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## MENX

Natalia S. Pellegata

Institute of Pathology, Helmholtz Zentrum München-German Research Center for Environmental Health, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany

### Abstract

Multiple endocrine neoplasias (MEN) are a group of hereditary disorders characterized by tumors arising in more than one neuroendocrine tissue. There are two major forms which can occur in humans, MEN type 1 (MEN1) and MEN type 2 (MEN2). These syndromes are transmitted as autosomal dominant traits with high penetrance and have a different tumor spectrum. MEN1 and MEN2 are caused by germline mutations in the *MEN1* and *RET* genes, respectively. Recently, a variant of the MEN syndromes was discovered in a rat colony and was named MENX since affected animals develop tumors with a spectrum that shares features with both MEN1 and MEN2 human syndromes. Extensive genetic studies identified a germline mutation in the *Cdkn1b* gene, encoding the p27 cell cycle inhibitor, as the causative mutation for MENX. Capitalizing on these findings, heterozygous germline mutations in the human homologue, *CDKN1B*, were searched for and identified in patients with multiple endocrine tumors. As a consequence of this discovery, a novel human MEN syndrome, named MEN4, was recognized which is caused by mutations in p27. Altogether these studies identified *Cdkn1b/CDKN1B* as a novel tumor susceptibility gene for multiple endocrine tumors in both rats and humans. Here I review the phenotypic features and the genetics of the MENX rat syndrome. I briefly address the main functions of p27 and how they are affected by the MENX-associated mutation. Finally, I present examples of how this animal model might be exploited as a translational platform for preclinical studies of pituitary adenomas.

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### Résumé

Les néoplasies endocriniennes multiples (NEM) sont un groupe de pathologies héréditaires caractérisées par des tumeurs se développant dans plusieurs tissus neuroendocrines. On décrit deux formes majeures chez l'homme : la NEM de type 1 (NEM1) et la NEM de type 2 (NEM2). Ces syndromes sont transmis de manière autosomique dominante avec une pénétrance élevée et ont un spectre tumoral différent. Les NEM1 sont en rapport avec des mutations germinales du gène *MEN1* et les NEM2 liées à des mutations germinales du gène *RET*. Récemment, un variant de syndrome de NEM a été découvert dans une lignée de rats et a été appelé NEMX car les animaux touchés développaient des tumeurs rappelant à la fois les syndromes humains NEM1 et NEM2. Des études génétiques extensives ont identifié une mutation germinale dans le gène *Cdkn1b* qui code pour l'inhibiteur du cycle cellulaire p27 comme étant la mutation causale de la NEMX. À partir de ces données, des mutations germinales hétérozygotes dans l'homologue humain *CDKN1B* ont été recherchées et identifiées chez des patients ayant des tumeurs endocrines multiples. Après cette découverte, un nouveau syndrome NEM, appelé NEM4, a été reconnu. Il est en rapport avec des mutations de p27. Toutes ces données identifient *CDKN1B* comme un nouveau gène de susceptibilité tumorale pour les NEM aussi bien chez le rat que chez l'homme. Cette revue reprend les caractéristiques phénotypiques et génétiques du syndrome NEMX et fait le point sur les fonctions principales de p27 ainsi que sur la façon dont la mutation associée à NEMX perturbe ces fonctions. Enfin, elle donne l'occasion, à partir d'exemples, de voir comment ce modèle animal peut être exploité comme plateforme translationnelle pour les études précliniques des adénomes hypophysaires.

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### 1. Phenotypic features of MENX

In 2002, we discovered a rat strain that spontaneously and consistently develops multiple neuroendocrine tumors with high penetrance and at young age. Animals affected by this disease

show phenotypic overlap with both the MEN1 and MEN2 syndromes, developing multifocal pituitary adenoma (incidence: 100%), bilateral pheochromocytoma (100%), multifocal thyroid C-cell hyperplasia (78%), parathyroid hyperplasia (65%), extra-adrenal pheochromocytoma (paragangliomas) and pancreatic islet cells hyperplasia ([1] and unpublished results). This multitumor syndrome was termed MENX and it was reported to be inherited as a recessive trait, meaning that affected rats

