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1 Pigment-dispersing activities and cortisol-releasing activities of melanocortins
2 and their receptors in xanthophores and head kidneys of the goldfish *Carassius*
3 *auratus*
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33 Abstract

34 The five subtypes of melanocortin receptors (MCRs) mediate the functions
35 of α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone
36 (ACTH). In fish, these hormones are involved in pigment dispersion and cortisol
37 release, respectively. α-MSH-related peptides exhibit ACTH-like activity in
38 certain fishes. We recently found that multiple *Mcr* transcripts are expressed in
39 some cell types in the barfin flounder, which is related to regulation of α-MSH
40 activities. Similar results were also observed for the cortisol-releasing activity
41 of α-MSH-related peptides in the head kidney. The present study was
42 undertaken to assess relationship between the expression of multiply expressed
43 *Mcrs* and α-MSH activities using goldfish. We also determined if α-MSH-related
44 peptides exhibit ACTH-like activity in goldfish. The transcripts of *Mc1r,* but not
45 those of other subtypes, were observed in xanthophores. α-MSH, which has an
46 acetyl group at the N-terminus, was found to disperse pigment in a dose-
47 dependent manner in xanthophores. This potency was found to be slightly
48 greater than that of desacetyl-α-MSH. These results support our findings that
49 MCR has a higher affinity for α-MSH when single *Mcr* subtype is expressed. On
50 the other hand, transcripts of *Mc2r,* but not those of other subtypes, were
51 observed in the head kidney. $ACTH_{1-24}$-stimulated cortisol release was observed
52 in a dose-dependent manner, while α-MSH-related peptides showed no activity.
53 It therefore appears that MC2R also acts as an ACTH-specific receptor in goldfish
54 and that association of α-MSH-related peptides upon release of cortisol is
55 uncommon in fishes.

56

57

58 Keywords: adrenocorticotropic hormone (ACTH); cortisol-release; goldfish;
59 melanocortin; melanocortin receptor; melanocyte-stimulating hormone (MSH);
60 pigment-dispersion.

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1. Introduction

α-Melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) are peptide hormones liberated from a common precursor known as proopiomelanocortin (POMC) [54–56]. While POMC is biosynthesized in both the pars distalis (PD) and the neurointermediate lobe (NIL) of the pituitary in teleost fish, ACTH is mainly produced in the PD, and α-MSH in the NIL in a manner that depends upon tissue-specific proteolytic cleavage [6, 21, 35, 39, 51]. While ACTH is generally composed of 39 amino acid residues, α-MSH is identical to *N*-acetyl-$ACTH_{1\text{-}13}$-amide. ACTH, α-MSH, and their related peptides are collectively classified as melanocortins (MCs) on the basis of the presence of the common amino acid sequence—His-Phe-Arg-Trp [12].

MC receptors (MCRs) are members of the G protein-coupled receptor (GPCR) family. The members of this family have seven transmembrane domains [16, 33, 34]. MC system consisting of POMC and MCRs in fish have been shown to be similar to that of mammals. For example, the ACTH signal is mainly mediated by the MC2 receptor (MC2R), which is one of the five subtypes of MCRs [23, 48]. While MC2R selectively binds ACTH, ACTH can bind to other MCRs in addition to MC2R [1, 23, 48]. Although α-MSH does not bind to MC2R, the signal of α-MSH, is mediated by other MCRs. The representative biological activities of ACTH and α-MSH (including cortisol release from interrenal cells [57 60] and pigment dispersion [12, 14, 15], respectively) are related to tissue-specific expression of different MCR subtypes [33]. However, the studies on the biological activities of the MC system using barfin flounder, *Verasper moseri*, a teleost, have shown interesting relationships between different molecular forms of α-MSH-related peptides and MCRs [25–27, 56].

The barfin flounder is a large commercially important flatfish that inhabits the Pacific coast of northern Japan. We demonstrated the existence of pigment-dispersing activities of α-MSH-related peptides in skin parts [26]. Interestingly, while α-MSH modified with a monoacetyl group at N-terminus was found to mediate dispersion of pigments in xanthophores, this peptide exhibited negligible pigment-dispersing activities in melanophores. On the contrary, desacetyl (Des-Ac)-α-MSH, which lacks the acetyl group, has pigment-dispersing activities in

both xanthophores and melanophores, while the activities in xanthophores were found to be lower than the activities caused by α-MSH. Subsequently, we identified expression of only *Mc5r* in xanthophores and concomitant expression of *Mc1r* and *Mc5r* in melanophores [27]. Similar relationships between the degree of acetylation and biological activities were also observed for *in vitro* cortisol-releasing activities in the barfin flounder. While Des-Ac-α-MSH–stimulated cortisol release from interrenal cells was observed, α-MSH showed negligible effects. Moreover, both *Mc2r* and *Mc5r* were expressed in the interrenal cells [25].

There is a growing body of evidence indicating that many GPCRs form heterodimers that may affect ligand affinity [2, 5, 13, 28, 32, 36, 38, 43, 47]. Therefore, the concomitant expression of the different *Mcr* subtypes in melanophores and interrenal cells led to the assumption that a heterodimer consisting of MC1R and MC5R in melanophores, or MC2R and MC5R in interrenal cells, may have low binding affinity for α-MSH [25, 27]. This assumption conversely suggests that expression of only one *Mcr* subtype may lead to enhancement of biological activity of α-MSH relative to that of Des-Ac-α-MSH. In fact, acetylation enhances the activities of α-MSH-related peptides via MCRs expressed in human embryonic kidney-293 cells [44–46]. The present studies were undertaken to examine these possibilities using pigment cells and head kidney tissues from goldfish, *Carassius auratus*. We also determined if the cortisol-releasing activities of α-MSH-related peptides are common to goldfish by determining whether other *Mcrs*, in addition to *Mc2r*, are expressed in goldfish head kidney.

