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Original Paper

Ectopic Vasopressin Expression in MMTV-*v-Ha-ras* Transgenic Mice Delays the Onset of Mammary Tumorigenesis

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Neuropeptides are often ectopically expressed by non-endocrine tumours. We used transgenic mice to assess the effect of ectopic expression of the neuropeptide, vasopressin, in mammary tumours induced by the transgenetic expression of an activated *ras* oncogene. Mice bearing a mouse mammary tumour virus-vasopressin (MMTV-VP) fusion transgene synthesise authentic VP in mammary ducts and alveoli. Bitransgenic mice bearing both MMTV-VP and MMTV-*v-Ha-ras* transgenes developed tumours that were histologically indistinguishable from those of single MMTV-*v-Ha-ras* animals. However, tumour onset was significantly delayed in the bitransgenic animals. These data provide evidence that an ectopic neuropeptide can slow the development of *ras* tumours *in vivo*.

Key words: *v-Ha-ras*, mammary tumorigenesis, vasopressin, mouse mammary tumour virus, transgenic mice
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INTRODUCTION

THE PRODUCTION and secretion of neuropeptides by tumours has long been recognised through the appearance of characteristic clinical syndromes. Inappropriate secretion of vasopressin (VP), a nine amino acid neuropeptide hormone, was first postulated in 1967 [1], following earlier reports of an association between hyponatraemia and lung tumour [2]. In 20–40% of patients with small cell carcinoma of the lung (SCCL), VP peptide released into circulation causes the syndrome of inappropriate antidiuresis (SIAD; refs [1, 3]), the clinical features of which include hyponatraemia and hypo-osmolality of plasma concurrent with increased urine osmolality. Besides its involvement in clinical SIAD, there is also evidence to suggest that VP may play a direct role in carcinogenesis. In some SCCL cell lines, treatment with VP stimulates growth [4] and Ca^{2+} mobilisation [5, 6]. These findings, together with the demonstration of VP receptors in SCCL cells [7], support the hypothesis that VP is an autocrine factor in SCCL.

It has also been suggested that VP may be able to regulate the growth of breast carcinomas [8]. Evidence has been presented that VP is involved in normal mammary function and growth [9] and V1-type VP receptors are present in the mammary gland of the rat [10]. Specifically, it has recently been shown that the gene encoding the V1b class of receptor is expressed in mouse mammary tissue [11]. Both the rat mammary cell line WRK1 [12] and the human mammary cell line MCF-7 [8] bear V1 VP receptors, and the administration of VP to the latter cell line results in mitogenic effects, characterised by increases in cell number, protein synthesis and inositol phosphate accumulation [8].

Transgenic mice bearing mouse mammary tumour virus long terminal repeat (MMTV-LTR)-fusion oncogenes, which are efficiently expressed in the mammary gland, have been widely used in the study of neoplasia [13]. Mice bearing hybrid MMTV-oncogene transgenes inevitably develop tumours, directly demonstrating the involvement of these genes in tumour development *in vivo*. Further, "bitransgenic mice", bearing two oncogenes, have been used to demonstrate oncogene co-operativity, providing evidence that cancer is a multistep process. Compared with expression alone, mammary tumour development has been shown to be more rapid when the *Wnt*-1 and *int-2* [14], or *ras* and c-*myc* [15] oncogenes were expressed together.

We have used transgenic mice to study the growth regulatory properties of VP *in vivo*, and the involvement of VP in carcinogenesis. We ectopically expressed the VP gene in the mammary gland of mice under the control of the MMTV long terminal repeat (LTR) and showed that, whilst the production of authentic VP on its own has no pathological effect, it can profoundly affect tumour development when co-expressed with the *Wnt*-1 oncogene [16]; mammary tumours in VP-*Wnt*-1 double transgenic mice grew more aggressively as judged by mitotic index and agrophylic nucleolar organiser region counts. In this report, we have extended these studies to examine the consequences of VP co-expression with *ras* to tumour development. Surprisingly, co-expression of VP with *ras* in the mammary gland results in delayed tumour development.

