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REVIEW

Advances in paraganglioma–pheochromocytoma cell lines and xenografts

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Abstract

This review describes human and rodent-derived cell lines and xenografts developed over the last five decades that are suitable or potentially suitable models for paraganglioma–pheochromocytoma research. We outline the strengths and weaknesses of various models and emphasize the recurring theme that, despite the major challenges involved, more effort is required in the search for valid human and animal cell models of paraganglioma–pheochromocytoma, particularly those relevant to cancers carrying a mutation in one of the succinate dehydrogenase genes. Despite many setbacks, the recent development of a potentially important new model, the RS0 cell line, gives reason for optimism regarding the future of models in the paraganglioma–pheochromocytoma field. We also note that classic approaches to cell line derivation such as SV40-mediated immortalization and newer approaches such as organoid culture or iPSCs have been insufficiently explored. As many existing cell lines have been poorly characterized, we provide recommendations for reporting of paraganglioma and pheochromocytoma cell lines, including the strong recommendation that cell lines are made widely available via the ATCC or a similar cell repository. Basic research in paraganglioma–pheochromocytoma is currently transitioning from the analysis of genetics to the analysis of disease mechanisms and the clinically exploitable vulnerabilities of tumors. A successful transition will require many more disease-relevant human and animal models to ensure continuing progress.

Key Words

- paraganglioma
- pheochromocytoma
- succinate dehydrogenase
- models
- cell lines
- xenografts
- SV40
- MPC
- MTT
- imCC
- hPheo1
- PC12

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Introduction

Paraganglioma–pheochromocytoma

Paragangliomas and pheochromocytomas are neuroendocrine tumors that arise mainly in the adrenal medulla or paraganglia of the head and neck, but may also develop in abdominal or thoracic paraganglia. Benign paragangliomas frequently retain the general histological morphology of normal paraganglia, and comprise several cell types of which the most predominant are the 'chief' or 'chromaffin' cells, also known as type I cells (strictly speaking 'chromaffin' is a misnomer for paraganglioma cells of the head and neck as the traditional potassium dichromate chromaffin reaction, based on the oxidation of stored catecholamines, is generally negative in these cells as catecholamine production is too low to produce a noticeable color shift. Nevertheless, the term is now widely used to describe all paraganglioma–pheochromocytoma tumor cells). These cells are usually arranged in rounded cell nests and typically have a relatively large cell nucleus in proportion to the

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pale cytoplasm. The second prominent cell type is the sustentacular cell (type II cell), with an elongated nucleus and an extended cytoplasm, surrounding a 'nest' of chief cells. Together these cells dominate the characteristic 'cell ball' structures of the paraganglion, traditionally referred to as 'zellballen', which are often encapsulated by a dense stroma. The characteristic appearance of normal paraganglia is frequently maintained even in very large tumors, suggesting that the chief/chromaffin tumor cell component may control the expansion of other, non-neoplastic cell types. By contrast, metastatic tumors often consist primarily of chromaffin cells. Chromaffin cells are the only neoplastic component of paragangliomas, and sustentacular cells in head and neck paragangliomas remain diploid (Douwes Dekker *et al.* 2004, Powers & Tischler 2020). Loss of heterozygosity and loss of SDHB are confined to chromaffin/chief cells (Douwes Dekker *et al.* 2003, Hensen *et al.* 2004, van Nederveen *et al.* 2009). Furthermore, the aberrant methylation found in paragangliomas and pheochromocytomas (Cervera *et al.* 2009, Letouze *et al.* 2013) is only present in the chief cell component (Hoekstra *et al.* 2015).

Genetics

Pheochromocytomas were originally associated with mutations in genes that cause syndromic diseases such as multiple endocrine neoplasia type 2 (MEN2) (*RET* gene), neurofibromatosis type 1 (*NF1* gene) or von Hippel-Lindau disease (*VHL* gene). By the 1990s paragangliomas and pheochromocytomas had also been recognized in non-syndromic families and the underlying genetic cause in many of these families was later shown to be a mutation in succinate dehydrogenase (SDH) subunit D (Baysal *et al.* 2000) or another SDH subunit genes such as *SDHA*, *SDHB*, *SDHC* or *SDHAF2* (Niemann & Muller 2000, Astuti *et al.* 2001). Subsequent genetic analysis of PPGL patients has led to the identification of a heterogeneous collection of both germline and somatic variants in up to 19 genes to date (Fishbein 2019, Neumann *et al.* 2019). In addition to *RET*, *VHL*, *NF1* and the *SDH* genes, suspected or confirmed PGL-associated genes now include *HRAS*, *EPAS1* (HIF2A), *FH*, *MDH2*, *IDH1*, *IDH2*, *DLST*, *SLC25A11*, *GOT2*, *TMEM127* and *MAX*. It is worth noting that most clinical PPGL cases are caused by variants in metabolism-related genes, which currently include *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *FH*, *MDH2*, *IDH1*, *IDH2*, *DLST*, *GOT2* and *SLC25A11*.

Following the pioneering work of Dahia *et al.* (2005), it became clear that these tumors form two distinct clusters in terms of gene expression patterns. Cluster 1 paragangliomas and pheochromocytomas (mutated in *SDH* genes and *VHL*) were characterized by gene expression associated with angiogenesis, hypoxia, coordinated suppression of oxidoreductase enzymes and the reduced expression of *SDHB* (Dahia *et al.* 2005). By contrast, Cluster 2 (*RET* and *NF1*) tumors showed gene expression patterns related to translation initiation, protein synthesis and kinase signaling. *RET* and *NF1* both share an ability to activate the RAS/RAF/MAP kinase signaling pathway and the outcomes of activated RAS signaling may determine the distinctive expression profile in these tumors. Subsequently identified germline-mutated genes associated with paraganglioma–pheochromocytoma, such as *MDH2* (Cascon *et al.* 2015) or *TMEM127* (Qin *et al.* 2010), also tend to associate with one or other of these clusters. It is worth noting that virtually all cell lines and pheochromocytomas identified to date in a wide variety of mouse and rat backgrounds appear to associate with cluster 2 rather than cluster 1 tumors. Why spontaneous or induced (genetically or chemically) cluster 1-related animal tumors fail to develop and why human cluster 1 tumors fail to give rise to cell lines is still not understood.

Rodent-derived cell models

In this review, we first discuss rodent-derived cell lines, followed by several cell lines derived from human sources, and then provide a brief overview of xenograft models. Cell models are listed briefly in Table 1 and are more extensively summarized in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

When discussing paraganglioma and pheochromocytoma cell culture it is important to draw a sharp distinction between the culture of these two entities and their occurrence in experimental animals. While pheochromocytomas are relatively rare in animal models, they do occur on a regular basis, both spontaneously or as a result of chemical or radiological induction in rats and mice (in fact, the toxicology literature contains important information often overlooked by researchers) and on a wide variety of genetically modified backgrounds in mice (Warren & Chute 1972, DeLellis *et al.* 1973, Pellegata *et al.* 2006, Greim *et al.* 2009). Paragangliomas on the other hand, defined here as non-adrenal tumors originating in any paraganglia, are relatively rare in rats and mice (van Zwieten *et al.* 1979, Hall *et al.* 1987, Pirak *et al.* 1988, Li *et al.* 2013, Powers *et al.* 2020) and the systematic development of these tumors has only been reported in