2. Materials and Methods

2.1. Fish

Immature goldfish, *C. auratus*, were obtained from a commercial dealer in Shizuoka, Japan, and all experiments were conducted according to the Guidelines for the Care and Use of Animals of Kitasato University. The fish were reared in indoor tanks with circulating freshwater under a natural

129 photoperiod. The average body sizes of the fish used for molecular cloning and
130 gene expression tests and pigment-dispersing activities were 4.9 cm, standard
131 length (SL), and 3.9 g body weight (BW). For these experiments, tissue samples
132 were collected from fish anesthetized with 0.2% 2-phenoxyethanol, and
133 subsequently frozen in dry ice/ethanol bath. Skin samples used for
134 measurements of pigment-dispersing activities and for cell dispersion were
135 collected from fish (4.9 cm SL, 4.1 g BW on average) anesthetized with ice-cold
136 water. Head kidneys used in experiments for cortisol-releasing activities were
137 collected from fish (13.5 cm SL, 87 g BW on average) anesthetized with 0.2% 2-
138 phenoxyethanol.

139

140 *2.2. Peptides*

141 α-MSH was purchased from the Peptide Institute (Osaka, Japan).
142 Diacetyl (Di-Ac)-α-MSH was purchased from Sigma Chemical (St. Louis, MO,
143 USA). Des-Ac-α-MSH and $ACTH_{1-24}$ were synthesized and purified according to
144 the previously described methods [53]. The amino acid sequence of $ACTH_{1-24}$,
145 which is identical to that of barfin flounder ACTH-A, salmon ACTH-A, and tuna
146 ACTH [52], differs by one residue with respect to the sequence of goldfish ACTH
147 at position 20 (Ile in goldfish *vs* Val in others) [11].

148

149 *2.3. Molecular cloning*

150 *2.3.1. Nucleic acid preparation for sequence determination*

151 Total RNA was extracted from brain and head kidney using Isogen (Nippon
152 Gene, Tokyo, Japan). First-strand cDNAs were synthesized from total brain
153 RNA for amplification of *Mc1r* and *Mc3r*, cDNA, and from head kidney RNA for
154 *Mc2r* cDNA with the SMART RACE cDNA Amplification Kit (BD Biosciences,
155 Palo Alto, CA, USA). Custom oligonucleotides were synthesized at Nihon Gene
156 Research Labs Inc. (Sendai, Japan).

157

158 *2.3.2. Amplification of DNA fragments for sequence determination*

159 Polymerase chain reaction (PCR) using a thermal cycler (MJ Mini, BIO-
160 RAD, Hercules, CA, USA) under conventional conditions was performed to

161 amplify the DNA fragments with HotStar Taq Master Mix (Qiagen, Hilden,
162 Germany), or Takara LA Taq (Takara, Otsu, Japan). Amplification procedures
163 were common for all three *Mcr* cDNAs. First, the middle segment of the cDNA
164 of each *Mcr* was amplified from first-strand cDNA by PCR using HotStar Taq
165 DNA polymerase with primers designed from fish *Mcr* nucleotide sequences.
166 Then, the 3′ region of each *Mcr* cDNA was amplified from first-strand cDNA by 3′
167 rapid amplification of cDNA ends (3′RACE) using HotStar Taq DNA polymerase
168 with a gene-specific primers and Universal Primer A Mix (UPM) provided in the
169 SMART RACE cDNA Amplification Kit. The 5′ region of each *Mcr* cDNA was
170 amplified from first-strand cDNA by 5′RACE using HotStar Taq DNA polymerase
171 with UPM and gene-specific primers. Finally, each *Mcr* cDNA containing the
172 full-length reading frame was amplified from first-strand cDNA by PCR using
173 HotStar Taq DNA polymerase with gene-specific primers. Primer sequences are
174 listed in Table 1.
175
176 *2.3.3. Sequence determination and data processing*
177 PCR-amplified DNA was purified by agarose gel electrophoresis (NuSieve
178 GTG Agarose; Cambrex Bio Science, Rockland, ME, USA). DNA was extracted
179 from the agarose gel using a QIAEX II Gel Extraction Kit (Qiagen), ligated into
180 plasmid pT7 Blue T-Vector (Novagen, Madison, WI, USA) or pSTBlue-1 AccepTor
181 Vector (Novagen) and transfected into JM109-competent cells. Recombinant
182 plasmid DNA was prepared using the alkaline-SDS method and both strands
183 were sequenced using a capillary DNA sequencer (3130-Avant Genetic Analyzer;
184 Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator Cycle
185 Sequencing Ready Kit ver. 3.1. DNASIS-Pro (Hitachi Software Engineering,
186 Yokohama, Japan) was used to process nucleotide and amino acid sequences, to
187 calculate amino acid sequence identity, to align amino acid sequences, and to
188 construct a phylogenetic tree by the neighbor-joining (NJ) method [37].
189 Transmembrane domains were predicted using a program for the prediction of
190 transmembrane helices in proteins "TMHMM Server v2.0"
191 (http://www.cbs.dtu.dk/services/TMHMM-2.0/).
192

193 *2.4. Reverse transcription (RT)-PCR for tissue distribution*

194 Dorsal skin, caudal fin, and head kidney tissue were taken from three
195 goldfish. A sample of RNA was prepared using Isogen (Nippon Gene). RNA
196 was then treated with TURBO DNase (Ambion, Austin, TX, USA) for 4 h at 37°C.
197 RNA yield was estimated by spectrophotometry. For each tissue, an equal
198 amount of total RNA (100 ng) from three individuals was combined and subjected
199 to amplification using a One-Step RT-PCR kit (Qiagen) with primer sets shown in
200 Table 2. *β-actin* cDNA was used as a positive control. PCR products were
201 electrophoresed on 3% agarose gel (Agarose S, Nippon Gene) and visualized with
202 0.025% ethidium bromide. Photographs were taken using a Densitograph (Atto,
203 Tokyo, Japan).