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MATERIALS AND METHODS

Bitransgenic mice

MMTV-*v-Ha-ras* transgenic mice [15] were purchased from Du Pont (Boston, Massachusetts, U.S.A.) and bred with C57BL/6J × CD 1 F1 mice to form a local subline. Transgenic offspring were identified by Southern blotting using clone HB-11 (American Type Culture Collection, Rockville, Maryland, U.S.A.), which contains 2.2 kb of the Harvey Sarcoma Virus, as a probe. MMTV-VP (MV-A) transgenic mice have been described [16]. MMTV-*v-Ha-ras* transgenic mice were mated with MV-A mice to produce bitransgenic mice. DNA was extracted from tail biopsies of female pups and Southern blot analysis was used to identify mice carrying the VP transgene as previously described [16]. Mice carrying the MMTV-*v-Ha-ras* transgene were identified as described above. Obligate heterozygotes were used in all studies.

Analysis of transgene expression by Northern blot hybridisation

Total RNA isolation and Northern blotting have been described [16]. The probes used were: TJ (transgene junction), a 35-mer complementary to sequences at the MMTV-VP transgene junction that recognises the MMTV-VP transgene RNA, but not the endogenous VP transcript; GAPDH, a 45-mer complementary to the rat glyceraldehyde 3-phosphate dehydrogenase mRNA; 18S, a 17-mer complementary to 18S rRNA; HR, a 33-mer corresponding to amino acids 7–18 of the transforming protein of Harvey murine sarcoma virus.

VP radioimmunoassay

Approximately 10–20 mg of mammary gland or mammary tumour tissue were weighed and disrupted in 0.1 M HCl with a sonicator. After incubation on ice, samples were dried under vacuum and resuspended in 0.5 ml of 0.1 M Tris–HCl buffer (pH 7.4). Appropriate dilutions of unextracted supernatant were used for radioimmunoassay. All samples were assayed in duplicate. Radiolabelled ^{125}I iodotyrosyl2 VP [Arg 8] and the VP antiserum were purchased from Amersham (U.K.). The VP standard was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). The intra- and interassay coefficients of variation were 9.1 and 10.4%, respectively.

Gross and microscopic examination of mammary tumours

Animals were examined weekly for onset of mammary tumour development. The age at which a tumour was first detected was recorded. Tumours were allowed to grow until approximately 2–3 cm in diameter before the animals were sacrificed. Animals were thus sacrificed at the same stage of disease development. Blood was collected for plasma osmolality determination (Wescor Vapor Pressure Osmometer). Mammary tumours and adjacent normal mammary tissue were used in either RNA analysis (see above), radioimmunoassay (see above) or histological examination. An attempt was made to monitor the rate of tumour growth by measuring the length and width of tumours at regular intervals. However, this method was found to be inadequate for two reasons. Firstly, the tumours grew into irregular shapes and estimations of area and volume by linear measurements of length, width and height were at best only approximations. Secondly, at postmortem examination, many of the tumours were found to contain cysts or necrotic cavities. Thus, some rapidly enlarging masses were due to accumulating blood or fluid. For these reasons, linear measurements of tumour growth were abandoned in favour of assessment by histological criteria. For histology, tissue was fixed in 10% buffered formalin. Tumours were classified according to Dunn [17]. In type-A mammary adenocarcinomas, the epithelial cells are small, usually cuboidal and arranged to form small round cavities or small elongated tubes. Type-B mammary adenocarcinomas are characterised by a "varied" structure. There are acinar areas, cysts with blood or fluid, intracystic papillary projections, solid cords, sheets or nests of cells with no sign of glandular differentiation. The proliferative characteristics of the tumours were assessed using mitotic index and agyrophilic nucleolar organiser region (NOR) counts. The mitotic index was obtained by counting the number of mitoses in five random (400× magnification) microscopic fields of each tumour section examined. For demonstration of the NOR in tumour cell nuclei, the agyrophilic NOR method of Chiu and colleagues [18] was used. The silver stained black particles in 50 cells of each tumour were counted and the NOR number per cell was calculated. All counts were carried out without knowledge of tumour origin.

Immunocytochemistry

Ha-ras was detected in formalin-fixed, paraffin-embedded sections by immunoperoxidase staining, using rat monoclonal IgG1, clone Y13-259 (Oncogene Science, Cambridge, Massachusetts; U.S.A.), which recognises both *c-Ha-ras* and *v-Ha-ras*, in accordance with the instructions of the supplier. Sections were counterstained with haematoxylin.

Statistical analysis

All data are expressed as the mean ± S.E. Statistical evaluation of data was performed by Student's *t*-test.