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Table 1 Cell models

Species	Acronym	Tissue of origin	Benign/metastatic	In cell repository?	Reference(s)
Rat	PC12	Adrenal medulla	NR (benign?)	Yes	Greene & Tischler 1976
Rat	MAH	Adrenal medulla precursor cells	Benign	No	Birren & Anderson 1990
Rat	RAD5.2	Adrenal medulla precursor cells	Benign	No	Eaton 2000
Rat	RS0/RS1/2	Adrenal medulla	Benign	No	Powers 2020
Bovine	BADA.20	Adrenal medulla precursor cells	Benign	No	Eaton 2000
Mouse	?	Adrenal medulla	Benign	No	Tischler *et al*. 1995
Mouse	PATH.1/PATH.2	Adrenal medulla	Benign	No	Suri *et al.* 1993
Mouse	?	Adrenal medulla	Benign		Cairns *et al.* 1997
Mouse	MPC	Adrenal medulla	NR (benign?)	No	Jacks *et al.* 1994, Powers *et al.* 2000, 2002, 2004
Mouse	tsAM5D	Adrenal medulla	Benign	No	Murata *et al.* 2003
Mouse	MTT	Adrenal medulla	Metastatic	No	Martinova *et al.* 2009
Mouse	imCC	Adrenal medulla	Non-tumor cell	No	Letouze *et al.* 2013
Human	EPG1	Carotid body	Metastatic	No	Stuschke *et al.* 1992, 1995
Human	KNA	Adrenal medulla	Benign	No	Pfragner *et al.* 1998
Human	KAT45	Adrenal medulla	Benign	No	Venihaki *et al.* 1998
Human	PTJ64p	Jugulotympanic	Benign	No	Cama *et al.* 2013, Florio *et al.* 2017
Human	hPheo1	Adrenal medulla	Benign	No	Ghayee *et al.* 2013

B6/CD1 B-Raf$^{+/LSLV600E}$ mice, occurring exclusively on an F1 hybrid B6/CD1 background (Urosevic *et al.* 2011).

The proper definition of a cell line is a 'cell population derived from a primary culture at the first sub-culture' (McAteer 2002). However, in common parlance, many scientists use the term to describe continuous or immortal cell lines and many official sources, such as the NCI Dictionary of Cancer Terms, also adhere to this essentially inaccurate definition. When describing a cell line it is therefore important to accurately define its growth characteristics.

PC12

The study that indisputably established the field of pheochromocytoma cell culture resulted from a collaboration between Lloyd A Greene and Arthur S Tischler at Harvard Medical School. In the resulting paper, published in 1976, Greene and Tischler described PC12, a noradrenergic clonal line originating from rat adrenal pheochromocytoma cells derived from a solid tumor that arose in an irradiated parabiotic rat (Warren & Chute 1972) and was passaged subcutaneously in New England Deaconess Hospital strain white rats (Greene & Tischler 1976).

A primary characteristic of this cell line, which has gone on to form a mainstay of neurological research worldwide, is its ability to develop neurites similar to those of sympathetic neurons upon exposure to NGF. Removal of NGF leads to the degeneration of neurites and resumption of cell division. PC12 cells also exhibit dense core chromaffin-like granules and they synthesize and store the catecholamine neurotransmitters dopamine and norepinephrine (Greene & Tischler 1976).

The manner in which this cell line was established provided a model for all subsequent efforts to propagate relatively pure paraganglioma or pheochromocytoma cells in that Greene and Tischler recognized that newly dissociated pheochromocytoma cells adhere poorly to plastic culture dishes. Cells were initially plated on plastic tissue culture dishes. The next day, lightly-adherent pheochromocytoma cells were mechanically (rather than enzymatically) dislodged using forceful aspiration with a Pasteur pipette and moved to culture dishes coated with collagen. After a number of passages on collagen-coated dishes, cells were again passaged to plastic dishes. PC12 cells are now available from a wide range of cell repositories and have been used in a very broad range of studies not only related to adrenal function and catecholamine production, but also in neuronal differentiation and other aspects of neurological development and function. These cells still largely maintain the phenotype and morphology of chromaffin cells, despite decades in culture, and can be a useful control when culturing primary tumor cells (Fig. 1). In addition to their widespread use in 2D cell culture, PC12 cells have also been used to produce mouse xenografts for a wide variety of purposes, including the study of malignant behavior of pheochromocytomas

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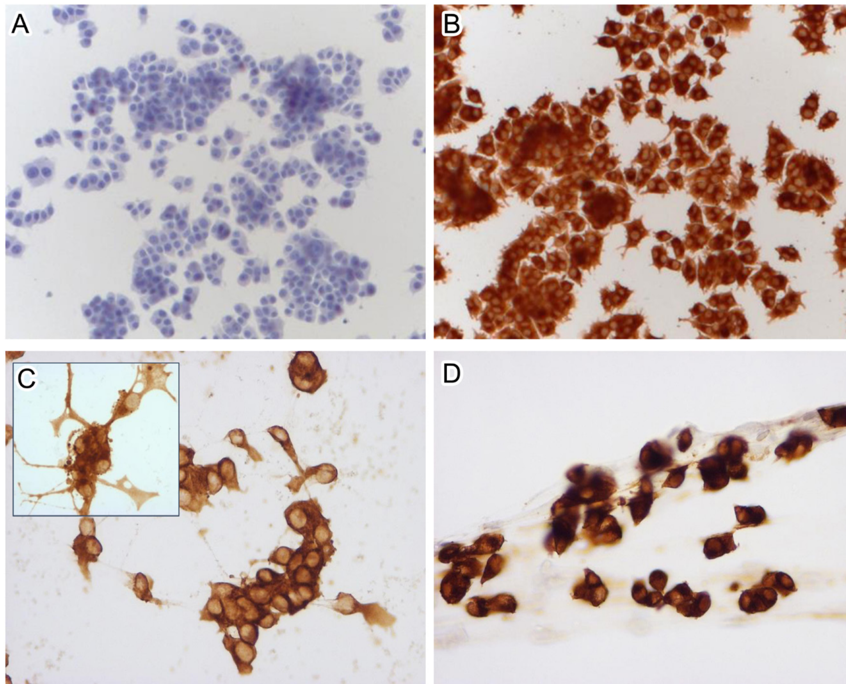

Figure 1
(A) Rat PC12 cells in culture (hematoxylin, 200x) show a characteristic primitive or partially differentiated chromaffin morphology, mainly evident in the high nucleus to cytoplasmic ratio, the compact appearance of the cells and the frequent appearance of short neurites when adherent to plastic. (B) PC12 cells (200x) show strong expression of the synaptophysin protein (anti-synaptophysin antibody, LEICA NCL-L-SYNAP-299). (C) Synaptophysin protein expression in primary chromaffin cells of a pheochromocytoma (4-week culture, 400x) or (D) a carotid body tumor (16-month culture, 400x) is broadly similar to PC12 cells. Chromaffin cells in short-term primary cultures also frequently display more differentiated characteristics (C, inset, 200x).

(Zielke *et al.* 1998), the efficacy of ^{131I}MIBG targeted radiotherapy (Rutgers *et al.* 2000) and the efficacy of the receptor tyrosine kinase (RTKs) inhibitors sunitinib and sorafenib (Denorme *et al.* 2014). Interestingly, these latter two studies provided convincing evidence of the efficacy of therapeutic approaches which was not fully reflected in later clinical studies. Neither ^{131I}MIBG (Pryma *et al.* 2019) nor sunitinib (O'Kane *et al.* 2019) therapy produced the clinical improvements suggested by the outcomes in this model system, indicating that more relevant clinical models may be required.