204

205 *2.5. Skin cell dispersion and RT-PCR*

206 Skin cell dispersion was performed as described previously [26, 27].
207 Small parts of caudal fin or dorsal skin were rinsed in Hanks' balanced salt
208 solution (HBSS). The samples were allowed to stand for 20 min at room
209 temperature in a dissociation medium [DM: 1 mg/mL collagenase type III
210 (Worthington, Freehold, NJ, USA), 1×10^{-4} M epinephrine (Sigma-Aldrich, St.
211 Louis, MO, USA), 2 mg/mL bovine serum albumin (Sigma-Aldrich), 0.1 mg/mL
212 soybean trypsin inhibitor (Roche, Indianapolis, IN, USA), and 5 U/mL DNase I
213 (Takara)] and then gently agitated for 10 min in the same solution. The DM
214 was removed, and the samples were rinsed three times with HBSS. Finally,
215 during the gentle agitation in fresh DM, dispersed single xanthophores and other
216 nonchromatophoric dermal cells were isolated using glass capillaries under a
217 microscope. cDNA from an isolated cell was synthesized using the Super-Script
218 III CellsDirect cDNA Synthesis System (Invitrogen, Carlsbad, CA, USA),
219 according to the manufacturer's instructions. The cDNA obtained from three
220 cells was dissolved in 90 μL H_2O, and a 5-μL aliquot was analyzed using PCR to
221 detect *Mcr* mRNAs. As a control, 2 μL was analyzed to detect *β-actin* mRNA.
222 The primer sets for *Mcrs* and *β-actin* mRNA were the same as those used in
223 section 2.4. The PCR conditions programmed into the thermal cycler (MJ Mini;
224 BIO-RAD) included reverse transcription at 50°C for 50 min followed by

225 amplification of the appropriate cDNA fragment with activation of the enzyme at
226 95°C for 15 min followed by 40 cycles of (i) denaturation for 15 s at 94°C, (ii)
227 annealing for 30 s at 60°C, and (iii) extension for 40 s at 72°C. The PCR
228 products were electrophoresed and visualized as described above. The series of
229 experiments from cell dispersion to PCR was repeated three times.

230

231 *2.6. Incubation of fish scales with MC peptides for measurements of pigment-*
232 *dispersing activities*

233 Scales removed from the dorsal skin were incubated in HBSS for 1 h at
234 20°C. After changing HBSS, the scales were incubated under the same
235 conditions. Subsequently, the scales were incubated in HBSS containing serially
236 diluted MSH at final concentration of 1 nM to 1 μM for 1 h. Photographs were
237 taken using a light microscope equipped with a digital still camera (PDMCII,
238 Olympus, Japan), and subsequently ten randomly selected xanthophores from
239 five skin parts were observed for each peptide concentration. The xanthophore
240 index (XI) was calculated to evaluate the pigment-dispersing activity of each
241 peptide by analogy with melanophore index [11] . An average XI obtained from
242 10 xanthophores represented XI of each skin part (n = 5).

243

244 *2.7. Incubation of head kidney parts with MC peptides for measuring cortisol-*
245 *releasing activities*

246 L-15 medium was used for incubation. The washing and incubation
247 temperature was 20°C. Head kidney tissues dissected from several goldfish
248 were diced to approximately 1 mm^3 and combined. A flask containing the diced
249 tissue (1 g/100 mL) was incubated for 30 min with occasional shaking. This
250 incubation for washing was repeated three times and the medium was exchanged
251 at the end of each incubation period. Following the transfer of approximately 20
252 mg of the diced tissues to each well of a 48-well plate, the tissue was
253 preincubated for 90 min in 0.5 mL medium. After changing the medium, the
254 tissue was incubated for an additional 60 min in 0.5 mL medium containing MC
255 peptides ($ACTH_{1-24}$, Des-Ac-α-MSH, α-MSH, or Di-Ac-α-MSH). Final
256 concentrations ranged from 10 nM to 100 nM. Control experiments were carried

257 out for the same time periods without including hormone in the medium. Each
258 assay was performed in duplicate (n = 5). Preincubation and incubation media
259 were used for the cortisol assay. Cortisol levels after incubation were expressed
260 as percentage of the levels present in the preincubation media. Cortisol was
261 extracted from the medium as described previously [63]. The cortisol levels
262 were measured using a time-resolved fluoroimmunoassay for cortisol [62].
263
264 *2.8. Statistics*
265 All data are expressed as the mean ± standard error values. The
266 variances of the xanthophore index were tested by the Kruskal–Wallis test [50], a
267 nonparametric test analog to one-way analysis of variance (ANOVA). When
268 significant differences were detected, comparisons of treatments versus the
269 control were performed with a post hoc multiple comparison test for the Kruskal–
270 Wallis test. The amounts of cortisol in the head kidney were compared by one-
271 way ANOVA. Significance was determined at 5% level.
272
273
274 3. Results
275 *3.1. Nucleic acid sequence determination*
276 Because *Mc4r* and *Mc5r* cDNAs have been cloned in goldfish [8, 10], the
277 nucleotide sequences of *Mc1r*, *Mc2r*, and *Mc3r* cDNAs were determined in the
278 present study. Fig. 1 shows the assembly of cDNA fragments amplified by RT-
279 PCR for each *Mcr* cDNA. Nucleotide sequences were determined using these
280 fragments.
281
282 *3.1.1. Nucleic acid sequence of goldfish Mc1r cDNA*
283 The nucleotide sequence of a cDNA amplified from goldfish brain total
284 RNA was found to encode a reading frame consisting of 321 amino acid residues
285 (Fig. 2). Phylogenetic analysis revealed that the new sequence is localized in a
286 clade of MC1R of various species in a phylogenetic tree for the five different
287 MCRs (Fig. 3). This indicates that the new receptor sequence encodes MC1R of
288 goldfish. The locations of the seven transmembrane domains were predicted as

