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PROOPIOMELANOCORTIN HETEROZYGOUS AND HOMOZYGOUS NULL MUTANT MICE DEVELOP PITUITARY ADENOMAS

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Abstract – Mice lacking all pro-opiomelanocortin (POMC)-derived peptides have been created by gene targeting of the POMC locus in embryonic stem cells. Phenotypes of the POMC null homozygous mutants include obesity, pigmentation defects, and adrenal insufficiency. Here, we report that both POMC null homozygous and heterozygous mutants also develop pituitary gland tumors, which result in their premature death. The tumors occur with 100% penetrance in both POMC heterozygous and homozygous genotypes. Histological examinations reveal that tumors start from hyperplastic focal points of melanotrophic cells within the intermediate lobe. Based on the morphological and immunohistological features, we have classified the tumors as non-invasive, non-secreting, intermediate lobe adenomas. These findings uncover potential novel roles of melanocortins in the regulation of cell proliferation.

Key words: proopiomelanocortin, pituitary adenoma, melanotrophs, melanocortin receptors.

INTRODUCTION

The mouse pituitary gland is comprised of three distinct lobes. The predominant anterior lobe contains a variety of hormone secreting cells, including corticotrophs. The posterior lobe consists of hypothalamic neurosecretory terminals and the relatively small intermediate lobe primarily consists of melanotrophs. Two cell types, the corticotrophs and the melanotrophs, express the POMC gene. Post-translational processing of the POMC prohormone leads to the production of adrenocorticotropic hormone (ACTH) in the corticotrophs, and of α-melanocyte stimulating hormone (α-MSH) and β-endorphin in the melanotrophs (1,17,23,27). This tissue specific processing is achieved through differential expression of prohormone convertases 1 and 2 (PC1 and PC2; 31). PC1 cleaves POMC into ACTH as well as other derivatives, and PC2 subsequently cleaves ACTH into α-MSH and corticotropin-like intermediate lobe peptide (CLIP). Thus, PC1 is expressed in the corticotrophs of the anterior lobe, whereas both PC1 and PC2 are expressed in the melanotrophs of the intermediate lobe (5,26).

Abbreviations: **ACTH**: adrenocorticotrophic hormone; **α-MSH**: alpha melanocyte stimulating hormone; **CLIP**: corticotropin-like intermediate lobe peptide; **CRH**: corticotropin releasing hormone; **DA**: dopamine; **FSH**: follicle stimulating hormone; **GH**: growth hormone; **LH**: luteinizing hormone; **PC1/2**: prohormone convertase 1/2; **POMC**: proopiomelanocortin; **PRL**: prolactin; **TSH**: thyroid stimulating hormone.

In humans, pituitary adenomas are common neoplasms accounting for approximately 15% of all intracranial tumors (3,18). These tumors are characterized by cell type of origin, hormonal secretion, and size (2,11). For example, somatotopinomas over express growth hormone (GH), causing acromegaly in adults, and corticotropinomas lead to ACTH hypersecretion and adrenal steroid overstimulation (Cushing disease). While the majority of pituitary adenomas are hormone producing, clinical studies consistently identify adenomas that produce no biologically active hormone (null-cell adenoma; 19,29).

We have generated a mouse mutant that completely lacks all POMC-derived peptides by targeted deletion of the entire POMC coding region (30). As in humans with POMC deficiency (14), the mouse displays the triad of obesity, pigmentation defects, and adrenal insufficiency. Unexpectedly, homozygous and heterozygous POMC null mutant mice develop pituitary tumors. In this study, we characterize the phenotype of these tumors.

MATERIALS AND METHODS

Mice

Generation of POMC knockout mice through homologous gene targeting in embryonic stem cells has been described previously (30). Mice were maintained on the inbred 129SvEv-Tac background. Heterozygous mice were mated to maintain the

colony and generate experimental animals of all three genotypes (wild type, heterozygous null, homozygous null). Mice were genotyped by PCR analysis of tail DNA. Mice were euthanized at specific ages or when showing signs of malaise, and pituitaries were dissected and processed for analysis. All procedures were approved by the OMRF IACUC.

