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Review

Animal models of pheochromocytoma

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Summary. Pheochromocytomas are neuroendocrine tumors of adrenal chromaffin cells. They are rare in all species except rats but occur with increased frequency in several human familial tumor syndromes. Concurrence of pheochromocytoma with other tumors sometimes parallels these human syndromes in rats, bovines, horses and dogs but a shared genetic basis for human and spontaneously occurring animal pheochromocytomas has thus far not been established. Pheochromocytomas are inducible in rats by a variety of non-genotoxic substances that may act indirectly by stimulating chromaffin cell proliferation. They are not known to be similarly inducible in other species but arise with increased frequency in transgenic and knockout mice that to varying degrees recapitulate human tumor syndromes. Preliminary evidence suggests that homologous somatic genetic changes might contribute to pheochromocytoma development in humans and some mouse models. The nerve growth factor-responsive PC12 cell line, established from a rat pheochromocytoma, has for almost 30 years served as a research tool for many aspects of neurobiology involving normal and neoplastic conditions. Recently developed pheochromocytoma cell lines from neurofibromatosis knockout mice supplement the PC12 line and have generated additional applications. Advantages of the mouse lines include expression of substantial levels of the epinephrine-synthesizing enzyme, phenylethanolamine N-methyltransferase and expression of high levels of the receptor tyrosine kinase, Ret, which is characteristic of sporadic and familial human pheochromocytomas but not of PC12 cells. Disadvantages include an apparently less stable phenotype. It is difficult to establish pheochromocytoma cell lines from any species, although the tumor cells persist in culture for many months. Understanding of factors that permit pheochromocytoma cells to proliferate might itself provide important insights for tumor biology.

Key words: Adrenal medulla, Pheochromocytoma, chromaffin cell, Mouse, Rat

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Introduction

The study of pheochromocytomas is reminiscent of the tale of the wise men and the elephant. Investigators who work with these interesting tumors do so from many perspectives and often see the results in their own light. Pheochromocytomas may be studied as a disease, as a model for embryological development or as a tool for a variety of scientific disciplines. Nevertheless, the whole is more than its separate parts and each discipline contributes to advancement of the others.

Pheochromocytomas are neuroendocrine tumors of adrenal chromaffin cells. They are extremely rare in humans, with an annual incidence of less than 1 per million (Beard et al., 1989), but occur with increased frequency in a number of familial tumor syndromes including multiple endocrine neoplasia types 2A and 2B (MEN2A, MEN2B), Von Hippel-Lindau (VHL) disease, neurofibromatosis type 1 (NF1) and familial paraganglioma syndromes associated with mutations of the mitochondrial respiratory enzymes, succinate dehydrogenase B and D (SDHB, SDHD) (Neumann et al., 2002).

The rarity of pheochromocytomas is notable across species, with the exception of the rat. In some strains of laboratory rats, upwards of 30% of males spontaneously develop pheochromocytomas in lifespan studies (Haseman et al., 1998). In contrast, the lifetime frequency of the tumors is typically around 1% in wild-type laboratory mice (Tischler and Sheldon, 1996; Tischler et al., 1996b), but much higher in several genetically engineered mouse models. Occasional pheochromocytomas are encountered in other laboratory, domestic and wild animals. Concurrence of pheochromocytoma with other tumors is sometimes suggestive of MEN2 in bovines (Wilkie and Krook,

1970) and horses (De Cock and MacLachlan, 1999) or of mixed MEN syndromes in rats (Fritz et al., 2002; Lee et al., 1982), ferrets (Fox et al., 2000) and dogs (Barthez et al., 1997). Pheochromocytomas have been shown to be inducible by hormones and other non-genotoxic agents in many studies of rats (Tischler and DeLellis, 1988) and occasional studies of guinea pigs (Lupulescou, 1961), suggesting possible mechanisms by which environmental influences could affect the frequency of the tumors in genetically predisposed individuals.

The development of experimental applications of animal models of pheochromocytoma has to a large extent been initially driven by intriguing observations made with human tumor tissue. Individual animal models have subsequently found their own applications, while at the same time contributing, in different ways and varying degrees, to understanding of human pathology.

One application currently of great potential interest is the molecular genetics of pheochromocytomas. It is not known how the diverse germline genetic defects in familial tumor syndromes predispose to the development of pheochromocytomas. However, variable frequencies of pheochromocytoma in each syndrome suggest cooperative interactions with additional genes. Genomic scanning studies of human pheochromocytoma tissue employing comparative genomic hybridization (CGH) demonstrate somatic gains and losses in the majority of tumors and particularly point to losses in chromosomes 1p and 3q as possible common denominators in both sporadic and familial tumors (Edstrom et al., 2000). Specific genes remain to be identified and their functions remain to be elucidated. CGH studies of recently developed mouse pheochromocytoma models (You et al., 2002) show losses of mouse chromosome 4, which is homologous to human chromosome 1p. Additional homologies have also been identified, suggesting that genetic mechanisms in the genesis of pheochromocytomas may be similar with respect to humans and mice.

Numerous studies have resulted from the discovery of the phenotypic plasticity of pheochromocytoma cells. During embryogenesis, both chromaffin cells and sympathetic neurons are derived from pluripotent precursors that originate in the neural crest. Commitment of those precursors to differentiate in the chromaffin cell or neuronal lineage is determined at least in part by environmental cues, but the mechanisms of commitment remain poorly understood. For example, it is not clear how adrenal medullary neurons persist in the same environment as chromaffin cells in most species (Holgert et al., 1996a; Wong, 2003), or why some pheochromocytomas contain areas of ganglioneuroma or ganglioneuroblastoma (Tischler, 2000). The finding in 1975 that human pheochromocytoma cells retain the ability to respond to neurotrophic signals and transdifferentiate into sympathetic neurons in cell culture (Tischler et al., 1976) led to the establishment of the widely studied, nerve growth factor-responsive, PC12 pheochromocytoma cell line (Greene and Tischler, 1976; Tischler and Greene, 1975) from a transplantable rat pheochromocytoma (Warren and Chute, 1972) that had previously shown a small amount of spontaneous neuronal differentiation *in vitro* (DeLellis et al., 1973). Interestingly, but unappreciated at the time, comparable plasticity had previously been reported in primary cultures of an extra-adrenal human paraganglioma (Costero and Chevex, 1962) and in intra-ocular transplants of adult rat chromaffin cells (Olson, 1970). It is now recognized that neonatal or adult chromaffin cells from most species, including adult humans (Tischler et al., 1980), are to varying degrees able to differentiate into neurons and those properties are reflected in pheochromocytomas from every species thus far examined. Parallel studies of pheochromocytoma and normal chromaffin cells therefore provide opportunities to study mechanisms that regulate normal sympathoadrenal development and to determine how regulation becomes abnormal during the development and progression of neoplasia.

