligonucleotide-treated cells, respectively (Figure 1a). The induction of apoptosis was rapid, as annexin V staining was detected in the antisense-treated UACC-62 melanoma cells within 12 h after treatment. The proliferation of both

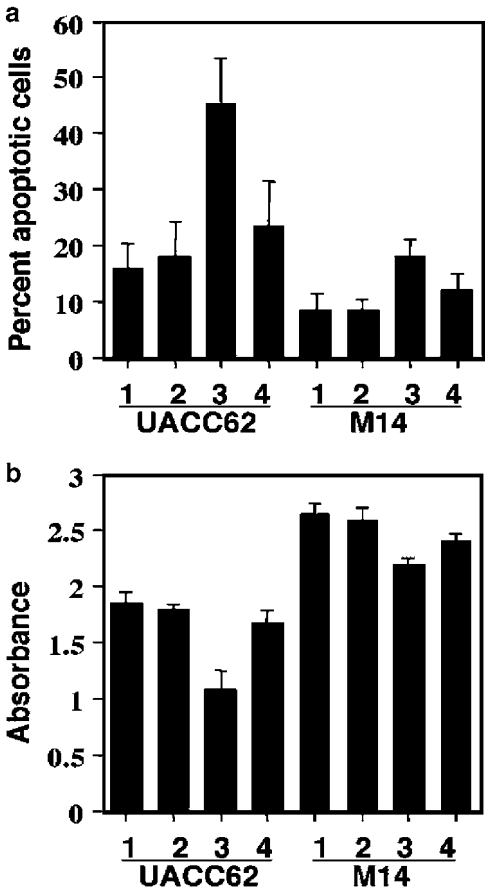

Figure 1 Cell proliferation and apoptosis in UACC-62 and M14 melanoma cells treated with antisense or sense *PAX3* oligonucleotides. (**a**) Cells were treated with media only (1), lipofectin (2), or transfected with *PAX3* antisense oligonucleotide (3), or *PAX3* sense oligonucleotide (4), and analysed at 12, 24, 48, and 72 h. A significant increase in apoptosis was observed in M14 and UACC62 cells after transfection with *PAX3* antisense oligonucleotides (3), but not after treatment with sense *PAX3* oligonucleotides (4), lipofectin only (2) or media only (1) at 24 h. Apoptotic cells were stained with fluorescein annexin-V conjugate and analysed as in Figure 5, below. (**b**) Cell mass was measured by MTT assay after exposure to media only (1), lipofectin (2), antisense *PAX3* (3), or sense *PAX3* oligonucleotides (4) at 24 h. Results represent the average of three separate experiments. Error bars show standard deviations

UACC-62 and M14 cells was significantly inhibited at 24 h following treatment (Figure 1b).

PAX genes are frequently expressed in cancer

These experiments confirm that *PAX3* is required for the survival of melanoma cell lines, but extending this observation to other *PAX* genes, and whether *PAX* genes are expressed frequently in cancer in general, has not been investigated. A comprehensive analysis of gene expression profiles by microarray in the NCI-60 panel did not include *PAX* genes, although the genes expressed in each cell line were characteristic of the particular tumor type (Ross *et al*., 2000). To characterize the approximate frequency with which *PAX* genes are expressed in a cross-section of common human

tumors, we carried out quantitative analysis of steady-state levels of mRNA of *PAX1–PAX9* (except *PAX4*) in 54 cancer cell lines, including 51 cell lines of the NCI-60 cell line panel, using real-time PCR. Relative to baseline levels of expression, 48 cancer cell lines (89%) expressed at least one *PAX* gene, while 35 cell lines (65%) expressed three *PAX* genes or more (Figure 2). The *PAX3* gene was consistently expressed in all seven melanoma cell lines, and in many other cancer cell lines, including breast cancer. *PAX6* was expressed at high levels in brain, breast, and other cancer cell lines. The most frequently expressed *PAX* genes were *PAX3*, *PAX6*, *PAX8*, and *PAX9*. As verification of the accuracy of the real-time PCR, *PAX* gene expression profiles of *PAX2*, *PAX3*, *PAX6*, *PAX7*, and *PAX8* were also carried out in 14 normal nonirradiated fetal or adult kidney tissues that were obtained from histologically verified normal kidney tissue adjacent to Wilm's tumors or renal-cell carcinomas following tumor resection, and these experiments did not show expression of *PAX3*, *PAX6*, or *PAX7* (data not shown). Expression of *PAX2* and *PAX8* was detected in the samples, which was expected as *PAX2* and *PAX8* are expressed in kidney tissues (Eccles *et al*., 1992).

We then analysed whether the expression of *PAX* mRNA, as detected by real-time PCR, was reflected by expression of protein, focusing primarily on PAX2. *PAX2* is expressed in several human cancer types (Dressler and Douglass, 1992; Eccles *et al*., 1992; Gnarra and Dressler, 1995; Silberstein *et al*., 2002), and PAX2 expression in several human tumors has been shown previously using an anti-Pax2 antibody (Dressler and Douglass, 1992; Gnarra and Dressler, 1995; Silberstein *et al*., 2002). The expression of *PAX2* mRNA concurred with the expression of PAX2 protein, as detected by Western blot, in most of the cell lines analysed (Figure 3a, b, and data not shown). However, in several cell lines with detectable PAX2 protein, *PAX2* mRNA levels were below the cutoff level of detection by real-time PCR (Figure 3a, b), suggesting that the real-time PCR may have underestimated the true frequency of *PAX* gene expression. If the cutoff for real-time PCR was changed from 30 cycles to 32 cycles, making the baseline of detection less stringent, then more cell lines that were positive for PAX2 by Western blot were also positive by real-time PCR, but more than 96% of cancer cell lines expressed at least one *PAX* gene at this level of baseline detection.