Histological Examinations

Immediately after euthanasia, pituitary glands were carefully removed and fixed in 10% formalin in phosphate-buffered saline. They were then dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. A series of 5µm thick sections were cut from each pituitary and stained with hematoxylin and eosin, or used for immunohistochemical stainings.

Immunohistochemistry

Immunohistochemical analysis was carried out with formalin-fixed paraffin sections. Rabbit anti-sera against ACTH (IHC 8502) and α-MSH (IHC7251) were obtained from Peninsula Laboratories, Inc., and rabbit anti-sera against luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), and prolactin (PRL) were kindly provided by Dr. A.F. Parlow through the NIDDK National Hormones and Pituitary Program. Immunoreactivity was visualized by the avidin biotin complex method (Vectastain ABC-AP Kit, Vector Laboratories).

In situ Hybridization

Pituitaries were either flash-frozen in 2-methylbutane and sectioned on a cryostat (Leica CM3050) or formalin-fixed, paraffin-embedded, and sectioned on a microtome. For cRNA probes of PC2 a 500bp fragment (accession number NM_008792, position 610 - 1140) was cloned into the pDrive vector (PCR Cloning Kit; Qiagen Inc., Valencia, CA). Sense and antisense DIG- labeled riboprobes were synthesized using the DIG RNA Labeling Kit (SP6/T7; Roche Diagnostics Corporation, Indianapolis, IN). Anti-digoxigenin AP Fab Fragments (Roche) were applied to the hybridized sections and NBT/BCIP (Roche) used to visualize the signal.

RESULTS

The survival rate of POMC heterozygous null mutant mice, both male and female, is 50% of wild-type at 42-45 weeks of age and less than 5% beyond 52 weeks of age; no survivors were found past 60 weeks (Fig. 1A). POMC homozygous null mutant mice, when aged past 30 weeks, show the same survival rates. Upon anatomical analysis of older POMC heterozygous null mutant mice, large pituitary gland tumors were found. These same tumors were also found in POMC homozygous null mutant mice. The tumors grow at a variable rate and vary in mass, some up to 100-fold larger by weight than a normal pituitary gland. The majority of tumors extracted from mice upon death are approximately 10 to 15-fold larger by weight than normal pituitary glands (Fig. 1 B).

Further examination revealed that both POMC null homozygous and heterozygous mutant mice 7 months of age and older consistently develop macroscopically visible pituitary tumors (Fig. 2). In mice of either genotype, 5 months of age and younger, no tumors could be found macroscopically, however, some mice displayed enlarged pituitary glands. Frequently, the tumors are highly vascularized, as seen by their deep brown color (Fig. 2). Their growth results in the compression of the overlying brain; dissection of the tumors revealed a large depression in the ventral surface of the brain, but showed no evidence of invasion of surrounding tissues.

