Morphology, Biochemistry, and Pathophysiology of MENX-Related Pheochromocytoma Recapitulate the Clinical Features

Tobias Wiedemann, Mirko Peitzsch, Nan Qin, Frauke Neff, Monika Ehrhart-Bornstein,† Graeme Eisenhofer, and Natalia S. Pellegata

Institute for Diabetes and Cancer (T.W., N.S.P.) and Institute of Experimental Genetics (F.N.), Helmholtz Zentrum München, 85764 Neuherberg, Germany; Institute of Clinical Chemistry and Laboratory Medicine (M.P., N.Q., G.E.), University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, and Department of Internal Medicine III (M.P., N.Q., G.E.), University Hospital Carl Gustav Carus, and Division of Molecular Endocrinology (M.E.-B., G.E.), Medical Clinic III, Technische Universität Dresden, 01307 Dresden, Germany

Pheochromocytomas (PCCs) are tumors arising from neural crest-derived chromaffin cells. There are currently few animal models of PCC that recapitulate the key features of human tumors. Because such models may be useful for investigations of molecular pathomechanisms and development of novel therapeutic interventions, we characterized a spontaneous animal model (multiple endocrine neoplasia [MENX] rats) that develops endogenous PCCs with complete penetrance. Urine was longitudinally collected from wild-type (wt) and MENX-affected (mutant) rats and outputs of catecholamines and their O-methylated metabolites determined by mass spectrometry. Adrenal catecholamine contents, cellular ultrastructure, and expression of phenylethanolamine N-methyltransferase, which converts norepinephrine to epinephrine, were also determined in wt and mutant rats. Blood pressure was longitudinally measured and end-organ pathology assessed. Compared with wt rats, mutant animals showed age-dependent increases in urinary outputs of norepinephrine ($P = 0.0079$) and normetanephrine ($P = 0.0014$) that correlated in time with development of tumor nodules, increases in blood pressure, and development of hypertension-related end-organ pathology. Development of tumor nodules, which lacked expression of N-methyltransferase, occurred on a background of adrenal medullary morphological and biochemical changes occurring as early as 1 month of age and involving increased adrenal medullary concentrations of dense cored vesicles, tissue contents of both norepinephrine and epinephrine, and urinary outputs of metanephrine, the metabolite of epinephrine. Taken together, MENX-affected rats share several biochemical and pathophysiological features with PCC patients. This model thus provides a suitable platform to study the pathogenesis of PCC for preclinical translational studies aimed at the development of novel therapies for aggressive forms of human tumors. (Endocrinology 157: 3157–3166, 2016)

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare neuroendocrine tumors derived from chromaffin cells of the adrenal medulla and paraganglia of the autonomic nervous system, respectively. PCCs/PGLs occur sporadically or as a result of an inherited germline mutation in one of at least 14 genes (35%–40% of cases), including VHL, NF1, RET, SDHA, SDHB, SDHC, SDHD, SDHAF2, HIF2α, TMEM127, MAX, FH, PHD2, and MDH2 (1). Somatic mutations of several of the above-mentioned genes also appear to be responsible sporadic PCC/PGL, in particular, activating mutations of HIF2α are rare in the germline and occur mainly as somatic mu-

† Deceased, October 24, 2015.

Abbreviations: BP, blood pressure; H&E, hematoxylin and eosin; MENX, multiple endocrine neoplasia; PAS, periodic acid-Schiff; PCC, pheochromocytoma; PGL, paraganglioma; PNMT, phenylethanolamine N-methyltransferase; wt, wild type.
tations. Recently integrative genomic approaches have identified other common alterations in sporadic PCCs that now await functional validation (2, 3). Gene expression profiling of human PCCs/PGLs has demonstrated that transcriptomic signatures of these tumors reflect the underlying driver mutation (4, 5). Indeed, PCCs and PGLs can be divided into two main clusters, designated as cluster 1 and cluster 2; cluster 1 comprises tumors having germline mutations in VHL, SDHx, FH, and probably HIF2α, whereas cluster 2 includes tumors associated with mutations in NF1, RET, MAX, and TMEM127 as well as most of the sporadic cases (1).