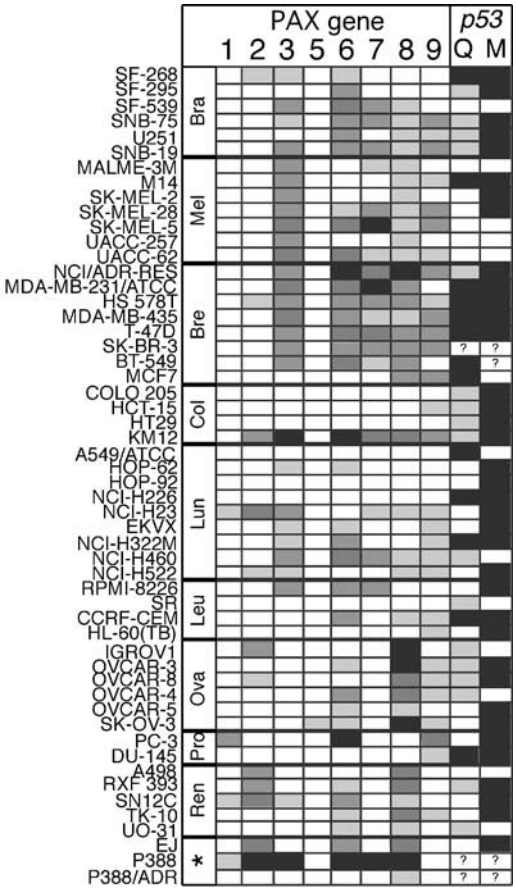

Figure 2 Detection of *PAX* gene mRNA in 54 cancer cell lines. The mRNA level of eight *PAX* genes (*PAX1-PAX3* and *PAX5-PAX9*) was analysed using quantitative real-time PCR in 54 cell lines (named on the left side of the figure). The cell lines comprised 51 cell lines of the NCI-60 panel, including brain (Bra), melanoma (Mel), breast (Bre), colon (Col), lung (Lun), leukemia (Leu), ovarian (Ova), prostate (Pro), and renal (Ren) cancer, plus three additional lines (*) comprising EJ bladder carcinoma cells, and P388 and P388/ADR leukemia cells. The relative expression level of *PAX* mRNA is represented by shading as follows, no expression (white), low (light grey), moderate (medium grey), high (dark grey), very high (black). In the two columns on the right is shown the level of *TP53* mRNA expression (Q), and *TP53* mutation status (M) for each cell line. Data for the mutation status and expression level for p53 for each cell line were obtained from the website, http://dtp.nci.nih.gov/mtargets/mt_browse.html, or as reported (Rieger *et al*., 1995). The relative *TP53* expression level was interpreted as undetectable (white), low (light grey), and high (black). Mutation status is shown as nonmutant (white), and mutant (black)

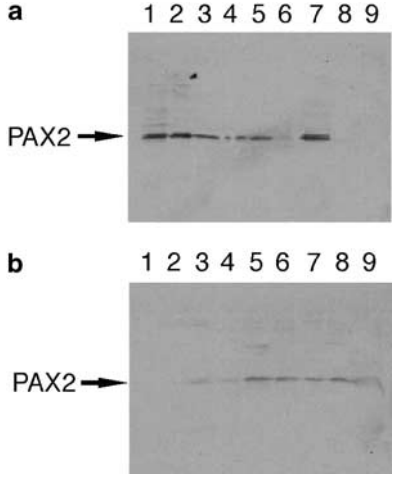

Figure 3 Expression of PAX2 protein in cancer cell lines. (**a, b**) PAX2 protein expression was analysed in a series of tumor cell lines by Western blot. Equal amounts of cell lysates were resolved by SDS–PAGE, transferred onto nitrocellulose, and probed for PAX2 using rabbit anti-Pax2 antibody. (**a**) Lane 1: IMCD; lane 2: EJ; lane 3; PC-3; lane 4: TK-10; lane 5: SF 268; lane 6: UACC 62; lane 7: P388; lane 8: P388/ADR; and lane 9: COS-7. (**b**) Lane 1: SK-MEL-2; lane 2; OVCAR-3; lane 3: OVCAR-8; lane 4: OVCAR-4; lane 5; OVCAR-5; lane 6: SK-OV-3; lane 7: A498; lane 8: RXF-393; and lane 9: IGROV1

The *PAX* gene expression patterns in the cell lines did not correlate with expression of other cancer-related genes, including *TP53* (Figure 2) or members of the *BCL2* superfamily, despite evidence suggesting that regulatory networks of these genes exist in cells (Stuart *et al*., 1995a; Hewitt *et al*., 1997; Barton *et al*., 2000; Margue *et al*., 2000). In many of the cancer cell lines in this study, the presence or absence of *PAX* gene expression did not necessarily match the type of fetal or adult tissues in which *PAX* genes are normally expressed. In one report, overexpression of *PAX6* in human colon and bladder cancers was associated with hypermethylation of exon 5 of the *PAX6* gene (Bender *et al*., 1999; Salem *et al*., 2000). However, the precise mechanisms leading to persistent *PAX* gene expression in cancer are poorly understood.