MPC/MTT model system

MPC (mouse pheochromocytoma cells) and the later derived MTT (mouse tumor tissue) cells, developed in the labs of Arthur Tischler and Karel Pacak, respectively, were derived from pheochromocytomas arising in the adrenal medulla of the *Nf1* knockout mouse originally described by Tyler Jacks in 1994 (Jacks *et al.* 1994, Tischler *et al.* 1995, Powers *et al.* 2000, Martiniova *et al.* 2009). The *NF1* gene encodes neurofibromin, a GTPase that plays a role in negatively regulating the RAS/MAPK pathway. Although neurofibromin is widely expressed, defects in *NF1* disrupt cell growth and neural development in particular. The resulting condition in humans, neurofibromatosis type 1, is primarily characterized by cutaneous neurofibromas, café au lait spots, neurofibromas, optic nerve gliomas and various skeletal defects. Pheochromocytomas and paragangliomas are also present in up to 7.7% of NF1 syndrome cases (Kepenekian *et al.* 2016) but head and neck paragangliomas have never been reported.

The designation 'MPC' covers at least six cell lines, each derived from an independent adrenal tumor, but the most studied is the 4/30/PRR cell line. Martinova *et al.* used this cell line to develop a more aggressive line, referred to as MTT (mouse tumor tissue), by a serial passage in nude mice (Martiniova *et al.* 2009). Compared to the progenitor 4/30/PRR MPC pheochromocytoma cell line, MTT produces greater liver infiltration, referred to as 'hepatic metastases' by the authors (100 vs 4–20 lesions), shows faster development of these lesions (3 vs 4–5 weeks), and mice show decreased median survival (median 25 days vs median 68 days for MPC).

MPC and MTT

Heterozygous animals of the *Nf1* knockout mouse strain used to produce MPC cells are viable and develop a variety of tumors similar although not identical to human *NF1* syndrome. One difference is the higher frequency, at 18%, of adrenal pheochromocytomas in this model (Tischler *et al.* 1995). Tumors in these mice arose on an F1 hybrid inbred genetic background, following the crossing of heterozygous (*Nf1*+/$Nf1^{n31}$) 129SV males with C57BL/6 females, somewhat reminiscent of F1 hybrid B6/CD1 *B-Raf*+/^{LSLV600E} mice (Urosevic *et al.* 2011). To aid derivation of cell lines from *Nf1* tumors, Powers *et al.* used

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4 Gy gamma irradiation to accelerate the occurrence of adrenal pheochromocytomas, and five of the six cell lines eventually established were derived from irradiated mice (Powers *et al.* 2000).

Similarly to the procedure used in the establishment of PC12 cells, during the establishment of MPC Tischler *et al.* transferred weakly attached cells to replicate dishes with and without a collagen coating. Cell proliferation and identity were determined by bromodeoxyuridine labeling and immunohistochemical staining for tyrosine hydroxylase (TH), followed by multiple rounds of differential plating and detachment to eliminate fibroblasts and other possible contaminating cells, followed by serial passage by trypsinization. Characterization of the cell lines showed the expected morphologies of both primitive and more differentiated chromaffin cells, and four of the six cell lines expressed phenylethanolamine *n*-methyltransferase (PNMT), the enzyme that converts norepinephrine into epinephrine. Numerous dense core vesicles consistent with both epinephrine and norepinephrine production were visible on electron microscopy and most cell lines produced epinephrine.

MPC cells strongly express the receptor tyrosine kinase, Ret, and the GDNF receptor, GFRalpha1 (Powers *et al.* 2002). This was unexpected because both of these receptors are normally limited to developmental stages, perhaps suggesting that these tumors arise in tissues arrested at an early developmental stage.

In addition, MPC cell lines show variable patterns of chromosomal gain and loss, with either loss or gain of chromosome 4 (orthologous to human chromosome 1p) being equally common, and overall chromosomal instability, with both hypodiploid and hyperdiploid near tetraploid patterns present (Powers *et al.* 2005). Microarray gene expression patterns showed a clear distinction between normal adrenal medulla and MPC samples, but also between MPC tumor lines individually (Powers *et al.* 2007). In addition to *Ret* and *GFRalpha1*, MPC cell lines express many developmentally regulated genes with a role in the CNS and peripheral nervous system, and nearly 20% of overexpressed genes were reportedly involved in early neural development, consistent with the interesting idea that pheochromocytomas develop from neural progenitors that do not normally persist beyond early development.

These cell lines were further characterized in a paper by Ohta *et al.* (2008) in which the authors compared the cultured 4/30/PRR MPC cell line to tumors arising after subcutaneous and intravenous injection of the cells into nude mice. Subcutaneous injection produced local tumors in all mice, confirming the ongoing tumorigenic potential of these pheochromocytoma cells, while intravenous injection resulted in hepatic infiltration. Comparative gene expression analysis revealed significantly lower expression of five genes (*Metap2*, *Reck*, *S100a4*, *Timp2*, and *Timp3*) in hepatic infiltrates compared to subcutaneous tumors and cultured MPC cells.

Martinova *et al.* subsequently used the MPC cell line 4/30/PRR to develop a more aggressive line, referred to as MTT (mouse tumor tissue), by serial passage in nude mice (Martiniova *et al.* 2009). After determining the optimal conditions for MPC tumor development with the maintenance of a pheochromocytoma-like phenotype, Martinova *et al.* studied in vitro gene expression in MTT vs MPC cell lines. Of 338 genes differentially expressed between the two cell lines, 47 were also differentially expressed in benign vs malignant human pheochromocytomas. Interestingly, the five metastasis-related genes identified by Ohta *et al.* were apparently not found in this comparison. Seven of the 47 genes were then selected for further validation due to their association with the same biological network. However, when these seven genes (*MMP14*, *FOS1*, *FRK*, *GATA2*, *KRT8*, *MMP2*, and *NTS1*) were cross-validated in an independent set of human metastatic and benign pheochromocytomas they failed to show comparable differences in expression.

Experimental studies

The MPC and MTT mouse cell lines have formed the basis of a variety of studies, including the investigation of PI3K/AKT, mTORC1 and RAS/RAF/ERK signaling (Nolting *et al.* 2012), the action of lovastatin and 13-cisretinoic acid (Nolting *et al.* 2014), evaluation of the topoisomerase I inhibitor, LMP-400 (Schovanek *et al.* 2015), and the patterns and reproducibility of metastatic spread (Ullrich *et al.* 2018).

Although paraganglioma–pheochromocytoma show few signs of an innate anti-tumor response and little potential for modern immunotherapies based on somatic mutations and tumor neoantigens (Wood *et al.* 2018), two groups have used MPC/MTT-based models to explore possible alternative immunotherapeutic strategies. Papewalis *et al.* investigated the utility of chromogranin A (CgA), a widely used marker protein for neuroendocrine tumors, as a specific target in a mouse model of pheochromocytoma (Papewalis *et al.* 2011). Caisova *et al.* opted to explore the enhancement of innate immunity-mediated antitumor responses as an

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anti-pheochromocytoma strategy (Caisova *et al.* 2019). Although paraganglioma–pheochromocytoma may show less potential for modern immunotherapy than many other cancer types, the above papers perhaps indicate that in the absence of other potent treatment options these strategies may be worth exploring.