Although usually benign, approximately 10% of PCC and 35% of PGL cases are malignant as defined by the presence of distant metastases (6). The first-line therapy for patients with localized disease is surgery (7). For patients with metastatic disease systemic conventional chemotherapy has been tested without clear benefit on overall survival. Radiotherapy with the radiopharmaceutical 131I-metaiodobenzylguanidine was shown to have positive therapeutic effects, but tumor regression occurred in only 30% of patients (8). Some targeted therapies have also been evaluated: treatment with the tyrosine kinase inhibitor, sunitinib, has shown some efficacy in patients with progressive disease (9), whereas the mammalian target of rapamycin inhibitor everolimus had limited efficacy with progressive disease (9), whereas the mammalian target of rapamycin inhibitor everolimus had limited efficacy (10, 11). There thus remains considerable clinical need for more effective therapies against aggressive forms of this tumor entity.

Animal models of cancer have been instrumental to our understanding of disease progression and spread in elucidating gene function and for identifying and testing novel therapeutic approaches. For rare tumors, such as PCCs/PGLs, having suitable animal models that are representative of the situation in patients is essential, particularly because obtaining large cohorts for molecular studies or clinical trials is a challenge. Transgenic mice developing a PCC alone or in combination with other malignancies have been described and include mice heterozygous for Rb1 gene deletion (12), with the combined deletion of Rb1 and p53 (13), heterozygous for a loss-of-function mutation in Nf1 (14), homozygous for the deletion of both p27 and p18 (15). Monoallelic inactivation of the tumor suppressor gene PTEN, alone or in combination with p27 inactivation, predisposes animals to bilateral PCCs (16). Due to often incomplete tumor penetrance and phenotypic differences between engineered mouse strains and the corresponding human disease, these animal models have provided limited value for elucidating the molecular pathogenesis or evaluating antitumor agents against PCCs/PGLs.

Allograft and xenograft models of the mouse MPC 4/30/PRR (MPC) PCC cell line (17) and its aggressive derivative mouse tumor tissue-derived (MTT) cell lines (18) have been established to evaluate the efficacy of novel compounds or to set up imaging protocols (19–21). These models have the limitation that the tumorigenic process is accelerated and not physiological.

The multiple endocrine neoplasia (MENX) multiple endocrine neoplasia syndrome is a multitumor syndrome that spontaneously arose in a rat strain due to a germline homozygous mutation in p27. MENX-affected (mutant) rats develop endogenous bilateral PCCs with complete penetrance and with time-dependent progression from hyperplasia to tumor nodule formation (22). Rat PCCs share similarities with their human counterpart in terms of histopathological features, gene copy number variations (23), expression signatures (24), and uptake of radiolabeled tracers for imaging (25, 26). MENX rats may therefore provide a valuable tool to elucidate PCC tumorigenesis and to perform preclinical trials. Exploiting this PCC animal model for preclinical and translational studies requires not only comprehensive characterization of the underlying tumor biology but also of the associated biochemical and pathophysiological features.

With the above-mentioned information in mind, we have further characterized MENX rats with a particular focus on key features in patients with PCCs, including catecholamine production, metabolism and secretion, chromaffin cell ultrastructure, blood pressure (BP), and end-organ pathological changes. We here report that MENX mutant rats show catecholamine-related biochemical features also observed in patients with PCCs. Affected rats further display elevated BP and morphological changes in the heart and kidneys similar in nature to those observed hypertensive patients. The similarities between the phenotypic features of MENX rats and patients with PCCs indicate that this animal model provides a valuable tool to study tumorigenesis of adrenomedullary cells and from this develop new therapeutic strategies to target aggressive forms of PCC/PGL.

Materials and Methods

Animals

The features of the MENX rat strain have previously been reported (27). For all studies, male and female MENX-affected and wild-type Sprague Dawley rats were group housed under controlled conditions (temperature 23°C, 12 h light, 12 h dark cycle). Animals had access to standard rodent chow (1314 TPF, Altromin) and water ad libitum. All procedures were approved by the local authorities (AZ 55.2–1-54–2532-225–13) and complied with German animal protection laws.
Biomaterial collection and processing

Homozygous mutant rats used in this study were killed at 1, 2, 3, 4, 6, and 8 months of age, and wild-type littermate rats were killed in parallel for comparison. Immediately after the euthanasia, trunk blood was removed and collected. All rats were inspected for the presence of macroscopically visible lesions, which were sampled, weighed, and immersion fixed in 4% buffered formalin. Later they were embedded in paraffin. Similarly, relevant internal organs (ie, endocrine glands, liver, lung, heart, kidneys, spleen, thymus) were collected, fixed in formalin, and embedded in paraffin. Urine was collected from wild-type and mutant rats at different ages and snap frozen in liquid nitrogen. Samples were stored at −80°C until analysis. Rat adrenal tissues were collected at necropsy, weighed, snap frozen, and stored at −80°C until used.