Dependence of ovarian and bladder cancer cell lines on PAX2 *for survival*

As multiple *PAX* genes are frequently expressed in cancer, it was pertinent to determine whether multiple members of the *PAX* gene family are required for survival when expressed in cancer. In previous studies, renal cancer cells exhibited reduced proliferation capacity upon downregulation of *PAX2* using an antisense oligonucleotide, although cell survival was not examined (Gnarra and Dressler, 1995). To determine whether *PAX2* is required for cell survival in cancer cell lines, we designed a *PAX2* small inhibitory double-stranded RNA (siRNA) and used this to silence *PAX2* expression in IGROV1 ovarian cancer cells, which express *PAX2* and *PAX8*, and in EJ bladder carcinoma cells, which express *PAX2*, *PAX6*, and *PAX8*. Following a single transfection of cells with the *PAX2* siRNA, we observed a large and sustained inhibition of PAX2 protein expression lasting 7 days (Figure 4a, b). In EJ cells, PAX2 protein levels returned to normal by day 7, but remained silenced at 7 days in IGROV1 cells. Cell proliferation was strongly inhibited, as an increase in total cell mass in the *PAX2* siRNA-treated cultures was inhibited for more than 7 days in both EJ cells and IGROV1 cells in comparison to control treatments following a single treatment with *PAX2* siRNA (Figure 4c–e), irrespective of the status of *TP53* or *HRAS* mutations in the cells (Parada *et al*., 1982; Rieger *et al*., 1995) (http://dtp.nci.nih.gov/mtargets/mt_browse.html) (Figure 2). In contrast, treatment with the *PAX2* siRNA did not affect the growth of COS-7 cells, which do not express PAX2 protein (Figure 4e).

A striking increase in annexin V positive cells was identified in both EJ and IGROV1 *PAX2* siRNA-treated cells, but not in COS-7 cells, suggesting that cells

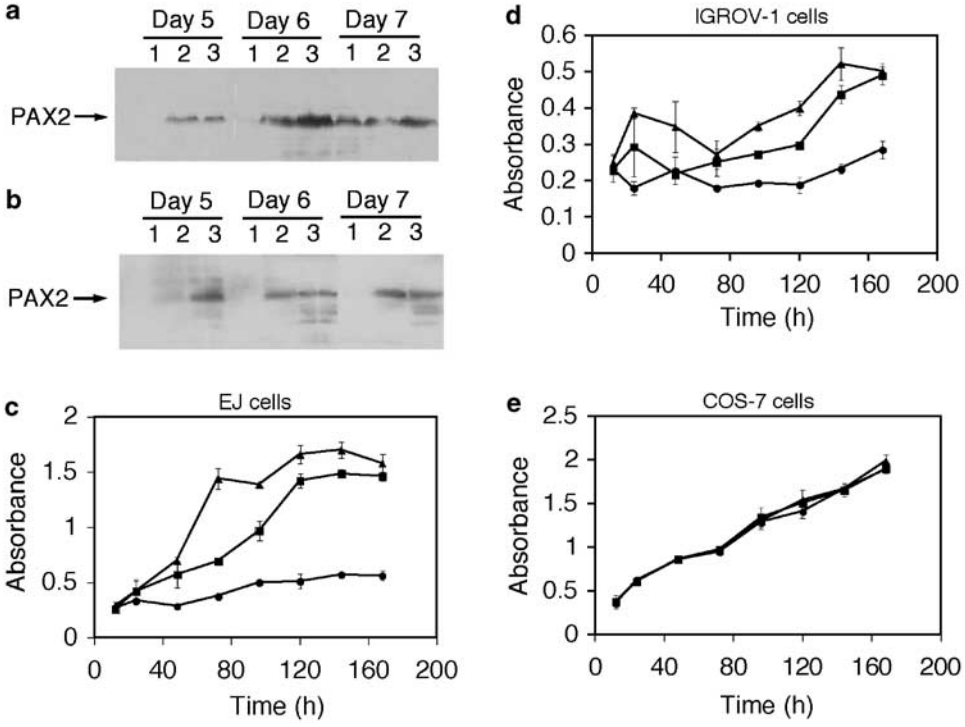

Figure 4 Silencing of PAX2 protein expression and reduced cell proliferation in EJ and IGROV-1 cells treated with *PAX2* siRNA. (**a**) Western blot of EJ cells transfected with *PAX2* siRNA duplex (1), treated with lipofectamine 2000 only (2), and with media only (3). (**b**) Western blot of IGROV-1 cells transfected with *PAX2* siRNA duplex (1), treated with lipofectamine 2000 only (2), and with media only (3). RNAi of *PAX2* was carried out for 7 days, during which PAX2 protein expression was detected with a Pax2-specific antibody. Only results from days 5, 6, and 7 are shown, as days 1–4 were identical to day 5. (**c**–**e**) Cell proliferation of cells transfected with *PAX2* siRNA duplex (•), treated with lipofectamine 2000 only (■) and with media only (▲). Cell mass was measured by MTT assay after continuous exposure to siRNAs, lipofectamine 2000 or media only for 7 days. The results represent the average of at least five separate experiments. Error bars show standard deviations

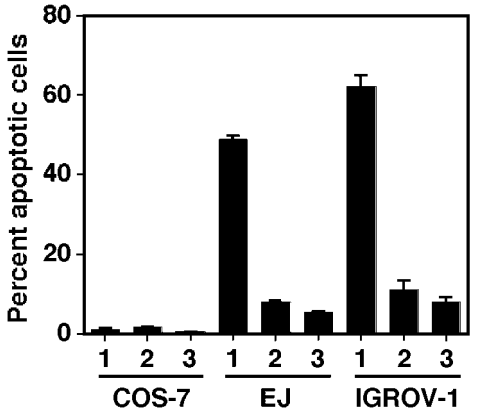

Figure 5 Cell death following *PAX2* silencing. Apoptosis was observed in cells expressing *PAX2* (EJ and IGROV1 cells), after transfection with *PAX2* siRNAs (1), but not after treatment with lipofectamine 2000 only (2) or media only (3). COS-7 cells have no detectable PAX2 expression and were used to show the specificity of the *PAX2* siRNA for cells expressing *PAX2*. Apoptotic cells were stained with fluorescein annexin-V conjugate 24, 48 or 72 h post-transfection. Cell death was analysed by flow cytometry. Results are shown for 72 h, but significant levels of apoptosis were observed at other time points, including 24 h post-transfection. Results represent the average of five separate experiments. Error bars show standard deviations