Although sympathetic neurons and chromaffin cells are developmentally related and functionally similar, a defining functional difference between the two cell types is that only chromaffin cells express the epinephrine - synthesizing enzyme, phenylethanolamine N-methyltransferase (PNMT) (Wong, 2003). Analyses of pheochromocytomas from most species, and particularly from rats, indicate that PNMT, which is the last enzyme in catecholamine biosynthesis and the last catecholamine-synthesizing enzyme to be expressed during embryogenesis, is also the first to be lost or diminished during neoplastic transformation. The lability of PNMT expression in tumors may reflect physiological mechanisms that tightly regulate epinephrine synthesis and limit it to only a few cell types. Pheochromocytoma cells are currently utilized in a variety of studies of PNMT regulation and may provide insights into development and maintenance of the normal chromaffin cell phenotype.

Mechanistic studies of cell growth and differentiation are greatly facilitated by the availability of pheochromocytoma cell lines and have thus far been conducted mostly with PC12 cells. Comparisons of PC12 cells to normal rat chromaffin cells have been informative because the normal chromaffin cells show very little baseline proliferation but may be stimulated to proliferate *in vivo* and *in vitro*, while PC12 cells proliferate autonomously and can be stimulated to undergo neuronal differentiation. One intriguing and thus far unexplained observation is that several agents that are mitogens for normal chromaffin cells, including nerve growth factor (NGF) (Lillien and Claude, 1985; Tischler et al., 1994), are anti-proliferative and induce neuronal differentiation of PC12 cells (Greene and Tischler, 1976). It is now possible to conduct similar studies with mouse pheochromocytoma lines recently established (Powers et al., 2000) from neurofibromatosis knockout mice (Jacks et al., 1994). Similarities and

differences between the rat and mouse models suggest both parallel and unique applications for each and also raise questions of which model is more relevant to various aspects of human tumor biology. Advantages of the mouse model include genetic resemblances to human pheochromocytomas, expression of substantial levels of the epinephrine-synthesizing enzyme, phenylethanolamine N-methyltransferase (PNMT) (Powers et al., 2000) and expression of high levels of the receptor tyrosine kinase, Ret (Powers et al., 2002), which is characteristic of sporadic and familial human pheochromocytomas (Takaya et al., 1996) but not of PC12 cells (Pachnis et al., 1993; Powers et al., 2002). Disadvantages include an apparently less stable phenotype. There are currently no adequately documented human pheochromocytoma cell lines, despite many attempts to establish them and despite several initially promising reports.

This paper presents a brief overview of animal models of pheochromocytoma, including their distinctive and shared characteristics and their current and potential applications.

Rat pheochromocytomas

Many strains of rat develop pheochromocytomas, either spontaneously during aging (Tischler and Coupland, 1994), or after prolonged exposure to a variety of drugs, foods, chemicals and other agents (Tischler et al., 1989a). The lesions are more frequent in males than in females and are frequently bilateral and multifocal. They have at times posed problems in presentation of data from toxicological testing studies of food and drugs to regulatory agencies, although most agencies now accept that the relevance of the lesions to human toxicology is minimal (Lynch et al., 1996).