Histology and immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were sectioned (3 μm) and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or Elastin-Van Gieson’s trichrome according to established protocols. Immunohistochemistry was performed on an automated immunostainer (Discovery XT; Ventana Medical Systems) as previously described (28). Staining of 3-μm rat tissue sections was performed with antibodies against phenylethanolamine N-methyltransferase (PNMT) (Enzo Life Science) (Table 1) (24). Images were recorded using a Hitachi camera HW/C20 installed in a Zeiss Axioplan microscope with Intelliscan software (Zeiss MicroImaging). Stainings were reviewed by an experienced pathologist (F.N.).

Measurements of catecholamines and O-methylated metabolites

Urinary norepinephrine, epinephrine, dopamine, and their respective O-methylated metabolites, normetanephrine, metanephrine, and 3-methoxytyramine, were determined by liquid chromatography with tandem mass spectrometry (30). In brief, analytes were extracted from urine by an offline solid-phase extraction procedure and subsequently directly injected into the liquid chromatography with tandem mass spectrometry system. Intraassay coefficients of variation were, respectively, determined at 7.1%, 10.3%, and 5.1% for norepinephrine, epinephrine, and dopamine, and 9.0%, 5.7%, and 4.5% for normetanephrine, metanephrine, and 3-methoxytyramine. Concentrations were volume corrected using urinary creatinine excretion levels. Tissue catecholamines were measured by liquid chromatography with electrochemical detection (30).

BP measurements

Blood pressure and heart rate were measured by a noninvasive computerized tail-cuff method using the MC4000 BP analysis systems (Hatteras Instruments) (31). Measurements were carried out at 4, 6, and 8 months of age with 10 measurement runs in each session.

Ultrastructural analysis

For electron microscopy, rat adrenomedullary fragments (~1 mm³) collected at necropsy were fixed in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer pH 7.4 (Science Services), post-fixed in 2% aqueous osmium-tetraoxide (pH 7.3), dehydrated in gradual ethanol (30%-100%) and propylene oxide, and embedded in epoxy resin. Semithin sections were cut and stained with toluidine blue. Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate and examined at 80 kV under a Philips (Electronic Instruments) electron microscope (model 301).

Statistical analysis

Statistical analyses used the JMP statistics software package (SAS Institute Inc). Impact of genotype was established using multivariate analyses that included consideration of age with a longitudinal repeated measures design and additional correction for influence of gender. Results of the BP and tissue catecholamine measurements are shown as the mean ± SEM. A paired two-tailed Student’s t test was used to detect significance between two series of data, and P < .05 was considered statistically significant.

Results

Urinary biochemistry of adult rats

A characteristic feature of functional PCCs is production and metabolism of catecholamines to their O-methylated metabolites within tumor. Dopamine is metabolized to methoxytyramine, norepinephrine to normetanephrine, and epinephrine to metanephrine. Thus, measuring production of the metabolites, rather than the precursor catecholamines, provides the recommended approach for diagnosis. To determine whether PCCs in the rat MENX model show similar catecholamine-related biochemical features to humans, we analyzed a series of 11 homozygous mutant rats (four males and seven females) and 14 wild-type (wt) age-matched rats (eight males and six females) for their urine outputs of norepinephrine, epinephrine, dopamine, normetanephrine, metanephrine, and 3-methoxytyramine. Urine samples were collected consecutively from each animal at 4, 6, and 8 months of age.