MTT-Sdhb

MTT has also been used recently in conjunction with shRNA-mediated knockdown (KD) of *Sdhb* (D'Antongiovanni *et al.* 2017, Richter *et al.* 2018). This strategy effectively combines aspects of cluster 1 and cluster 2 tumors, together with an unknown genetic contribution from the original irradiation of the MPC cell line and other poorly understood characteristics of that cell line, particularly the continued expression of neurofibromin. *Sdhb* knockdown in these cells led to an approximately 60% reduction in *Sdhb* expression, so these cells are actually closer in phenotype to a human heterozygous carrier of an *SDHB* mutation than to an *SDHB*-negative tumor. Nevertheless, studies using this model have produced interesting results and show certain physiological correlates with *SDH*-mutated tumors. The Manelli–Rapizzi group in Florence has shown that *Sdhb* knockdown spheroid cultures (MTT cells grown in low-attachment conditions) develop neurites reminiscent of human paraganglioma–pheochromocytomas cells in culture and exhibit markedly different migration patterns compared to spheroids without *Sdhb* knockdown. In addition, these investigators identified a role for exogenous, fibroblast-derived lactate in modulating the motility of *Sdhb* knockdown cells.

Relevance of MPC/MTT

Overall, the MPC/MTT cell lines and their use in mouse models represent the most relevant pheochromocytoma model system currently available. It has been claimed that an MPC-based model 'provides an appropriate model for pre-clinical investigations on metastatic PPGLs' (Ullrich *et al.* 2018). Although we agree that better alternatives were not yet available at the time, it is important not to lose sight of the serious shortcomings of this model, especially regarding *SDHB*-related metastatic PPGLs. If a paraganglioma or pheochromocytoma once metastasized is no longer dependent on the initiating mutation, Ullrich *et al.* may well be correct. However, if the initiating genetic insult and the tissue of origin (thoracic or abdominal extra-adrenal tissues) are important factors in metastatic behavior, and more importantly in responses to possible therapeutics, this model may be less relevant to the study of most metastatic tumors. We know that a non-adrenal origin and *SDHB* mutations predispose to metastatic paraganglioma. Neither of these preconditions are a component of any MPC-based model. Equally, a whole range of rodent models develop adrenal pheochromocytomas including c-Mos transgenics (Schulz *et al.* 1992), RET Met918 transgenics (Smith-Hicks *et al.* 2000), Cdkn1b-mutated Sprague–Dawley rats (Pellegata *et al.* 2006), Rb1/Trp53 dual knockouts (Tonks *et al.* 2010), ceramide synthase 2 knockout mice (Park *et al.* 2015), ErbB2 transgenics that develop bilateral adrenal pheochromocytomas (Lai *et al.* 2007), connexin 32 knockouts (King & Lampe 2004), PTEN knockouts (Korpershoek *et al.* 2009) that develop metastatic pheochromocytoma, and B-Raf transgenics that develop both adrenal pheochromocytomas and extra-adrenal paragangliomas (Urosevic *et al.* 2011). None of these rodent models have been used to derive cell lines, even though they might arguably be as relevant as the MPC/MTT cell lines, perhaps even more so in the case of B-raf which is the only animal model that develops extra-adrenal paragangliomas, and is, therefore, a potentially important model for *SDHB*-related paraganglioma as we know that tumor location is a major factor in disease behavior.

The relevance of MPC/MTT cell lines is a question that will need to be answered before the therapeutic strategies investigated in these models, which could potentially have negative consequences for patients, can move to clinical trials. It is also worth reiterating that the relatively aggressive and genetically ill-defined *Nf1* mouse-derived MPC/MTT cell model has obvious limitations in terms of relevance to human *SDH*-associated tumors. Better models are needed, particularly human-derived cell models and models demonstrably based on *SDH* mutations. Only in comparison to these new models will we be able to accurately assess the strengths and weaknesses of the MPC/MTT system.

RS0 cell line

Very recently an interesting and potentially important model was reported, again from the Tischler/Powers lab (Powers *et al.* 2020). Due to the many challenges facing the use of mice in pheochromocytoma research, Tischler and Powers chose to use a rat xenograft model with *SDH*-deficient pheochromocytoma as a stepping stone for cell line development. The basis of the model

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was a heterozygous knockout of the rat *Sdhb* gene using the now defunct TALEN (transcription activator-like effector nuclease) technique in Sprague–Dawley rats. Of the rat mutants obtained, a 13-bp deletion in exon 1 of *Sdhb* was chosen for further study and animals carrying this mutation were then exposed to 5 Gray of gamma irradiation 1 week postnatally, an approach successfully used in the development of the rat PC12 (Greene & Tischler 1976) and mouse MPC cell lines (Powers *et al.* 2000). Upon necropsy, small macroscopic pheochromocytomas of around 0.3 to 0.6 cm were found in three irradiated and one non-irradiated rat. One irradiated rat even developed a carotid body paraganglioma, an extremely rare tumor in rats. In addition, multiple microscopic lesions were found in the adrenal medulla of a number of other animals. Interestingly, Tischler and Powers used a similar approach in Sdhb+/– mice from the Maher lab (Tishler AS & Powers JF, unpublished observations) but even though four tumors were found in 54 irradiated Sdhb+/– or WT mice, all tumors were Sdh-positive and none gave rise to cell lines.

The pheochromocytomas from the rat RS0 model were then used to establish xenografts in NSG mice by subcutaneously injecting tissue from five apparently viable PCs, which resulted in two distinct, serially transplantable, PC xenograft lines designated RS0 (Sdhb+/–) and RS1/2 (*Sdhb*+/+). Histologically, RS0 xenografts exhibit a well-defined 'Zellballen' architecture, stain negative for SDHB protein, and closely resemble human paragangliomas, while RS1/2 shows a more diffuse growth pattern. The ultrastructural features of RS0 are also somewhat reminiscent of human *SDH*-deficient tumors, with relatively sparse secretory granules and cytoplasmic vacuoles, but the typical mitochondrial swelling and degeneration found in many human tumors are absent. To explain this difference the authors cited data suggesting that rodent *Sdh*-null cells may be less bioenergetically compromised than cells from other species, an explanation that might very well underlie the relative resistance of rodent cells to induction of pheochromocytomas and paragangliomas.

To generate primary cell cultures, the RS0 and RS1/2 tumors were harvested, minced, dissociated in collagenase/trypsin and used to establish two cell lines, designated as RS0 and RS1/2, respectively, which were subsequently characterized by double immunocytochemical staining for tyrosine hydroxylase (TH) and BrdU.

An important and innovative aspect of this study was the cell culture approach used to establish cell lines. In preliminary studies, neither xenograft model yielded a cell line when cultured in routine RPMI culture medium (10% horse serum/5% fetal bovine serum) under a standard 95% air/5% CO_2 atmosphere, with RS0 cells dying at around 2 weeks while RS1/2 cells slowly dwindled over many months. The situation improved with culture in 5% O_2, perhaps indicating hypersensitivity to O_2 (Walker *et al.* 2006), but changing to a low-to-absent serum medium together with stem cell-promoting supplements finally allowed RS0 cells to proliferate as a continuous cell line on uncoated plastic culture dishes, appearing as free-floating spheres with an approximately 14-day doubling time.