289 shown in Fig. 2. The DRY motif is present at a position homologous to that of
290 MC1R of the other species [33]. There are three potential sites for *N*-linked
291 glycosylation [4] and eight potential sites for phosphorylation by protein kinase C
292 [61] (Fig. 2).
293
294 *3.1.2. Nucleic acid sequence of goldfish Mc2r cDNA*
295 The nucleotide sequence of a cDNA amplified from goldfish head kidney
296 total RNA was found to encode a reading frame consisting of 302 amino acid
297 residues (Fig. 2). Phylogenetic analysis revealed that the new sequence is
298 grouped into a clade together with the remaining MC2Rs (Fig. 3). This indicates
299 that the new receptor sequence encodes for the goldfish MC2R. The locations of
300 the seven transmembrane domains are shown in Fig. 2. The DRY motif is
301 present at a position homologous to that of MC2R of the other species. There is
302 one potential site for *N*-linked glycosylation as well as three potential sites for
303 phosphorylation by protein kinase C (Fig. 2).
304
305 *3.1.3. Nucleic acid sequence of goldfish Mc3r cDNA*
306 The nucleotide sequence of a cDNA amplified from total RNA of goldfish
307 brain was found to encode a reading frame consisting of 327 amino acid residues
308 (Fig. 2). Phylogenetic analysis revealed that the new sequence is related to the
309 MC3Rs in other species (Fig. 3). This indicates that the new receptor sequence
310 encodes a goldfish MC3R. The locations of the seven transmembrane domains
311 were predicted as shown in Fig. 2. The DRY motif is present at a position
312 homologous to that of MC3R of the other species. There are three potential sites
313 for *N*-linked glycosylation and two potential sites for phosphorylation by protein
314 kinase C (Fig. 2).
315
316 *3.2. Expression of Mcrs in goldfish skin, fin and head kidney*
317 Fig. 4 shows the detection by RT-PCR of all cloned goldfish *Mcrs* in the fin,
318 skin and head kidney. The negative control for the expression of each *Mcr* and
319 amplification of positive control *β-actin* are also shown. Expression of *Mc1r* was
320 observed in skin and fin. Expression of *Mc2r* was observed in head kidney.

321 When RT-PCR was performed for the all five goldfish *Mcrs* by using total RNA
322 extracted from single cells of the fin and skin, a cDNA fragment of *Mc1r* was
323 amplified from the total RNA derived from xanthophores (Fig. 5). None of the
324 cDNA fragments of the five subtypes of *Mcr* were amplified from the total RNA
325 derived from nonchromatophoric dermal cells. Because of the limitations of the
326 kit used in this experiment, the absence of genomic DNA in the total RNA
327 samples was indirectly evaluated using *β-actin* amplification. The *β-actin*
328 fragment containing the intron was never amplified (Fig. 5).
329
330 *3.3. Biological activities of α-MSH-related peptides*
331 *3.3.1. Pigment-dispersing activities*
332 Fig. 6 shows the effects of α-MSH-related peptides on *in vitro* pigment
333 dispersion in goldfish scales. Des-Ac-α-MSH was found to stimulate the
334 dispersion of pigments in xanthophores in a dose-dependent manner (Fig. 6A).
335 α-MSH and Di-Ac-α-MSH also showed similar effects on pigment dispersion (Figs.
336 6B and C). At a concentration of 1 nM, the effect of α-MSH was greater than
337 that of Des-Ac-α-MSH. At a concentration of 100 nM, the effect of Di-Ac-α-MSH
338 was greater than that of Des-Ac-α-MSH.
339
340 *3.3.2. Cortisol-releasing activities*
341 The effects of MC peptides on *in vitro* cortisol release from the head
342 kidney of goldfish are shown in Fig. 7. $ACTH_{1–24}$ was found to stimulate cortisol
343 release in a dose-dependent manner. However, Des-Ac-α-MSH, α-MSH, and Di-
344 Ac-α-MSH were found to have negligible effects on cortisol release.
345
346
347 4. Discussion
348 *4.1. Phylogenetic distribution of MCR and the MC system in goldfish*
349 The presence of MCR has been demonstrated across a wide spectrum of
350 vertebrate classes, including Cephalaspidomorphi (lampreys), Chondrichthyes
351 (sharks), Sarcopterygii (lobe-finned fish including tetrapods), and Actinopterygii
352 (ray-finned fish) [17, 18, 24, 48]. While mammals and chickens possess five

353 MCR subtypes (MC1R to MC5R), zebrafish is the only fish species in which the
354 five subtypes were demonstrated with MC5R subdivided into MC5Ra and MC5Rb
355 [30]. *Fugu* lacks *Mc3r*, while four *Mcr* subtypes have been identified by genomic
356 studies [23, 30]. In goldfish, the presence of *Mc4r* and *Mc5r* has been shown in
357 our previous studies [8, 10]. Therefore, the identification of *Mc1r, Mc2r*, and
358 *Mc3r* cDNA provides a second line of evidence for the presence of five MCR
359 subtypes in fish. The cypriniformes including goldfish and zebrafish are a
360 rather primitive group of ray-finned fish when compared to tetradontiformes [37].
361 It is therefore possible that the five subtypes of MCR may have appeared in a
362 common ancestor of ray-finned fish and tetrapods. Subsequently, in the branch
363 leading to derived group of ray-finned fish such as tetradontiformes, *Mc3r* may
364 have been deleted during the course of evolution.
365 In goldfish, the primary structure of POMC has been reported previously
366 [11]. MC peptides such as Des-Ac-α-MSH, α-MSH, Di-Ac-α-MSH, and some
367 ACTH variants released from pituitary cells have been identified [58]. Moreover,
368 primary structures of agouti-signaling protein and agouti-related protein as
369 endogenous antagonists for α-MSH have been reported in goldfish [7, 9]. Here,
370 we demonstrated the presence of five MCR subtypes thus showing that the
371 goldfish MC system possesses a molecular repertoire comparable to that of
372 tetrapods.
373
374 *4.2. Effects of α-MSH-related peptides on pigment dispersion*
375 The pigment-dispersing activity of Des-Ac-α-MSH, α-MSH, and Di-Ac-α-
376 MSH are almost equal in goldfish, but the activity of α-MSH is somewhat greater
377 than that of Des-Ac-α-MSH. These results are similar to those observed in
378 xanthophores of barfin flounder [26]. In both species, the xanthophores express
379 only one *Mcr* subtype, i.e. *Mc1r* and *Mc5r* in goldfish and barfin flounder,
380 respectively. On the other hand, the response of goldfish xanthophores to α-
381 MSH is quite different from that of melanophores of barfin flounder in which α-
382 MSH has negligible effects on melanin dispersion in a range from 1 nM to 1 μM.
383 Goldfish xanthophores are also different from barfin flounder melanophores
384 because two different subtypes of *Mcr—Mc1r* and *Mc5r*—are expressed in the