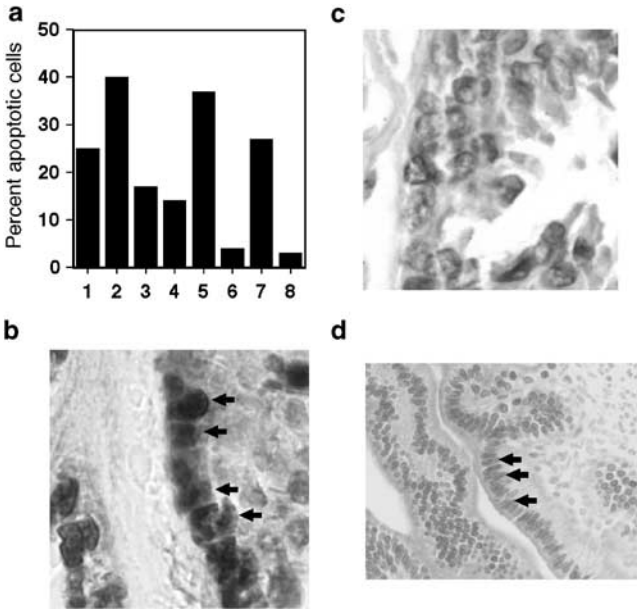

Figure 6 Frequent expression of PAX2 protein in primary human tumors. (**a**) Graph showing percentage of primary tumors expressing PAX2 protein by immunohistochemistry. Positive expression of PAX2 was scored if strong nuclear staining was observed in greater than 10% of tumor cells in the section. 1: brain cancer, 2: breast cancer, 3: colon cancer, 4: lung cancer, 5: lymphoma, 6: melanoma, 7: ovarian cancer, 8: prostate cancer. (**b**) Expression of PAX2 (arrows) in an ovarian cancer detected using anti-Pax2 antibody (magnification $\times 500$) (**c**) Immunohistochemistry using anti-TBTP antibody as a negative control. (**d**) Expression of Pax2 in epithelial cells adjacent to the lumen of an adult mouse oviduct (magnification, $\times 200$), detected by immunohistochemistry with anti-Pax2 antibody

treated with *PAX2* siRNAs were eliminated by apoptosis (Figure 5). Even though two or three different *PAX* genes were expressed in the IGROV1 and EJ cells, up to 62% of IGROV1 cells and 50% of EJ cells underwent apoptosis by 72 h (Figure 5), while 25–47% of cells underwent apoptosis by 24 h following treatment (data not shown). In contrast, less than 3% of COS-7 cells underwent apoptosis following treatment. These data suggest that the expression of multiple *PAX* genes did not interfere with induction of apoptosis following the silencing of *PAX2* expression alone. This was unexpected, as several studies have suggested that *PAX* gene function is redundant to the concurrent expression of other *PAX* family members (Mansouri and Gruss, 1998; Bouchard *et al*., 2000). In contrast, crossregulation and evidence for hierarchical regulation of *PAX* genes during development (Pfeffer *et al*., 1998; Borycki *et al*., 1999) may explain the nonredundant effects of silencing *PAX2* expression.

PAX2 is frequently expressed in primary human cancers

To determine whether the expression of *PAX* genes in the cancer cell lines accurately represents the situation in primary tumors, positive immunoreactivity for PAX2 protein was analysed in 406 primary tumors, representing eight common tumor types (Figure 6, Table 1). PAX2 was expressed in at least 25% of tumor sections in brain, breast, lymphoma, and ovarian cancer (Figure 6). In each section, PAX2 immunopositive expression was detected in a varying proportion of tumor cells (not shown). The frequency of PAX2 expression in primary tumors was similar to that of *PAX2* mRNA expression in the cell lines, except breast cancer in which *PAX2* mRNA was observed in none of the cell lines, but PAX2 protein was expressed in 27/68 (40%) primary breast cancer tissues. Silberstein *et al*. (2002) also found that *PAX2* is expressed in approximately 53% of primary breast cancers. The discrepancy in the PAX2 expression between the tumors and the cell lines in this study may simply be a reflection of the particular cell lines in the NCI-60 panel, or that the cells that ultimately give rise to the breast cancer cell lines are not the cells in the tumors that express PAX2.

