Post-translational Processing of Proopiomelanocortin (POMC) in Mouse Pituitary Melanotroph Tumors Induced by a POMC-Simian Virus 40 Large T Antigen Transgene*

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Mice harboring a transgene composed of proopiomelanocortin (POMC) gene promoter sequences (nucleotides −706 to +64) ligated to the simian virus (SV) 40 early gene encoding large T antigen developed large POMC-expressing pituitary tumors. Histologically the tumors arose from the intermediate lobe, contained nuclear SV40 T antigen and POMC peptides, but did not express other pituitary hormones. POMC processing in the pituitary tumors was indistinguishable from normal mouse intermediate lobe melanotrophs and was characterized by high proportions of acetylated and carboxyl-terminal shortened β-endorphins, and amino-terminal acetylated α-melanocyte-stimulating hormone, and virtually no adrenocorticotropic hormone (ACTH) (1–39), β-lipotropin, or POMC. The tumors contained abundant levels of mRNA for the prohormone convertase PC2 and undetectable levels of PC1. Normal mouse neurointermediate lobe also has a high ratio of PC2/PC1 expression that is distinct from the relative abundance of PC1 in anterior lobe and AtT-20 corticotroph cells. In contrast, extracts from tumors transplanted subcutaneously in nude mice contained predominantly nonacetylated forms of β-endorphin (1–31) and (1–27), very little ACTH (1–39), almost no corticotropin-like intermediate peptide or α-melanocyte-stimulating hormone, and higher proportions of intact POMC. Surprisingly, despite the less efficient proteolytic cleavage, a transplanted tumor expressed both PC1 and PC2. These studies are the first biochemical documentation of a melanotroph pituitary tumor in a rodent species and provide a new model for the investigation of pituitary oncogenesis and the molecular basis of tissue-specific prohormone post-translational processing.

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‡‡§§ The abbreviations used are: POMC, proopiomelanocortin; ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone; SV40, Simian virus 40; Tag, large T antigen; RP-HPLC, reversed-phase high performance liquid chromatography; PC, prohormone convertase; LPH, lipotropin.

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appears to be capable of high specific activity cleavage at the paired basic residues at the ACTH amino terminus and at the γ-LPH/β-endorphin junction as well as at the KKRR residues within ACTH, although there is controversy about its activity at the KR site at the carboxyl terminus of ACTH (21, 22). PC2 is expressed abundantly in the intermediate lobe (18, 22, 23) consistent with its putative role as a major POMC processing enzyme. However, the proteolytic preferences of the processing enzymes identified to date are insufficient to account for all of the known processing steps in the generation of the cell-specific bioactive peptides derived from the prohormone POMC, in particular the KK cleavage at the β-endorphin carboxyl terminus required for generation of β-endorphin (1-27) and (1-26) (21).

Targeted tumorigenesis utilizing a tissue-specific gene promoter and the Simian virus (SV) 40 large T antigen has been a frequently used strategy to induce neoplasms in endocrine glands in transgenic mice. The resulting tumors have been used to characterize the multistep processes involved in tumor evolution (24), as a sensitive reporter of gene expression from promoter fragments (25), and as a source of tissue for the propagation of novel immortalized cell lines (25). A particular advantage of the transgenic approach is the transformation of cells that rarely produce spontaneous tumors. For example, both gonadotrophs and a precursor cell type for somatomammotrophs in the mouse pituitary have been immortalized and provided an enriched source of cell-type specific transcription factors for the analysis of pituitary hormone gene expression (27, 28).

The purpose of our study was to characterize the effects of pituitary cell transformation on the post-translational processing of POMC by producing transgenic mice with a fusion gene encoding SV40 large T antigen under the transcriptional control of a rat POMC promoter. Three independent founder mice developed pituitary tumors and a single transgenic pedigree has been maintained for multiple generations. HPLC and radioimmunoassay for POMC-derived peptides showed that the processing patterns in the tumors were virtually identical to those characteristic of normal intermediate lobe melanotrophs. After serial subcutaneous transplantation in nude mice, extracts of the pituitary tumors showed processing enzymes identified to date are insufficient to account for all of the known processing steps in the generation of the cell-specific bioactive peptides derived from the prohormone POMC, in particular the KK cleavage at the β-endorphin carboxyl terminus required for generation of β-endorphin (1-27) and (1-26) (21).