385 flounder melanophores [27]. We recently observed that *Mc1r* and *Mc5r* are
386 expressed in melanophores of Japanese flounder where α-MSH showed no effects
387 on pigment dispersion, while Des-Ac-α-MSH is effective. Moreover, both α-MSH
388 and Des-Ac-α-MSH are effective in xanthophores where only *Mc5r* is expressed
389 (manuscript in preparation). Taken together, these results indicate that there is
390 a good correlation between pigment-dispersing activities, the degree of
391 acetylation, and the specific *Mcr* subtype(s) expressed in chromatophores. α-
392 MSH-related peptides exhibit pigment-dispersing activities irrespective of the
393 degree of acetylation when one type of *Mcr* is expressed and monoacetylation,
394 which leads to generation of α-MSH, cancels the activities when two types of
395 *Mcrs* are concomitantly expressed.
396 Monoacetylation of the N-terminus may contribute to an increase in
397 pigment-dispersing activity of α-MSH-related peptides in goldfish because the
398 activity of α-MSH was found to be slightly but significantly higher than Des-Ac-α-
399 MSH when their effects at the concentration of 1 nM were compared.
400 Pharmacological studies on sea bass MC1R have revealed that α-MSH is more
401 effective than Des-Ac-α-MSH in stimulation of cellular activities [44].
402 Acetylation-mediated augmentation of the binding affinity of α-MSH-related
403 peptides was also observed during pharmacological studies with human and
404 mouse MC1R [3, 49]. Taken together, these results suggest that α-MSH may
405 have higher activity than Des-Ac-α-MSH as a result of increased affinity for an
406 MCR when only one MC subtype (at least MC1R or MC5R) is present. Similar
407 enhancing effects of pigment-dispersion caused by monoacetylation have also
408 been observed in grass carp, tilapia, and frogs [12, 22, 59]. According to our
409 data, it is reasonable to propose that only one Mcr subtype may be expressed in
410 melanophores of these species.
411 The pigment-dispersing activity of α-MSH was found to be
412 indistinguishable from that of Di-Ac-α-MSH in the goldfish xanthophores. In
413 pharmacological studies of sea bass, α-MSH was found to have efficacy similar to
414 that of Di-Ac-α-MSH for stimulation of cellular activities via MC1R [45]. Given
415 that goldfish MC1R may have similar properties to sea bass, it is likely that α-

416 MSH and Di-Ac-α-MSH interact with goldfish MC1R with similar binding
417 affinities.
418
419 *4.3. Effects of ACTH and α-MSH-related peptides on cortisol-release*
420 Recently, we reported on interesting relationships among molecular forms
421 of α-MSH-related peptides with respect to N-terminal acetylation, cortisol-
422 releasing activity, and *Mcr* subtypes expressed in interrenal cells of barfin
423 flounder [25]. First, Di-Ac-α-MSH and Des-Ac-α-MSH were found to stimulate
424 cortisol release, and the former showed greater activity than the latter, while the
425 activity of α-MSH was negligible. Second, transcripts of *Mc2r* and *Mc5r* were
426 detected in the interrenal cell. These relationships are similar to those observed
427 for pigment-dispersing activities of α-MSH-related peptides in melanophores of
428 this specie [26, 27]. Hence, we assumed that a heterodimer consisting of MC2R
429 and MC5R is associated with the differences in the activity of these peptides in
430 the interrenal cells. The present study was undertaken to characterize the
431 relationships between all the three players in the goldfish. The *Mc2r* was found
432 to be the only *Mcr* expressed in head kidney containing interrenal cells and,
433 supporting our hypothesis, the three α-MSH-related peptides were found to not
434 have cortisol-releasing activities.
435 Among the five MCR subtypes, MC2R has been shown to be an ACTH-
436 specific receptor because MC2R exclusively binds ACTH but not α-MSH-related
437 peptides [1, 23, 48]. A classical study has shown that a central region of the
438 ACTH molecule, which consists of basic amino acid residues, is important for
439 binding to the receptor [19, 40]. These properties have been confirmed using
440 native and expressed ACTH-receptors (namely MC2R) [19, 20, 41]. In the
441 present study, it was found that $ACTH_{1-24}$, which consists of an N-terminal region
442 corresponding to α-MSH, a central basic region corresponding to $ACTH_{15-18}$, and a
443 short C-terminal region corresponding to $ACTH_{19-24}$, exhibits dose-dependent
444 cortisol releasing activity. These results confirm that ACTH is a major
445 secretagogue of cortisol in goldfish and MC2R the main link of the pituitary-
446 interrenal axis.