Our results show that *PAX* gene expression is required for the growth and survival of multiple cancer cell types, and that inhibiting expression of *PAX2* or *PAX3* genes in cancers leads to the rapid induction of apoptosis. Furthermore, *PAX* genes are expressed in a high proportion of common human tumors, but only in a limited number of adult tissues (Dahl *et al*., 1997). Of particular interest is the expression of *PAX2* in a high proportion of breast and ovarian cancers. Our results concur with those of a recent study, which showed *PAX2* expression in more than 50% of breast cancers (Silberstein *et al*., 2002). *PAX2* is also expressed in the tissues of the genital tract (Fickenscher *et al*., 1993) including oviduct, and *PAX2* and *PAX8* are frequently expressed in ovarian cancer cell lines. It is likely that overexpression of other members of the *PAX* gene family also contribute to cancer cell growth and survival in specific types of cancer, since chromosomal

Table 1 PAX2 immunopositive staining in cancer tissues

Cancer tissue	*Cancer histological subtype*	n *(Total = 406)*	n *PAX2 positive*	*Comments*
Brain	Glioblastoma Multiforme	25	5	Strong staining in dispersed individual cells
Breast	Ductal adenocarcinoma	56	20 (6/9 Estrogen-receptor positive, 7/9 progesterone-receptor positive, and 1/7 c-*erb*-B2 positive)	Strong staining in patches of positive and negative cells
	Lobular adenocarcinoma	8	6 (one Estrogen-receptor positive, one progesterone-receptor positive)	Strong staining, in patches of positive and negative cells, and also in individual cells
	Ductal carcinoma *in situ*	1	1	Strong staining in patches
	Poorly differentiated	3	0	
Colon	Tubular adenoma	2	2	Strong staining in patches of positive and negative cells
	Mucinous adenocarcinoma	4	0	
	Adenocarcinoma	46	7	Strong staining in patches of positive and negative cells
Lung	Squamous cell	35	6	Moderate to strong staining in dispersed individual cells, or in diffuse patches
	Mucinous adenocarcinoma	2	0	
	Adenocarcinoma	30	4	Strong staining in dispersed individual cells or in diffuse patches
	Broncheoalveolar carcinoma	2	0	
Lymphoma	B or T cell	35	14	Strong staining in a high percentage of individual cells, or in patches of positive and negative cells
	Hodgkins	5	1	Strong staining in patches
	Follicular	1	0	
Melanoma	Nonmetastatic	20	1	Strong staining in dispersed cells
	Metastatic	5	0	
Ovarian	Mucinous adenocarcinoma	5	0	
	Serous papillary adenocarcinoma	36	12	Strong staining either in arcades of cells, or in patches of positive and negative cells
	Endometrioid	5	0	
	Clear cell	7	2	Strong staining in dispersed individual cells
Prostatic	Adenocarcinoma	73	2	Medium to strong staining in patches of positive and negative cells

rearrangements lead to the translocation of heterologous promoter sequences into the 5′ regulatory regions of the *PAX5* or *PAX8* genes, resulting in constitutive expression of the *PAX* gene mRNA in some lymphomas and thyroid cancer, respectively (Morrison *et al*., 1998a; Kroll *et al*., 2000; Cazzaniga *et al*., 2001).

Although the constitutive expression of *Pax* genes in adult tissues may not in itself be oncogenic (Dressler *et al*., 1993; Steinbach *et al*., 2001), the observation that a very high proportion of tumors express *PAX* genes irrespective of their tissue of origin suggests that *PAX* gene expression confers a strong advantage to cancer cell growth, and that cancer cells have exploited this expression pattern for tumor progression rather than tumor initiation.

Recent studies show that *PAX* genes are required only at certain stages of tissue development or organogenesis, suggesting that there are cell-specific *PAX* gene functions (Porteous *et al*., 2000; Ridgeway and Skerjanc, 2001; Bouchard *et al*., 2002; Chi and Epstein, 2002). In relation to this, the immunohistochemical staining patterns of PAX2 expression in primary tumor sections revealed that only a variable fraction of the tumor cells expressed PAX2. This does not necessarily imply that not all cells in a tumor are susceptible to the apoptotic effects of inhibiting *PAX2* expression, as it is possible that *PAX* genes induce the expression of essential autocrine or paracrine survival factors in tumors, and that the silencing of *PAX* gene expression therefore modulates the expression of these survival factors.

The identification of tumor-specific molecules that serve as targets for the development of new cancer drugs is a major goal in cancer research. The results presented here suggest that *PAX* genes exhibit several attributes that would be expected of a suitable cancer target, including a role in cancer cell survival, and widespread expression in cancer.

Materials and methods

Cell culture, RNA isolation, transfections, and RNAi

Cells were grown at 37°C and 5% CO_2 in humidified atmosphere, in either RPMI 1640 medium supplemented with 5% inactivated FBS for cell expansion and RNA isolation, or in Dulbecco's modified Eagle's medium supplemented with 10% FBS for transfections. All cell culture media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. RNA was isolated from cell lines using Trizol reagent, according to the manufacturer's instructions (GIBCO-BRL). At 6–24 h before transfection at 60–80% confluency, the cells were trypsinized, diluted 1 : 3 with fresh medium ($1–5 \times 10^5$ cells/ml) and transferred to 24-well plates (500 μl per well). Cells were either transfected with single-stranded sense or antisense oligonucleotides against human *PAX3*, or with annealed siRNAs against human *PAX2*. RNAi transfections contained Lipofectamine 2000 (Invitrogen, 1 μl per well) and 0.50 μg of each *PAX2* siRNA and GL2 siRNA duplex per well. Oligonucleotide transfections contained Lipofectin (Invitrogen, 4 μl per well) and 1 μM sense or antisense *PAX3* oligonucleotide. Control cell incubations were carried out with Lipofectin or Lipofectamine 2000 only and with media only. After 5–12 h, the transfection medium was replaced with serum-containing medium. Cell incubations were carried out for up to 7 days following transfection with siRNA. Single transfections were shown to be just as efficient as multiple transfections; there was no further change in the level of Pax2 protein expression with multiple transfections, and although there was a slight increase in apoptosis of the cells, this was probably due to toxicity induced by the increased levels of lipofectamine following multiple transfections. PAX2 expression was subsequently detected by Western blotting using a polyclonal antiPax2 antibody (Zymed). Cell proliferation was monitored by the MTT assay (Roche) and cell death was assayed by flow cytometry. For RNAi, 21-nucleotide RNAs were chemically synthesized, de-protected and purified by Dharmacon Research (Lafayette, CO, USA). Complementary short interfering ribonucleotides (siRNAs) targeting human *PAX2* mRNA (Accession No. NM_003989.1) were synthesized, corresponding to the coding region 1455–1473 relative to the first nucleotide of the start codon (5′-rGrUrCrGrArGrUrCrUrArUrCrUrGrCrArUrCrCTT-3′ and 5′-rGrGrArUrGrCrArGrArArUrArGrA rCrUrCrGrArCTT-3). A second set of complementary siRNAs targeting the *GL2* luciferase mRNA (Accession No. X65324) was synthesized, corresponding to the coding region 153–173 (5′-rCrGrUrArCrGrCrGrGrArArUrArCrUrUrCrGrATT-3′ and 5′-rUrCrGrArArGrUrArUrUrCCrGrCrGrUrArCrGTT-3). The *GL2* siRNAs were used as an internal control to test for the specificity of *PAX2* siRNAs. For annealing of siRNAs, 35 μM of single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES–KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h incubation at 37°C. For oligonucleotide transfections, antisense and sense *PAX3* phosphorothioate oligonucleotides were synthesized (Genset, Singapore) corresponding to nucleotides 123–141 of human *PAX3* mRNA: antisense (5′-GCGTGGTCATCCTGGGGGC-3′), sense (5′-GCCCCCAGGATGACCACGC-3′) (Bernasconi *et al*., 1996).