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RESULTS

Phenotype and Morphology—Three founder transgenic mice from a total of nine identified by dot-blot analysis of tail DNA for SV40 large T antigen sequences developed obvious pituitary tumors. Founder mouse No. 427 was bred successfully prior to succumbing to its pituitary tumor and established a pedigree that has maintained the pituitary tumor phenotype unchanged for 15 generations through successive crosses to outbred Swiss Webster females. Microscopic abnormalities of the pituitary intermediate lobes were evident as early as 3 weeks of age and consisted of multifocal nodules of SV40 Tag immunoreactive melanotrophs. Tag immunopositive cells were also demonstrated occasionally in the anterior lobes (Fig. 1 and 2). The neoplastic melanotrophs had a reduced cytoplasmic to nuclear ratio and pleomorphic nuclei. Colocalization of SV40 Tag and ACTH immunoreactivity was found in variable proportions of both the melanotrophs and corticotrophs, although the most intensely immunofluorescent Tag positive cells were devoid of ACTH staining.

At 6 weeks of age many of the mice had a diffuse hyperplasia of the intermediate lobe easily visible to the unaided eye, however, the general architecture of the pituitary remained intact. Serial examination of transgenic mice at 3-week intervals clearly revealed a progressive growth of the intermediate lobes culminating in tumor masses overflowing the sella turcica and compressing the overlying brain. Any incipient anterior lobe nodules in addition to the normal anterior lobes were engulfed and destroyed by the intermediate lobe tumors, presumably by competition for nutrient supply by the more rapidly dividing transformed melanotrophs. Mice succumbed to massive tumors of the pituitary gland (ranging to 150 mg wet weight compared to a normal gland weight of 2 mg) by 12–20 weeks. Larger tumors sometimes contained tiny residual islands of immunohistochemically normal anterior lobe cells buried within them but there was no evidence of hormone expression other than POMC peptides in the neoplastic cells (data not shown). Transformed cells from the large tumors varied widely in their content of Tag, ACTH-like and ß-endorphin-like peptides by immunohistochemical staining (Fig. 2).

Two founder mice and approximately 20% of the 427 pedigree mice developed massive thymic hyperplasia in addition to or preceding their pituitary tumors. No POMC mRNA or ß-endorphin peptides were detected in the hyperplastic thymus glands. No tumors of the brain, reproductive tract, or any other extrapituitary site were found in the transgenic mice.

Transgenic mice with pituitary tumors characteristically had a 50% increase in adrenal weight compared to sibling controls (7.4 ± 0.5 versus 4.7 ± 0.2 mg, × ± S.E., n = 9). Histologically the enlarged adrenals had cortical hyperplasia in the zona fasciculata and reticularis (Fig. 3). The tumor bearing mice invariably rapidly lost weight in their final week and never developed any increased adipose tissue. Tissue content of ß-endorphin and plasma ß-endorphin and corticosterone levels in normal and transgenic mice are compared in Table I.

Dissociated pituitary tumor cells readily formed subcutaneous tumors when implanted into nude mice but not outbred Swiss Webster or hybrid B6D2 F1; mice. Tumors were serially transplanted up to three times for a total growth period of 8 months following dissociation of the original primary tumors.

Fig. 1. ACTH-like immunoreactivity (A and C) and SV40 T antigen (B and D) in a pituitary gland from a 3-week-old transgenic mouse. The intermediate lobe (IL) is enlarged and both the intermediate and anterior lobes (AL) have multifocal nodules of T antigen positive cells. An arrow points at a nodule in the anterior lobe. The intermediate lobe is a patchwork of cells demonstrating all combinations of strong and weak expression of both T antigen and POMC (simultaneous dual immunofluorescence technique; NL, neural lobe; the outlined areas in A and B are enlarged in C and D, respectively; scale bar represents 300 μm).

Fig. 2. At 3 weeks of age (left) multifocal nodules of abnormal cells are present in a normal sized intermediate lobe (arrowsheads). Pituitary tumor cells from an advanced neoplasm contain widely variable cytoplasmic immunopositivity for ACTH (center) and nuclear localization of SV40 T antigen (right). Left, hematoxylin and eosin stains, scale bar represents 100 μm; center and right, avidin-biotin-peroxidase complex technique, differential interference contrast, scale bar represents 50 μm.