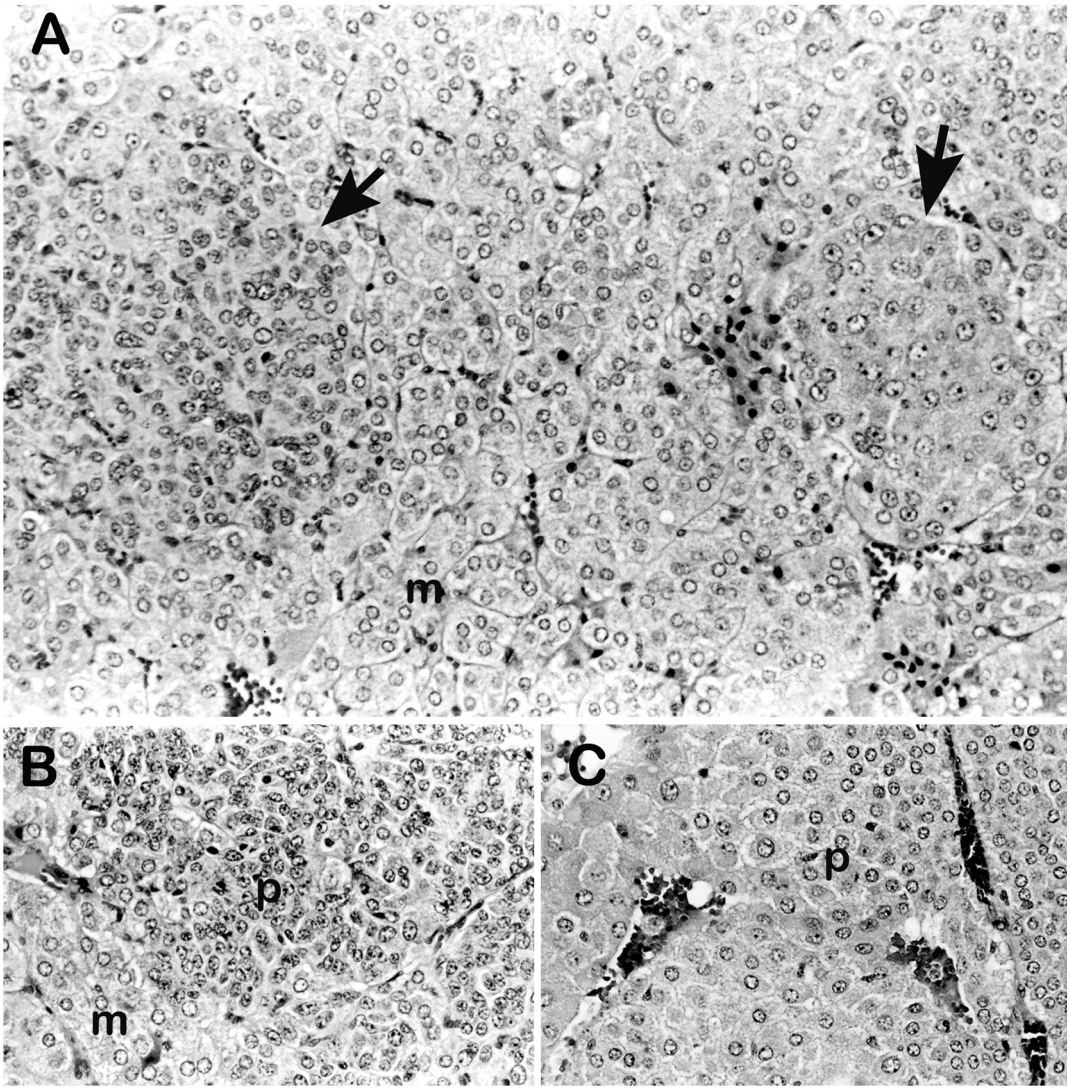

Fig. 1. Hematoxylin and eosin-stained sections of typical adrenal medullary lesions from aged rats. **Panel A** shows two hyperplastic nodules (arrows) separated by normal adrenal medulla (m). The nodule at left, consisting of basophilic cells slightly smaller than normal chromaffin cells, exhibits the most frequently encountered morphology. The nodule at right consists of cells slightly larger than normal chromaffin cells, with vesicular nuclei and focally prominent nuclei. **Panels B and C** show portions of large pheochromocytomas with comparable morphology to the nodules in **panel A**. A small amount of normal medulla is present in **A**, lower left. A, x 200; B, C, x 100

Reported frequencies of pheochromocytoma in rats have declined over several decades, probably the result both of improved animal care standards and of arbitrary classification of small lesions that do not compress the adjacent parenchyma or reticulin framework as hyperplastic nodules. According to the criteria of toxicologic pathology, lesions that compress or infiltrate adjacent adrenal tissue are classified as benign pheochromocytomas, while lesions that invade locally through the capsule of the adrenal are classified as malignant, irrespective of whether metastases are present (Strandberg, 1996). The latter convention is unfortunate in obscuring the fact that metastatic pheochromocytoma is uncommon in rats, as in humans.

"Hyperplastic nodules" are typically composed of monomorphous populations of cells representing morphologies identical to those in larger tumors (Fig. 1). They are also functionally indistinguishable from larger tumors in showing greatly reduced or undetectable expression of PNMT in immunohistochemical studies, while retaining other catecholamine biosynthetic enzymes, including tyrosine hydroxylase (TH) (Tischler and Coupland, 1994; Tischler et al., 1990, 1999). They might therefore hold clues to the earliest events in neoplastic transformation.

Non-genotoxic agents that induce adrenal medullary hyperplasia and pheochromocytomas in rats include the anti-hypertensive agent reserpine (DHHS, 1980), which increases trans-synaptic stimulation of chromaffin cells (Sietzen et al., 1987), and vitamin D3 (Tischler et al., 1999), which perturbs Ca^{++} homeostasis. Several relatively short-term studies in our laboratory showed that reserpine stimulates chromaffin cell proliferation *in vivo*, leading us to hypothesize that pheochromocytomas are induced secondarily to increased cell turnover that facilitates DNA damage (Tischler et al., 1988, 1989a,b; 1991, 1995b, 1996a). This hypothesis is attractive in part because chromaffin cells might be particularly vulnerable to oxidative damage by products of catecholamine metabolism (Baez and Segura-Aguilar,

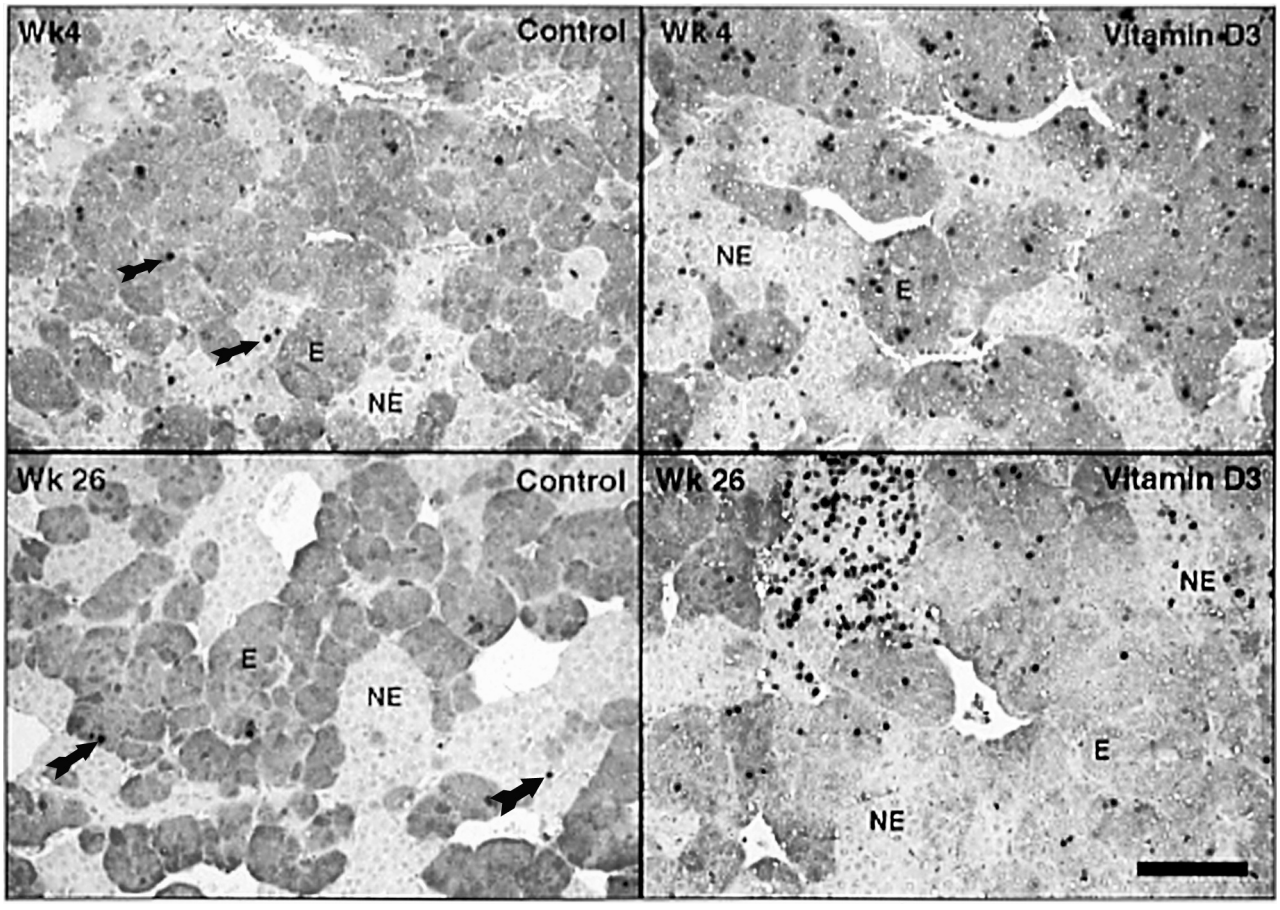

Fig. 2. Representative sections of rat adrenal glands stained for BrdU incorporation (black nuclei, arrows) and PNMT (dark cytoplasm) after ingesting a diet containing vitamin D3 (20,000 IU/kg/day) or control solvent for 4 or 26 wks. A marked vitamin D_3 -stimulated increase in labeling of E cells (PNMT-positive) and NE cells (PNMT negative) is seen at wk 4. By wk 26, the response is diminished overall, but intense labeling is evident in a PNMT negative hyperplastic nodule at top left. Bar: 100 um. Reprinted from (Tischler et al., 1999).