RESULTS

A total of 77 female animals were obtained from mating MV-A mice with MMTV-*v-Ha-ras* mice; 25 were double transgenics (VP-*v-Ha-ras*), 18 were *v-Ha-ras* single transgenics (*v-Ha-ras*), 21 were VP single transgenics (VP) and 13 were wild-type (WT). The expression of the VP transgene in VP-*v-Ha-ras* bitransgenic mice was similar to that previously observed in VP single transgenic animals (Figure 1a; [16]). Abundant MMTV-VP transgene mRNA was present in mammary tumours that developed in VP-*v-Ha-ras* mice (Figure 1a). Immunoreactive VP peptide was elevated in the mammary gland and in mammary tumours of VP-*v-Ha-ras* bitransgenic mice compared with *v-Ha-ras* single transgenic mice (Table 1). The level of *v-Ha-ras* transgene RNA expression in mammary tumours of VP-*v-Ha-ras* bitransgenic mice was perhaps slightly lower than in tumours from *v-Ha-ras* mice (Figure 1b). However, semiquantitative analysis of transcripts in tumours taken from animals is very difficult because tumours can vary with respect to cysts, necrotic cavities and fluid and blood accumulations. Thus, RNA extracted from a tumour mass will inevitably be a mixture, derived from varying amounts of tumour and other, ill-defined, material. Further, depending on growth rate and metabolism, tumour masses are heterogeneous with regard to the levels of control transcripts. However, immunocytochemical detection of Ha-ras protein in mammary glands (Figure 2) and mammary tumours (not shown) of VP-*v-Ha-ras* and *v-Ha-ras* mice revealed positive staining for *v-Ha-ras* in ducts and myoepithelial cells of the mammary gland of *v-Ha-ras* and VP-*v-Ha-ras* mice. In contrast, mainly epithelial cells stained positive for *c-Ha-ras* in normal mammary tissue from VP and wild-type mice.

VP co-expression had no effect on tumour differentiation as determined by histological examination (Table 2). Nor did VP expression affect tumour growth rate as determined either by

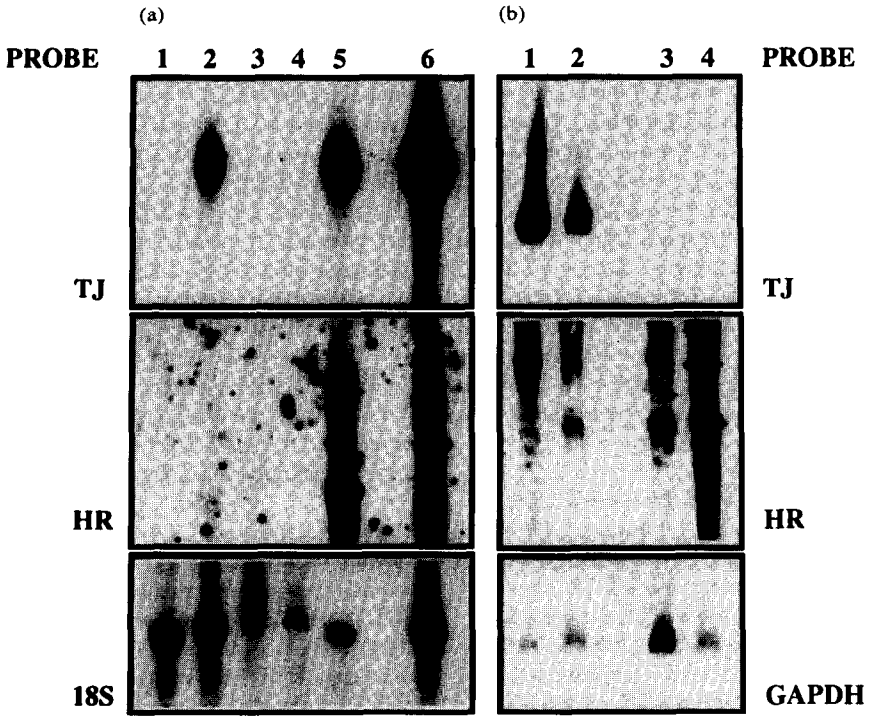

Figure 1. (a) Northern blot analysis of RNA extracted from tissues of VP-*v-Ha-ras* bitransgenic mice. Approximately 5 μg of total RNA from each tissue was used. The filter was sequentially probed with the following radiolabelled oligonucleotides: TJ (4 days exposure); HR (1 day exposure) and 18S (4 h exposure). The tracks are: 1, hypothalamus; 2, parotid gland; 3, submaxillary gland; 4, spleen; 5, mammary gland (adjacent to tumour); 6, mammary tumour. Note that the *v-Ha-ras* RNA is consistently revealed as a smear. (b) Northern blot analysis of RNA extracted from mammary tumours of VP-*v-Ha-ras* bitransgenic mice (lanes 1 and 2) and *v-Ha-ras* single transgenic mice (lanes 3 and 4). Approximately 10 μg of total RNA from each tissue was used. The filter was sequentially probed with the following radiolabelled oligonucleotides: TJ (4 days exposure); HR (17 h exposure) and GAPDH (6 days exposure).

