Endocrine, *vol. 22, no. 3, 257–265, December 2003* 0969–711X/03/22:257–265/$25.00

Agouti-Related Protein (AGRP) Is Conserved and Regulated by Metabolic State in the Zebrafish, *Danio rerio*

Youngsup Song,[1] Gregory Golling,[2] Theresa L. Thacker,[2] and Roger D. Cone[1]

[1]*Vollum Institute, Oregon Health and Science University, Portland, OR, and* [2]*Znomics, Inc., Portland, OR*

Agouti-related protein (AGRP) and proopiomelanocortin (POMC) genes encode secreted hypothalamic factors regulated by metabolic state in mammals and are involved in energy homeostasis. The zebrafish, *Danio rerio*, is a model system for forward genetics in vertebrates: POMC and AGRP in this organism have not been well characterized. Toward this end, AGRP and POMC were cloned from zebrafish. Zebrafish AGRP cDNA encodes a 127-amino-acid protein 36% and 40% identical to human and mouse AGRP, respectively. Zebrafish POMC cDNA encodes a 222-amino-acid preprohormone. Sequence identity to the mammalian ortholog is about 50%. Zebrafish AGRP and POMC transcripts were detected at 24 h post-fertilization (hpf) by RT-PCR, and *in situ* hybridization demonstrated zebrafish AGRP mRNA exclusively in hypothalamus and POMC mRNA in hypothalamus and pituitary. Fasting did not alter POMC transcript levels, while AGRP transcripts were significantly upregulated. The ratio of AGRP/POMC transcripts in adult brain was increased up to threefold by fasting. These results demonstrate that the melanocortin system is regulated by metabolic state in zebrafish, and this finding thus provides a vertebrate system for the genetic analysis of the role of the melanocortin system in energy homeostasis.

Key Words: Agouti-related protein (AGRP); zebrafish; melanocortin; energy homeostasis; feeding.

Introduction

The central melanocortin system, consisting of hypothalamic arcuate nucleus neurons expressing POMC or AGRP and the downstream targets of these neurons *(1)*, has been demonstrated to play a critical role in energy homeostasis in a variety of mammalian species including both humans and rodents *(2–5)*. In the brain, cell bodies of POMC neurons are localized in hypothalamic arcuate nucleus and nucleus tractus solitarius (NTS) of the caudal medulla. Axon terminals containing POMC immunoreactivity are found in over 100 brain regions, including hypothalamic regions known to be involved in the regulation of energy balance, such as arcuate nucleus (ARC), paraventricular nucleus (PVN), dorsomedial nucleus (DMN) and lateral hypothalamus area (LHA). Melanocortin peptides cleaved from the POMC prohormone act by binding to the melanocortin-3 and melanocortin-4 receptors (MC3-R and MC4-R) in the CNS *(6,7)*. Stimulation of the MC4-R inhibits food intake and energy storage *(8,9)*, while the MC3-R appears to act, at least in part, as an inhibitory autoreceptor on the system *(10–12)*. Agouti-related protein (AGRP) is related to Agouti protein and exclusively expressed in adrenal gland and arcuate nucleus of hypothalamus in brain *(13,14)*. AGRP is expressed exclusively in arcuate neurons expressing the orexigenic neuropeptide Y (NPY) *(15)*. The projection sites of AGRP neurons, such as ARC, PVN, DMN, and LHA are largely overlapping with POMC neuron targets *(16,17)*. Hypothalamic AGRP is an antagonist of MC3R and MC4R, and by blocking melanocortin signaling, increases appetite and leads to weight gain. Fasting decreases hypothalamic POMC expression about 50%, and increases hypothalamic AGRP expression about 500–1000% *(13,15)*. The ratio of POMC to AGRP expression in arcuate nucleus of hypothalamus thus determines the relative activity of melanocortin target sites, which in turn regulate the levels of energy intake and expenditure.

Many fish orthologs of peptides involved in the central regulation of energy homeostasis in mammals, for example, NPY, POMC and AGRP, have been cloned from fish and demonstrated to be highly conserved in terms of primary sequence, structure, and function (for review, see ref. *18*). POMC has been cloned from several fish *(19–21)* and POMC immunoreactivity was detected in pituitary and lateral tuberal nucleus (NLT) of the hypothalamus, believed to be a homolog of the mammalian arcuate nucleus *(22)*. Receptors for the melanocortin peptides cleaved from POMC have been cloned from fugu fish (MC1, 2, 4, and 5) and zebrafish (MC1–5R) *(23,24)*, and also appear highly conserved. Although zebrafish MC4R was found in the brain, unlike mammals it was also detected in the eye, gastrointestinal tract, and ovaries by RT-PCR *(24)*. Recently, goldfish melanocortin

Received July 31, 2003; Revised September 22, 2003; Accepted October 6, 2003.

Author to whom all correspondence and reprint requests should be addressed: Roger D. Cone, PhD, Senior Scientist, Vollum Institute, Director, Center for the Study of Weight Regulation, and Associated Disorders, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239-3098. E-mail: cone@ohsu.edu

receptor 4 was cloned, and its distribution in brain was mapped by *in situ* hybridization *(25)*. Consistent with zebrafish MC4R expression pattern, RT-PCR detected goldfish MC4R transcripts in peripheral tissue, but strongly conserved central distribution of goldfish MC4R in lateral tuberal nucleus (NLT), ventral telencephalon (Vv), parvo- and magnocellular (NPO) neurons, homologs of mammalian ARC, lateral septal nucleus, SCN, and PVN. Importantly, intracerebroventricular administration of MTII in the goldfish inhibited feeding, while the MC4-R antagonist AGRP stimulated food intake; these data strongly argue that the central melanocortin system also regulates food intake in fish. AGRP has also recently been cloned from goldfish, and has been demonstrated to be regulated by fasting in this species *(26)*. While the goldfish is an important model system for neuroanatomical and physiological studies in the fish, being of a similar size to rodents, the zebrafish has been developed as a simple vertebrate system for large-scale forward genetic analysis. In this study, we have cloned zebrafish AGRP and zebrafish POMC, and characterized their spatial and temporal expression and their regulation by fasting.

