Models of pheochromocytoma: what's on the horizon?

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Pheochromocytomas (PCCs) and extra-adrenal paragangliomas (PGLs) are the most hereditarily driven of all human tumors. Germline mutations of at least 15 genes are now known to confer susceptibility to these tumors in nearly half of affected patients [1]. Somatic mutations of the same genes and others account for a sizable percentage of the tumors that are sporadic [1,2]. Genotype–phenotype correlations associated with each driver mutation determine tumor location, biochemical function, multiplicity, metastatic potential and development of syndromically associated abnormalities. Although these correlations profoundly affect clinical decisions and patients' outcomes, development of effective treatments has unfortunately lagged behind the rapid advances in genetics of PCC/PGL. For patients in whom the tumors have metastasized, there is currently no cure.

The development of new treatment modalities will require new experimental models for both basic and translational research. Rodent models, human and

animal cell lines, primary cell cultures and xenografts are complementary sources of information and could each play a role. The development of those models is particularly challenging given the tumors' diverse causes and manifestations. Nonetheless, progress has been made, in some cases by revisiting old models for new purposes. Rapidly evolving technologies also suggest that additional models might soon be available.

Potential animal models have existed since the 1960s in veterinary, toxicology, radiobiology and pathology literature. Historically, however, for the most part they were either regarded as curiosities or were utilized for research only tangentially related to clinical aspects of PCC or PGL as diseases. PCCs and, rarely, extra-adrenal PGLs, were reported to occur spontaneously in a variety of wild and domestic animal species, sometimes accompanied by hyperplasia or neoplasia in multiple endocrine glands in the same combinations seen in hereditary human endocrine neoplasia syndromes [3]. For most of the spontaneous models the genetic basis

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was not determined and relevance to human disease remains unknown. An exception is the MENX rat, which corresponds to MEN4 in humans and harbors a germline mutation in *Cdkn1B* [4,5].

Overall, spontaneous PCCs are unusually common in rats, particularly in aging males, but are rare in mice. It is therefore somewhat surprising that the tumors arise in a variety of genetically engineered mice, only some of which carry mutations known to be associated with human PCCs [3]. Further, some mice with relevant mutations do not display the phenotypes associated with those mutations in humans [6] or display incomplete or modified versions of those phenotypes [7]. A particular disappointment in that regard has been the absence of PCCs or PGLs in mice with succinate dehydrogenase gene mutations [6,8]. Because *SDHB* mutations are the single most common cause of metastasizing PGLs in humans, *SDHB* models are sorely needed. Extra-adrenal PGLs are rare in both wild-type and genetically altered animals.

The cell lines currently most widely utilized as PCC models are PC12 [9], derived from an irradiated parabiotic rat, MPC 4/30/PRR (commonly abbreviated to simply MPC), from an irradiated heterozygous *Nf1* knockout mouse [10] and MTT (mouse tumor tissue derived), a relatively aggressive derivative of MPC established from disseminated tumor developing after tail vein injection of MPC cells in a nude mouse [11]. Both PC12 and MPC proved to be genetically relevant to human PCC more than a decade after their development. PC12 cells were found early on to harbor a *Max* deletion [12], 17 years before *MAX* was identified as a human PCC susceptibility gene [13]. Similarly, 12 years after the establishment of MPC cells it was discovered that *NF1* is the most frequently mutated gene in sporadic human PCC [14]. There are currently no human PCC or PGL cell lines, despite many efforts to establish them and several reports that were not subsequently confirmed. One existing human line, obtained by transducing primary cultures of sporadic PCC cells with hTERT, so far appears to be at best minimally functional and was reported as a progenitor cell line [15].