Table 1. *Immunoreactive VP in tissues of transgenic mice*

	Immunoreactive peptide (pg/mg)			
	VP-*v-Ha-ras*	(*n*)	*v-Ha-ras*	(*n*)
Normal mammary tissue (adjacent to tumour)	33.7 ± 13.8*	5	3.97 ± 1.5	7
Mammary tumour	216 ± 83.9*	5	6.07 ± 2.06	7

* $P < 0.05$ (VP-*v-Ha-ras* versus *v-Ha-ras*).

mitotic index (Table 2) or NOR count (Table 2). Most VP-*v-Ha-ras* and *v-Ha-ras* mice developed only one mammary tumour. The age of tumour onset was analysed firstly by determining the T50, or the age at which 50% of the population developed tumours (Figure 3). Secondly, we determined the mean age at which tumours developed (Table 2). Using both methods, the onset of tumour development in VP-*v-Ha-ras* mice was found to be significantly delayed compared with *v-Ha-ras* mice.

DISCUSSION

We have adopted a transgenic approach to the study of the growth regulatory properties of neuropeptides *in vivo*. The expression of a transgene consisting of rat VP structural gene under the control of the MMTV-LTR in mammary tissue, resulted in the production of authentic VP [16]. Co-expression of VP with *v-Ha-ras* in bitransgenic mice resulted in a significant delay in *v-Ha-ras* induced tumour onset.

In MMTV-VP transgenic mice, ectopic VP production had no detectable effect on the physiology of the transgenic mice; plasma osmolality remained normal and neither VP single transgenic mice [16] nor bitransgenic VP-*v-Ha-ras* mice carrying mammary tumours presented symptoms resembling SIAD (not shown). Further, ectopic VP production had no detectable effect on the development and function of the mammary gland in VP single transgenic mice [16]. However, we were surprised to find that VP co-expression significantly delayed the onset of tumour development that results from *v-Ha-ras* expression. This effect is probably mediated by the VP peptide, although we cannot rule out a role for other products of the VP propeptide, namely neurophysin or glycopeptide. These data provide further compelling *in vivo* evidence that ectopic neuropeptide production can modulate the development of tumours *in vivo*.

There are several possible explanations for this effect. There is the possibility that genetic background could result in a difference between tumour development in VP-*v-Ha-ras* bitransgenic mice and *v-Ha-ras* single transgenic mice. We compared the T50 and mean age of tumour onset of *v-Ha-ras* single transgenic mice produced from the MMTV-*v-HA-ras* × MV-A cross (T50 = 142 days; mean age of tumour onset = 174 + 40 days; $n = 18$) with the original MMTV-*v-HA-ras* animals described by Leder (T50 = 168 days; $n = 52$; 15) and with our local MMTV-*v-HA-ras* colony (T50 = 105 days; mean age of tumour onset = 151 + 19 days; $n = 26$). Age of tumour onset did not differ between these groups of animals. This suggests that genetic background is not an important determinant of the time of tumour initiation.