1994; Masserano et al., 1996). However, the finding of a diffuse proliferative response involving both adrenergic and noradrenergic chromaffin cells (Tischler et al., 1989a) was not reconciled with the noradrenergic phenotype of most proliferative lesions (Tischler et al., 1990). We subsequently found that vitamin D_3, which had not previously been associated with adrenal medullary toxicity, was the most potent stimulus to in vivo chromaffin cell proliferation yet identified (Tischler et al., 1996a). This finding was consistent with work from other investigators showing that vitamin D_3 induces TH in chromaffin cells similarly to trans-synaptic stimulation and suggesting that the two inducers may act by converging mechanisms (Puchacz et al., 1996). We then utilized vitamin D_3 as a model to prospectively study early events in the pathogenesis of proliferative lesions, examining the phenotype of proliferating chromaffin cells and their association with cholinergic nerve fibers (Tischler et al., 1999). Rats receiving varied doses of vitamin D_3 were maintained for up to 26 weeks, with a final week of labeling with bromodeoxyuridine (BrdU) as a proliferation marker (Tischler, 1995; Shi et al., 1992). Paraffin sections of the adrenal glands were double-stained immunohisto-chemically for BrdU and TH or PNMT to identify replicating chromaffin cells and discriminate E-cells from NE-cells. The distribution of nerve endings was evaluated by staining for vesicular acetylcholine transporter (VAchT), which permits visualization of cholinergic terminal fields in the peripheral nervous system with finer resolution than is obtainable with other markers (Schafer et al., 1998). The salient findings in this study (Tischler et al., 1999) were: 1. Vitamin D_3 caused a maximal 4-5 fold increase in mean BrdU labeling at week 4, diminishing to a two-fold increase at week 26. 2. During the 26 weeks an initial preponderance of labeled E cells gave way to a preponderance of labeled NE cells. 3. Despite the sustained mitogenic effect of vitamin D_3, there was very little increase in total chromaffin cell number at week 26. However, by week 26, a spectrum of BrdU-labeled focal proliferative lesions including microscopic "hot spots", hyperplastic nodules and pheochromocytomas was seen in the adrenal medullas of almost 90% of rats receiving high dose vitamin D_3, vs. no lesions in controls. Hot spots were defined as loose clusters of five or more BrdU-labeled cells that stood out sharply against the background population of sparsely labeled cells and were not detected by initial screening of H&E sections. Lesions were usually multicentric and bilateral and almost all were PNMT-negative. The lesions were not innervated and thus contrasted sharply with normal chromaffin cells, which reside within a network of nerve terminals. Almost 90% of the lesions were at the corticomedullary junction (Fig. 2).

Several components of a, thus-far, tenable model for induction of pheochromocytomas in the rat are suggested by the above findings: 1. Most proliferating chromaffin cells die. 2. Although innervation may provide mitogenic signals to both E cells and NE cells, proliferating NE cells might have a selective survival advantage, possibly due to different regulatory peptides in subsets of cholinergic nerve fibers that selectively innervate different chromaffin cell populations (Holgert et al., 1996b) and may exert anti-apoptotic effects (Journot et al., 1998). 3. Cells comprising even the smallest of focal proliferative lesions proliferate independently of innervation and may have undergone neoplastic transformation. 4. Survival of cells that give rise to proliferative lesions may be favored by corticosteroids. A need for this protection would most likely be greatest immediately after neoplastic transformation, as autonomously proliferating chromaffin cells lose the protective effects of signaling pathways normally activated by neurotransmitters or growth factors delivered from neurons by anterograde transport.

Common denominators associated with development of pheochromocytomas in routine toxicological studies of rats are not as readily apparent as might be expected from the above experimental studies. Nevertheless, some associations do exist. Development of the tumors correlates, albeit imperfectly, with co-existing chronic renal failure (Nyska et al., 1999) or with severe lung injury causing chronic hypoxemia (Ozaki et al., 2002). Although renal failure is initially associated with decreased production of active vitamin D and with hypo- rather than hyper-, calcemia, hypercalcemia resulting from secondary hyperparathyroidism may occur in advanced renal disease (Nyska et al., 1999), as in the experimental vitamin D model. Rat pheochromocytoma cells have been demonstrated to posess oxygen sensing ability and to respond to hypoxia with activation of signaling pathways also activated by neurally derived signals (Seta et al., 2002). Systemic stress leading non-specifically to reflex neural stimulation of chromaffin cells might also be a contributing factor in some studies.

Although the rat appears to be an interesting model for induction of pheochromocytomas in genetically susceptible individuals, it should be noted that there is no known genetic parallel to human pheochromo-cytomas and the genetic basis of the tumors in rats is unknown. Mutations of ret and abnormalities of other genes associated with human pheochromocytomas have been searched for and not found (Zheng et al., 1996; Fritz et al., 2002).

Mouse pheochromocytomas

In lifespan studies of different strains of laboratory mice, pheochromocytomas occur with reported frequencies of 0-5%. The striking male predilection seen in rats is not paralleled in mice and, also in contrast to rats, the tumors in mice are usually solitary (Tischler and Sheldon, 1996; Tischler et al., 1996b). One potential model for inherited susceptibility to multiple pheochromocytomas in mice reported almost 50 years ago was apparently not further developed (Jones and

Woodward, 1954). In toxicological studies, the frequency of pheochromocytomas in mice is only slightly increased by a small number of chemicals with no apparent common denominators (Hill et al., 2003). Adrenal medullary tumors with some features of pheochromocytoma arise in approximately 10% of mice injected postnatally with polyoma virus (Tischler et al., 1993).