Quantitative real-time PCR

First-strand cDNA was produced from 2 μg of random hexamer primed total RNA using reverse transcription reactions (20 μl) containing 15 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgCl_2$, 0.05% Tween-20, 500 μM dNTPs (Roche), 0.2 mM DTT, 10 μM random hexamer primer (Roche), 1 U DNase I (Roche), 1 U RNasin (Promega), and 200 U Superscript II reverse transcriptase (Life Technologies). Reactions were incubated at 42°C for 50 min. Relative quantitation by real-time PCR was carried out using SYBR-green detection of PCR products in real time using either the ABI PRISM 7700 or ABI PRISM 7000 Sequence Detection System (Applied Biosystems Inc.). In each experiment, the human *β2 microglobulin* (B2 M) housekeeping gene was amplified as a reference standard. Primers for B2 M were B2MF, 5′-AGATGAGTATGCCTGCCGTGT-3′ and B2MR, 5′-GCTTACATGTCTCGATCCCACTTA-3′. In all real-time PCR experiments, RT and genomic DNA samples were included as negative controls. Template RNAs were treated with DNase I prior to amplification, according to the manufacturer's instructions (Applied Biosystems Inc.). Each real-time PCR reaction (25 μl) contained 2.5 μl of cDNA, 12.5 μl of 2X SYBR Green Master Mix (Applied Biosystems Inc.), including Amplitaq polymerase (Perkin-Elmer), and primers at a final concentration of 20 μM. Primers for each *PAX* gene were designed (for technical reasons primers for *PAX4* did not efficiently amplify transcripts and so were not used in the analysis) using Primer Express (Applied Biosystems Inc.), and tested empirically for efficiency of amplification using RNA from cell lines known to express the *PAX* gene of interest. Primers were as follows: PAX1F, 5′-AATGGGCTAGAGAAACCTGCCT-3′; PAX1R, 5′-CCCACGGCAGAGAGGGT-3′; PAX2F, 5′-CCCAGCGTCTCTTCCATCA-3′; PAX2R, 5′-GGCGTTGGGTGGAAAGG-3′; PAX3F, 5′-TCCATACGTCCTGGTGCCAT-3′; PAX3R, 5′-TTCTCCACGTCAGGCGTTG-3′; PAX5F, 5′-AGTACAGCAGCCACCCAACC-3′; PAX5R, 5′-GCGTCACGGAGCCAGTG-3′; PAX6F, 5′-GACTTCGGTGCCAGGGC-3′; PAX6R, 5′-TGGTATTCTCTCCCCCTCCTTC-3′; PAX7F, 5′-AGGCCTTTGAGAGGACCCAC-3′; PAX7R, 5′-CTGAACCAGACCTGCACACG-3′; PAX8F, 5′-CGCTCAGCCTGGCAGC-3′; PAX8R, 5′-GTCAATGCTTAGTCGGCAGCT-3′; PAX9F, 5′-GGTTGGAGAAGGGAGCCC-3′; PAX9R, 5′-ACAGCTGGGAGACCATTTGG-3′. Reactions were prepared in duplicate and heated to 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 58°C for 60 s, with a final incubation at 95°C for 15 min. To detect the log phase of amplification, the fluorescence level (quantification of product) was determined at each cycle. The cycle at which the fluorescence reached threshold (CT) was recorded, averaged between duplicates, and normalized to the averaged CT value for B2 M. The normalized values were then subtracted from 30, being a cutoff CT value at which we determined expression from each gene was undetectable, the difference of which was deemed to be related to the expression level of each *PAX* gene. The relative expression level for each *PAX* gene in the cell lines was then calculated according to the manufacturer's instructions (Applied Biosystems Inc.). To represent the relative expression level, the following categories were delineated; 0–1 units, no expression (white box), 2–19,

low (light grey), 20–199, moderate (medium grey), 200–1999, high (dark grey), and >2000, very high expression (black).