Results

Cloning Zebrafish AGRP and POMC cDNA

Human and mouse C-terminal fragments of AGRP (83–132) cDNA sequences were used to perform a BLAST search from zebrafish and fugu genome databases. Based on these BLAST analyses, we designed primers to amplify the reported fragment of zebrafish AGRP (zAGRP). RT-PCR produced a 200 bp partial zebrafish AGRP cDNA. Based on this sequence, we then amplified 5' and 3' ends of zebrafish AGRP cDNA from a zebrafish brain cDNA library in a λgt 11 vector (a kind gift from Dr. Christian Goblet), with gene- and λgt 11 vector–specific primers. 5' and 3' PCR products were cloned into pCR4-TOPO vector (Invitrogen). A full-length zebrafish AGRP cDNA sequence was then amplified by PCR from the zebrafish brain cDNA library with primers designed based on the 5' and 3' end sequences obtained. The cDNA sequence of AGRP is 877 bp. The complete open reading frame of 381 bp encodes 127 amino acids (Fig. 1A). When the deduced zebrafish AGRP amino acid sequence was compared to those of human and mouse, its sequence identity to human was 36% with a sequence homology of 43%, with 40% sequence identity and 48% sequence homology to the mouse. When we compared the C-terminal fragment of human or mouse AGRP (aa83–132), which is sufficient for the pharmacological actions of AGRP *(27)*, to that of zebrafish AGRP, sequence identity increased to 58% with 65% sequence homology. We also found the spatially conserved nine cysteine residue repeat at the c-terminal domain of zebrafish AGRP (Fig. 2A).

By BLAST search analysis of the zebrafish genome database using human and mouse POMC cDNA sequences, we found the partial POMC genomic DNA sequences. Based on the genomic DNA sequences, we designed primers to clone the partial zebrafish POMC cDNA sequence. Based on the cDNA sequence obtained from RT-PCR, we designed primers to obtain 5' and 3' ends of zebrafish POMC cDNA sequence by PCR from a zebrafish brain cDNA library. The cDNA sequence of POMC is 737 bp and the complete open reading frame of zebrafish POMC cDNA was 666 bp, encoding a protein of 222 amino acids (Fig. 1B). Deduced zebrafish POMC amino acid sequence has 51% and 48% sequence identity to those of human and mouse POMC amino acid sequences, respectively, and 58% and 55% sequence homology to those of human and mouse. Based on the deduced processed peptide sequences from POMC, the predicted α-melanocyte-stimulating hormone (α-MSH) peptide is 100% identical to the mouse and human peptide. POMC amino acid sequence identity between fish is high. For example, the deduced zebrafish POMC amino acid sequence is identical to that of the carp, *Cyprinus carpio*, is 81% (Fig. 2B).

Temporal and Spatial Expression of Zebrafish AGRP and POMC Transcripts During the Early Developmental Stages

We examined the temporal expression of zebrafish AGRP and POMC transcripts by RT-PCR analysis. Both AGRP and POMC transcripts were detected by RT-PCR at 24 hpf and the expression of both AGRP and POMC transcripts increased as development progressed (Fig. 3A). Spatial and temporal expression of those transcripts was also examined by whole mount *in situ* hybridization analysis (3B–E). Embryos staged at 24 hpf, 48 hpf, and 72 hpf were probed with digoxigenin (Dig)-labeled antisense or sense zebrafish AGRP and POMC cRNA probe. Figure 3B shows that AGRP transcripts were expressed in 48 hpf embryos. The transcripts were detected exclusively in hypothalamus in a bilaterally symmetrical pattern. The pattern of AGRP expression continued in 72 hpf embryos (Fig. 3C) and later stages of embryos (Fig. 4C). Zebrafish POMC transcripts were detected in hypothalamus and more strongly at both anterior and posterior pituitary (3D–E). Hypothalamic expression of POMC transcripts at 48 hpf was observed in a bilaterally symmetrical distribution comparable to AGRP, and at later development stages of embryos (Fig. 4A). We could not detect significant signal of either AGRP or POMC transcripts in 24 hpf embryos, possibly because of probe sensitivity, since RT-PCR detected these transcripts at that stage. Both zebrafish AGRP and POMC sense cRNA did not show any signal (data not shown).

Regulation of AGRP and POMC mRNA Expression by Metabolic State in Early Development and in Adult Zebrafish

Wild-type embryos were divided into two groups. Since, by d 5, baby fish have consumed all the nutrition from their own yolk cells, one group was fed from d 6 post-fertilization (dpf), and the other group was kept in filter-sterilized

A

```
1   acgcgggggagcctgggacgtgagcactacagtgatacatccagatcacacacaaaaagt

61  gtggctaaagtttatctctttttttgagtctgagtgattatgatgctgaacacagtaatc
                                              M  M  L  N  T  V  I
121 tttggctggtttttggtgaatgttgtggtgatggcatctcatccacacctgagacgcaga
     F  G  W  F  L  V  N  V  V  V  M  A  S  H  P  H  L  R  R  R
181 gaaaactcattcattctgacatctgacacagactcactgcctgaaatggagcaccttgaa
     E  N  S  F  I  L  T  S  D  T  D  S  L  P  E  M  E  H  L  E
241 ataaactcagcagaagaaaagatactagaagaccttgaagcctatgatgaggatctgggc
     I  N  S  A  E  E  K  I  L  E  D  L  E  A  Y  D  E  D  L  G
301 aaagctgtccacctgcagagaagaggcacacgctccccgagccgctgtatccctcatcaa
     K  A  V  H  L  Q  R  R  G  T  R  S  P  S  R  C  I  P  H  Q
361 cagtcctgtctgggtcatcatctgccctgctgcaacccctgcgacacctgctactgccgc
     Q  S  C  L  G  H  H  L  P  C  C  N  P  C  D  T  C  Y  C  R
421 ttctttaaggccttctgttactgccgcagcatggacaacacctgcaaaaacgaatatgca
     F  F  K  A  F  C  Y  C  R  S  M  D  N  T  C  K  N  E  Y  A
481 tagagacaagttctgccttcagatagcaaatggtagcattatatttacagttttaagagc
     -
541 tactgaatgtatttgtggacagtgcaatcataaactcgcacagagaatccacagagaagt
601 tttttgttataatagagaagcacaaagcatagagtgcaccattggctgcggttttaaggt
661 gtaatttaattgcaatattatttactcaaatgttgtcttaaatattttgtatatatgcat
721 tcaataaactctgattattgcaattgtgtagagtattaactgaaagagctataaatgatg
781 acaacatgcagacacattggattgtgagaaatgtgccttaaatgcaattaaattattatt
841 aagggaatatattattaggcaaaataaaataaaatat
```