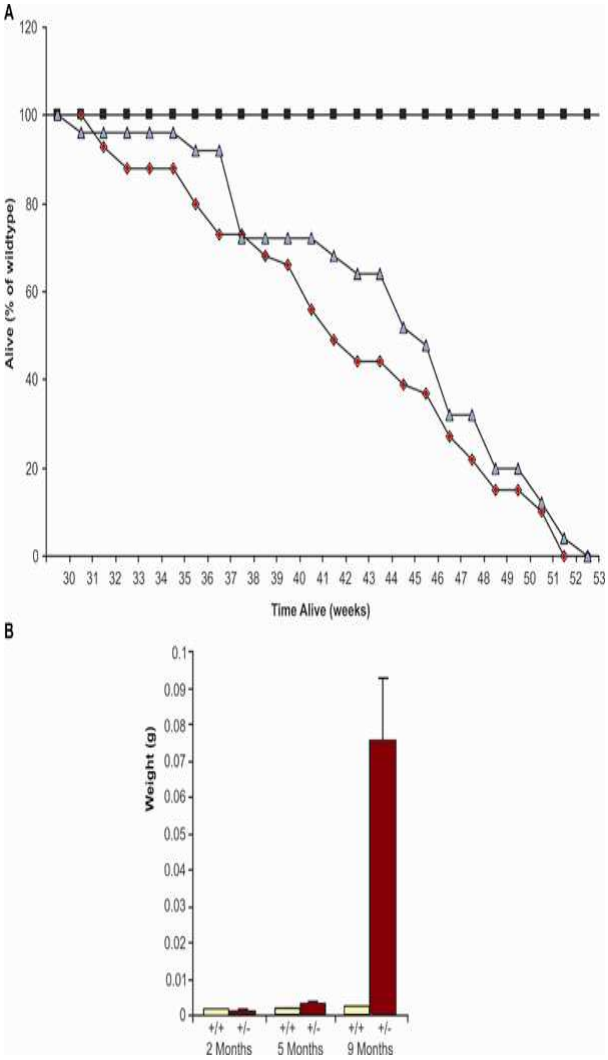

Fig. 1 Decreased survival of POMC heterozygous null mutant mice with increased pituitary tumor growth. (A) Age at time of death was recorded for mice from 30 to 53 weeks after birth. Presented are the percentages of mice alive at the end of each week. Starting population were 25 mice per group, each for POMC heterozygous null and wildtype males and females. Data for wildtype mice of both genders are represented in one graph: closed squares; heterozygous males: open triangles; heterozygous females: open diamonds. (B) Average weight of pituitary glands extracted from POMC heterozygous null mutant mice and wildtype littermates. Values represent the mean ± SEM for 5-9 mice per group. Average weights are 0.00134±0.0001g at 2 months, 0.00282±0.0006g at 5 months, 0.0753±0.0173g at 9 months for heterozygous mutants and 0.00143±0.0001g at 2 months, 0.00143±0.0001g at 5 months, 0.00237±0.0001g at 9 months for wildtype animals.

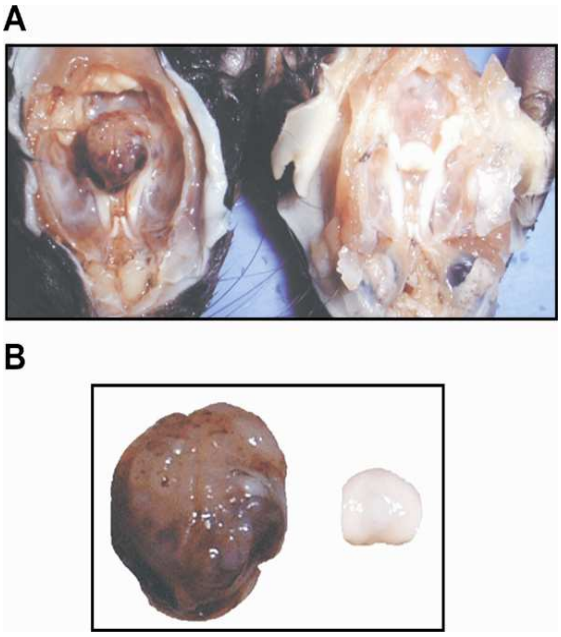

Fig. 2 Gross appearance of pituitary tumors in POMC heterozygous null mutant mice. (A) Basal views of a pituitary from an 8-month-old POMC heterozygous null mutant mouse (*left*) and from a wildtype littermate (*right*), photographed *in situ*. (B) Macroscopic appearance of dissected tumor showing size difference and color variation next to wildtype pituitary.