447 Because MC2R is a specific receptor for ACTH, the other MC peptides
448 never interact with this receptor [23, 33, 48]. Therefore, the finding that there
449 are no cDNAs for *Mcr* subtypes other than *Mc2r* cDNA is in agreement with the
450 lack of cortisol-releasing activities of α-MSH-related peptides in goldfish head
451 kidney tissues. Also in carp, ACTH stimulates cortisol release, but α-MSH does
452 not [31]. The presence and absence of the MC2R and MC5R, respectively, have
453 been shown for this species, while investigations of the other MCR subtypes have
454 not been reported. Even though cypriniformes such as goldfish and carp
455 generally have only the MC2R subtype in interrenal cells, two MCR subtypes
456 may heterodimerize in tilapia, rainbow trout and barfin flounder because
457 cortisol-releasing activities of α-MSH-related peptides have been observed in
458 these species [25, 29, 42]. Interestingly, tilapia has some similarities to barfin
459 flounder in relation to potency of cortisol-releasing activities and degrees of
460 acetylation. Di-Ac-α-MSH has greater activity than Des-Ac-α-MSH, and α-MSH
461 exhibits the lowest activity among the three α-MSH-related peptides. Similar
462 results have also been observed in investigations of rainbow trout, while data are
463 not available for Di-Ac-α-MSH.
464
465
466 5. Conclusions
467 We recently hypothesized that coexpression of different types of *Mcr* leads
468 to the formation of heterodimers of MCRs, which decrease the pigment-
469 dispersing activities of α-MSH. The activity of this peptide is enhanced only
470 when one subtype of MCR is present [26, 27]. In goldfish xanthophores, where
471 only *Mc1r* was expressed, α-MSH displayed similar activity to that of Des-Ac-α-
472 MSH and Di-Ac-α-MSH; moreover, the activity of α-MSH was greater than that
473 of Des-Ac-α-MSH. These results support our assumption. On the other hand,
474 $ACTH_{1-24}$, but not any α-MSH-related peptides, was found to stimulate cortisol
475 release from goldfish head kidney where only the *Mc2r* subtype is expressed.
476 These results indicate that MC2R is specific for ACTH and the link member in
477 the pituitary-interrenal axis of the goldfish.
478

479

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795 Figure legends

796 Figure 1. Schematic representation of the relative positions of the
797 DNA fragments of *Mc1r* (A), *Mc2r* (B), and *Mc3r* (C) in goldfish. DNA fragments
798 were amplified from brain or head kidney cDNA. Boxes show reading frames.
799 Horizontal arrow shows relative positions and direction of primers. The
800 numbers show positions on each cDNA. For details regarding the nucleotide
801 sequences, see Accession No. AB618067 for *Mc1r*, AB618068 for *Mc2r*, and
802 AB618069 for *Mc3r*.

803

804 Figure 2. Amino acid sequences of five subtypes of goldfish MCRs. Common
805 amino acids with MC1R are shaded. Transmembrane domains (TM) were
806 deduced for each MCR subtype. Italicized amino acids show potential *N*-
807 glycosylation motifs. Lowercase letters show potential protein kinase C
808 phosphorylation motifs. Numbers in parentheses show sequence identity with
809 MC1R sequence. ###DRY motif.

810

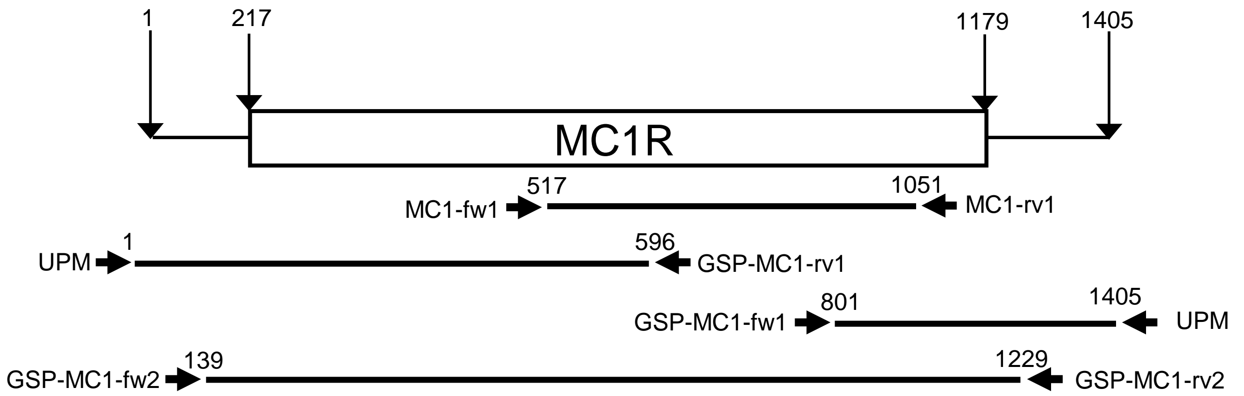
811 Figure 3. Phylogenetic tree for MC receptors of fish, chickens, and mammals
812 including 5 MCR subtypes of goldfish constructed by the NJ method. Accession
813 numbers of MC1R for *Fugu*: AAO65548, flounder: AB287974, goldfish: AB618067,
814 zebrafish: NP_851301, chicken: P55167, mouse: BAD16661, and human:
815 Q01726; MC2R for *Fugu*: AO65550, flounder: AB541411, goldfish: AB618068,
816 zebrafish: NP_851302, chicken: BAA24002, mouse: NP_032586, and human:
817 AAH69074; MC3R for goldfish: AB618069, zebrafish: NP_851303, chicken:
818 BAA32555, mouse: NP_032587, and human: AAH69599; MC4R for *Fugu*:
819 AAO65551, flounder: AB287975, goldfish: CAD58853, zebrafish: NP_775385,
820 chicken: AAT73773, mouse: P56450, and human: AAH69172; MC5R for *Fugu*:
821 AAO65553, flounder: AB540951, goldfish: CAE11349, zebrafish-a: NP_775386,
822 zebrafish-b: NP_775387, chicken: BAA25640, mouse: P41149, and human:
823 AAH69545.