B

```
1   ctttggttactgacttctttctgtgcaaaagagatggtgaggggagtgaggatgttgtgt
                                        M  V  R  G  V  R  M  L  C
61  cctgcttggctcttggctctggctgttctctgcgcaggaggatctgaagtcagagctcag
     P  A  W  L  L  A  L  A  V  L  C  A  G  G  S  E  V  R  A  Q
121 tgttgggaaaatgcccgctgtcgagacctcagcacagaggagaacatcttggaatgcata
     C  W  E  N  A  R  C  R  D  L  S  T  E  E  N  I  L  E  C  I
181 caattatgcaggtctgaacttacagatgaaaccccgtctaccctggagaaagccatcta
     Q  L  C  R  S  E  L  T  D  E  T  P  V  Y  P  G  E  S  H  L
241 cagcctccctccgagccggagcaaatcgacctcctcgcacacctttcccctgtagcactc
     Q  P  P  S  E  P  E  Q  I  D  L  L  A  H  L  S  P  V  A  L
301 gcagcccctgaacagatagagccggagtccggccctcgacacgaccacaagcgctcctac
     A  A  P  E  Q  I  E  P  E  S  G  P  R  H  D  H  K  R  S  Y
361 tccatggagcacttccggtggggcaaaccggtcggccgcaaacgcagacccatcaaggtg
     S  M  E  H  F  R  W  G  K  P  V  G  R  K  R  R  P  I  K  V
421 tacacgaacggcgtggaagaggaatccgccgaaacgcttccggcagagatgagacgcgag
     Y  T  N  G  V  E  E  E  S  A  E  T  L  P  A  E  M  R  R  E
481 ctggcaaataacgaggtcgactatccgcaagaagagatgcctttaaacccactggggaag
     L  A  N  N  E  V  D  Y  P  Q  E  E  M  P  L  N  P  L  G  K
541 aaggaccccccctacaaaatgacccatttccgctggagcgtcccgccggctagcaagcgc
     K  D  P  P  Y  K  M  T  H  F  R  W  S  V  P  P  A  S  K  R
601 tatggaggcttcatgaagtcctgggacgagcgtgctcagaaaccactgctcacactcttc
     Y  G  G  F  M  K  S  W  D  E  R  A  Q  K  P  L  L  T  L  F
661 aaaaacgtaatgcataaagaccaaccgaggaaggatgagtgagtggctttaaggggaga
     K  N  V  M  H  K  D  Q  P  R  K  D  E  -
721 ggttgttatagggggtc
```

Fig. 1. Full-length cDNA and deduced amino acid sequences of zebrafish AGRP and POMC. The bar indicates the stop codon. (**A**) Zebrafish AGRP cDNA sequence; (**B**) zebrafish POMC cDNA sequence.