Mutant rats at 3–4 months of age show adrenomedullary cell hyperplasia, which then progresses to PCC by 8 months of age (24). Using a statistical model considering gender and presence of mutations as independent variables and urinary outputs of amines as dependent repeated variables, we established that mutant rats show age-associated changes and differences in urinary outputs compared with wt animals, consistent with early adrenal med-

Table 1. Antibody List

| Protein Name | Host    | Company          | Catalog Number | Dilution |
|--------------|---------|------------------|----------------|----------|
| PNMT         | Rabbit  | Enzo Life Science| BML-PZ1040-0050| 1:500    |


ular hyperplasia followed by the development of tumors (Figure 1). Specifically, mutant rats showed progressive increases in urinary outputs of norepinephrine (P = .0079), normetanephrine (P = .0014), and 3-methoxytyramine (P < .0001) with advancing age compared with wt animals. Urinary outputs of norepinephrine and normetanephrine were not different at 4 months but were more than 2-fold higher (P < .002) at 8 months of age, when all mutant animals showed PCCs at necropsy compared with the absence of neoplastic lesions in wt rats.

In contrast to norepinephrine and normetanephrine, urinary outputs of dopamine, epinephrine, and metanephrine did not increase with advancing age in mutant vs wt rats (Figure 1). Urinary outputs of dopamine, methoxytyramine, and metanephrine, but not epinephrine, were already significantly increased (P < .01) in mutant vs wt animals at 4 months of age and remained elevated at 6 and 8 months. Urinary outputs of metanephrine in particular were greater than 2.5-fold higher in mutant than wt animals at all ages. Interestingly, epinephrine did not show the same pattern as its O-methylated metabolite, metanephrine, which showed no significant difference when compared with wt animals (Figure 1).

Biochemistry and morphology of young rats

Due to the pronounced increase in urinary outputs of metanephrine in 4-month-old mutant compared with wt rats, we extended our measurements of catecholamines and metanephrines to younger animals. We collected urine samples from 19 1-month-old mutant rats (11 females, eight males) and from nine age-matched wt littermates (five females, four males) and measured catecholamines as reported above. The results showed that indeed metanephrine excretion is significantly higher (P < .0001) in mutant rats already at 1 month of age compared with wt rats (Figure 2, A and B). In contrast, urinary outputs of the other metabolites and their precursor catecholamines were not significantly different among the two groups of animals at this age.

Additionally, we determined the amount of norepinephrine, epinephrine, and dopamine in adrenal gland tissues of 1-month-old mutant (n = 4) and wt (n = 5) littermates. We established that the adrenal glands of mutant 1-month-old rats contained higher relative total levels of catecholamines than those of wt animals (Figure 2C). At this stage, the adrenal glands of mutant and wt rats are highly similar in terms of size and weight, but the medulla can already be hyperplastic (Figure 2D).

Altogether, in mutant rats, urinary outputs of norepinephrine, normetanephrine, and 3-methoxytyramine increase with tumor progression. In contrast, the levels of metanephrine are high at all ages tested, already starting at 1 month of age, indicating that this phenotype is genetically determined.
Figure 2. Catecholamine levels, adrenal gland histology, and adrenal medulla ultrastructure in young rats. A and B, Measurement of catecholamine levels in the urine of 1-month-old wild-type (Wt/wt) and mutant (Mut/mut) littermates. Samples were obtained from 19 mutant and nine wt rats. DA, dopamine; EPI, epinephrine; MN, metanephrine; 3-MTY, methoxytyramine; NMN, normetanephrine; NOREPI, norepinephrine. EPI and MN are shown separately due to the different scale. **, P < .001. C, Measurement of epinephrine and norepinephrine amount in the adrenal gland. D, H&E stain of adrenal glands from wild-type and mutant rats. E, High-magnification images of the adrenal medulla from wild-type and mutant rats.
In agreement with data of catecholamine content, ultrastructural analysis of adrenal chromaffin cells of mutant rats showed that they contain highly elevated numbers of dense core vesicles of ovoid shape, whereas they seem to lack the moderately electron-dense vesicles of rounded shape seen in the cells of wt rats (Figure 2E).

**Immunohistochemistry**

The adrenal medulla of rodents contains two cell populations of adrenergic and noradrenergic chromaffin cells that can be distinguished based on the expression of the enzyme PNMT, which is expressed only in the former cell type. We previously reported that the tumors in MENX rats are not immunoreactive to the anti-PNMT antibody (24). We here analyzed the adrenal glands of mutant and wt rats at the ages also used for the biochemical and ultrastructural analyses, ie, at 1.5, 4, 6, and 8 months. Representative stainings are shown in Figure 3.