824

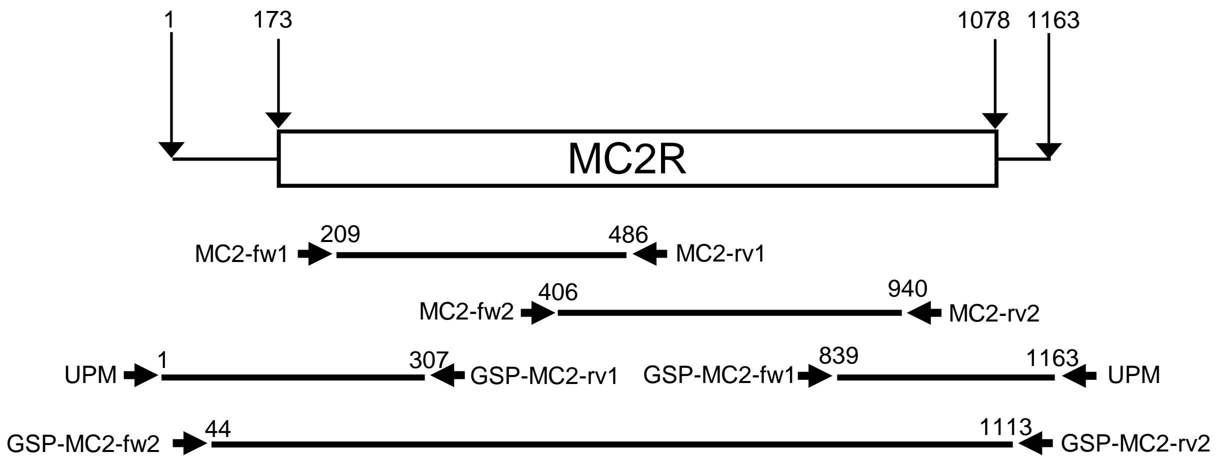
825 Figure 4. Expression of *Mcrs* in caudal fin (A), dorsal skin (B), and head kidney
826 (C). Total RNA samples prepared from these tissues were subjected to RT-PCR

827 (RT+) using specific primers for *Mcrs* (see Table 2 for primer sequences).
828 Numbers indicate *Mcr* subtypes. "RT−" indicates negative control (PCR for *Mcrs*
829 in total RNA). "Positive control" shows amplification of *β-actin* fragment in each
830 tissue.
831
832 Figure 5. Expression of *Mcrs* in cells isolated from the caudal fin (A) and dorsal
833 skin (B). RT-PCR was performed using the total RNA extracted from
834 xanthophores and nonchromatophoric dermal cells (see Table 2 for primer
835 sequences). Numbers indicate *Mcr* subtypes. Total RNAs prepared from three
836 single cells were combined. *β-Actin* was used as an internal control.
837 Amplification of the *β-actin* fragment from xanthophores "X," and
838 nonchromatophoric dermal cells "D" was not observed. Genomic DNA was use
839 as a template in "G." The amplified DNA for *β-actin* contained one intron.
840
841 Figure 6. Pigment-dispersing activity of synthetic Des-Ac-α-MSH, α-MSH, and
842 Di-Ac-α-MSH on xanthophores. XI, xanthophore index analogous to the
843 melanophore index. Asterisks show significant differences compared to the
844 control value determined by a post hoc comparison test for the Kruskal–Wallis
845 test at $P < 0.05$. Alphabetical letters indicate the differences in potency among
846 the three peptides at one dose according to the results of the same test at $P <$
847 0.05 (n = 5). For example, the pigment-dispersing activity of 1 nM α-MSH was
848 greater than that of 1 nM Des-Ac-α-MSH.
849
850 Figure 7. Effects of MC peptides, including $ACTH_{1-24}$, Des-Ac-αMSH, α-MSH,
851 and Di-Ac-α-MSH on *in vitro* cortisol release from the head kidney of goldfish.
852 Head kidney tissue parts were preincubated for 1 h and then followed by
853 incubation with each MC peptide for 1 h. The concentration of cortisol in the
854 incubation medium was estimated relative to that of the preincubation medium.
855 Asterisks show significant differences compared to the control value by ANOVA
856 at $P < 0.05$ (n = 5).
857

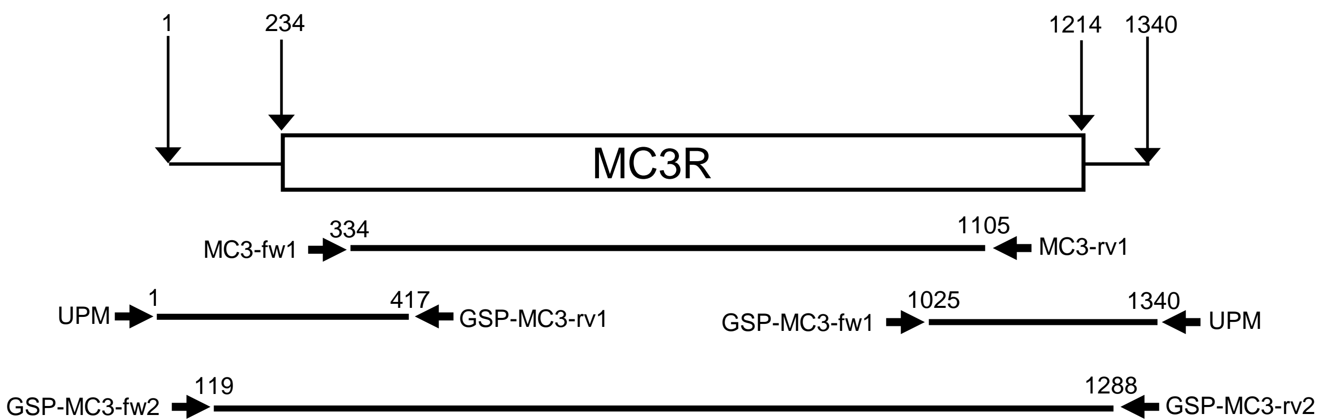
A



B



C



Kobayashi et al. Fig. 1

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MC1R    MNDssrHYFsmkHMDYIYNIDNNITLNTTLGEMNATGIAQIMIPQELFLMLGLISLVENILVVAAIIKN  69
MC2R    MNSS-----------------------TEALSTHPTDCAEVQVPSQVFMAIAVASLSENILVILAVIKN  46
MC3R    MNDSYLQFLKGQKPANSTSLPPNGSTVDPPAG---ALCEQVQIQAEVFLTLGIVSLLENILVILAVVKN  66
MC4R    MNTSHHHGPHHsyrNHSQGALPVGKPDQGERGSTSGCYEQLLISTEVFLTLGLVSLLENILVIAAIIKN  69
MC5R   MMNTSEATLSLWAISANSSPVLDLLNTTETPSHAKPKACEQLNIATEVFLILGIISLLENILVICAIVKN  70
                                                              TM1