There is also the possibility that the delay in development of

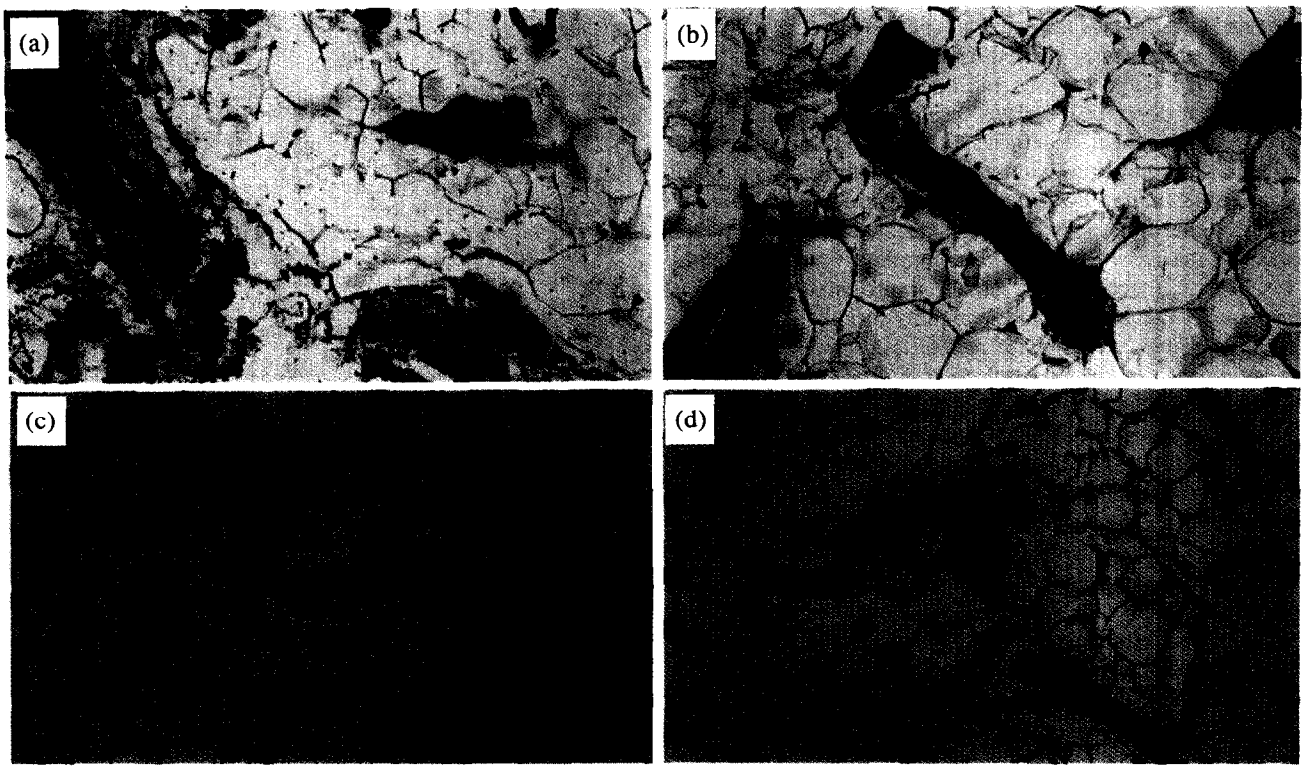

Figure 2. Immunohistochemical detection of Ha-ras in normal mammary gland sections from wild-type (a), VP (b) and *v-Ha-ras* (c) single transgenic mice and VP-*v-Ha-ras* (d) bitransgenic mice. Magnification 250×. The antigen is revealed by the brown stain over the blue haematoxylin counterstain.

Table 2. Tumour development and histological properties of tumours in v-Ha-ras *and* VP-v-Ha-ras *transgenic mice*

	VP-*v-Ha-ras*	(*n*)	*v-Ha-ras*	(*n*)
Age of tumour onset (days)	310.0 ± 36.2*	21	174.0 ± 39.8	13
Histological type	B	11	B	9
	A	2	A	2
Mitotic index (type-B tumours)	6.2 ± 1.3	11	7.7 ± 2.3	9
NOR (type-B tumours)	3.5 ± 0.4	8	4.6 ± 0.7	7

* $P < 0.025$ (VP-*v-Ha-ras* versus *v-Ha-ras*).

tumours in VP-*v-Ha-ras* transgenic mice is a consequence of downregulation of the expression of the *v-Ha-ras* transgene. However, *v-Ha-ras* RNA and protein continues to be made in mammary gland and mammary tumours expressing the VP transgene.

A third possible explanation for the delay in tumour development is gametic imprinting. Imprinting affects the expression of certain genes depending on whether they are inherited from the male or female parent. This concept is supported by studies demonstrating that both the paternal and maternal genomes are necessary for normal embryonic development [19]; the two parental genomes play complementary but non-equivalent roles during embryogenesis [20]. Information imparted by passage of some genes through the male and female parent can affect their expression. For example, differential expression of a transgene introduced into mice due to imprinting has been described [21].