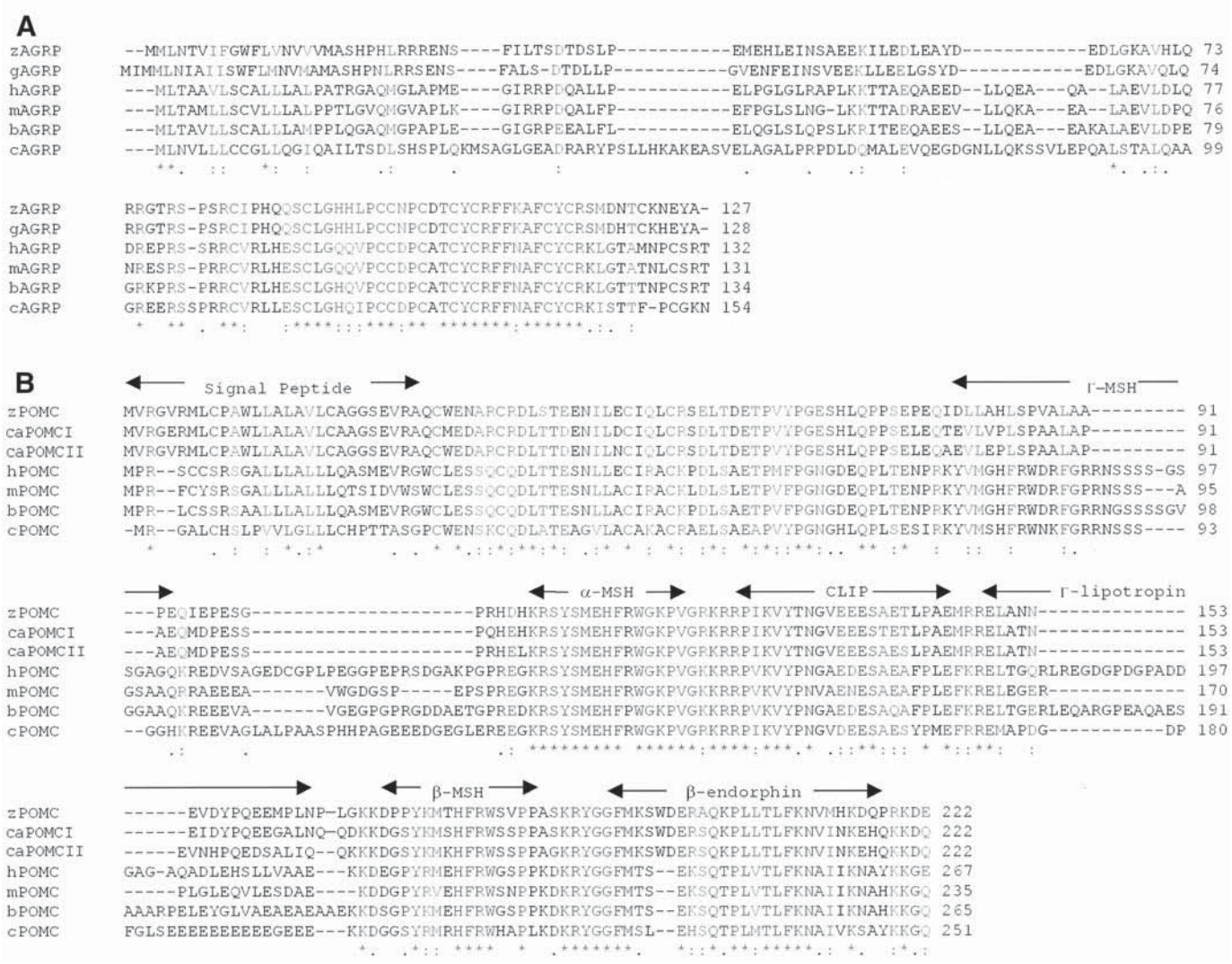

Fig. 2. Comparison of the zebrafish AGRP and POMC peptide sequence across different species. (**A**) Comparison of deduced zebrafish AGRP peptide sequence (zAGRP) to goldfish (accession number not yet available), human (hAGRP: gi15384342), mouse (mAGRP: gi6671529), cow (bAGRP: gi2511603), and chicken (cAGRP: gi5381214) AGRP sequence. (**B**) Comparison of deduced zebrafish POMC (zPOMC) peptide sequence to deduced amino acid sequence from carp POMC I (caPOMC I: gi14194542), II (caPOMC II: gi3819101), human (hPOMC: gi13637253), mouse (mPOMC: gi116882), cow (bPOMC: gi27806467), and chicken (cPOMC: gi3869133), using the cluster W program operated by SDSC biowork bench 3.2. (*, conserved across all species; :, conservation of strong groups; ., conservation of weak groups; - no consensus)

fish system water without feeding. Baby fish from both control fed group and fasted group were fixed at 10 dpf (5 d fed or fasted), and 19 dpf (14 d fed or fasted) and subjected to whole mount *in situ* hybridization. The expression pattern of POMC transcripts in hypothalamus and pituitary in the fasted groups were comparable to that of the control group; no significant difference in hypothalamic POMC transcript level was detected (Fig. 4A,B). AGRP transcripts were detected in hypothalamus in a bilaterally symmetrical pattern in both control and fasted groups. Although there was some variation of AGRP transcript levels in controls, AGRP transcripts in fasted group were highly upregulated compared to those in control fed groups (Fig. 4C–F). It appeared that the 14 d fast produced a greater upregulation of AGRP than the 5 d fast (Fig. 4C–F). Because we found that AGRP expression is upregulated in early zebrafish, we next quantitate the regulation of AGRP transcripts. We performed this experiment in larger adult organisms, because it is not readily feasible to dissect the pituitary away from the CNS in the young fish, and the pituitary is a source of very high levels of POMC mRNA. And because we observed the effect qualitatively in both sexes, we did not feel it was necessary to perform the quantitation in both sexes. We divided 5–9 mo-old female fish into control, 2 d fasted, 5 d fasted,

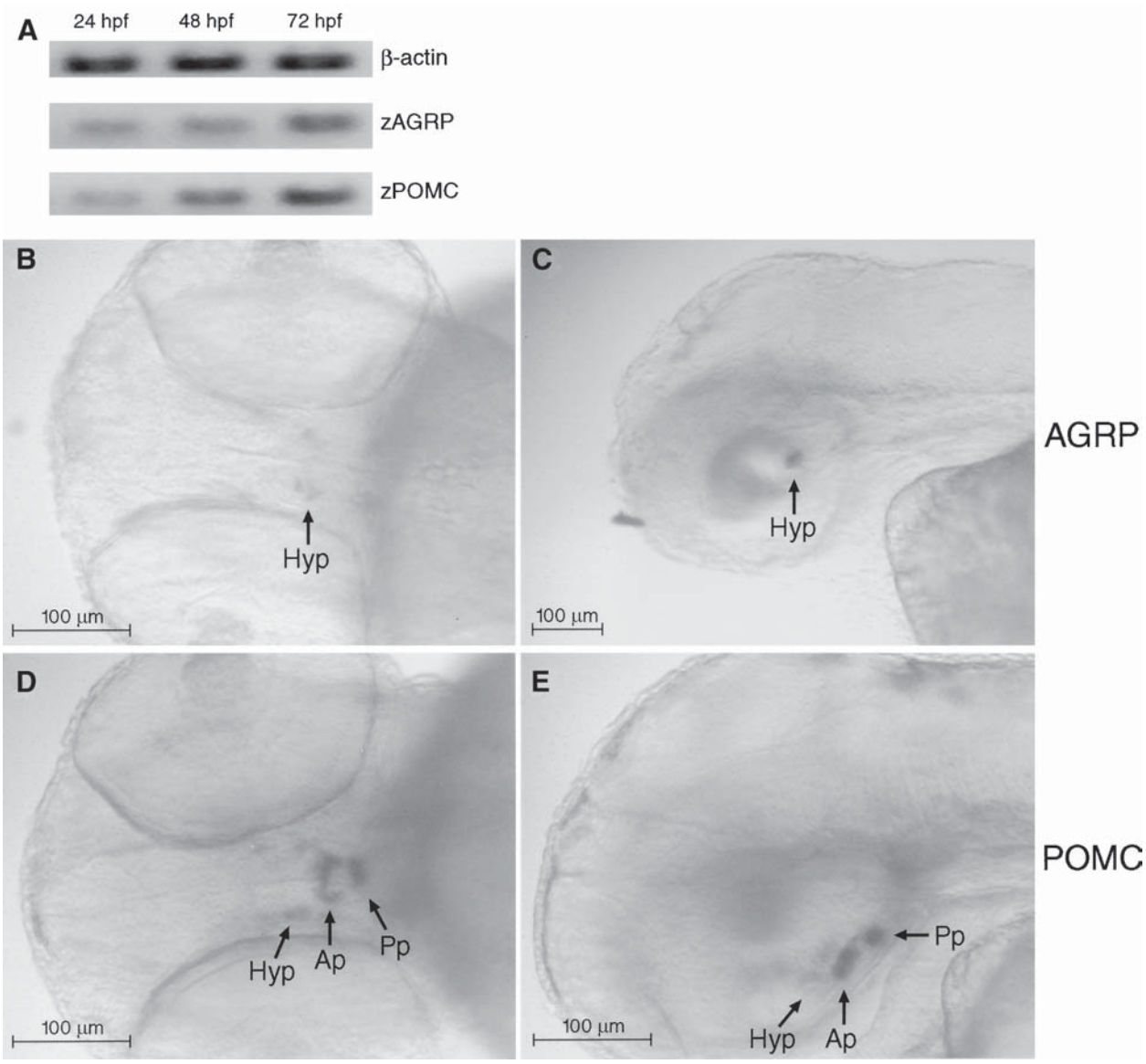

Fig. 3. Temporal and spatial expression pattern of zebrafish AGRP and POMC transcripts at different stages of early development. (**A**) RT-PCR analysis of AGRP, POMC transcripts. Total RNA from whole zebrafish staged at 24 hpf, 48 hpf, and 72 hpf was extracted, reverse, transcribed and amplified with gene-specific primers of β-actin, AGRP, and POMC. (**B–E**) Whole mount *in situ* hybridization analysis using zebrafish AGRP and POMC antisense cRNA probes. (**B**) 48 hpf dorsal view of AGRP; (**C**) 72 hpf lateral view of AGRP; (**D**) 48 hpf ventral, view of POMC; (**E**) 48 hpf lateral view of POMC. Hyp = hypothalamus; Ap = anterior pituitary; Pp = posterior pituitary.