Histological examination of pituitary glands extracted from younger mutant mice without macroscopically visible tumors revealed intermediate lobe hyperplasia consisting of single basophilic foci composed of small, dense hyperchromatic cells with no change in vasculature, while the anterior and posterior lobes' architecture remained normal (Fig. 3A). Hyperplasia of the intermediate lobe has been seen as early as 2.5 months of age in POMC heterozygous mutant mice and is always restricted to the intermediate lobe, initially at a single focal point in both null and heterozygous mutants. In almost all POMC homozygous and heterozygous null mutant mice 7 months of age and older, a tumor had developed with cells displaying various degrees of atypical nuclei and an overall increase in vascularity of the tumor tissue; the tumors had clusters of cells which gave the appearance of nodules (Fig. 3B). At that point, no distinction could be made between the different lobes, in that the tumor had pushed the anterior and posterior lobes to a thin band at the margin of the tumor. Production of anterior lobe hormones was unaffected. It is also important to note that despite a difference in genotype, pituitary tumors that develop in POMC null homozygous versus heterozygous mutant mice are, macroscopically and microscopically, morpho-logically consistent.

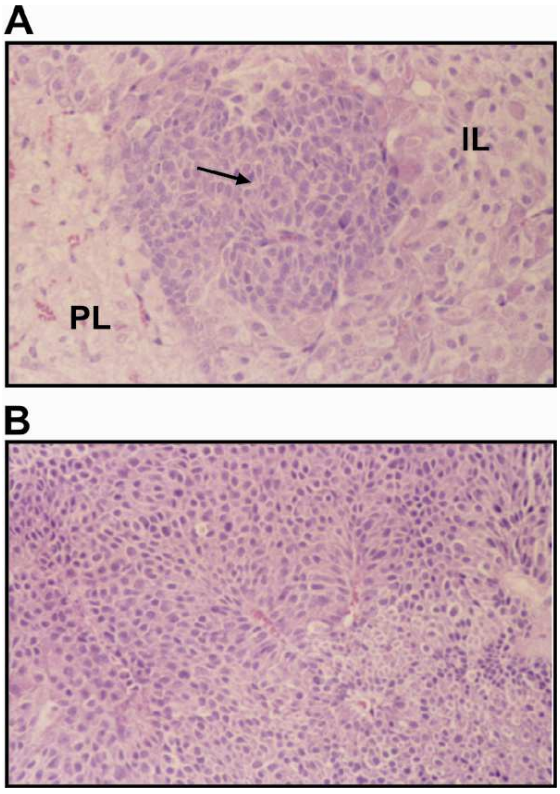

Fig. 3 Intermediate lobe cell morphology in POMC heterozygous null mutant mice comparing early hyperplasia and late stage tumors. Pituitary gland sections stained with hematoxylin and eosin from POMC heterozygous null mutant mice 2.5 months of age (A) and 10 months of age (B). Black arrow: center of hyperplastic focus of cells with altered morphology surrounded by normal intermediate lobe tissue; IL: intermediate lobe; PL: posterior lobe.

Immunohistological examination of tumor tissue taken from POMC heterozygous mutant mice displaying late-stage tumors, i.e. lacking defined architecture of intermediate and posterior lobes, revealed that transformed cells varied in their content of immunoreactive α-MSH. In some cases immunoreactivity for α-MSH was found at the margin of the tumor, while in most cases all immunoreactivity was absent (Fig. 4A). However, pituitary gland sections taken from POMC heterozygous mutant mice displaying early hyperplasia showed relatively homogeneous staining with antibodies to α-MSH (Fig. 4B). Staining against all other anterior lobe hormone producing cell types revealed normal hormone production of GH, LH, FSH, TSH, and PRL. An example is shown in Figure 4A.

In situ hybridization analysis of POMC heterozygous mutant pituitary glands displaying early hyperplasia showed a lack of PC2 mRNA expression within the transformed cells (Fig. 4C). However, subsequent immunohistochemical examination revealed that those same cells do stain positively with antibodies to α-MSH (Fig. 4C).