SDS–PAGE and immunoblotting

Cell samples were lysed in 20 μl loading buffer (50 mM Tris, 4% SDS, 12% glycerol, 2% 2-mercaptoethanol, 0.01% coomassie brilliant blue) and were separated on a 12.5% Tris-glycine gel. Gels were then either fixed and stained with coomassie brilliant blue (0.1% (w/v) coomassie brilliant blue R-250, 45% (v/v) methanol and 10% (v/v) acetic acid), or electrotransfered onto 0.2 m nitrocellulose (100 V, 1 h) in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) and then blocked with 2% (w/v) fat-free milk powder in TBS (5 mM Tris-HCl, pH 7.4, 20 mM NaCl), 0.1% Tween-20. Rabbit anti-Pax2 polyclonal antibody (Zymed, Cat No. 71-6000) diluted 1 : 1000 in TBS, 0.1% fat-free milk powder, and 0.1% Tween-20 was used for immunodetection with overnight incubation. After 3 × 10 min washes in TBS, 0.1% Tween-20, horseradish peroxidase-conjugated goat anti-rabbit IgG (1 : 10 000, Biorad) was used as a secondary antibody. The incubation with secondary antibody was for 1 h at room temperature, followed by 3 × 10 min washes in TBS and visualized by chemiluminescence using a Pierce Super Signal R chemiluminescence substrate with Kodak X-OMATTM AR imaging film.

PAX2 immunostaining

For PAX2 immunostaining, either paraffin-embedded tumor tissues were sectioned at 10 μM and mounted onto L-lysine-coated slides, or tumor tissue array slides (TARP4) were obtained from the National Cancer Institute (Bethesda). Tumor sections were deparaffinized and incubated with polyclonal rabbit primary anti-Pax2 antibody (10 μg/ml) (Zymed, Cat No. 71-6000), or polyclonal rabbit primary anti-thiobutyltriphenylphosphonium (TBTP) antibody (1 : 1000 dilution) (Lin *et al*., 2002) for 30 min at room temperature in PBS supplemented with 20 mM glycine and 1% bovine serum albumin. Primary antibodies were detected with Vectastain ABC anti-rabbit IgG kit (Vector Laboratories) as described by the manufacturer, followed by incubation with diaminobenzidine (DAB) substrate (Sigma). Negative control incubations using the secondary anti-rabbit IgG antibody from the ABC kit omitting the primary anti-Pax2 antibody were also carried out, and as expected gave negative results, Sections were washed in PBS, counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher).

MTT assay

Cell proliferation of EJ, IGROV-1, M14, UACC62, and COS-7 cells was measured using the cell proliferation kit I (MTT) supplied by Roche. Cells were seeded in 96-well plates (10^4 cells per well) with 100 μl DMEM containing 10% FBS. Following transfection, as described above, the cells were incubated for 12 h, after which 10 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37°C. The resulting formazan within the cells was dissolved in 100 μl of 10% SDS in 0.01 M HCl. Optical densities were read immediately at 620 nm using a PolarStar Optima microplate reader. This procedure was repeated every 24 h for up to 7 days following treatment.

Flow cytometry

Cells (EJ, IGROV-1, M14, UACC62, and COS-7) grown in 24-well plates were treated with *PAX2* siRNAs, *PAX3* sense or antisense oligonucleotides, lipofectamine 2000 or lipofectin only or media only as described above. These treatments were carried out for 12, 24, 48, and 72 h, after which the cells were prepared for flow cytometry. Following each incubation period, the medium was collected from the wells (to retain any detached cells) and combined with PBS used to wash the wells twice. The cells were then harvested by trypsinization and combined with the detached cells and PBS. The cells were pelleted at 4°C (1000 *g*, 5 min), washed in PBS twice and resuspended in 100 μl binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) supplemented with 2 μl fluorescein annexin-V conjugate (Molecular Probes, OR, USA). The cells were incubated for 15 min on ice in the dark, diluted in 400 μl binding buffer and analysed using a Becton Dickinson fluorescence-activated cell sorter. Apoptotic cell death was quantitated using the Cell-Quest software (Becton Dickinson, San Jose, CA, USA).

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References

Barr FG, Galili N, Holick J, Biegel JA, Rovera G and Emanuel BS. (1993). *Nat. Genet.,* **3,** 113–117.
Barton K, Leiden JM and Margue CM. (2000). *Dev. Biol.,* **222,** 110–123.
Bender CM, Gonzalgo ML, Gonzales FA, Nguyen CT, Robertson KD and Jones PA. (1999). *Mol. Cell. Biol.,* **19,** 6690–6698.
Bernasconi M, Remppis A, Fredericks III WJ, FJR and Schafer BW. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 13164–13169.
Borycki AG, Li J, Jin F, Emerson CP and Epstein JA. (1999). *Development,* **126,** 1665–1674.
Bouchard M, Pfeffer P and Busslinger M. (2000). *Development,* **127,** 3703–3713.
Bouchard M, Souabni A, Mandler M, Neubuser A and Busslinger M. (2002). *Genes Dev.,* **16,** 2958–2970.
Cazzaniga G, Daniotti M, Tosi S, Giudici G, Aloisi A, Pogliani E, Kearney L and Biondi A. (2001). *Cancer Res.,* **61,** 4666–4670.
Chi N and Epstein JA. (2002). *Trends Genet.,* **18,** 41–47.
Dahl E, Koseki H and Balling R. (1997). *BioEssays,* **19,** 755–765.
Davis RJ, D'Cruz CM, Lovell MA, Biegel JA and Barr FG. (1994). *Cancer Res.,* **54,** 2869–2872.
Dressler GR and Douglass EC. (1992). *Proc. Natl. Acad. Sci. USA,* **89,** 1179–1183.
Dressler GR, Wilkinson JE, Rothenpieler UW, Patterson LT, Williams-Simons L and Westphal H. (1993). *Nature,* **362,** 65–67.
Eccles MR. (1998). *Ped. Nephrol.,* **12,** 712–720.
Eccles MR, Wallis LJ, Fidler AE, Spurr NK, Goodfellow PJ and Reeve AE. (1992). *Cell Growth Differ.,* **3,** 279–289.