10 d fasted, and 15 d fasted groups. In each group, four or five female fish were fed or fasted at the designated day, were sacrificed, and whole brain tissue was dissected. Total RNA was extracted from brain tissues and reverse transcribed and relative levels of AGRP to POMC transcripts were quantified by real-time quantitative RT-PCR. The AGRP/POMC transcript ratio was then normalized to the ratio of the control fed groups. As shown in Fig. 5, AGRP/POMC ratio in fasted groups increased steadily compared to control fed group as a function of the length of the fast; 2, 5, 10, and 15 d fasting elevated the AGRP/POMC ratio about 40%, 60%, 100%, and 200%, respectively, compared to control group.

Discussion

AGRP has been cloned from human, mouse, rat, cow, and chicken, and these, along with another recent report on the goldfish *(26)*, represent the first reports of this protein from fish. The complete AGRP peptide encodes 127 amino acids and the pharmacologically active C-terminal domain (aa78–127) is highly conserved (58% identity) when compared to the comparable portion of the mouse sequence. In mammals, 10 cysteine residues are present at the C-terminus of AGRP and disulfide bond formation of all 10 cysteine residues is important for the structural stability and

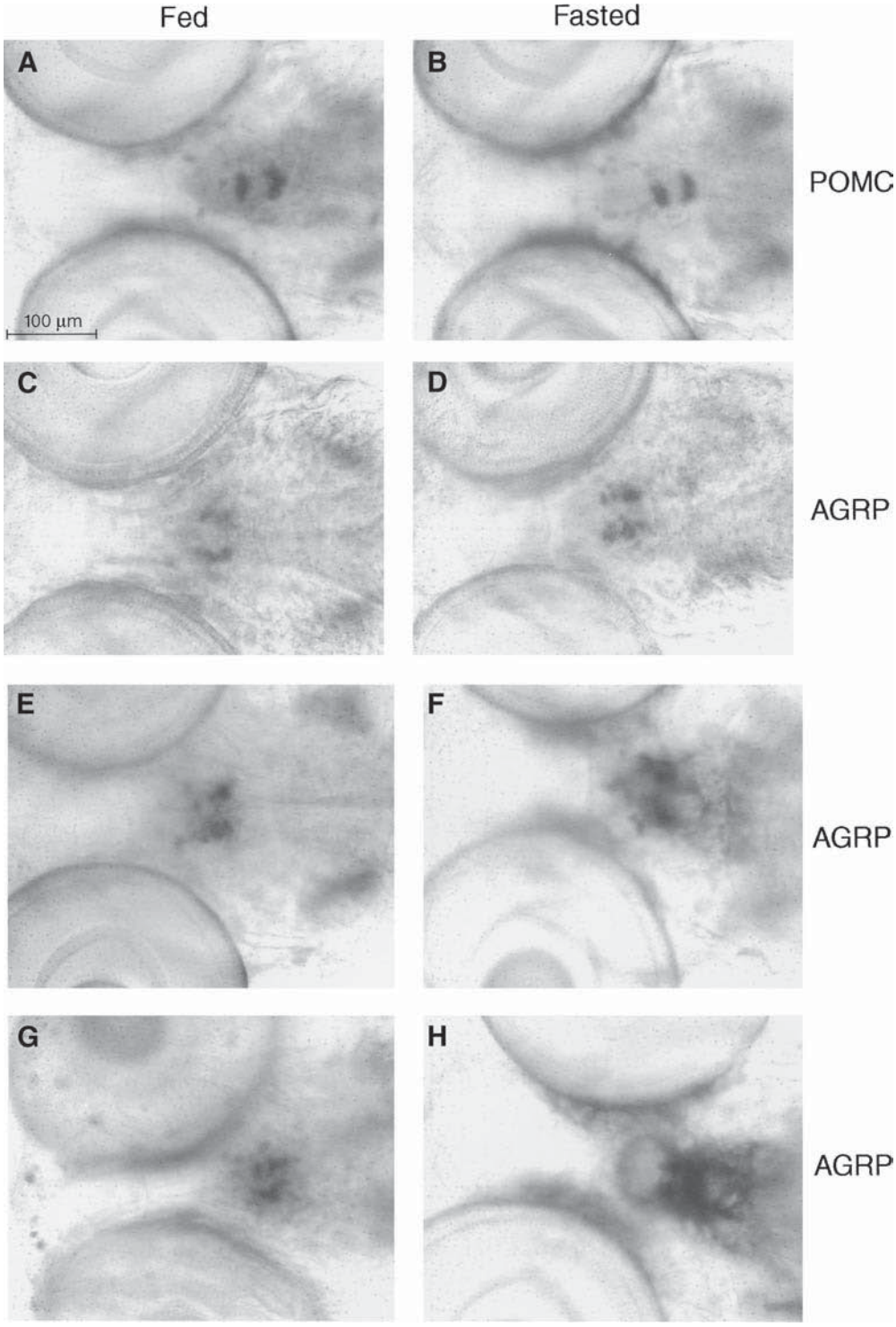

Fig. 4. Analysis of feeding-dependent regulation of AGRP and POMC transcripts by whole mount *in situ* hybridization. Wild-type embryos were divided into two groups, one group was fed from d 6 and the other group was fasted either for five more days (10 dpf) or 14 more days (19 dpf). Embryos were collected at d 10 and 19, fixed and hybridized with DIG-labeled antisense cRNA probe of zebrafish AGRP or POMC. First row represents 14 d fed (left) or fasted (right). Rows 2–4 are from fish fed (left) or fasted (right) for 5 (C–D), or 14 (E–H) d. Experiments were performed in parallel.

biological function of the protein *(28)*. Ten cysteine residues are also found in the zebrafish protein, and all but the last are spatially conserved as well. An RFF motif, which is comparable to HFRW motif of α-MSH, and important for AGRP's activity as an antagonist of the MC4-R, was also conserved in zebrafish AGRP *(29)*.