Where do we stand today and where are we headed? As complementary models, MPC and MTT cells are already used in preclinical studies to test candidate drugs for treating metastatic PCC. Their use for that purpose is likely to increase. PC12 cells, for years studied mainly in

the domain of neuroscience, are also increasingly used for drug testing. However, a caveat is that all three cell lines proliferate rapidly compared with human PCCs, which typically grow slowly *in vivo* and not at all in culture [16]. Because of the increased sensitivity of dividing cells, the efficacy of many drugs tested thus far is likely to be overestimated by testing against these cell lines, as illustrated by a recent study comparing cytotoxicity of topoisomerase 1 inhibitors against MPC, MTT and primary human PCC/PGL cell cultures [16]. These rodent cell lines and newer ones will increasingly be used to search for more tumor-specific targeted therapies. At a more basic level, PC12 and MPC cells are being utilized to dissect signaling pathways responsible for specific aspects of tumor phenotype. In elegant studies Qin *et al.* have shown that introduction of *Max* to PC12 cells, which are noradrenergic, increased PNMT expression and slowed proliferation while introduction of *Hif2a* to MPC cells, in which that gene is lacking, caused impaired PNMT expression and increased proliferation [17].

Although PCC/PGL cell lines are not yet available to represent the entire gamut of mutations, lines of other cell types harboring those mutations will help to fill the gap. Dahia *et al.* have studied fibroblasts from a new *Tmem127* knockout mouse and *TMEM127*-mutated human renal cell carcinoma to show how loss of TMEM127 causes accumulation of active mTOR in lysosomes, suggesting possible therapeutic strategies targeting endosomal/lysosomal pathways [18]. Favier *et al.* have utilized nonfunctional immortalized *Sdhb*^{-/-} cells from mouse adrenal medulla to show that increased succinate accumulation in these cells inhibits DNA and histone demethylation, leading to a hypermethylator phenotype comparable to that seen in *SDHB* mutated human PGL and supporting therapeutic use of demethylating agents [19]. These cells have a slow proliferation rate associated with increased migratory and invasion abilities and have been established in a xenograft model in nude mice [FAVIER LABORATORY, UNPUBLISHED DATA]. A revolutionary technological advance likely to speed the development of new models is the advent of the genome-editing techniques TALEN and CRISPR, which permit precise insertion or deletion of genes or portions of genes at specific sites [20]. These new techniques can both speed the development and lower the cost of animal models by directly modifying the

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genome in an ovum, in contrast to conventional methods starting with embryonic stem cells. In cell cultures they permit complete knockout of genes, in contrast to knockdown obtainable with siRNA, and probably with fewer off-target effects [20]. In one project already underway, heterozygous *Sdhb*^{+/-} rats have been developed using the TALEN technique [POWERS JF, TISCHLER AS, UNPUBLISHED DATA]. *A priori*, rats might be expected to be a more promising model than mice because of their innate proclivity to develop PCC and, occasionally, PGL. Further, the incidence of PCC in rats is increased by diet, hormones, drugs and other chemicals [3]. If these animals do develop *Sdhb*-associated tumors they will therefore serve as both a source of needed cell lines and a novel model for studying tumor induction and progression at its earliest stages.

Despite many advances, it is inescapable that human PCC or PGL cell lines would be preferable to rodent lines or lines of phenotypically dissimilar cells. The absence of these cell lines presents both a source of frustration and a conundrum. Hundreds of attempts using many techniques and culture media since at least the early 1970s have demonstrated that human PCC or PGL cells do not proliferate in culture and do not take in mouse xenografts. The conundrum is why the tumor cells, even from tumors that grew rapidly *in vivo*, immediately cease to proliferate in culture but can often survive for a year or longer while maintaining tyrosine hydroxylase expression and other differentiated traits. Immunocytochemical staining for bromodeoxyuridine incorporation and tyrosine hydroxylase expression demonstrates

that cessation of DNA synthesis and tumor cell proliferation occurs less than one day *in vitro*, and is therefore likely attributable to causes other than cellular senescence. Importantly, neither hypoxia nor patient-derived serum is mitogenic. Current approaches to deciphering the conundrum include transduction of primary cultures with lentivirus libraries designed either to screen multiple kinases for ability to activate proliferation or to screen multiple siRNAs for ability to knock down inhibitors of proliferation. Systematic efforts to identify metabolomic and transcriptional differences between tumor tissue and cultured tumor cells are also in their early phases. Preliminary attempts have also been made in some laboratories to generate cell lines and animal models starting with induced pluripotent stem cells from patients with hereditary PCC or PGL. It remains to be seen whether any of these novel approaches will bear fruit.

The international PCC/PGL research consortium PRESSOR encourages and attempts to foster collaboration on development of new experimental models (for more information, please visit the PRESSOR Tumor Models Working Group webpage [21]).

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