E-mail address: natalia.pellegata@helmholtz-muenchen.de



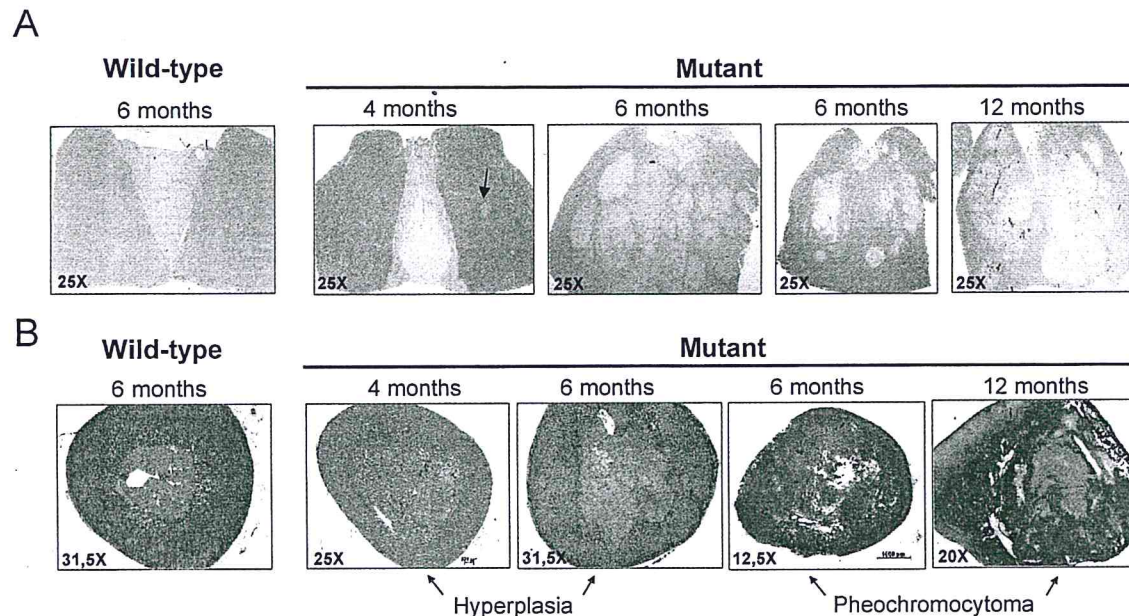


Fig. 1. Histology of pituitary and adrenal glands of wild-type and mutant rats at different ages. A. Lesions in the anterior pituitary (adenohypophysis) are visible in mutant rats at 4 months of age as small neoplastic nodules (arrow). Then, the lesions progress into multifocal, highly vascularized adenomas. B. Mutant rats show adrenal medullary hyperplasia at 4 months of age, which develops into pheochromocytoma with age. Original magnifications are reported.

are homozygous for the underlying genetic mutation (mut/mut) [1]. Gross histomorphological analysis of the rat tumors showed that they are similar to their human counterpart. Moreover, a clear progression of the lesions over time was observed in affected rats. Indeed, all mutant rats develop anterior pituitary tumors, which progress from small neoplastic nodules (about 4 months of age) to large adenomas (8–12 months of age) (Fig. 1A). Homozygous mutant rats also develop adrenal medullary hyperplasia at 3–4 months of age, which progresses to pheochromocytoma by 6–8 months of age (Fig. 1B). More than two-thirds of affected rats present with multifocal C-cell hyperplasia, which can eventually lead to locally invasive medullary thyroid carcinoma. In addition to the tumor phenotype, MENX-affected rats develop macroscopically visible bilateral juvenile cataracts, a phenotype that precedes the appearance of neoplastic disease and was therefore used as phenotypic marker [1]. Mutant rats are bigger in size than their wild-type littermates and show organomegaly, particularly of spleen and thymus. Affected rats have an average life span of  $10 \pm 2$  months whereas their wild-type littermates live approximately 24–30 months [2].

## 2. The genetic defect causing MENX

In an effort to identify the gene causing MENX, we performed linkage studies, which allowed us to initially map the *MENX* locus to an interval of approximately 22 cM on the distal part of rat chromosome 4, and, subsequently, to exclude the rat homologue of the *RET* gene (located on chromosome 4) as the candidate gene for the disease [3]. Linkage analysis of additional animals obtained by backcrosses allowed us to refine the mapping of the *MENX* locus to a ~3 Mb interval [3]. Several candidate genes located in this region were

screened for mutations. Among them was *Cdkn1b*, encoding the cyclin-dependent kinase (Cdk)-inhibitor p27. We found that MENX-affected rats are homozygous for a tandem duplication of eight nucleotides (c. 520–528dupTTCAGAC) in exon 2 of the *Cdkn1b* gene. This *Cdkn1b* mutation segregated with the disease phenotype in all the tested affected rats (>200), but it was never observed in unaffected littermates nor in DNA from control rats of seven commercially available strains. This mutation results in a frameshift after codon 176, predicting a novel C-terminal domain containing 42 p27-unrelated amino-acid residues [2].