In terms of metabolite profile, *SDH* deficiency in RS0 xenografts was accompanied by high levels of succinate and lactate accumulation, in contrast to RS1/2 and adrenal medulla. *In vivo* 13C-glucose labeling indicated that pyruvate dehydrogenase (PDH) and pyruvate carboxylase (PC) showed approximately equal activity, consistent with previous studies that found increased utilization of the anaplerotic pathway catalyzed by PC in *Sdh*-deficient mouse cell lines (Lussey-Lepoutre *et al.* 2015). The catecholamine profile of RS0 xenografts was reminiscent of some SDH-deficient human paragangliomas, predominantly producing dopamine, with low levels of norepinephrine and undetectable epinephrine.

Transcriptome analysis of RS0 xenografts showed a high expression of markers associated with the Hif2a regulatory network and with hereditary SDHB mutations. RNAseq also confirmed the almost complete loss of *Sdhb* mRNA in RS0 xenografts. Comparative analysis of TCGA study data, which defines four tumor subgroups including a kinase signaling, a pseudohypoxia, a WNT-altered and a cortical admixture subtype (Fishbein *et al.* 2017), together with RNAseq data from rat samples showed that all three (rat adrenal medulla, RS0 and RS1/2) clustered with the human pseudohypoxic cluster. The RS0 sample might have been expected to cluster differently from the other *Sdhb*-positive rat samples, but perhaps further analysis will provide greater insight into differences in gene expression between these samples.

This model was developed using an innovative combination of methods, and in addition to basic protein and immunohistochemical characterization, was subsequently characterized at the genomic, transcriptomic and metabolomic level. Importantly, the strategies used to derive the RS0 cell line may be broadly applicable to other, including human, *SDH*-deficient models. The RS0 model is not an exact replica of a human *SDHB* tumor, as the likely metabolic and genetic differences in rodent cells represent one intrinsic limitation. The dependence on irradiation to generate this model also introduces

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undefined but possibly crucial factors into the model that need to be further characterized. Although it is reassuring to see the loss of *Sdhb* in the RS0 xenograft/cell line, it remains possible that this is a bystander effect. In order to properly validate the model, complementation with WT *Sdhb* followed by a study of at least the growth and invasive properties of the cell line both in vitro and in vivo will be necessary. Clearly, any model will have limitations but these limitations need to be clearly defined by the proponents of a model and to be fully understood by those using the model. Based on its current characterization, the RS0 cell line appears to be the closest model to *SDHB*-mutated human pheochromocytoma now available and as such appears to be by far the most valid model currently available. In view of the significance of metabolic abnormalities to the wider cancer research community, eventually making this model available via a cell repository is recommended.

Other mouse-derived models

Another mouse cell model, dubbed 'immortalized mouse chromaffin cells' (imCC), was derived from an *Sdhb* knockout mouse (129S2/SvPas; MGI:5521531) (Letouze *et al.* 2013). Taking advantage of genetically modified mice carrying loxP recombination sites flanking endogenous mouse *Sdhb* exon 2, Letouze *et al.* generated *Sdhb*$^{lox/lox}$ mice and then isolated mouse chromaffin cells from the adrenal medulla of these mice. These mice did not develop pheochromocytomas or any other tumors. These cells, expressing normal levels of *Sdhb*, were then put into long-term culture but remained quiescent. However, after 6 months some cultures showed signs of growth and cells were then isolated and transduced with a Cre-recombinase expressing adenovirus, followed by limiting dilution cloning to obtain homozygous *Sdhb* null clonal cell lines. These cell lines, therefore, represent the first bona fide complete knockout model system of *Sdhb*.

The derived cells were reportedly deficient for SDHB protein and showed loss of Sdh/succinate cytochrome c reductase (SCCR) activity, accompanied by high levels of intracellular and secreted succinate. Letouze *et al.* also found other established characteristics of *Sdhb* loss in imCC, including elevated expression and nuclear translocation of HIF2a and a hypermethylation phenotype. The cells were then used to explore phenotypic behaviors including methylation-related modification of cell migration (Letouze *et al.* 2013).

This interesting model is accompanied by several important caveats. First, the cells from which *Sdhb* was deleted reportedly first underwent 'spontaneous immortalization', a phenomenon the authors made no effort to investigate. Secondly, Letouze *et al.* referred to these cell lines as mouse 'chromaffin cells' but presented no evidence to unequivocally establish chromaffin origin. Many other cell types are present in the mouse adrenal medulla besides chromaffin cells. Furthermore, imCCs appear in the available illustrations to exhibit a mesenchymal morphology, a characteristic acknowledged by Loriot *et al.* (2015). Human chromaffin cells in culture that express accepted markers such as chromogranin A, tyrosine hydroxylase or synaptophysin tend not have a mesenchymal appearance (Fig. 1). In our experience, cells of mesenchymal appearance are invariably negative for protein markers characteristic of chromaffin cells, suggesting that imCC cells may not be mature chromaffin cells. In light of the potential value of these cells as an *Sdhb* knockout model, the poor phenotypic characterization and lack of clear verification of the chromaffin status of these cells complicates the interpretation of any data obtained using these cells (Lussey-Lepoutre *et al.* 2015, Kluckova *et al.* 2020).

Nevertheless, imCCs were recently used together with MPC/MTT and primary pheochromocytoma tumor cultures to assess the efficacy of commonly used drug combinations. Fankhauser *et al.* showed that the PI3Ka inhibitor BYL719 and the mTORC1 inhibitor everolimus were effective in decreasing MPC/MTT and imCC cell viability at clinically relevant doses (Fankhauser *et al.* 2019). Despite the caveats attached to these models as discussed above, and the problem that primary pheochromocytoma cultures have an unknown vitality, complicating interpretation of any results, the comprehensive approach applied in this study was probably the best available at the time. It will be interesting to see if and how the moderately positive results of this study translate to a clinical setting.

Immortalized rodent cell models

As chromaffin cells have long been known to show little or no proliferation in vitro, one avenue to obtaining sufficient cells for study has been immortalization using viral oncogenes. An early attempt was described in 1990 by Birren and Anderson, in which these investigators derived the rat adrenal cell line, MAH, from a progenitor cell of the neural crest-derived sympathoadrenal lineage using a v-myc-containing retrovirus. These cells reportedly retained many of the properties of normal progenitor cells. Derived cells showed typical chromaffin cell morphology

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in culture and expressed high levels of TH, neurofilament protein, NCAM, and Thy-l, but lacked PNMT expression and showed no evidence of epinephrine production (Birren & Anderson 1990). These cells have mainly been used to study hypoxia in the chromaffin cell lineage (Nurse *et al.* 2009) and have not yet found an application as a model for study of tumorigenesis, although they might potentially be useful in the study of the hypothesis that tumors arise from cells arrested at an early development stage (Devilee *et al.* 2002, Powers *et al.* 2002) or that have escaped developmental culling (Lee *et al.* 2005).

The potent oncogene SV40 large T antigen (Tag), or a temperature-sensitive variant (tsTag) (Cairns *et al.* 1997), has frequently been used to immortalize mostly non-cancerous cell types. Expression of the WT large T protein simultaneously inactivates pRB and p53, leading to a defective G1/S cell cycle checkpoint, inhibition of apoptosis and obstruction of differentiation (Jha *et al.* 1998). The temperature-sensitive variant, tsTag, permits cell proliferation at 32–33°C but supposedly arrests proliferation and allows differentiation at 38–39°C, although the reliability of temperature shift-dependent cell cycle inhibition may be poor in some systems (Eaton & Duplan 2004, May *et al.* 2005).