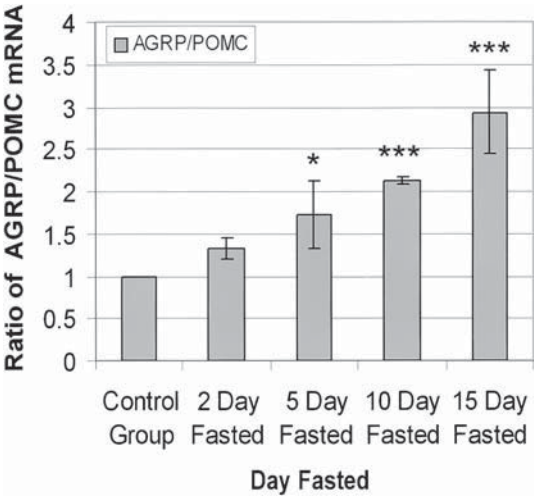

Fig. 5. Effect of food deprivation on the ratio of AGRP/POMC transcripts in adult female zebrafish. Adult female fish were grouped. Control group was fed normally and the other groups were deprived of food for the indicated days, then sacrificed for preparation of brain RNA. In each group four or five brains were dissected and pooled, and total RNA extracted. The Ct (number of cycles required for synthesizing a fixed threshold of product) was measured by real-time quantitative RT-PCR, and used to determine the relative ratio of AGRP/POMC transcripts, compared to the control value, set to 1, determined from fish fed *ad libitum*. Data show the mean and standard deviation from three independent experiments. Error bar indicates ± standard deviation, significance tested by one-way ANOVA (*$p < 0.05$, ***$p < 0.001$).

AGRP was a detected as early as 24 hpf by PCR, and could be observed in the hypothalamus by *in situ* hybridization at 48 hpf. AGRP transcripts were detected in hypothalamus in a bilaterally symmetrical pattern, and the overlap in distribution with POMC, mapped in several fish species to the lateral tuberal nucleus, suggests that AGRP cell bodies may map to this nucleus as well. The lateral tuberal nucleus is the teleost equivalent of the mammalian arcuate or infundibular nucleus, and AGRP mRNA has been mapped to the ventrobasal portion of this nucleus in the goldfish *(26)*. In contrast to AGRP, agouti has not been reported from any fish species yet, and it is interesting to speculate that mammalian agouti is diverged from AGRP. Interestingly, zebrafish and goldfish MC4R expression is also detected in peripheral tissues, although its major expression in hypothalamus is conserved, as are several of its pharmacological properties *(24,25)*.

POMC has been characterized from several fish *(20)* including zebrafish *(21,30)*. The 737 bp zebrafish POMC cDNA encodes a 222-amino-acid preprohormone. Sequence identity to human is about 53% and, when individual peptides are compared, sequence identity is increased, α-MSH: 100%; β-MSH: 71%; corticotropin-like intermediate lobe peptide (CLIP): 62%; and β-endorphin: 56%. Both α-MSH and β-MSH have the HFRW sequence motif, which is required for binding to melanocortin receptors. Paired basic POMC-peptide cleavage sites such as KR and KK motif were conserved between POMC-derived peptides in zebrafish, suggesting that zebrafish POMC is processed by similar post-translational modification. No γ-MSH or γ-lipotropin was found in zebrafish. We detected POMC transcripts at 24 hpf by RT-PCR, and its spatial expression pattern in hypothalamus and pituitary became evident by 48 hpf. Like AGRP, the POMC expression pattern in hypothalamus was bilateral and symmetric. The expression pattern of both AGRP and POMC continued at 20 dpf.

POMC has been widely studied in pituitary, but little has been reported about hypothalamic POMC function. We further characterized the role of POMC and AGRP in energy homeostasis in the zebrafish. After fasting, AGRP expression in the hypothalamus of 20 dpf fish was significantly upregulated. No detectable changes in POMC transcripts were observed between control fed group and fasted groups. We also examined the regulation of AGRP and POMC in adult fish. Because POMC mRNA levels undergo only modest changes in the mammal, and in the zebrafish as shown here, we normalized changing levels of AGRP transcripts to hypothalamic POMC transcript levels. After fasting, the AGRP/POMC transcript ratio gradually increased. By 15 d fasting, the AGRP/POMC transcript ratio increased as much as 300% in some experiments. AGRP mRNA has been reported to be upregulated as much as 13-fold in mice *(36)* and 2.2-fold in Japanese quail *(37)* by fasting. Fasting reduced POMC mRNA expression by 50% in mice *(36)*; however, in Japanese quail, POMC mRNA expression was not changed by fasting *(37)*. Previous work in the goldfish demonstrated that ICV injection of NPY increased food intake and fasting increased NPY expression in hypothalamus *(31)*. More recently, synthetic melanocortin agonists and antagonists have been demonstrated to inhibit or stimulate food intake, respectively, in this species *(25)*. Goldfish AGRP has recently been identified and demonstrated to be upregulated by fasting in the adult goldfish *(26)*. These data, together with those shown here, demonstrate that the endogenous hypothalamic melanocortin system is likely to regulate food intake in multiple fish species, that the system appears responsive to metabolic state in fish as young as 48–72 hpf, and that, as in mammals, the AGRP gene appears highly regulated while the POMC gene appears to play a more tonic role. Characterization of the conserved nature of this system in the young transparent zebrafish identify this species as a model system for genetic analysis of energy homeostasis in a vertebrate.

Materials and Methods

Zebrafish

Tab 5 and Tab 14 strains of zebrafish were raised and bred as described *(32)*; 10–15 adult fish were maintained in half gallon tanks of an AHAB System (Aquatic Habitats) at 26–28°C, under a 14-h light, 10-h dark cycle. The fish were fed with flakes (Eco-systems, Inc.) and supplemented

with brine shrimp. The embryos were obtained by natural crosses and fish embryos, and larvae stage was determined according to Kimmel et al. *(33)*.