Fig. 3. Adrenal gland from 12-week-old control (left) and transgenic littermate bearing a large pituitary tumor (right). The cortex is hyperplastic in the transgenic mouse while the centrally located medulla is unaffected. Hematoxylin and eosin stains, scale bar represents 500 μm.
TABLE I

| Tissue and plasma content of β-endorphin immunoreactivity and plasma corticosterone in normal mice, transgenic mice, and nude mice implanted subcutaneously with pituitary tumor cells |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|
| β-Endorphin immunoreactivity in   | Plasma β-endorphin | Plasma corticosterone |
| NIl (normal mice)*               | pmol/tissue      | pmol/mg wet weight | µg/ml            | ng/ml           |
| Transgenic mice primary tumors   | 696 ± 69         | 1393 ± 117       | <55              | 41 ± 7          |
| Nude mice with transplanted tumors | 1088 ± 169       | 12 ± 2           | 1007 ± 49        | 679 ± 100       |
| Tumors (transgenic or nude)      | 1052 ± 358       | 2 ± 0.6          | ND*              | >1000           |

* Mean ± S.E., n = 3–5.
† NIl, neurointermediate lobe.
‡ ND, not determined.

Northern blot analysis of mRNA extracted from normal pituitary glands, primary pituitary tumors, transplanted tumors, and cultured tumor cells showed a pattern of increasing abundance of Tag RNA and decreasing abundance of both POMC RNA and dopamine β-receptor RNA (Fig. 4). The relative abundance of POMC and Tag mRNA was confirmed in three separate experiments. Despite less efficient expression of the POMC gene, the transplanted tumors continued to store β-endorphin immunoreactive material and stimulate corticosterone release (Table I).

Molecular Forms of β-Endorphin and ACTH-related POMC Peptides in Normal Pituitary, Primary Pituitary Tumors, and Transplanted Tumor Tissue—The molecular forms of POMC-derived peptides were characterized in extracts of pituitary tumor tissue from descendants of transgenic founder mouse No. 427. All primary tumors examined (total of 5) showed essentially identical results and representative data are presented in the figures. Fig. 5 compares the β-endorphin-related peptides extracted from normal Swiss Webster mouse neurointermediate lobe (A), normal mouse anterior lobe (B), and two individual pituitary tumors (C and D). Qualitatively, the relative proportions of tumor β-endorphin(1–31), acetylated β-endorphin(1–31), acetylated β-endorphin(1–27), and acetylated β-endorphin(1–26) were very similar to those found in the normal mouse intermediate lobe and distinct from normal mouse anterior lobe. Peak 6 in normal anterior lobe (Fig. 5B) was identified as β-LPH by the coelution of β-endorphin- and γ-LPH immunoreactivity (data not shown). A minor, but possibly significant difference, was that tumor extracts contained a slightly higher percentage of unprocessed POMC than the normal tissues.

The pattern of post-translational processing was strikingly different in the six transplanted tumors compared to all the primary tumors that were analyzed. Fig. 6, A–D, shows the β-endorphin processing profile of four independent transplanted tumors. Notably, the transplanted tumor extracts contained a high percentage of β-endorphin(1–31) (peak 1) and variable quantities of two peaks (7 and 8) that eluted close to, but distinct from, acetylated β-endorphin(1–31). To further characterize these peptides, we pooled material from the three major peaks shown in Fig. 6D and analyzed them by ion exchange HPLC and immunoassay. Peak 1 eluting in fractions 43–45 was confirmed to be nonacetylated β-endorphin(1–31) (Fig. 7A). Peak 7 eluting in fractions 48–50 contained mostly nonacetylated β-endorphin(1–27) and minor amounts of acetylated β-endorphin(1–31) and nonacetylated β-endorphin(1–26) (Fig. 7B). Peak 8 eluting in fractions 52–53 was nonacetylated β-endorphin(1–26) (data not shown). Additionally, as in the primary pituitary tumors, there was relatively more unprocessed POMC (peak 5 in Fig. 6, A–D). These results indicate that relatively little acetylation of β-endorphin(1–31) was taking place and that carboxyl-shortening of β-endorphin was occurring, but with reduced efficiency.