## 3. The function of p27

p27 belongs to the KIP/CIP family of cell cycle inhibitors, and its main function is to regulate the cell cycle transition from the G1 to the S phase. Specifically, p27 inhibits CyclinE/Cdk2 complexes by binding to both proteins. This binding prevents Cdk2 from phosphorylating Rb, the Retinoblastoma protein. Rb usually binds transcription factors of the E2F family, which in turn induce the expression of genes necessary for the progression to the S phase. When p27 levels are absent or reduced, Rb is phosphorylated and cell cycle progression promoted, which ultimately leads to increased and uncontrolled cell proliferation [4]. In addition to its role in cell cycle progression, p27 is involved in apoptosis and cell differentiation.

In many cell types, p27 is a rate-limiting effector of cell cycle exit. Hence, the intracellular amount of p27 is tightly regulated, being high during quiescence and low during S phase, when the cells are committed to proliferation. This fine regulation of the levels of p27 is principally achieved through ubiquitin-dependent proteasomal degradation through two main pathways



mediated by the Skp2-dependent SCF (skp-cullin-f-box) E3 ligase and by the KPC ubiquitin ligase, respectively [5,6]. The function of p27 is also modulated by altering its intracellular localization. Indeed, p27 is mainly a nuclear protein and in this compartment can bind to and inhibit Cyclin/Cdk complexes, but it can be sequestered in the cytoplasm where it may have additional functions [7].

While mutation of *CDKN1B* is uncommon in human cancers, the down-regulation of the p27 protein is often observed in a great variety of human cancers, including those of the colon, breast, prostate, stomach and others, and this down-regulation is associated with poor prognosis [8]. In aggressive colorectal carcinoma samples, loss/reduction of p27 expression is caused by accelerated proteasome-dependent degradation of the protein, likely due to increased expression of the ubiquitin ligase SKP2 [9]. The observation that, in many human tumors, low p27 protein levels often associate with no change in *CDKN1B* mRNA expression has been considered indirect evidence that enhanced proteolysis is responsible for p27 down-regulation. Reduced p27 expression has also been demonstrated in various neuroendocrine tumors, including those of the gastrointestinal tract [10], parathyroid adenomas [11], pheochromocytomas [12] and all types of pituitary adenomas [13]. Among the pituitary tumors, p27 levels are especially reduced in corticotroph adenomas, where they correlate with aggressive tumor behaviour [13].

#### 4. Characterization of the p27 mutation causing MENX

Following the identification of the genetic mutation causing the MENX syndrome, the expression of the *Cdkn1b* gene in affected rats was explored in more detail. The analysis of various normal tissues of affected rats at young age (before they develop malignancies) and of age-matched wild-type littermates (controls) showed no significant difference in the level of *Cdkn1b* mRNA between the two groups [2]. Furthermore, correct splicing of the mutant mRNA was demonstrated in the tissues of mutant rats. When the expression of the encoded p27 protein was assessed by western blotting, a faint band corresponding in size to the predicted mutant p27 protein could be observed only in a few tissues (i.e. thymus and thyroid). Immunohistochemical staining performed with an anti-p27 specific antibody showed extremely low (thyroid, pituitary, thymus, parathyroid, and brain) or lack of (adrenals, lung, kidney, liver, and testis) p27 immunoreactivity in tissues of affected rats, while the same tissues presented with strong nuclear positivity for p27 in wild-type rats [2]. This suggests that the MENX mutation does not affect the transcription or processing of *Cdkn1b* mRNA, but affects the amount of mutant p27 protein by post-transcriptional or post-translational mechanisms. This situation is reminiscent of the reports on human tumors showing reduced p27 expression (see previous paragraph).