Isolation of Total RNA and cDNA Synthesis

Total RNA was extracted with the phenol/guanidinium thiocyanate method using Trizol LS reagent (Invitrogen) according to the manufacturer's instruction. To remove genomic DNA, purified total RNA was treated with RNase free DNase (Roche) at 37°C for 30 min and cleaned with Rneasy mini kit (Qiagen) according to the manufacturer's instructions; 500 ng of purified total RNA was reverse transcribed with oligo dT primers using a first-strand cDNA synthesis kit (Fermentas).

Cloning of Zebrafish AGRP and POMC cDNAs

BLAST searches of the zebrafish genome database (http://www.ensembl.org/Danio_rerio/) and fugu genome database (http://fugu.hgmp.mrc.ac.uk/) with existing AGRP and POMC sequences were performed. Based on the BLAST search results, primers were designed to obtain partial zebrafish AGRP and POMC cDNAs. PCR products were cloned into pCR4-TOPO vector (Invitrogen). To get full cDNA sequences of zebrafish AGRP and POMC, nested primers were designed based on the sequences of PCR products. PCR reactions were then carried out using a zebrafish brain cDNA library *(34)* as a template, gene-specific nested primers, and vector (λgt11)-specific nested primers in a thermal cycler (Gene AMP PCR system 2400, Perkin Elmer), using Pfu polymerase (Stratagene). Full-length zAGRP and zPOMC sequences were then amplified from a zebrafish brain cDNA library and cloned into pCR4-TOPO vector (Invitrogen) (zPOMC (forward): 5'CGGGATCCCTTTGGTTAC TGACTTCTTTC 3', zPOMC (reverse): 5' CGGGATCCG ACCCCCTATAACAACCTCTCC 3'; zAGRP (forward): 5' GGATCCGTCTGAGTGATTATGATGCTGAACAC 3', zAGRP (reverse): 5' GGATCCGCAGCCAATGGTGCA CTCTATG 3'; the first six nucleotides of each primer encode a *Bam*HI cloning site.

Whole Mount In Situ *Hybridization*

To generate antisense DIG-labeled AGRP cRNA probe, the plasmids were linearized by digestion with *Not*I and subjected to in vitro transcription with T3 RNA polymerase and for sense DIG-labeled cRNA probe, the plasmids were linearized by digestion with *Spe*I and subjected to in vitro transcription with T7 RNA polymerase according to the manufacturer's protocol (Roche). For generation of antisense DIG-labeled POMC cRNA probe, the plasmids were linearized by digestion with *Spe*I and subjected to in vitro transcription with T7 RNA polymerase and for sense DIG-labeled cRNA probe, the plasmids were linearized by digestion with *Not*I and subjected to in vitro transcription with T3 RNA polymerase as described above. Zebrafish embryos at different developmental stages were collected, manually dechorionated, and fixed in 4% paraformaldehyde in PBS at room temperature for 3–5 h. Whole mount *in situ* hybridization was performed as described previously *(35)*. Briefly, fixed embryos were treated with –20°C methanol and rehydrated with a series of descending methanol concentrations (75%, 50%, and 25%) in PBS. They were then washed with PBS and treated with proteinase K (Fermentas) for 8 min at room temperature at a concentration of 10 μg/mL in PBS up to 24 hpf, 20 μg/mL from 24 hpf to 72 hpf, and 50 μg/mL up to 15 dpf. Embryos were refixed with 4% paraformaldehyde in PBS at room temperature for 20 min, washed five times with PBS, prehybridized with hybridization buffer [50% formamide, 5X SSC, 50 μg/mL heparin (Sigma)], 500 μg/mL tRNA (Roche), 0.1% Tween-20, and 9.2 m*M* citric acid (pH.6.0) at 65°C for 3 h, then probed with either antisense or sense DIG-labeled zAGRP or zPOMC probe at 65°C overnight at 500 ng/mL in hybridization buffer. DIG-labeled cRNA probes were detected with 1:500 diluted alkaline phosphatase conjugated antidigoxigenin antibody (Roche) in 2% BMB (Roche), 20% lamb serum (Gibco BRL) in MAB (100 m*M* maleic acid, 150 m*M* NaCl, 0.1% Tween-20, pH 7.5) at 4°C overnight, followed by staining with NBT/BCIP solution (Roche) at room temperature for 2–5 h. After PBS washing, methanol was applied to the stained embryos to remove the nonspecific stain, and refixed in 4% paraformaldehyde in PBS. The embryos were mounted in 100% glycerol and pictures were taken by AxionVision (Ver3.1) software with a SemiSV11 Dissecting Microscope (Carl Zeiss).

Fasting and Feeding Experiments

For fasting experiments, to minimize contamination with microorganisms, fish system water was filter-sterilized with 0.2 μm supor membrane (PALL) and at 10 hpf, 0.003% propylthiouracil (PTU) was introduced to block the deposition of pigmentation. About 100 to 200 embryos in a 500 mL beaker were fed with commercially available artificial bacteria (Zeigler) from d 5 up to d 10, and from d 10 supplemented with brine shrimp. For the fasting group, embryos from d 5 were transferred to a 500 mL beaker in filter-sterilized system water and kept there until sacrifice. Adult fish were fed two times a day as previously described in flowing fish system water. For fasting adult fish, 5–9-mo-old female fish were grouped in one tank filled with filter-sterilized fish system water, and supplied with fresh filtered system water every other day. At d 2, 5, 10, and 15 of fasting, fish were anesthetized with ice and whole intact brain tissue was dissected. Dissected brain tissues were quickly frozen in liquid nitrogen and kept at –80°C until used for preparation of RNA.

Real-Time Quantitative PCR

Real-time quantitative PCR primers were designed by web-based "primer 3" software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/) to minimize primer self-dimerization. For AGRP, forward primer 5' GTCCAC

CTGCAGAGAAGAGG 3', reverse primer 5' GCCTTAA AGAAGCGGCAGTA 3' are used, and for POMC, forward primer 5' CCCCCTACAAAATGACCCAT 3', reverse primer 5' ATCCTTCCTCGGTTGGTCTT 3' are used. Real-time PCRs were performed with 4 μL of 100 μL first-strand cDNA that is diluted five times from 20 μL initial reaction volume as a template, 5 pmol of each of forward and reverse primers, 0.15 unit of UDP glycosidase (Invitrogen), 2X SYBR PCR mix (Applied Biosystems), and with deionized water to make final volume either 10 or 15 μL in a 384-well plate (Applied Biosystems).