Because acetylation of β-endorphins was markedly reduced we reassayed the HPLC column fractions to determine if there was a similar reduction in acetylated forms of α-MSH. Further analyses of the HPLC fractions were performed using a relatively acetyl-specific α-MSH assay and a carboxy-terminal specific ACTH assay. Fig. 8A shows that the primary pituitary tumors processing closely resembled normal mouse intermediate lobe α-MSH profiles. The primary tumors contained large relative amounts of diacetyl-α-MSH (peak 11) and there was a minor amount of intact ACTH(1–39) (data not shown). In contrast, the transplanted tumors from nude mice (Fig. 8B) contained no diacetyl-α-MSH and very little des- and monoacetyl-α-MSH (peaks 9 and 10). The profile of ACTH-related peptides in the transplanted tumors (Fig. 8C) showed no corticotropin-like intermediate peptide, almost no ACTH(1–39), unidentified peaks of later eluting material, and unprocessed POMC (peak 5). In other studies (40) we have demonstrated that the phosphorylated and glycosylated ACTH(1–39) contained in normal mouse anterior pituitary extracts elutes as four major peaks at 23–36 min on the system used here. Partial cleavage at the KR site between

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* Mean ± S.E., n = 3–5.
† NIl, neurointermediate lobe.
‡ ND, not determined.
Fig. 5. Molecular forms of β-endorphin containing molecules in normal mouse intermediate and anterior lobes and primary pituitary tumors. Mouse pituitary tissue was analyzed by RP-HPLC and β-endorphin immunoassay as described under "Experimental Procedures." The elution times of the peptides were as follows: 1, β-endorphin(1–31) (45–47 min); 2, acetyl-β-endorphin(1–31) (53–54 min); 3, acetyl-β-endorphin(1–27) (69 min); 4, acetyl-β-endorphin(1–26) (62 min); and 5, POMC (73–75 min). The elution time of peak 6 (β-LPH) is shown by the arrow and was confirmed using an immunoassay for γ-LPH (not shown). A, intermediate lobe, 1.5 neurointermediate lobes were fractionated and analyzed. B, anterior lobe, a small portion of one anterior lobe dissected completely free of melanotrophs was fractionated and analyzed. C, POMC-Tag pituitary tumor No. 1; and D, POMC-Tag pituitary tumor No. 2. 50 mg wet weight equivalent of the tumor extracts were fractionated.

ACTH and β-LPH sequences in the transplanted tumors was confirmed by the presence of γ-LPH detected with the γ-LPH radioimmunoassay (data not shown). The later eluting material (peaks between 50 and 65 min) was not characterized further, however, the other major ACTH containing material in normal pituitary tissues is the 20-kDa ACTH biosynthetic intermediate. Therefore these peaks most likely represent a mixture of the 20-kDa biosynthetic intermediate cleaved at the KR paired basic pair between ACTH and β-LPH sequences. The processing profiles of primary and transplanted melanotroph tumors are depicted schematically in Fig. 9 to illustrate the sites of efficient, partial, or absent endoproteolytic cleavage and the loss of acetylation function by the transplanted tumors.

Expression of PC1 and PC2 in Normal Pituitary Tissues and Melanotroph Tumors—To determine whether the change in POMC processing accompanying transplantation of the tumors was due to altered expression of PC1 or PC2, we analyzed total RNA from AtT-20 cells, mouse anterior lobes, neurointermediate lobes, primary pituitary tumors, and a transplanted tumor by Northern blots (Fig. 10). PC1 expression (Fig. 10A) was highest in AtT-20 cells, moderately abundant in normal anterior lobe and a transplanted tumor, low in normal neurointermediate lobes, and undetectable in three independent primary tumors. The identical pattern of PC1 expression was confirmed by an RNase protection assay (data not shown). PC2 expression (Fig. 10B) was highest in normal neurointermediate lobe, moderately but variably abundant in the primary and transplanted tumors, and barely detectable in AtT-20 cells and normal anterior lobes. Equivalent amounts of total RNA were loaded on the gels based on the pattern of ribosomal RNA staining (Fig. 10C).