SV40 immortalized cell lines could potentially serve as useful models in paraganglioma–pheochromocytoma research. The native phenotype of a parental cell line is often preserved in SV40-immortalized cell lines (Noonan *et al.* 1976, Katakura *et al.* 1998, Roberts *et al.* 2015, Furuya *et al.* 2017, Selt *et al.* 2017), and rat and bovine chromaffin cells transformed with SV-40 continue to show primary chromaffin cell markers (Eaton *et al.* 2000), a catecholaminergic phenotype, normal proliferation and contact inhibition (Eaton & Duplan 2004). However, careful characterization of possible changes in phenotype is a prerequisite of any reliable model, and a tightly controllable inducible system such as Tet-On seems preferable in the case of relatively indolent paraganglioma–pheochromocytoma cells.

SV40 has been successfully used to generate adrenal tumors in mice based on a TH-Tag transgene with adrenal-specific expression driven by 5′ flanking sequences from the rat tyrosine hydroxylase (TH) gene (Suri *et al.* 1993). These tumors were then used to derive the cell lines PATH.1 and PATH.2 (peripheral adrenergic TH-expressing) which could be passaged weekly for at least 2 years. These cell lines appeared relatively stable, both expressing variable levels of TH, dopamine and norepinephrine, and exhibited the classic morphology of chromaffin cells in culture, with clusters of rounded cells showing large nuclei and sparse cytoplasm. It is unclear why these cells received no further attention beyond initial publications. They are still be in existence, however, and possibly available via authors of the paper.

Using a similar approach to Suri *et al.,* Murata *et al.* reported the establishment of the clonal cell line, tsAM5D, from adrenal tumors that developed in mice expressing the tsSV40T transgene under control of the 5′-flanking promoter region of the human TH gene (Murata *et al.* 2003). Around 80% of mice developed adrenal tumors by 5–10 months of age, and the derived cell line tsAM5D showed morphology consistent with chromaffin cells and expressed mRNAs for TH, AADC, and chromogranins A and B, but little or no DBH or PNMT. Cells were dopaminergic, without expression of L-DOPA, norepinephrine or epinephrine. This cell line has primarily been used to study neuronal differentiation of chromaffin cells.

Transgenic mouse lines expressing SV40 tsTag driven by the promoter-enhancer region of GATA-1 somewhat surprisingly developed large uni- of bilateral adrenal tumors (Cairns *et al.* 1997). These tumors appeared to be poorly differentiated but yielded tumor cell lines that expressed chromogranins A and B, neurofilament protein (NF 160kd) and low norepinephrine levels. However, epinephrine was undetectable. These cell lines were apparently not further described in subsequent studies.

To date, none of the above cell lines have been utilized in published paraganglioma–pheochromocytoma research, although they may very well be worth exploring. One obvious criticism is the lack or poor quality of transcriptional control in all of the above systems. The subsequent development of the Tet On system based on the transactivator rtTA (reverse tetracycline-controlled transactivator), allowing tight control of gene activity by addition or removal of doxycycline (Gossen *et al.* 1995), has been shown to offer much better control of cell proliferation compared to the temperature-sensitive SV40 variant (May *et al.* 2005). More up-to-date SV40/viral chromaffin cell systems based on rtTA have not yet been described.

Another drawback of SV40 in the context of paraganglioma–pheochromocytoma research is that in some circumstances it may independently induce transformation, probably based on the inactivation of pRB and p53 (Tonks *et al.* 2010). Continued expression of SV40 in a cell system may therefore preclude unclouded analysis of another gene of interest, but a Tet-On system and/or use of a different oncogene would likely avoid this problem.

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Despite the possible disadvantages of oncogene-mediated immortalization, human chromaffin tumor cells steadfastly refuse to proliferate in culture, so the induction of proliferation by v-myc, large T or a similar approach, ideally driven by an inducible construct, remains an option that cannot be ignored.

Human cell lines

In contrast to diverse attempts, of varying success, to culture pheochromocytomas from either experimental animals or from human tumors, reports of paraganglioma cell culture are extremely sparse. Perhaps the earliest report of paraganglioma tissue culture is that of Costero & Chevez (1962), in which these authors described morphological aspects of the culture of two carotid body tumors. This was followed by a description by Gullotta & Helpap (1976) of the culture of three cases of extra-adrenal paragangliomas, including one carotid body tumor, in which little cell proliferation was observed. In 1981, Tischler *et al.* published the last report on the culture of exclusively paraganglioma cells (Tischler *et al.* 1981).

Paraganglioma

While all cell models for paraganglioma and pheochromocytoma discussed so far originated from rodents, there have been several published attempts to develop models from cultured human tumors. A human paraganglioma cell line was reported in the early 1990s, denoted as EPG1 (Stuschke *et al.* 1992, Stuschke *et al.* 1995), which was derived from a subcutaneous metastasis of a malignant carotid body paraganglioma. This cell line was included in various radiographic studies and was used to generate xenografts in nude mice (Budach *et al.* 1994). EPG1 was established from a tumor biopsy using standard methods and subsequently characterized on the basis of HLA class 1, fibronectin and vimentin expression (positive) and the expression of LDH isoenzymes, none of which clearly establishes cellular identity. Little further description was provided beyond its characteristically slow growth pattern both in vitro and in vivo.

Another attempt to establish a human paraganglioma cell line from *SDH*-mutated tumors has been described by Cama *et al.*, in which these authors cultured tissue from several tumors, including mainly jugulotympanic paragangliomas (Cama *et al.* 2013, Florio *et al.* 2017). These cell lines were first described in the course of a functional study and were used in ensuing experiments without meaningful validation of their identity or primary characteristics. When these cultures presumably ceased replication they were immortalized using retroviral transduction with hTERT and SV40 large T. There was no subsequent attempt to describe or validate this procedure with respect to the characteristics of derived cells compared to the original tumors. In light of risks to phenotypic integrity following retroviral transduction, this is a major oversight. Inspection of figures depicting morphology and immunohistochemistry results for these cell lines does not suggest that these cells are of neuroendocrine origin.

Pheochromocytoma

In the late 1990s, two groups reported the establishment of human pheochromocytoma cell lines, termed KNA and KAT45, respectively (Pfragner *et al.* 1998, Venihaki *et al.* 1998). Both cell lines were derived from sporadic pheochromocytomas and were clearly bona fide chromaffin cells, showing a close morphological resemblance to PC12 cells (Pfragner *et al.* 1998) (Fig. 1), with supporting evidence based on the production of catecholamines (Venihaki *et al.* 1998) or the expression of markers including chromogranin A, human neurofilament protein, S100 and NSE (Pfragner *et al.* 1998). However, nothing has been heard of these cell lines since, so they presumably failed to maintain proliferation at some point and had to be abandoned. The original authors have not responded to requests for further information.