The mouse adrenal has gained new importance in the era of genetic engineering. In the early 1990's, adrenal medullary neoplasms were reported in transgenic mice carrying SV40 or polyoma viral T-antigens driven by a variety of promotors (Aguzzi et al., 1990; Hammang et al., 1990; Giraldi et al., 1994; Helseth et al., 1992; Suri et al., 1993; Hammang et al., 1990), including those for TH (Suri et al., 1993) and PNMT (Hammang et al., 1990). Because they arose early in life and/or because of dedifferentiating effects of T-antigens, these tumors usually were relatively poorly differentiated. Some were classified as primitive neuroectodermal tumors (Fung et al., 1994) or neuroblastomas (Aguzzi et al., 1990). Better differentiated appearing pheochromocytomas and "hyperplastic nodules" have subsequently been reported to occur with high frequency in transgenic mice expressing *c-mos* (Schulz et al., 1992a,b) or MEN2B-type mutant ret (Met 918) (Smith-Hicks et al., 2000), and in *Rb* (Williams et al., 1994), Pten (You et al., 2002) or *Nf1* (Jacks et al., 1994) knockouts. Unfortunately, some of these mice have not been maintained and may be permanently lost as experimental models.

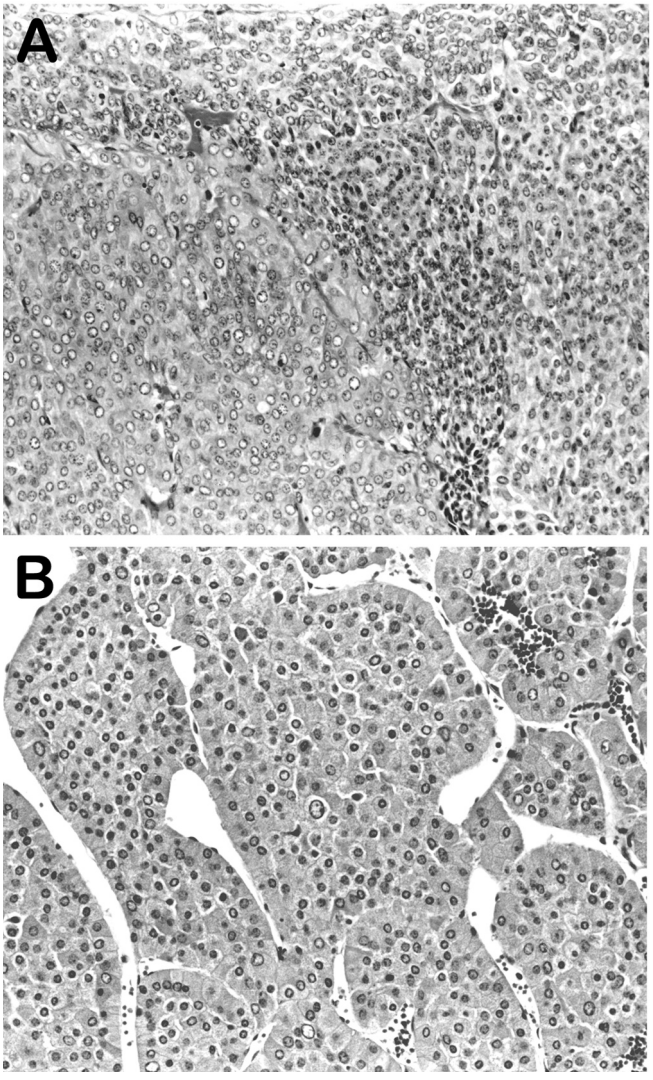

Fig. 3. Hematoxylin and eosin-stained sections of typical adrenal medullary tumors from aged mice. **Panel A**, which is from a large tumor, shows a mosaic-like pattern formed by cells with a variety of cytological features. **Panel B** shows an uncommon morphological variant consisting of relatively uniform cells polarized towards vascular channels. This variant, like the tumor in **A**, typically shows immunoreactivity for TH, which distinguishes it from adrenal cortical carcinoma (Tischler et al., 1996b). A, x 75; B, x 100

Histologically, pheochromocytomas in recently developed genetically engineered mice resemble their naturally occurring counterparts (Tischler and Sheldon, 1996). In general, mouse pheochromocytomas, particularly when large, tend to be more polymorphous than their counterparts in rats. A mosaic-like pattern formed by areas of cells with varied cytologic features may be seen. Tumor cells bordering vascular channels occasionally acquire columnar morphology with basal nuclei (Tischler et al., 1996b) (Fig. 3). Immunohistochemically, the tumors are consistently and diffusely positive for TH but variably and sometimes only focally positive for PNMT. PNMT expression is absent or greatly reduced in tumors associated with viral T-antigens (Suri et al., 1993; Tischler et al., 1993) and in a small series of MEN2B tumors (Smith-Hicks et al., 2000), but positive in tumors associated with aging (Tischler et al., 1996b) or toxic chemicals (Hill et al., 2003) and in tumors from *Nf1* knockout mice (Tischler et al., 1995a).

In both the *c-mos* and MEN2B models, multiple pheochromocytomas are associated with thyroid C-cell proliferations as in human MEN2A or 2B. However, some lines of *c-mos* mice develop exclusively pheochromocytomas or exclusively C-cell tumors, possibly due to differences in the transgene integration site. Interestingly, exclusively C-cell tumors have been reported in a transgenic mouse model of MEN2A (Kawai et al., 2000), perhaps for similar reasons. In both the *c-mos* and *Nf1* models, pheochromocytomas develop only in animals outbred to create a mixed genetic background (Schulz et al., 1992b; Tischler et al., 1996b). Pheochromocytomas occur with increased frequency in mice with a heterozygous knockout mutation of exon 31 of the murine *Nf1* gene (Jacks et al., 1994; Tischler et al., 1995a), but not in other neurofibromatosis mouse models (McClatchey and Cichowski, 2001). This finding initially suggested a possible specific association between exon 31 mutation and pheochromocyrtoma, but such an association has not been found in human

pheochromocytomas from patients with neurofibromatosis (Kimura et al., 2002). In the *Nf1* mouse tumors the wild-type *Nf1* allele is lost. The mutant allele preserves an open reading frame so that its mRNA is transcribed but is thought to produce an unstable neurofibromin protein that is undetectable (Jacks et al., 1994) or barely detectable (Powers et al., 2000) in the tumors.

Pheochromocytomas from *Nf1* knockout mice express high levels of wild-type Ret (Powers et al., 2002), often exceeding Ret expression by the tumors from MEN2B mice (J.F. Powers and A.S. Tischler, unpublished data). The levels of expression in both cases are somewhat surprising because little or no Ret is expressed in normal adult mouse chromaffin cells (Powers et al., 2002). A parallel situation exists in humans, where overexpression characterizes both sporadic and familial pheochromocytomas (Takaya et al., 1996). Although it is well established that Ret protein is detectable in the normal adult human adrenal (Nakamura et al., 1994), recent studies suggest that expression is limited to only a few cells that are often neuronal (Powers et al., 2003). These findings suggest that Ret activation may contribute to the development of both sporadic and familial pheochromocytomas but they are also paradoxical in raising the question of how a protein can cause tumors when it is minimally expressed. Among the possible explanations are that initiating events occur early in life and irreversibly damage immature sympathoadrenal cells that do express Ret, or that initiating events induce Ret, either physiologically or secondarily to somatic genetic damage.