Eccles MR, Yun K, Reeve AE and Fidler AE. (1995). *Am. J. Pathol.,* **146,** 40–45.
Fabbro D, Di-Loreto C, Beltrami CA, Belfiore A, Di-Lauro R and Damante G. (1994). *Cancer Res.,* **54,** 4744–4749.
Fickenscher HR, Chalepakis G and Gruss P. (1993). *DNA Cell Biol.,* **12,** 381–391.
Glaser T, Walton DS and Maas RL. (1992). *Nat. Genet.,* **2,** 232–238.
Gnarra JR and Dressler GR. (1995). *Cancer Res.,* **55,** 4092–4098.
Hewitt SM, Hamada S, Monarres A, Kottical LV, Saunders GS and McDonnell TJ. (1997). *Anticancer Res.,* **17,** 3211–3216.
Jordan T, Hanson I, Zaletayev D, Hodgson S, Prosser J, Seawright A, Hastie N and van Heyningen V. (1992). *Nat. Genet.,* **1,** 328–332.
Kozmik Z, Kurzabauer R, Dorfler P and Busslinger M. (1993). *Mol. Cell. Biol.,* **13,** 6024–6035.
Kroll TG, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM and Fletcher JA. (2000). *Science,* **289,** 1357–1360.
Lin TK, Hughes G, Muratovska A, Blaikie FH, Brookes PS, Darley-Usmar V, Smith RA and Murphy MP. (2002). *J. Biol. Chem.,* **277,** 17048–17056.
Mansouri A and Gruss P. (1998). *Mech. Dev.,* **78,** 171–178.
Margue CM, Bernasconi M, Barr FG and Schafer BW. (2000). *Oncogene,* **19,** 2921–2929.
Marques AR, Espadinha C, Catarino AL, Moniz S, Pereira T, Sobrinho LG and Leite V. (2002). *J. Clin. Endocrinol. Metab.,* **87,** 3947–3952.
Morrison AM, Jäger U, Chott A, Schebesta M, Haas OA and Busslinger M. (1998a). *Blood,* **92,** 3865–3878.
Morrison AM, Nutt SL, Thevenin C, Rolink A and Busslinger M. (1998b). *Semin. Immunol.,* **10,** 133–142.
Murer L, Caridi G, Della Vella M, Montini G, Carasi C, Ghiggeri G and Zacchello G. (2002). *Nephron,* **91,** 588–593.
Ostrom L, Tang MJ, Gruss P and Dressler GR. (2000). *Dev. Biol.,* **219,** 250–258.
Parada LF, Tabin CJ, Shih C and Weinberg RA. (1982). *Nature,* **297,** 474–478.
Pfeffer PL, Gerster T, Lun K, Brand M and Busslinger M. (1998). *Development,* **125,** 3063–3074.
Porteous S, Torban E, Cho NP, Cunliffe H, Chua L, McNoe L, Ward T, Souza C, Gus P, Giugliani R, Sato T, Yun K, Favor J, Sicotte M, Goodyer P and Eccles M. (2000). *Hum. Mol. Genet.,* **9,** 1–11.
Ridgeway AG and Skerjanc IS. (2001). *J. Biol. Chem.,* **276,** 19033–19039.
Rieger KM, Little AF, Swart JM, Kastrinakis WV, Fitzgerald JM, Hess DT, Libertino JA and Summerhayes IC. (1995). *Br. J. Cancer,* **72,** 683–690.
Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D and Brown PO. (2000). *Nat. Genet.,* **24,** 227–235.
Salem CE, Markl ID, Bender CM, Gonzales FA, Jones PA and Liang G. (2000). *Int. J. Cancer,* **87,** 179–185.
Sanyanusin P, Schimmenti LA, McNoe LA, Ward TA, Pierpont MEM, Sullivan MJ, Dobyns WB and Eccles MR. (1995). *Nat. Genet.,* **9,** 358–363.
Scholl FA, Kamarashev J, Murmann OV, Geertsen R, Dummer R and Schafer BW. (2001). *Cancer Res.,* **61,** 823–826.
Silberstein GB, Dressler GR and Van Horn K. (2002). *Oncogene,* **21,** 1009–1016.
St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A and Gruss P. (1997). *Nature,* **387,** 406–409.
Steinbach JP, Kozmik Z, Pfeffer P and Aguzzi A. (2001). *Int. J. Cancer,* **93,** 459–467.
Stuart ET, Haffner R, Oren M and Gruss P. (1995a). *EMBO J.,* **14,** 5638–5645.
Stuart ET, Kioussi C, Aguzzi A and Gruss P. (1995b). *Clin. Cancer Res.,* **1,** 207–214.
Tassabehji M, Read AP, Newton VE, Harris R, Balling R, Gruss P and Strachan T. (1992). *Nature,* **355,** 635–636.
Torban E, Eccles M, Favor, J. and Goodyer and P. (2000). *Am. J. Pathol.,* **157,** 833–842.
van Raamsdonk CD and Tilghman SM. (2000). *Development,* **127,** 5439–5448.
Winyard PJD, Risdon RA, Sams VR, Dressler GR and Woolf AS. (1996). *J. Clin. Invest.,* **98,** 451–459.