These stainings show that from a very early age (1.5 mo), the mutant rats have a higher proportion of PNMT-negative cells than the wt animals (Figure 3). With age (ie, with tumor progression), the amount of PNMT-negative cells in the mutant rats increases significantly (Figure 3). Given that in wt rats no changes in adrenal gland morphology and PNMT expression were observed between the 6- and the 8-month time points, we show the PNMT staining of only a representative 8-month-old mutant animal.

**Pathophysiology**

The most common sign of PCC is hypertension, found in most patients with symptomatic disease and resulting from increases in circulating catecholamines (32). To determine whether the high levels of excreted norepinephrine in MENX rats with PCCs associate with hypertension, we measured longitudinally and noninvasively systolic BP in a series of mutant (n/H110057) and wt (n/H110055) rats of both genders at ages of 4, 6, and 8 months. Given minimal association of gender with BP differences, the data from males and females of each genotype were combined. We observed that BP was significantly elevated in mutant rats at 4 months of age compared with age-matched wt animals (P/H11005/0.0017) and remained elevated at all subsequent ages (Figure 4). By 8 months of age, the mutant rats show on average a 57% increase in BP compared with wt age-matched animals (107.2 ± 3.3 mm Hg; 168.1 ± 30.6 mm Hg; P/H11005/.0016).

Persistent high BP in patients with PCC, if left uncontrolled, can cause damage to several organs and tissues, including arteries, heart, and kidneys (33–36). To determine the effects of hypertension in MENX-affected rats, we analyzed heart and kidney tissues at various ages using different histological staining procedures. In the myocardium of mutant rats older than 6 months of age, we observed a profusion of cells with elongated nuclei (Anitschkow- or caterpillar-like cells) and a thickening of blood vessel walls (Figure 5, A and B). Using PAS staining to detect proteoglycans in the basal membrane of blood vessels, a general disorganization of the cell layers around the blood vessel wall can be appreciated (Figure 5C). Tissue
Scarring was also present. Elastica-Van Gieson trichrome staining revealed perivascular fibrosis extending into the surrounding of the blood vessels (Figure 5D). These phenotypes were not seen in the myocardium of the wt rats up to 8 months of age (data not shown).

The kidneys of mutant rats 6 months old and older showed an accumulation of proteins in the ducts and thickening of the Bowmann’s capsule (Figure 5E). Additionally, all mutant rats showed severe proteinuria ($x \geq 500$ mg/dL) (data not shown). Evidence of glomerular damage was apparent from the presence of both hyper-plastic and hypoplastic glomeruli, with the occasional appearance of necrotic cells. Recruitment of inflammatory cells was also observed in mutant rat kidneys (Figure 5F). Altogether the pathological changes observed in the mutant rats are consistent with renal damage caused by sustained high BP. The wt rats showed no pathological changes in the kidneys up to 8 months of age (data not shown).

**Discussion**

PCCs are tumors of the adrenal medulla characterized by production of catecholamines leading to increased BP with associated end-organ pathology. Lack of suitable animal models for PCCs has presented an obstacle for preclinical studies directed at elucidating the pathophysiology or developing new therapies for these tumors. Here we characterize a spontaneous animal model (MENX) that develops PCC with complete penetrance. We show that this model displays morphological and biochemical features of the tumor with increases in BP and development of end-organ pathology that recapitulate the clinical characteristics of PCCs.

As now demonstrated, the MENX model is characterized by the development of tumors that produce increases in predominantly urinary norepinephrine and its O-methylated metabolite, normetanephrine. Additionally, in mutant rats we observed an age-independent substantial elevation of urinary metanephrine, the O-methylated metabolite of epinephrine, already at 1 month of age, well before the development of tumor nodules. Lack of parallel age-related increases in urinary excretion of dopamine with increases in its O-methylated metabolite, methoxytyramine, presumably reflects different sources of these amines; in particular, most urinary dopamine in both humans and rats is derived from renal decarboxylation of circulating L-dopa rather than extraction of circulating dopamine (37–39). Thus, as in patients with PCCs (40), measurements of methoxytyramine provide a more reliable indicator of tumoral dopamine production than urinary dopamine.