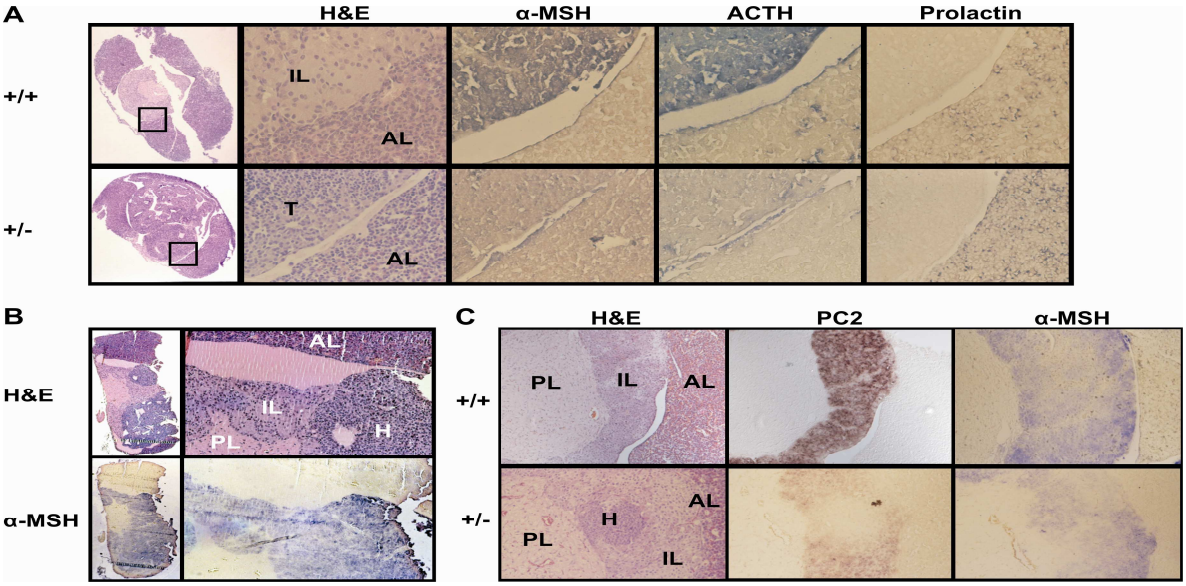

Fig. 4 Immunohistological and *in situ* hybridization analysis of pituitary tumors in POMC heterozygous null mutant mice. (A) Serial sections of pituitaries from wildtype (upper row) and POMC heterozygous null mutant (lower row) mice. The overview pictures (left panels) show the entire pituitary, the higher magnifications focus on the border of anterior lobe (AL) and intermediate lobe (IL) or tumor (T), respectively. Sections were stained with hematoxylin and eosin (H&E), and antibodies to α-MSH, ACTH, and Prolactin. (B) Serial sections of a pituitary taken from a POMC heterozygous null mutant mouse at 3 months of age and stained with hematoxylin and eosin (H&E; upper panels) and antibodies to α-MSH (lower panels). The higher magnifications (right panels) center on the discrete hyperplastic focus visible in the intermediate lobe in the left panel. AL: anterior lobe, IL: intermediate lobe, PL: the posterior lobe, H: hyperplasia. (C) Pituitary serial sections from 2.5 month-old POMC heterozygous null mutant mice (lower panels) and wildtype littermates (upper panels) were stained with hematoxylin and eosin (H&E), hybridized with riboprobes specific for prohormone convertase 2 mRNA (PC2), and stained with antibodies against α-MSH (α-MSH). PL: posterior lobe, AL: anterior lobe, IL: intermediate lobe, H: hyperplasia. All images are shown at the same magnification; the section from wildtype stained with α-MSH is a step section of the ones shown for H&E and PC2.