More recently, Ghayee *et al.* reported the establishment of a cell line referred to as a ‘progenitor’ (Ghayee *et al.* 2013). Designated hPheo1, this cell line was derived from a sporadic adrenal pheochromocytoma and immortalized using *hTERT*. Following the use of a neuronal differentiation regime consisting of BMP4, NGF and dexamethasone, the cell culture showed expression of markers including chromogranin A, PNMT and NCAM1 (CD56), but without significant expression of enzymes other than PNMT involved in catecholamine synthesis. A similar differentiation treatment can reportedly produce ‘sympathoadrenal progenitors’ from human pluripotent stem cells (Abu-Bonsrah *et al.* 2018), suggesting that while hPheo1 cells have certain neuroendocrine properties, it is not clear that these properties are derived from cells found in the original tumor. The morphological appearance of the cells also suggests that they are not primitive or differentiated chromaffin tumor cells but more closely resemble cells of mesenchymal origin. In addition, hPheo1

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cells exhibited a small chromosome 9p deletion resulting in loss of the p16 tumor suppressor protein, but no other cytogenetic changes. By contrast, the original tumor did not carry a 9p deletion but instead showed a range of cytogenetic changes affecting chromosomes 1, 3, 4, 11, and 17 that were not found in the hPheo1 cell line. These discrepancies led Ghayee *et al*. to propose that the hPheo1 line arose from a 'subclonal population of progenitor tumor cells' (Ghayee *et al.* 2013). Microarray expression analysis of hPheo1 cells further showed that they grouped together with the tumor and normal adrenal medulla, and were distinct from fibroblasts. However, it is not clear whether this grouping was the result of in vitro treatment with BMP4, NGF, and dexamethasone. Taken together, the data presented suggest hPheo1 did not originate from a differentiated neuroendocrine tumor cell but from another cell type present in the tumor, possibly even of non-neuroendocrine origin. Whether this cell originally derived from a 'population of progenitor tumor cells' therefore remains purely speculative. A particularly conspicuous aspect of the study by Ghayee *et al*. was the extent, clarity and openness of characterization, a manner of presentation that might reasonably be expected of all reports of new paraganglioma–pheochromocytoma cell lines.

As the above summary of attempts to develop human cell lines from paragangliomas and pheochromocytomas attests, the challenge of a human tumor-derived cell line of chromaffin origin has yet to be met. Discussion of this topic with researchers at any dedicated paraganglioma–pheochromocytoma congress will yield numerous anecdotes of fruitless efforts to culture these tumors. Nevertheless, closer questioning and inspection of the literature reveals that concerted efforts in this direction have been largely confined to a few dedicated enthusiasts. Although unsuccessful to date, an increasingly pressing need and new culture techniques perhaps suggest that the ambition of a human paraganglioma–pheochromocytoma cell line is still worth pursuing.

Human paraganglioma and pheochromocytoma mouse xenografts

In addition to the PC12-derived xenografts already discussed, several other cell lines and primary tumors have been used to produce xenografts. The human paraganglioma-derived EPG1 cell line was also used to establish xenografts in nude mice (Budach *et al.* 1993), which were subsequently used in radiographic studies to establish the tumor control dose in a comparative study together with other tumor xenografts. EPG1 xenografts were found to be relatively radio-resistant.

As paragangliomas in VHL patients are extremely rare, a highly unusual paraganglioma xenograft model (Gross *et al.* 1999) was based on a tumor obtained from a patient with VHL type 2A (p.Val166Phe pathogenic missense variant). These xenografts were established using paraganglioma tissue fragments subcutaneously transplanted in BALB/c nude mice. Tumors appeared by approximately 7 months in around 20% of the mice, and tissue fragments obtained from the tumor-bearing mice could be secondarily transplanted. These neoplasms were verified as being of chromaffin origin by immunohistochemical staining for chromogranin A and neuron-specific enolase. This model was developed to investigate the effectiveness of linomide (quinoline-3-carboxamide) in growth inhibition. Anti-tumor effects were reportedly mediated by the antiangiogenic properties of linomide, most prominently expressed through the inhibition of further expansion of tumor capillary bed volume and a consequent reduction in tumor blood flow.

More recently, Powers *et al.* (2017) used NOD-scid gamma (NSG) mice, which lack B and T-cells and are deficient in functional NK cells, to generate patient-derived xenografts (PDX) from a relatively large series of primary paragangliomas ($n = 11$) and pheochromocytomas ($n = 2$). This study aimed to evaluate NSG mice, which reportedly accept a broad range of primary human tumors, as a xenograft recipient, with the ultimate goal of establishing human cell lines by repeated passaging in NSG mice.

Following bilateral subcutaneous injection of dissociated tumor cells into the rear flanks of NSG mice, tumors developed from paraganglioma samples in 3 of the 13 mice (23%), emerging at around 11 months post-injection. Engrafted tumors included both *SDHB*-mutated and WT tumors, with grossly and microscopically identical bilateral tumors present in each successful case. Cellular identity was confirmed by analysis of morphology and protein markers, which showed maintenance of initial patterns of retained or lost tyrosine hydroxylase, chromogranin A and SDHB comparable to the original tumors. Tumors xenografts in NSG mice were characterized by prominent capillary and fibro-adipose tissue, with a variable presence of the cells comprising tumors, including tumorigenic chief cells and supporting sustentacular cells, arranged in typical 'cell nests'. One tumor consisted primarily of capillaries, including only very sparse tumor cells. Interestingly, the use of a human-specific anti-CD31 antibody suggested that the

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majority of tumor blood vessels were derived from human endothelial cells presumably co-injected with dissociated tumor cells, which appeared to have reconstituted their native architecture in the tumor once established in mice. However, it is possible that incompletely digested tumor fragments may have contributed to this impression. It was also unclear whether this vasculature was integrated with surrounding mouse vasculature and was thus functional and able to support tumor vitality and proliferation.

Verginelli *et al.* also recently described attempts to develop PDX models of paragangliomas (Verginelli *et al.* 2018), using a total of 90 PGL fragments from 16 patients and reporting an overall take rate of 89% (80/90). Xenografts were investigated 4.5–10 months post-transplantation and found to present as 4–6 mm nodules that infiltrated adjacent murine neurovascular bundles. PDX tissue, including vasculature, was of human origin, as demonstrated by human-specific antibodies and mtDNA analysis. Interestingly, human-derived vasculature was linked to the systemic murine circulation, as demonstrated by permeation with India ink solution after intracardiac perfusion. However, in contrast to the PDXs reported by Powers *et al.* (2017), the de novo-formed 'cell nests' described by Verginelli *et al.* were negative for accepted neuroendocrine markers such as CGA and SYP, although the authors did report that the cell nests were strongly reminiscent of the 'neuroepithelial PGL component', though no standard immunohistochemistry was presented to support this assertion. In terms of gross morphology, the PDXs presented by Verginelli *et al.* did not appear to be highly vascular, in contrast to those described by Powers *et al.* and native human tumors. One interesting finding was that the 'neuroepithelial-like cells' of the PDXs showed hyperplastic and swollen mitochondria with disrupted cristae, indicative of mitochondrial dysfunction and often found in chromaffin cells.

The most recent and by far the most successful attempt to generate xenografts has been the use of irradiated Sdhb+/− rat pheochromocytomas as a source of tissue for NSG mouse xenografts. This approach led to the development of the RS0 cell line described above (Powers 2020). The study described two distinct and serially transplantable PC xenograft models that the authors designated as RS0 (*Sdhb*−/−) and RS1/2 (Sdhb+/−), both of which yielded small but macroscopic pheochromocytomas following irradiation of rats. Histologically, RS0 pheochromocytomas exhibited the pronounced 'Zellballen' architecture found in many human tumors, accompanied by slightly clear cells and prominent blood vessels. It is significant that RS0 xenografts showed loss of *Sdhb* expression, but it remains possible that RS0 tumorigenesis is partly or wholly driven by mechanisms initiated by irradiation of donor animals and that loss of *Sdhb* is a bystander effect. Further detailed characterization of this potentially important model using complementation with WT *Sdhb* is therefore crucial.