The immunohistochemical staining of MENX-affected rat tissues for p27 suggests that the germline frameshift mutation in *Cdkn1b* behaves as a loss-of-function mutation in these animals. To better understand the link between p27 mutation and tumor predisposition in our animal model, we set out to functionally

characterize the encoded mutated p27 protein (hereafter referred to as p27fs177) *in vitro*. Studies performed using cycloheximide (CHX) to block new protein synthesis demonstrated that the p27fs177 protein is rapidly degraded in every phase of the cell cycle, including quiescence [14]. Using various proteasome-inhibitor drugs (MG132, Epoxomicin) we demonstrated that p27fs177 is in part degraded through proteasome-mediated proteolysis, just as wild-type p27, so that p27fs177 levels can be rescued by proteasome inhibition [14]. By means of siRNA gene knock-down, we also found that the pathways that mediate p27fs177 degradation in the various phases of the cell cycle involve the same molecules that degrade wild-type p27, specifically the SKP2 and the KPC1/2 ubiquitin ligases. However, p27fs177 is also degraded by ubiquitin-independent mechanisms. To study the degradation of p27fs177 in a more physiological system, we established primary fibroblasts from double mutant and wild-type rats. We also observed that in this experimental system p27fs177 is degraded very fast and it is barely detectable in fibroblasts from mutant rats. The expression of p27fs177 has no significant effect on cell proliferation of primary rat fibroblasts. In conclusion, in the MENX animal model, the fast degradation of p27fs177 causes the low level of the p27 protein we saw in the tissues of the mutant rats [14]. Based on our findings we postulate that reduced p27 levels, not newly acquired molecular phenotypes, predispose MENX-affected rats to tumor development. These observations establish parallels between rat and human neuroendocrine tumors, since, in both species, reduced p27 expression is observed and it is likely the result of increased protein degradation.

#### 5. MENX-associated pituitary adenomas

Rats affected by the MENX syndrome present with adenomas in the *pars distalis* (adenohypophysis) of the pituitary gland, just like most human patients. This tumor phenotype is observed in both male and female MENX-affected rats, but not in wild-type littermates at the same age. A prerequisite to employ the MENX model in translational studies of pituitary adenomas is the detailed characterization of the rat tumors. Thus, we thoroughly analyzed at the histological and immunohistological level these tumors.

At histological examination, we observed that the lesions in the adenohypophysis become apparent at 4 months of age as 3–4 bilateral nodules ranging in size between 150 and 200 microns. At the age of 6 months, the lesions increase in number to 8–10 and they reach the size of approximately 2 mm. In 8-month-old mutant rats, the lesions seem to merge to form macroscopically visible tumors and the size of the pituitary gland becomes 3–4 times bigger than that of wild-type pituitaries [15]. Adenomas at 8 months exhibit a significant increase in the number of vessels in comparison with early lesions. The network of reticular fibres is disrupted in all lesions, starting from the earliest stages, suggesting that the lesions are not preceded by hyperplasia but arise as nodules of neoplastic cells. Mitotic figures are present from the earliest lesions but they become especially evident in the larger, well-formed nodules (average  $3 \times 10$  high power fields). This finding is in agreement with the relatively high