DISCUSSION

Most phenotypes of the POMC null allele are recessive: obesity, insulin hypersensitivity, altered pigmentation, regression of adrenal glands. However, the heterozygous POMC null mutants do show a semi-dominant change in hormone levels, such as ACTH, corticosterone, aldosterone, and leptin (30). Here we report that a null allele of the POMC gene has a dominant phenotype: both homozygous and heterozygous POMC null mutant mice develop intermediate lobe pituitary tumors that result in death by 8 – 10 months of age. The tumors are non-invasive adenomas that originate as hyperplasia in α-MSH expressing melanotrophs. As the tumors develop they cease expressing α-MSH. These findings raise a number of questions: Why does reduction in POMC lead to tumor formation, and specifically to tumors of pituitary melanotrophs? What are possible mechanisms of pituitary tumor formation? And why is tumor formation a dominant phenotype in POMC null mutants?

The POMC prohormone is expressed in many different cell types throughout the organism. However, anatomical dissections of POMC heterozygous and homozygous null mutants at the time of their demise due to pituitary tumors did not reveal any macroscopically visible tumors in any other organs. Within the pituitary gland, POMC is expressed in melanotrophs of the intermediate lobe and in corticotrophs of the anterior lobe. Corticotrophs are under feedback control of hypothalamic corticotropin releasing hormone (CRH): low corticosterone levels lead to increased release of CRH, which stimulates corticotrophs to release more ACTH. POMC null heterozygous mutants have decreased levels of corticosterone and homozygous mutants completely lack corticosterone (30). It remains unexplained in the POMC mutant mice why tumorigenesis occurs in melanotrophs, while corticotrophs are unaffected. A possible mechanism would be that growth of melanotrophs is controlled by POMC peptides themselves, while growth of corticotrophs is controlled by other, non-POMC, factors.

While POMC-derived peptides are involved in the regulation of growth of the adrenal glands (6,22), there is no precedent for POMC-derived peptides as regulators of cell growth in the pituitary gland. However, the occurrence of intermediate lobe tumors in POMC null mutant mice raises the possibility that reduced negative feedback inhibition resulting from decreased POMC peptide expression, esp. α-MSH, plays a role in the abnormal growth. Such a regulation of melanotroph growth would explain tumor development in POMC homozygous and heterozygous null mutants. The feedback inhibition could be direct (autocrine) or indirect (paracrine), i.e. mediated by other factors in

addition to POMC peptides. There is evidence for the inhibitory role of hormones through an autocrine mechanism in the pituitary, specifically in the development of pituitary thyrotrope adenomas in patients with chronic primary hypothyroidism (9). However, POMC peptides regulating melanotroph proliferation in an autocrine regulation would require the presence of melanocortin receptors on these cells; this remains to be determined.

Another possibility is that POMC peptides are required to regulate factors outside the pituitary which in turn regulate melanotroph growth. One group of candidates is those hormones whose levels are known to vary in POMC heterozygous null mutant mice (leptin, corticosteroids). Furthermore, there is evidence that extrinsic factors play a role in the regulation of cell growth in the intermediate lobe of the pituitary gland. Specifically, it is well documented that dopamine (DA) plays an antiproliferative role in the intermediate lobe. The intermediate lobe is innervated by dopaminergic neurons (25). Dopamine has been shown to regulate not only POMC gene expression, but also melanotroph proliferation. Mice lacking DA D_2 receptors (D_2R) display intermediate lobe hyperplasia as well as increases in POMC transcription (24). However, D_2R null mutant mice show hyperplasia of the entire intermediate lobe, which is in contrast to the hyperplastic foci found within normal intermediate lobe tissue in the POMC model. Also, preliminary data using a highly sensitive radioimmunoassay show that dopamine levels do not differ between the pituitary glands in POMC null and wildtype mice (unpublished observations).