The availability of multiple tumors arising in genetically identical animals and of multiple cell lines derived from separate tumors (see "Pheochromocytoma Cell Lines", below) provides a powerful tool for identifying secondary genetic changes specifically involved in tumorigenesis. Pheochromocytomas in *Pten* knockout mice show consistent deletions in mouse chromosome 4 (You et al., 2002), which is homologous to human chromosome 1p, the most frequent deletion in human pheochromocytomas.

Pheochromocytomas in other species

Pheochromocytomas have been encountered in veterinary practice in dogs and cats (Maher and McNiel, 1997), cattle (Wright and Conner, 1968), horses (De Cock and MacLachlan, 1999), goats (de Gritz, 1997), ferrets (Fox et al., 2000), monkeys (Dias et al., 1996) and birds (Hahn et al., 1997). They occasionally are reported in various wild animals (Brack, 2000; Port et al., 1981; Reppas et al., 2001; Smith and Barker, 1983; Stetzer et al., 1981). Dogs in some series appear to show an exceptionally high frequency of metastatic pheochromocytoma (Gilson et al., 1994). Pheochromocytomas have been reported to be inducible by estradiol and growth hormone in guinea pigs (Lupulescou, 1961) and also by hormones (Kirkman, 1972) or by transplacental administration of N-ethyl-N-nitrosourea (Nakamura et al., 1989) in hamsters.

Pheochromocytomas are generally histologically similar across species. A notable variation is that the tumors in horses and bovines may exhibit striking cell polarity and carcinoid-like architecture that is otherwise seen only occasionally in mice (Tischler et al., 1996b) and extremely rarely in humans (Chetty, 1993). This morphology mimics features that may be seen in the normal bovine and equine adrenal medulla (Fig. 4). Pheochromocytomas in all mammals can almost always be shown to be diffusely positive for TH in properly fixed tissue but are variably positive for PNMT (Fig. 5).

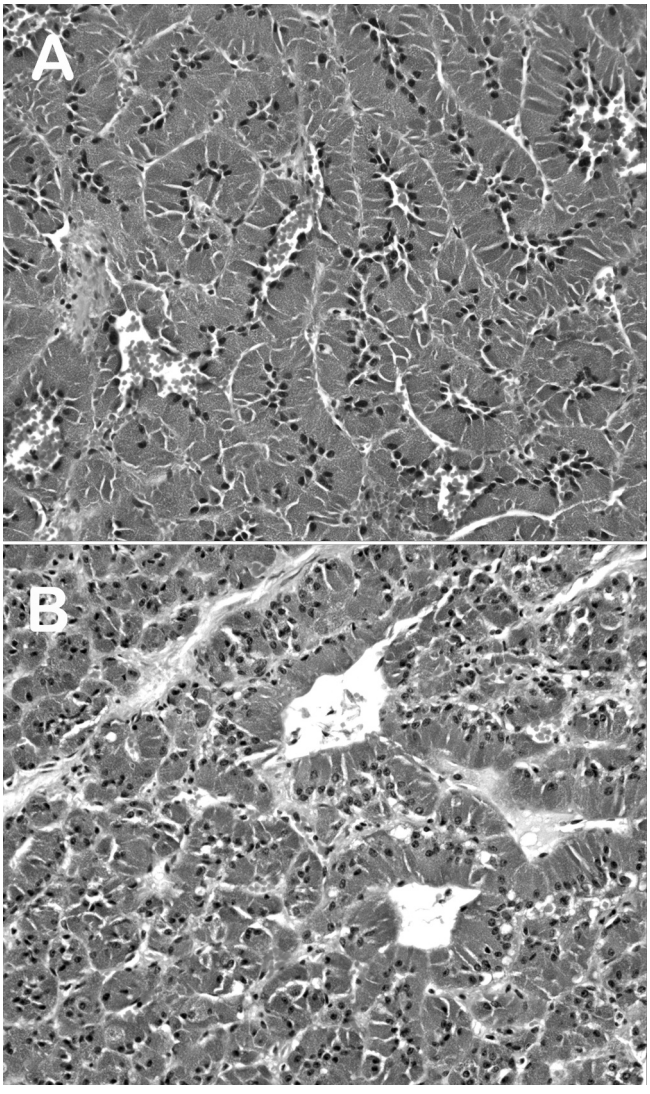

Fig. 4. Hematoxylin and eosin-stained sections of an equine pheochromocytoma (**Panel A**) and adjacent normal adrenal **(B)**. The tumor shows striking cell polarity and carcinoid-like architecture that are also seen focally in the normal horse adrenal. x 75

Immunostaining for chromogranin A, which may be a more stable antigen, can supplement staining for TH in diagnostically questionable cases but will not discriminate pheochromocytoma from other types of neuroendocrine tumor. Immunohistochemical studies in non-mammalian species may be limited by lack of appropriate antibodies.

Pheochromocytoma cell lines

Pheochromocytoma cells from humans, rats and mice tend to rapidly cease proliferation in primary culture. In addition, variable proportions of the tumor cell populations undergo spontaneous neuronal differentiation (Tischler et al., 1984, 1985, 1995a). Propensity for neuronal differentiation may in part reflect underlying genetic abnormalities and, in our experience, has been most pronounced in tumors from neurofibromatosis knockout mice (Tischler et al., 1995a).

Immunocytochemical staining for TH and BrdU after BrdU pulse-labeling (Tischler et al., 1992) provides a means for distinguishing neoplastic chromaffin cells from other cell types in primary cultures and for rapidly assessing the success of attempts to establish cell lines (Powers et al., 2000). In most instances, proliferation of TH-positive cells ceases with two weeks and does not resume, although the cells persist in cultures maintained for many months (Fig. 6). Use of the double-labeling method avoids the pitfall of propagating cells that are not neoplastic chromaffin cells while performing biochemical studies on persistent neoplastic chromaffin cells that are progressively diluted with successive cell passages.

The PC12 cell line, established from a representative rat pheochromocytoma in 1976 (Greene and Tischler, 1976), has become an important workhorse in many disciplines. A search of the PubMed database in July 2003 yields almost 7200 citations on this cell line. Initial applications of PC12 cells often involved NGF signaling and neuronal differentiation. Recent citations show many applications to human diseases, including neurodegenerative disorders (el-Agnaf and Irvine, 2002), viral infections (Su et al., 2002) and bipolar mood disorders (Detera-Wadleigh, 2001).