Recommendations for reporting of paraganglioma and pheochromocytoma cell lines

As must now be apparent from the discussion of currently available cell lines, many cell models have been inadequately characterized and as such represent weak foundations on which to base further research. Many researchers have resorted to the use of standard cell lines such as HEK293, but as we and others have experienced, different cell lines often yield conflicting results. Some findings in these cell lines have nonetheless been confirmed in tumor tissue, demonstrating that even standard cell lines can reveal bona fide tumor characteristics, such as the succinate accumulation or HIF-1 upregulation found in many paragangliomas and pheochromocytomas (Selak *et al.* 2005, MacKenzie *et al.* 2007). Other options are the cell lines described in this review, which besides problems of characterization are accompanied by problems of reproducibility when researchers are reluctant to share these models, a problem highlighted by the fact that the PC12 cell line is the only model discussed here that is available via an independent cell repository.

We strongly recommend that future models should not be introduced as an adjunct to a research study but should be presented separately and with adequate characterization, so that the model can be accurately appraised by the research community. Even a detailed characterization included in a study with a different focus might lead to an important cell line being overlooked by some researchers, especially those in other fields who might find use for such an important cell line, a scenario supported by the wide adoption of PC12. The growing interest in and importance of metabolism and hypoxia in cancer suggests that a tumor cell line with a deficiency in SDH would be of major interest. We, therefore, provide some suggestions for informative characterization in Table 2 and particularly urge researchers to provide a transparent characterization, so that the pros and cons of a model are readily apparent. We also strongly recommend that existing and future cell lines are made widely available via the ATCC, Coriell, DSMZ, JCRB or similar cell repository (https://web.expasy.org/cellosaurus/ or https://scicrunch.org/resources).

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Table 2 Recommendations for reporting of paraganglioma and pheochromocytoma cell lines

Recommended descriptive criteria	Suggested assay
Species	Species-specific PCR and Sanger sequencing, NGS or karyotype
Unique genotype (compared to existing cell lines)	Analysis of short tandem repeats (STR) and comparison to existing (database) cell line profiles
Confirmed pathologic diagnosis of paraganglioma or pheochromocytoma	The original tumor shows expected morphology and is positive for chromogranin A and/or tyrosine hydroxylase and/or synaptophysin and/or neuron-specific enolase proteins
Genomic alterations match the original tumor	Exome sequencing, high-density genotyping arrays, FISH or karyotype
In an SDHx-derived model	The cell line should show low or absent SDHB protein expression (*SDHA, B*, *C* & *D* mutated) or SDHA (in case of *SDHA* mutation)
Establish the identity of proliferating cells	Double staining for BrdU/Edu together with synaptophysin, chromogranin A and/or tyrosine hydroxylase
Number and rate of population doublings	Accurately describe number and rate of population doubling
To consider cell line immortal	At least 50 population doublings
Cryopreservation of early cell passages	In order to maintain early passage cultures and prevent phenotype drift
Suggested	
Expression of characteristic gene and/or protein profiles	PCR and Sanger sequencing, transcriptome profiling by RNA sequencing, immunohistochemistry, immunofluorescence or immunoblotting
Expected morphology and cellular features in culture	Light and/or electron microscopy, assessment of catecholamines
Regular monitoring of phenotype (strongly recommended prior to experimentation)	
Confirm expression of:	Chromogranin A and/or tyrosine hydroxylase and/or synaptophysin and/or neuron-specific enolase proteins
No/low expression of SDHB or SDHA protein	SDHB (*SDHB*, *C* & *D* mutated) or SDHA protein expression (*SDHA* mutation) should be low or absent
Rate of population doubling	Accurately describe current and original rate of population doublings
Strongly recommended: Deposit cell line with ATCC or similar cell repository	

Discussion

The title of this review, 'Advances in…', could be considered a misnomer, as recent 'advances' in paraganglioma–pheochromocytoma cell lines and xenografts have been sparse and of unclear relevance, with certain notable exceptions. While the development of mouse and cell models was originally 'plan A', as in any disease-related field of investigation, over the last two decades this task has proved more challenging than expected and has thus effectively been relegated to 'plan B' status by many groups. Nevertheless, seasoned figures in the field continue their efforts (Powers *et al.* 2017, Powers 2020) and others are adapting existing models to new circumstances (Richter *et al.* 2018, Ullrich *et al.* 2018), so research using these models continues and in light of hopeful recent developments from the Tischler/Powers lab, the coming years will hopefully see the introduction of new models from both rodent, and more importantly, human tumor sources.

Do we even need a model in a field in which the primary clinical challenge is metastatic *SDHB*-mutated paraganglioma? Perhaps a strategy of identification of the biological signatures of metastasis and utilization of existing therapeutics developed in other cancers (Calsina *et al.* 2019) will be sufficient to provide patients with new modalities? While this approach to research may prove fruitful, tumors rarely surrender easily to any one line of attack, so alternatives might be advisable. The downstream causal tumorigenic mechanisms in *SDH*-related tumors, in particular, have largely resisted elucidation over the last 15 years, suggesting that they may be dependent on novel cancer pathways and therefore require novel therapeutic approaches.

Updated classic approaches, such as oncogene-mediated immortalization coupled to tight control of gene expression, have been insufficiently explored and may represent the only practical way to obtain sufficient tumor cells for experimentation within a reasonable time interval. It is worth recalling that head and neck paragangliomas show an in vivo doubling time of 4 years (Jansen *et al.* 2000) and patients with malignant tumors display a 5-year overall survival rate of 85% (Hamidi *et al.* 2017), suggesting that successful culture of even these aggressive tumors may yield rates of proliferation too low to be practicable. Alternative approaches such as patient-derived tumor xenograft models, which are receiving renewed interest, the more recent development of patient-derived tumor organoid models (Bleijs *et al.* 2019), as well as the still unexplored possibilities of iPSCs combined

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with CRISPR/Cas as models of chromaffin-derived tumors (Suga 2019) suggest that new avenues may be opening.

We predict that little substantive progress will be made in basic science or in new therapeutics for paraganglioma–pheochromocytoma until a range of better rodent and human SDH-related models become freely available to the wider scientific community. As the field of tumor metabolism broadens these models may find unexpected applications in many other areas, and progress in other disciplines may eventually prove of benefit to paraganglioma–pheochromocytoma research.

Conclusions

We expect that the lack of SDH-specific paraganglioma–pheochromocytoma models, if it persists, will eventually become an insurmountable problem and as such should be given priority by both researchers and funding agencies. It can reasonably be argued that all functional and pre-clinical studies conducted to date are of disputable value at best, as they were inevitably conducted in models with only tenuous claims to relevance to human *SDHx* tumors. Although widely viewed as 'challenging', human *SDHx*-related paraganglioma and pheochromocytoma cell culture has been largely neglected (with notable exceptions) and the studies that have taken place have often remained unpublished, frustrating efforts to distinguish useful techniques and procedures from the less successful. The recent development of the RS0 xenograft model/cell line gives reason for optimism but will require further detailed characterization to confirm its relevance to *SDHB*-related human cancers. As the field of basic paraganglioma/pheochromocytoma research matures and moves from the study of genetics to the study of the molecular mechanisms driving tumorigenesis, the lack of numerous different human and animal models will continue to limit further progress.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/ERC-19-0434.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

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