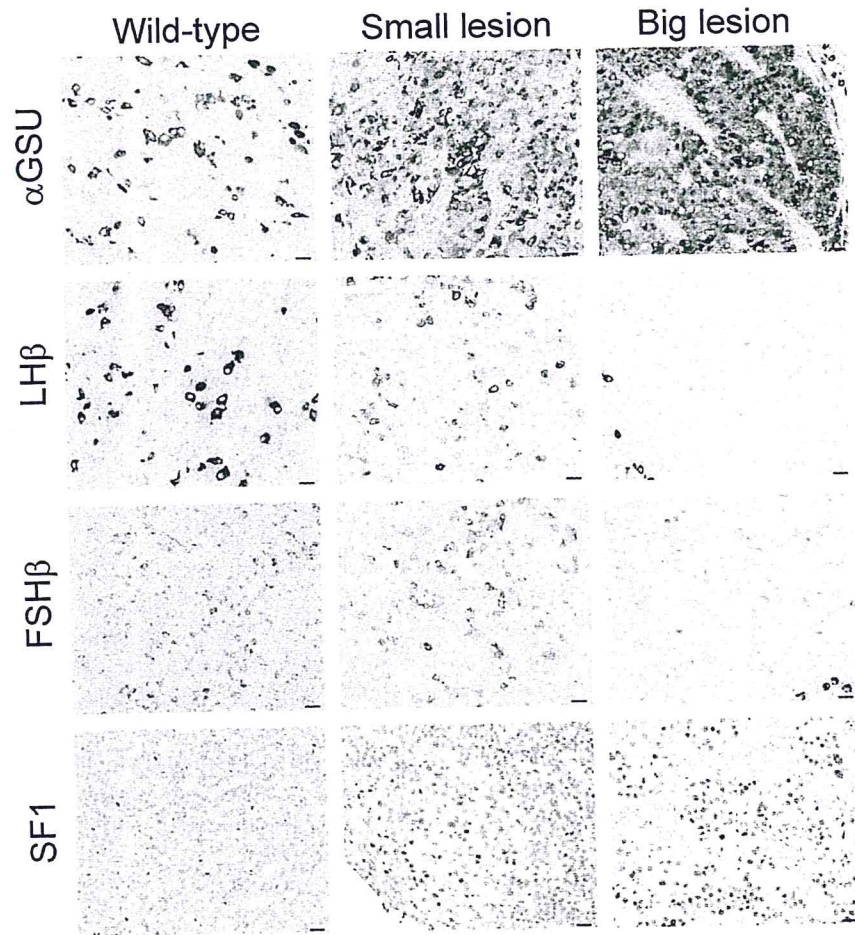


Fig. 2. Expression of gonadotroph-specific factors in MENX. Immunohistochemistry was performed on wild-type and mutant rat pituitary tissues. The antigens detected by specific antibodies are indicated on the left. Cells expressing the detected protein appear brown. Bars, 100  $\mu$ m.

immunoreactivity for the proliferation marker Ki67 we observed in the larger lesions (average 8%). Although adenomas reach a considerable size and tumor cells show sustained proliferation, no invasion of the skull base or brain tissue was observed and none of the lesions metastasized within or outside the cranio-spinal axis.

Immunohistochemistry was performed using antibodies against the seven pituitary hormone subunits GH, PRL, ACTH, LH $\beta$ , FSH $\beta$ , TSH $\beta$ , alpha-subunit ( $\alpha$ GSU) and showed variable expression of LH $\beta$  and FSH $\beta$  and widespread expression of the common glycoprotein subunit  $\alpha$ GSU in the rat tumors. Few lesions contained cells expressing PRL or GH. The number of cells expressing LH $\beta$  and FSH $\beta$  decreases with the increase in size of the nodule and becomes negligible in the largest adenomas, while neoplastic cells remain positive for the  $\alpha$ GSU (Fig. 2). All adenoma cells feature nuclear immunoreactivity for the transcription factor steroidogenic factor 1 (SF1), a marker of gonadotroph cells. Altogether, these data suggest that MENX-associated pituitary adenomas are derived from cells of the gonadotroph lineage and mostly resemble aggressive gonadotroph adenomas [15]. Given that animal models of gonadotroph adenomas are exceedingly rare, MENX-affected rats represent a unique platform for molecular and translational studies of this tumor entity.

## 6. Preclinical studies of MENX-associated pituitary adenomas

MENX-associated pituitary adenomas belong to the group of non-functioning pituitary adenomas (NFPAs). In humans, NFPAs represent about 30% of all pituitary adenomas. Although they are usually benign, NFPAs can grow to a considerable size causing signs and symptoms of mass effects and can extend or invade the parasellar structures causing severe morbidity to patients [16]. Transsphenoidal surgery is the treatment of choice but is rarely curative when tumors are invasive and if residual tumor is present, they require postoperative radiotherapy, which can cause complications [17]. Pharmacological treatment of NFPAs is still a matter of great debate and both somatostatin analogs and dopamine agonists have shown limited effect [18,19]. In this scenario, the identification of novel therapeutic approaches to treat NFPAs is of paramount importance.

Constitutive activation of the PI3K/AKT/mTOR signalling cascade occurs in a variety of human malignancies, where it sustains tumor cell proliferation and survival. Adenomas of the pituitary are among the tumors showing hyperactivation of this pathway [20], and therefore they should be sensitive to treatment with mTOR inhibitors. Rapamycin and its analog RAD001 (everolimus), inhibitors of mTOR, have shown antiproliferative



activity against pituitary adenomas grown as dispersed primary cultures *in vitro* [21,22], but a proportion of these tumors ranging from 30 to 70% were resistant to these compounds [23]. Resistance to rapamycin analogs has been in part ascribed to a feedback loop triggered by these compounds, which leads to the activation of the AKT kinase through the ribosomal S6 kinase S6K1, thereby counteracting the antitumor potential of mTOR inhibition [24].