DISCUSSION

The localization of tumors induced by the POMC-Tag fusion gene was highly tissue-specific for the pituitary gland of several independent lines of transgenic mice. Pituitary expression of the transgene was expected based on previous observations in transgenic mice produced by the microinjection of reporter genes containing identical regulatory sequences from the rat POMC gene (41, 42). Because the SV40 large T antigen is capable of transforming a wide range of murine cell types, including neurons (43-45), it was conceivable that the transgene used in these studies would be a more sensitive marker for expression of the POMC 5′-flanking sequences in the arcuate nucleus of the hypothalamus or reproductive tract (46, 47). The absence of expression in these tissues as characterized by immunohistochemistry and the lack of tumor formation is further evidence that the rat POMC gene sequences between −706 and +64 are not generally sufficient for productive transcriptional activity in extrapituitary locations.

Several transgenes encoding SV40 Tag under the transcriptional control of neuroendocrine gene promoters have induced thymic neoplasia with identical characteristics to the mice in
**Fig. 6.** The forms of β-endorphin containing molecules in nude mouse tumor tissue derived from POMC-Tag pituitary tumors. Pituitary tumor cells were propagated subcutaneously in nude mice. After several weeks the masses were removed and analyzed as described under "Experimental Procedures" and in the legend to Fig. 5. A-D are all RP-HPLC profiles. A, nude mouse tumor No. 1; B, nude mouse tumor No. 2; C, nude mouse tumor No. 5; and D, nude mouse tumor No. 12. 50 mg wet weight equivalents of the extracts were fractionated.

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There are several possible reasons why the tumors induced by the POMC-Tag fusion gene are derived from melanotrophs and not corticotrophs. Although the 5'-flanking sequences included in the fusion gene are sufficient for expression in both cell types in transgenic mice (41, 42), our previous data and recent analyses of other lines of transgenic mice (53) suggest that these sequences are expressed more highly under basal conditions in the melanotrophs. In addition, as the melanotroph tumors increase in size, two feedback mechanisms favor expression of the oncogene from the POMC promoter in melanotrophs relative to corticotrophs. Compres- sion of the hypothalamic stalk and a loss of the tonic dopaminergic inhibition would increase POMC-Tag expression in the intermediate lobe while the elevated circulating corticosterone levels documented in the mice would inhibit POMC-Tag expression in the anterior lobe. We have no evidence that a subpopulation of mice dies prematurely from corticotroph tumors and no mouse have ever developed the phenotype of obesity described by Furth (54) in the original studies of the corticotroph tumors used to derive AtT20 cells or in transgenic mice overexpressing corticotropin-releasing hormone (55). The difference in phenotype may be due to the marked differences in POMC products secreted by corticotrophs versus melanotrophs.

The processing phenotype of the tumors changed consistently after one to three passages in transplanted nude mice. The changes were all in the direction of less complete processing and demonstrated a lack of acetylated β-endorphin(1-31), a lack of acetylated α-MSHs or mature ACTH(1-39), and an increased proportion of unprocessed prohormone and precursor forms of ACTH. In contrast, the cleavages at the KR sites within β-lipotropin to produce β-endorphin(1-31) and between ACTH and β-lipotropin were maintained. Cleavage at the KK site at the carboxyl terminus of β-endorphin(1-31), characteristic of melanotrophs, was also partially retained. The resulting complement of POMC peptides most
closely resembles the processing profile found in the developing mouse or rat pituitary at late embryonic stages or postnatal day 1 (56, 57). During development, the carboxy-terminal shortening of β-endorphin(1–31) and the aminoterminal acetylation of α-MSH and β-endorphins appear after establishment of the endoproteolytic cleavages at KR sites (57). A recent report found that proglucagon processing in SV40 Tag-induced intestinal carcinomas in transgenic mice also changed markedly with progressive tumor growth suggesting that processing plasticity may be a common feature of neoplastic endocrine cells (58).