Similar to the situation for methoxytyramine and dopamine, but for largely different reasons, measurements of
metanephrine provide a more reliable indicator of tumoral or chromaffin cell epinephrine synthesis than measurements of epinephrine itself. Metanephrine is produced largely from epinephrine leaking from storage vesicles of adrenal medullary cells and thereby reflects the size of chromaffin cell epinephrine stores (41–43). The substantially elevated urinary excretion of metanephrine in MENX compared with wt rats at an early age (ie, before development of predominantly norepinephrine producing tumors) thus reflects increased stores of adrenal medullary epinephrine. Lack of parallel increases in urine epinephrine reflects the dissociation of epinephrine exocytotic secretion from epinephrine synthesis, storage, and metabolism as described elsewhere (44).

By immunohistochemical staining for PNMT, two populations of chromaffin cells were discerned at all ages in both wt and MENX mutant rat adrenals, a finding consistent with previous observations of two populations of epinephrine- and norepinephrine-producing chromaffin cells in rats (45). From the biochemical and immunohistochemical findings, it can be concluded that despite a background of enhanced chromaffin cell epinephrine production, the chromaffin cell tumors that develop in MENX mutant rats lack PNMT and produce mainly, if not exclusively, norepinephrine. Altogether it seems that the noradrenergic PNMT-negative tumor nodules, responsible for the age-dependent increase in urinary nor epinephrine and normetanephrine, develop on a background of adrenal medullary hyperplasia involving increased adrenal medullary contents of epinephrine and production of metanephrine.

Among patients with PCCs, those tumors that produce exclusively dopamine, norepinephrine, or both catecholamines show distinctly different global patterns of gene expression than those that additionally produce epinephrine (4, 46, 47). The former have been termed cluster 1 tumors and form in patients with predisposing gene mutations that differ from those leading to epinephrine-producing cluster 2 tumors (46, 47). Norepinephrine-producing cluster 1 PCCs secrete the catecholamines more continuously than epinephrine-producing tumors, reflecting a more immature secretory phenotype with less control to restrict exocytosis (44, 48). This is also consistent with the norepinephrine-producing PCCs in MENX-mutant rats in which the elevated urinary excretion of norepinephrine was associated with consistent elevations of BP at all measured time points compared with wt rats.

In mutant rats, PCC-related, organ-specific, hypertensive complications were also found that closely resemble the lesions among patients with these tumors. Vasoconstriction, resulting from the action of catecholamines on α-adrenergic receptors and leading to high BP, forces the heart to work harder than necessary to pump blood to provide adequate organ perfusion. Moreover, high levels of circulating catecholamines may directly contribute to end-organ pathology by structurally changing the vasculature and increasing extracellular matrix proteins, including collagen and fibronectin (33). Catecholamines can also exert direct toxic effects on the myocardium and directly promote hypertrophy, further contributing to the severe cardiovascular complications of pheochromocytoma (49). In the MENX model, pathological changes included thickening of the blood vessels, myocardial fibroses, and myocardial hypertrophy, all features also observed in patients with PCCs (49).

In addition to pathological changes in the heart, we also observed pathological changes in the kidneys of mutant rats. These included protein casts in the ducts due to both vascular stiffening and damage to the basal lamina of the filtration barrier, likely caused by elevated BP. Additionally, necrosis of the glomerular tuft could be demonstrated, again probably caused by sustained high BP, with subsequent hypoperfusion and resulting hypoxia. These are changes consistent with those previously reported in the kidneys of patients with endocrine forms of secondary hypertension (50). Although the end-organ pathological findings in mutant rats reported here are consistent with changes associated with high BP in human PCC patients, we cannot formally exclude that some of these phenotypes might be related to the genetic mutation in p27. Mice defective for p27 (p27−/−) develop pheochromocytoma in 23.8% of cases (15). In these mice, no pathological alterations similar to those we observed in MENX mutant rats have been reported (51); however, the number of animals analyzed was low.

In summary, the MENX rat model recapitulates the most relevant pathophysiological features of human PCC. Because, similar to human patients, MENX rats are characterized by increased catecholamine secretion and sustained hypertension, they provide a promising model for elucidating the pathophysiology of PCCs and for preclinical and translational studies aimed at developing novel targeted therapies for these tumors.

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Address all correspondence and requests for reprints to: Natalia S. Pellegata, PhD, Institute for Diabetes and Cancer, Helmholtz Zentrum München, Ingolstaedter
Landstrasse 1, 85764 Neuherberg, Germany. E-mail: natalia.pellegata@helmholtz-muenchen.de.

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