The phenotype of the PC12 line has been remarkably stable during almost 30 years of propagation. However, diminution of NGF responsiveness, decreased numbers of large secretory granules or loss of other desired traits has occurred in some laboratories, emphasizing the importance of freezing and storing early passages of any cell line. The characteristics of the cells have also been affected by culture conditions, most notably a switch made in some laboratories early in the history of the cell line from RPMI 1640 medium to Dulbecco's modified

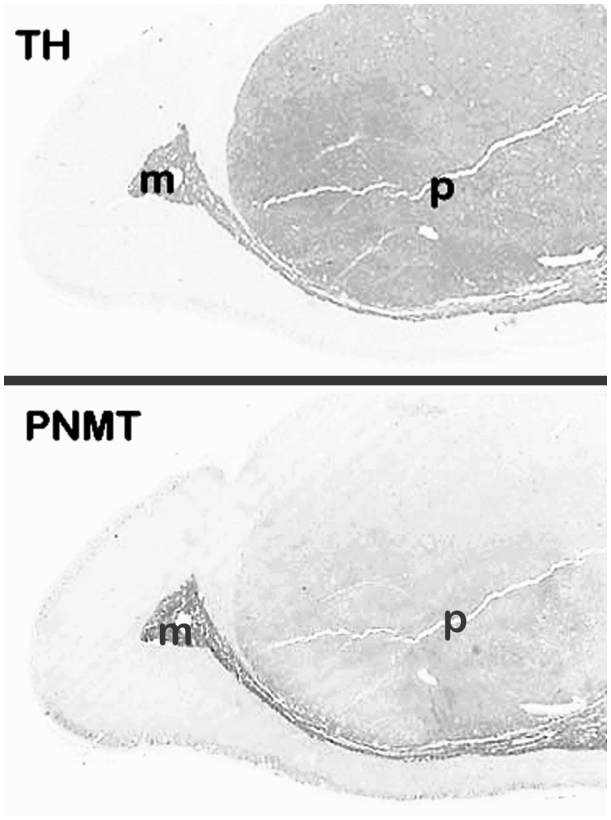

Fig. 5. Adjacent low magnification sections of the adrenal shown in Figure 4, stained for TH and PNMT. Immunoreactivity for TH, the sine qua non for catecholamine biosynthesis, is strong and diffuse in the pheochromocytoma (p) and normal medulla (m), while PNMT is expressed mostly in the normal tissue. x 3.0

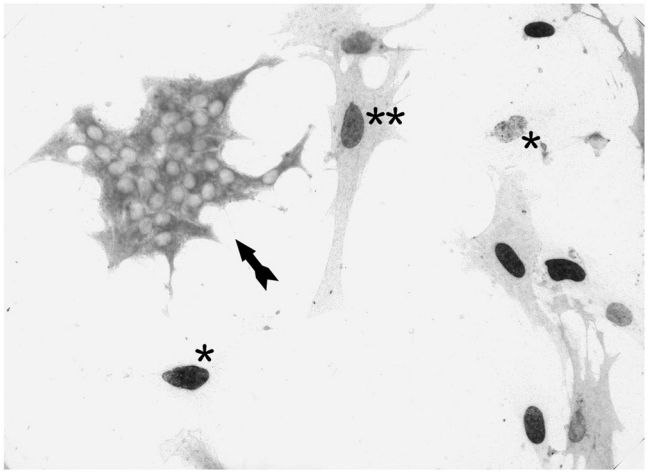

Fig. 6. Primary cell culture of a human pheochromocytoma stained for TH and BrdU incorporation after 2 weeks in vitro. A cluster of 28 neoplastic chromaffin cells (arrow) shows no BrdU labeling (nuclei are small clear circles), while labeled nuclei (* or **) are present in every other cell in the field. Cytoplasm is invisible in cells marked with (*) but faintly visible due to weak endogenous alkaline phosphatase activity in those marked with (**), which are probably endothelial cells. x 250

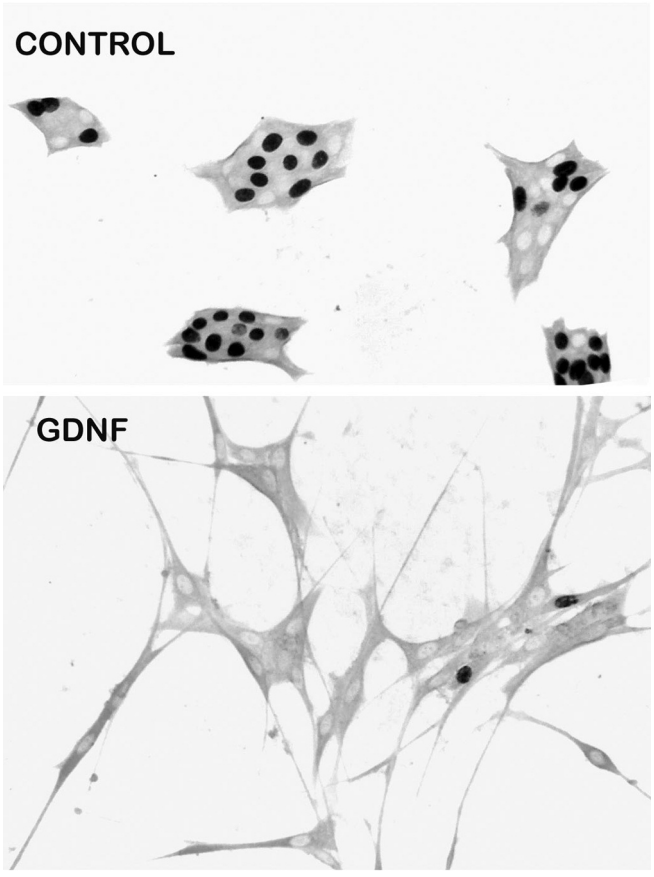

Fig. 7. Culture of MPC cell line 862L stained for TH and BrdU incorporation after 5 days in control medium or medium with GDNF (50 ng/ml), which arrests cell proliferation and causes neuronal differentiation (Powers et al., 2002). x 250

Eagle's medium (DMEM), which increases cell flattening and cell-substratum adhesion because of its higher Ca^{++} concentration. In our experience, PC12 cells maintained in DMEM often regain characteristics of canonical PC12 cells when re-introduced to RPMI.