We decided to take advantage of our MENX rat model and perform preclinical therapy-response studies to evaluate two inhibitors of the PI3K/AKT/mTOR pathway for their efficacy against gonadotroph adenomas. Immunohistochemical analysis showed that MENX-associated pituitary adenomas express high levels of phosphorylated Akt [25], indicating that Akt signalling is activated in these tumors, similarly to human pituitary adenomas. Therefore, MENX mutant rats are a suitable model to test the efficacy of antitumor drugs inhibiting the PI3K/AKT/mTOR signalling cascade. For our therapy-response studies, we established *ex vivo* primary cultures from the pituitary tumor cells of MENX-affected rats. Based on gene expression studies, pituitary adenoma cells grown as primary cultures are representative of the original primary tumors [15] and can therefore be exploited for preclinical *in vitro* studies of these neoplasms.

We tested the dual PI3K/mTOR inhibitor NVP-BEZ235 for its efficacy against MENX-associated pituitary adenomas, and then compared the results with the antitumor potential of the single mTOR inhibitor RAD001. NVP-BEZ235 belongs to a new class of compounds able to inhibit both mTOR and the upstream PI3K kinase, thereby preventing the negative feedback loop and AKT activation. We observed that NVP-BEZ235 is the most effective antineoplastic agent in our experimental system, since it can inhibit the viability of 100% of the rat pituitary adenomas grown as primary cultures. In contrast, RAD001 elicits only partial response from the rat pituitary adenomas. Consistently, at the molecular level, NVP-BEZ235 prevented the negative feedback activation of Akt, which was instead observed after treatment with RAD001 [25].

Based on our results, we would predict that human NFPAs might successfully respond to treatment with NVP-BEZ235. Indeed, recent data on these human tumors grown as dispersed primary cultures demonstrated that NVP-BEZ235 is extremely potent at inhibiting pituitary tumor cell survival [26].

Due to the genetic defect causing MENX, we also assessed whether the level of p27 plays a role in the sensitivity of rat pituitary adenoma cells to NVP-BEZ235. To this aim, we generated several genetically-defined cell lines characterized by different amounts of endogenous p27 from p27-deficient mice or MENX mutant rats. Interestingly, we observed that the amount of p27 positively correlates with the efficacy of NVP-BEZ235 as anti-tumor agent, suggesting that the expression of p27 in tumor cells may be a predictor of response to NVP-BEZ235. In our pituitary adenoma model, the use of the proteasome-inhibitor bortezomib, to stabilize mutant p27fs177, together with NVP-BEZ235 leads to a synergistic antiproliferative effect of the drugs [25].

Human NFPAs express p27 but at reduced levels compared with normal pituitary cells [27,28], and this is likely caused by post-translational mechanisms [28], similarly to what we

observed in MENX-affected rats. Therefore, our studies suggest that NFPAs might successfully respond to combined therapy with NVP-BEZ235 and bortezomib.

## 7. Conclusions

The rat MENX syndrome has provided us with a novel tumor susceptibility gene for multiple neuroendocrine tumors in both rats and humans: *Cdkn1b/CDKN1B* (p27). This discovery gave new impetus to studies focusing on the role of p27 in regulating neuroendocrine cell proliferation. On the other hand, this animal model provides access to a unique array of neuroendocrine tumors for molecular and translational studies. Rats affected by the MENX multiple endocrine neoplasia syndrome develop multiple tumors in the adeno-hypophysis with complete penetrance. These tumors are gonadotroph adenomas and develop through temporal and histological stages, similarly to what happens in human tumors. The Akt signalling cascade is activated in rat pituitary adenomas, just like in human NFPAs, and consequently the rat tumors respond very well to dual PI3K/mTOR inhibition by NVP-BEZ235, much like human NFPAs. Therefore, pituitary tumors in both species share important molecular and physiological features. In conclusion, the MENX animal model represents a useful platform to study cell type-specific carcinogenesis associated with a multistage pathway of tumor development. Studying this animal model will advance our understanding of the molecular pathogenesis of gonadotroph adenoma formation. MENX rats may also be exploited to develop and evaluate new treatment modalities against gonadotroph adenomas.

## Disclosure of interest

The author declare that he has no conflicts of interest concerning this article.

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