PC2 gene expression was relatively abundant in the primary pituitary tumors consistent with its presumptive role in normal melanotrophs in the post-translational processing of POMC (21, 22). Interestingly, despite the loss or reduction in processing at several paired basic amino acid sites of POMC in the transplanted tumors, both PC2 and PC1 were expressed at the mRNA level. These data suggest that the active enzymes may not be efficiently produced in the transplanted tumors either because of a block in translation or in activation of the proenzymes. Alternatively, other changes in the cell biology of the transplanted tumor cells involving the intracellular transport of the prohormone may alter either the accessibility or residence time in an appropriate convertase-rich compartment. A further possibility is that an enzyme distinct from PC1 and PC2 is physiologically relevant for POMC processing in melanotrophs and that this novel enzymatic activity is lost after tumor transplantation. Direct studies of the enzymatic activities in addition to PC gene expression are necessary to distinguish between these possibilities.

Our data does not allow us to make definitive conclusions concerning the molecular events responsible for the change in POMC processing. Transplantation of the tumors is necessarily associated with an increase in the total number of cell divisions and age of the neoplasm. The altered processing
POMC Processing in Mouse Melanotroph Tumors

Fig. 9. Summary and comparison of POMC processing in normal mouse melanotrophs, primary pituitary tumors, and subcutaneously transplanted pituitary tumors. A, in normal mouse melanotrophs and primary pituitary tumors five paired basic amino acids are cleaved efficiently, giving rise to the peptides depicted. The majority of α-MSH and β-endorphin peptides are amino-terminally acetylated. B, in the transplanted tumors specific endoproteolytic cleavages are markedly reduced in these melanotroph-derived cells. Amino-terminal acetylation of β-endorphin is also reduced and diacetyl-α-MSH is absent. Thus, specific post-translational processing steps are lost after transplantation. N, amino terminus; C, carboxyl terminus; K, lysine; R, arginine; solid arrow, efficient cleavage; open arrow, partial cleavage; -, absent cleavage; N.D., not determined.

may be due to general effects of transformation induced by SV40 Tag and would also occur in the primary tumors in situ after a sufficient length of time if the tumors were not fatal as a result of mass effect. Arguing against a simple loss of all differentiated function, however, was the emergence of PC1 expression at a level comparable to normal anterior lobe. Alternatively, the extracellular environment of the subcutaneously transplanted tumors may directly alter the processing phenotype either because of a loss of factors present in the tumors’ normal location or exposure to novel factors. Previous studies have suggested that POMC processing patterns can be altered in vivo by glucocorticoids (59), vasopressin (60), and interleukin-2 (61). Furthermore, in the neurointermediate lobe both PC1 and PC2 are regulated in response to dopaminergic agents and in AtT-20 cells PC1 is regulated in response to glucocorticoids and corticotropin-releasing hormone (23).

This study is the first biochemical documentation of a melanotroph pituitary tumor in mice. It was recently reported that vasopressin-SV40 large T antigen transgenic mice occasionally developed intermediate lobe tumors that stained with an antibody to ACTH, in addition to somatotroph tumors, but the forms of POMC peptides have not been characterized (62). Intermediate lobe pituitary adenomas are relatively common, however, in canine and equine species (8, 9). In the dog, two types of melanotrophs have been described. Type A cells produce α-MSH and β-endorphin similarly to mouse melanotrophs while type B cells are more like corticotrophs in their complement of POMC peptides (63, 64). Tumors of either cell type are capable of producing Cushing’s syndrome in dogs, however, with elevated glucocorticoid levels. The melanotroph tumors induced in transgenic mice are most like the canine type A. The processing pattern is identical to normal mouse intermediate lobe cells and the mice have striking elevations in plasma β-endorphin and corticosterone with histological evidence of adrenocortical hyperplasia. Although plasma ACTH levels were not measured in this study, it is likely that the melanotroph tumors secrete enough ACTH to cause the adrenocortical hyperplasia as has been found in canine Cushing’s syndrome. Large molecular weight products of POMC in combination with ACTH could also be responsible for stimulation of the adrenal gland (65).

The establishment of a line of transgenic mice that reproducibly develops melanotroph pituitary tumors provides a new tool for the study of pituitary oncogenesis and the biochemical processes regulating POMC post-translational processing. It should also be possible to derive immortalized cell lines representing the melanotroph cell lineage that will complement the existing AtT20 mouse corticotroph cell line in studies of
the cell-specific regulation of POMC gene expression and processing.

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