Real-time RT-PCRs were performed using an ABI 7900HT (Applied Biosystems). The PCR cycle was 55°C, 2 min for UDP glycosidase reaction and then initial denaturation step at 95°C for 5 min, and 40 cycles of 95°C 30 s, 60°C 30 s, 72°C 30 s, followed by 81.5°C for 1 min at each cycle for the detection of the product formation. At the end of the cycles melting curves of the products were verified for the specificity of PCR products. All measurements were performed in triplicate. SDS 2.1 software was used for the interpretation and analysis of data.

Acknowledgments

This work was supported by a research grant from Neurocrine Biosciences, Inc. (RDC). Dr. Cone and OHSU have a significant financial interest in Neurocrine Biosciences, Inc. and Znomics, Inc., companies that may have financial interests in the results of this research and technology. This potential conflict of interest has been reviewed and managed by the OHSU Conflict of Interest in Research Committee.

References

1. Cone, R. D. (1999). *Trends Endocrinol. Metab.* **10,** 211–215.
2. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J., and Cone, R. D. (1997). *Nature* **385,** 165–168.
3. Huszar, D., Lynch, C. A., Fairchild-Huntress, V., et al. (1997). *Cell* **88,** 131–141.
4. Vaisse, C., Clement, K., Guy-Grand, B., and Froguel, P. (1998). *Nat. Genet.* **20,** 113–114.
5. Yeo, G. S. H., Farooqi, I. S., Aminian, S., Halsall, D. J., Stanhope, R. G., and O'Rahilly, S. (1998). *Nat. Genet.* **20,** 111–112.
6. Roselli-Rehfuss, L., Mountjoy, K., Robbins, L., et al. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 8856–8860.
7. Mountjoy, K., Mortrud, M., Low, M., et al. (1994). *Mol. Endocrinol.* **8,** 1298–1308.
8. Butler, A. A. and Cone, R. D. (2002). *Neuropeptides* **36,** 77–84.
9. Cone, R. D., Cowley, M. A., Butler, A. A., Fan, W., Marks, D. L., and Low, M. J. (2001). *Int. J. Obes. Relat. Metab. Disord.* **25(Suppl. 5),** S63–S67.
10. Bagnol, T., Lu, X. Y., Kaelin, C. B., et al. (1999). *J. Neurosci.* **19,** RC26.
11. Cowley, M. A., Smart, J. L., Rubinstein, M., et al. (2001). *Nature* **411,** 480–484.
12. Marks, D. L., Butler, A. A., Turner, R., Brookhart, G., and Cone, R. D. (2003). *Endocrinology* **144,** 1513–1523.
13. Shutter, J. R., Graham, M., Kinsey, A. C., Scully, S., Luthy, R., and Stark, K. L. (1997). *Genes Dev.* **11,** 593–602.
14. Ollmann, M. M., Wilson, B. D., Yang, Y.-K., et al. (1997). *Science* **278,** 135–137.
15. Hahn, T. M., Breininger, J. F., Baskin, D. G., and Schwartz, M. W. (1999). *Nat. Neurosci.* **1,** 271–272.
16. Haskell-Luevano, C., Chen, P., Li, C., et al. (1999). *Endocrinology* **140,** 1408–1415.
17. Broberger, C., Johansson, C., Schalling, M., and Hokfelt, T. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 15043–15048.
18. Lin, X., Volkoff, H., Narnaware, Y., Bernier, N. J., Peyon, P., and Peter, R. E. (2000). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **126,** 415–434.
19. Takahashi, A., Amemiya, Y., Sarashi, M., Sower, S. A., and Kawauchi, H. (1995). *Biochem. Biophys. Res. Commin.* **213,** 490–498.
20 Dores, R. M. (1990). *Prog. Clin. Biol. Res.* **342,** 22–27.
21. Hansen, I. A., To, T. T., Wortmann, S., et al. (2003). *Biochem. Biophys. Res. Commun.* **303,** 1121–1128.
22. Chiba, A. (2001). *Gen. Comp. Endocrinol.* **122,** 287–295.
23. Logan, D. W., Bryson-Richardson, R. J., Pagan, K. E., Taylor, M. S., Currie, P. D., and Jackson, I. J. (2003). *Genomics* **81,** 184–191.
24. Ringholm, A., Fredriksson, R., Poliakova, N., et al. (2002). *J. Neurochem.* **82,** 6–18.
25. Cerda-Reverter, J. M., Ringholm, A., Schioth, H. B., and Peter, R. E. (2003). *Endocrinology* **144,** 2336–2349.
26. Cerda-Reverter, J. M. and Peter, R. E. (2003). *Endocrinology* **144,** 4552–4561.
27. Rossi, M., Kim, M. S., Morgan, D. G., et al. (1998). *Endocrinology* **139,** 4428–4431.
28. Bures, E. J., Hui, J. O., Young, Y., et al. (1998). *Biochemistry* **37,** 12172–12177.
29. Tota, M. R., Smith, T. S., Mao, C., et al. (1999). *Biochemistry* **38,** 897–904.
30. Liu, N. A., Huang, H., Yang, Z., et al. (2003). *Mol. Endocrinol.* **17,** 959–966.
31. Narnaware, Y. K., Peyon, P. P., Lin, X., and Peter, R. E. (2000). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **279,** R1025–R1034.
32. Westerfield, M. (1995). The zebrafish book. Eugene, OR: The University of Oregon Press.
33. Kimmel, C., Ballard, W., Kimmel, S., Ullmann, B., and Schilling, T. (1995). *Dev. Dyn.* **203,** 253–310.
34. Imboden, M., Devignot, V., Korn, H., and Goblet, C. (2001). *Neuroscience* **103,** 811–830.
35. Willett, C. E., Zapata, A. G., Hopkins, N., and Steiner, L. A. (1997). *Dev. Biol.* **182,** 331–341.
36. Mizuno, T. M., Makimura, H., Silverstein, J., Roberts, J. L., Lopingco, T., and Mobbs, C. V. (1999). *Endocrinology* **140,** 814–817.
37. Phillips-Singh, D., Li, Q., Takeuchi, S., Ohkubo, T., Sharp, P. J., and Boswell, T. (2003). *Cell Tissue Res.* **313,** 217–225.