Mouse pheochromocytoma (MPC) cell lines have recently developed from heterozygous *Nf1* knockout mice (Powers et al., 2000). MPC lines differ significantly from PC12 cells in that they show little or no expression of the NGF receptor TrkA and do not respond to NGF (Powers et al., 2000). Instead, all MPC lines express high levels of Ret and some respond to the Ret-activating ligand, glial cell line–derived neurotrophic factor (GDNF), by ceasing to proliferate and undergoing neuronal differentiation similarly to NGF-treated PC12 cells (Powers et al., 2002) (Fig. 7). The lines may therefore be uniquely valuable for studies of Ret signaling. The differentiating and antiproliferative effects of Ret activation in these cells are consistent with studies in which human pheochromocytoma cells were treated with GDNF in primary cultures (Powers et al., 1998) or human RET with an activating mutation was transfected into PC12 cells (Rossel et al., 1997). These findings raise interesting questions about how Ret signaling leads to adrenal medullary hyperplasia and pheochromocytomas in patients with MEN 2 syndromes.

All MPC lines have been demonstrated to show loss of the wild-type Nf1 allele (Fig. 8), contrary to a previous speculation that the wild-type allele might be retained (Powers et al., 2000).

In a genomic scanning analysis of four MPC lines by comparative genomic hybridization all of the lines showed losses of most or all of chromosome 9, while three lines lost most or all of chromosome 4 (JF Powers,

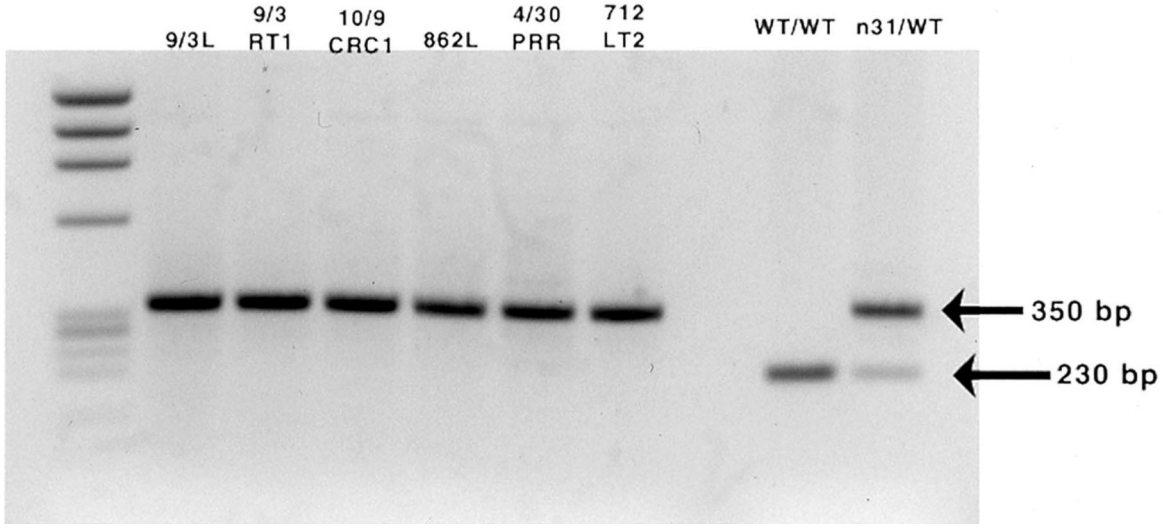

Fig. 8. PCR genotyping of six MPC cell lines (Powers et al., 2002) derived from separate tumors arising in heterozygous *nf1* knockout mice using protocol described by Jacks et al. (1994). All six cell lines show loss of the 230 base pair wild type nf1 allele and contain only the 350 bp modified allele, which contains a neomycin resistance cassette in exon 31 (n31). Tail DNA from wild type mice (WT/WT) and heterozygous knockouts (n31/WT) is shown in lanes at right.

AS Tischler and R Naeem, in preparation). Mouse chromosome 9 shows large areas of homology to human 3p, 3q and 11q, which are also frequently deleted (Dannenberg et al., 2000, 2003). These striking comparisons, together with the losses of mouse chromosome 4 described in pheochromocytoma tissue in the *Pten* knockout model (You et al., 2002), suggest that secondary genetic abnormalities may be critical for the development of pheochromocytomas in mice as well as in humans and may be similar across species. MPC cell lines may prove valuable for studying those changes.

Although MPC cells have been available for only a short time, they have already been used for several additional novel applications. One of those is to study how expression of the epinephrine -synthesizing enzyme PNMT is regulated. In normal tissues, PNMT is expressed in chromaffin cells but not in sympathetic neurons (Wong, 2003). MPC cells differ from PC12 cells in being able to express full length PNMT promoter constructs and relatively high levels of endogenous PNMT mRNA and protein (Powers et al., 2000). They may be particularly valuable for testing the hypothesis that PNMT expression is transcriptionally silenced during neuronal differentiation (Evinger, 1998). Hypoxia has been shown to be an inducer of PNMT in MPC cells (Evinger et al., 2002).

Decades of efforts to establish human pheochromocytoma cell lines have led to a few promising reports (Pfragner et al., 1998; Venihaki et al., 1998). However, continuously replicating human cells that maintain a chromaffin cell phenotype have not become available. Although MPC cells are attractive experimental models by virtue of their genetic and functional similarities to human pheochromocytomas, a drawback compared to PC12 cells is a greater tendency to phenotype drift. This may in part be due to the same factors that cause mouse pheochromocytomas to appear polymorphous in histologic sections or it may reflect cell culture artefact. For example electron micrographs show very sparse secretory granules in MPC cells in culture (unpublished data) compared to tumor tissue, in which granules were numerous (Powers et al., 2000). At present PC12 cells and MPC cells are therefore best regarded as complementary systems. Cell lines have also been established from several adrenal medullary tumors induced by viral T-antigens (Suri et al., 1993; Tischler et al., 1993) but those lines have not been extensively studied.

With the exception of tumors induced by viral T-antigens, the establishment of pheochromocytoma lines has been a challenging task. It should be borne in mind that PC12 cells (Warren and Chute, 1972) and five of six lines of MPC cells (Powers et al., 2000) arose from animals that had been irradiated prior to tumor formation, probably with resultant genetic damage that permitted the lines to be established. Pheochromocytoma cells from aged rats (Tischler et al., 1985), non-irradiated *Nf1* knockout mice (Powers et al., 2000), MEN2B transgenic and Rb knockout mice (both A.S. Tischler and J.F. Powers, unpublished data) as well as benign or malignant human pheochromocytomas persist in primary cultures but do not proliferate. This experience indicates that caution is warranted in drawing general conclusions from any single cell line, but also suggests that understanding of factors that permit pheochromocytoma cells to proliferate night itself provide important insights for tumor biology.

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