One possibility yet to be investigated is that POMC peptides interact, directly or indirectly, with the retinoblastoma (*Rb*) tumor suppressor pathway to regulate cell proliferation of melanotrophs. There are only three other mouse models described that develop intermediate lobe pituitary tumors. Mice with a single allele disruption in the tumor suppressor gene *Rb* were shown to develop intermediate lobe pituitary tumors, characterized as adenocarcinomas, at 8 - 10 months of age; tumorigenesis in $Rb^{+/-}$ mice results from a loss of the non-mutated *Rb* allele (12,28,21). The other two models with intermediate lobe tumors are mice homozygous mutant for $p27^{Kip1}$(7,13,20) or for $p18^{INK4c}$ (8). $p27^{Kip1}$ and $p18^{INK4c}$ are members of the CDK inhibitory protein family which block cyclin-CDK kinase activity, regulating entry of cells into S phase of the cell cycle (10). Both proteins are assumed to participate in the Rb tumor suppressor pathway, mediating two separate pathways that collaborate to inhibit CDK activity which in turn regulates Rb function and thus suppresses pituitary tumorigenesis (8). Homozygous $p27^{Kip1\ -/-}$ and $p18^{INK4c\ -/-}$ mice initially show generalized intermediate lobe hyperplasia, followed by development of adenomas at 8-11 months; double homozygous $p27^{Kip1\ -/-}/p18^{INK4c\ -/-}$ mutants develop adenomas at 3.5 months of age (7,8,13,20). The similar characteristics with respect to growth and dedifferentiation found in all the intermediate lobe tumor models supports the argument that the tumors all develop through a common mechanism.

In heterozygous POMC null mutants, hyperplastic cells during early tumor development show uniform staining with α-MSH, while in later stage tumors they do not. This observation of hormone production at early stages followed by lack thereof at late stages is not uncommon in cases of pituitary adenomas in general. For instance, $p27^{Kip}$ null mice display homogeneous staining of intermediate lobe hyperplastic cells with antibodies to POMC peptides at 11 weeks, while only isolated cells still stain positive at 30 weeks (13). A similar result was found in anterior lobe gonadotroph adenomas in mice, where FSHβ and LHβ expression can be found at early, but not at late stages of tumor growth (4,15).

At early tumor stages, intermediate lobes of POMC mutants display relatively homogeneous immunoreactivity to antibodies specific for α-MSH, including the hyperplastic cells. In these same hyperplastic cells, there was a lack of the PC2 mRNA transcript. PC2 is necessary for post-translational processing of POMC, specifically the processing of POMC into α-MSH in pituitary melanotrophs; PC2 null mice lack pituitary α-MSH (16). In every pituitary tissue sample from POMC heterozygous null mutants examined, PC2 mRNA expression was absent in cells from early hyperplastic foci, while α-MSH was present. The antibody used is specific for α-MSH in that is does not cross-react with the processed form of ACTH and does not cross-react with the pro-peptide, as it does not stain corticotrophs. Assuming antibody specificity, the data suggests that loss of α-MSH production during dedifferentiation of melanotrophs is preceded by down regulation of PC2 transcription. Further evaluation is necessary in order to fully understand these results.

The positive staining for the POMC-derived peptide α-MSH in early hyperplastic cells of POMC heterozygous null mice indicates the presence of a functioning POMC allele. Thus, loss of heterozygosity due to a somatic mutation occurring in the wild-type allele of the POMC heterozygous mouse as an explanation for tumorigenesis in POMC heterozygous mutants is unlikely.

A more likely explanation of tumorigenesis in heterozygous POMC null mutants is a gene dosage effect, especially a threshold effect. We have shown that lack of one allele of POMC already has effects on hormone levels (leptin, corticosterone, aldosterone; 30). We do not know presently if, in heterozygous POMC

null mutants, levels for other hormones and factors are changed and to what extent.

Even though at the present stage our analysis of pituitary tumor development in POMC null mutant mice raises more questions than it answers, we propose the following model: In the mouse pituitary proliferation of melanotrophs is under negative feedback inhibition through secretion of α-MSH and its subsequent binding to a melanocortin receptor expressed by melanotrophs. The inhibition of proliferation involves the Rb pathway and is POMC gene dosage dependent. While this is not the only model to fit the data, it allows formulation of a number of specific hypotheses which can be tested experimentally.

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