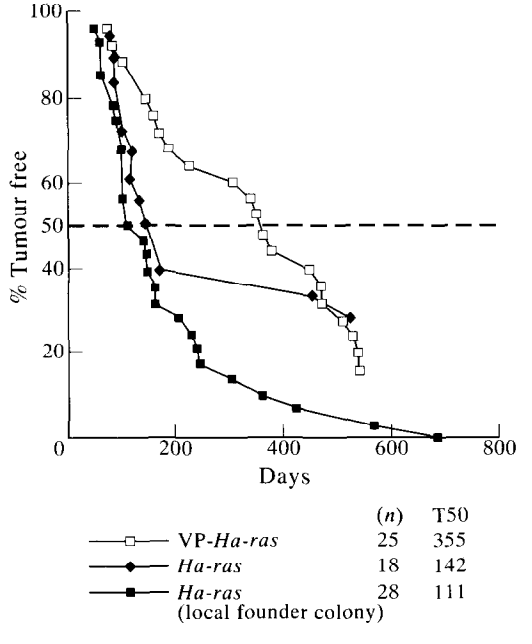

Figure 3. Development of mammary tumours in bitransgenic mice (VP-*v-Ha-ras*) bearing both MMTV-VP and MMTV-*v-Ha-ras* transgenes, and in single transgenic mice (*v-Ha-ras*), bearing only the MMTV-*v-Ha-ras* transgene, derived from the same crosses as the bitransgenic mice, or from the local *v-Ha-ras* colony.

We examined the breeding records of the VP-*v-Ha-ras* and *v-Ha-ras* mice. It was found that female bitransgenic and single transgenic mice were derived from both male and female MMTV-*v-Ha-ras* parents. In addition, many of the VP-*v-Ha-ras* mice that developed "delayed" tumours had, as their litter mates, single transgenic *v-Ha-ras* mice that developed tumours earlier. Thus, imprinting cannot account for the different age of tumour onset in the VP-*v-Ha-ras* and *v-Ha-ras* mice.

Finally, there is the possibility that delayed tumorigenesis is a result of interference between the *ras* and VP signalling pathways. The mitogenic action of VP is mediated by V1-type receptors [8, 12]. Ligand binding rapidly stimulates phospholipase C mediated hydrolysis of inositol phospholipids and the generation of the messenger molecules inositol 1,4,5-triphosphate and 1,2-diacylglycerol, which in turn result in Ca^{2+} mobilisation and protein kinase C (PKC) activation [22]. Our data are consistent with the hypothesis that the chronic occupation of VP receptors by transgene encoded ectopic VP generates signals that interfere with the downstream signal transduction pathway(s) of activated *ras*, thus preventing tumorigenesis. A secondary event might overcome this block in those mice that go on to develop tumours.

Evidence for interference between the pathway downstream of the G-protein coupled V1 receptor and *ras*-linked signals has been provided by *in vitro* experiments. Prior chronic administration of VP to Swiss 3T3 cells resulted in attenuation of the mitogenic effect caused by subsequent administration of bombesin [23]. The block was at a postreceptor level, as VP did not interfere with or diminish the binding of bombesin to its receptor. The affinity of VP for its own receptor was also not diminished, although there was a decrease in the number of binding sites. A similar effect by VP has been reported for PDGF stimulated early signals in Swiss 3T3 cells [24]. Although these findings provide evidence that prolonged occupation of the VP G-protein coupled receptor can diminish the effects of a tyrosine kinase receptor, the molecular mechanics of this effect remain unknown.

Ras is a core component of pathways that transmits growth signals from the receptor to the cell nucleus [25]. The ras pathway leads to the activation of MAP kinase [26]; a central molecule stimulated by pathways downstream of multiple ligands, amongst them, those which act through tyrosine kinase receptors and G-protein linked receptors. Whilst the details of cellular transformation by ras oncoproteins remain to be elucidated, it has been shown that MAP kinases are constitutively activated in *ras* transformed cells [27], and that ras oncoprotein introduced into quiescent cells directly activates MAP kinase without further requirement for external signals [28]. An inhibitory signal from VP could potentially act on components of this pathway in a similar way to the inhibition of signal transmission from *ras* to *raf*-1 by cAMP [29, 30]. Alternatively, it is possible that the antagonistic effect of VP on *ras* transformation could be mediated in the nucleus through transcription factors such as JunD, which negatively regulates fibroblast growth and antagonises transformation by *ras* [31].

This is the first *in vivo* demonstration that *Ha-ras*-induced tumorigenesis can be impeded by an ectopic ligand. Whilst the mechanisms of this pathway remain to be established, our findings suggest that there may be merit in the inclusion of VP agonists in the therapy of tumours bearing both activated *ras* oncogenes and VP receptors. The availability of VP agonists that can distinguish between V1- and V2-receptor types [32] means that proliferative effects could be inhibited without perturbing renal function.

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