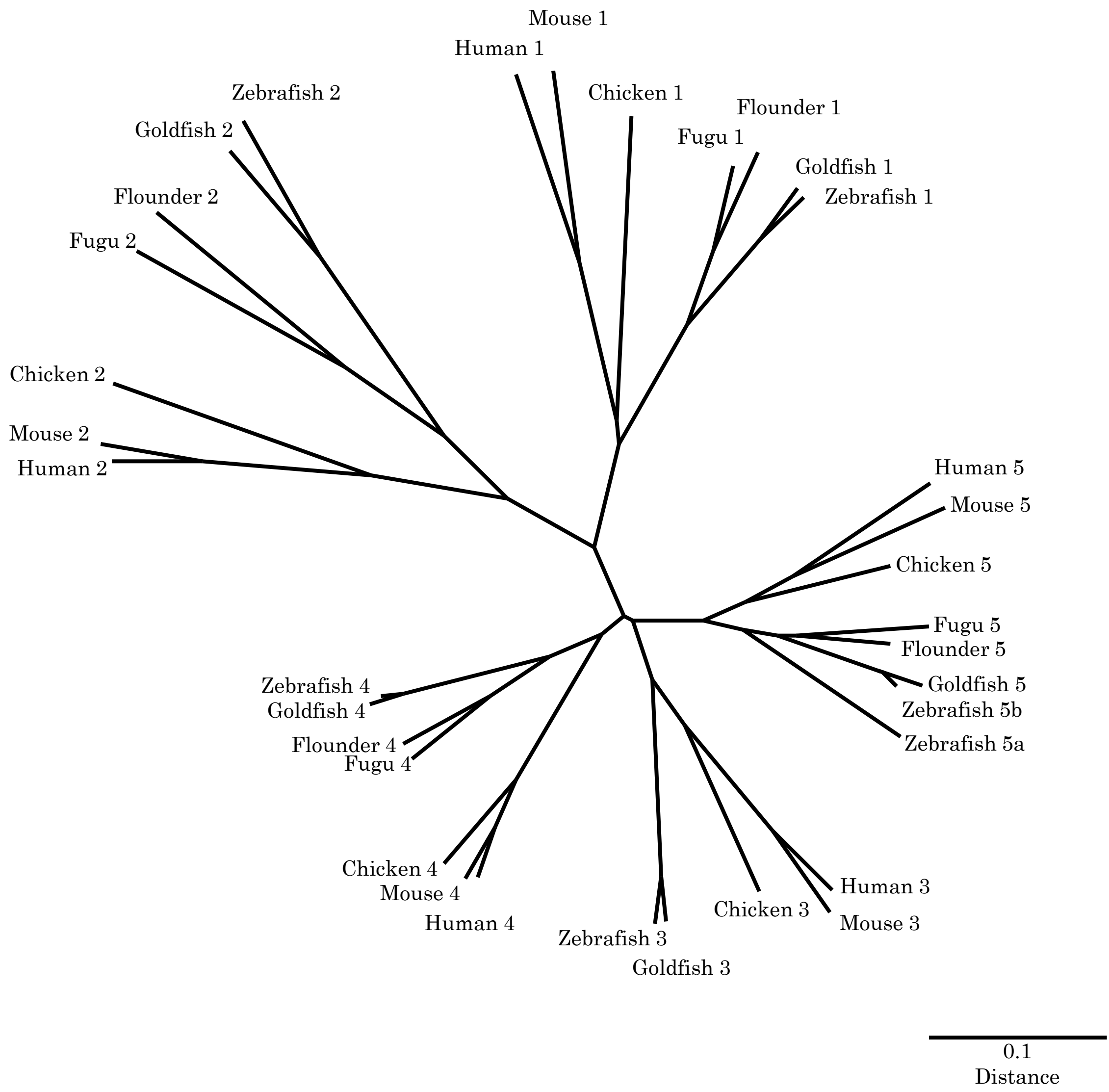
MC1R   RNLHSPMYYFICCLAVSDMLVSVSNVVETLFMLLKEHGLLLVtakMLQHLDNVIDIMICSSVVSSLSFLC 139
MC2R   RNLHSPMYCFICNLAVFNTISSLCKSLETILLLFKEAGHLN--GRFELNIDDIMDSLLCMCFLGSIFSIL 114
MC3R   KNLHSPMYFFLCSLAAADMLVSVSNSLETIVIAVLNSRLLVASDHFVRLMDNVFDSMICISLVASICNLL 136
MC4R   KNLHSPMYFFICSLAVADLLVSVSNASETVVMALITGGNLtyrESIIKNMDNIFDSMICSSLLASIWSLL 139
MC5R   KNLHSPMYFFVCSLAVADMLVSVSNAWETIVIYLLtnrQLVVEDHFIRQMDNVFDSMICISVVASMCSLL 140
                 TM2                                                  TM3

MC1R   TIAADRYITIFYALRYHSIMTtqrAVAIIAVVWLTSITSSSLFIVYHTDNAVIACLVTFFGLTLVFTAVL 209
MC2R   TIAVDRYISIFHALRYHTLMtmrRVVVTLSTIWVFCGTSGVLMIGFSNAAtvkIFFVVLFFTALLLILLL 184
MC3R   AIAVDRYVTIFYALRYHSIVtvrRALVAIAGIWLVCVVCGIVFIVYSESKTVIVCLITMFFAMLVLMATL 206
MC4R   AIAVDRYITIFYALRYHNIMtqrRAGTIITCIWTLCTVSGVLFIVYSESTTVLICLISMFFTMLALMASL 209
MC5R   AIAVDRYVTIFYALRYHNIMtvrRAAFIIGGIWTFCTSCGIVFIIYSDNTSVIVCLVSMFFIMLALMASL 210
          ###                         TM4                                 TM5

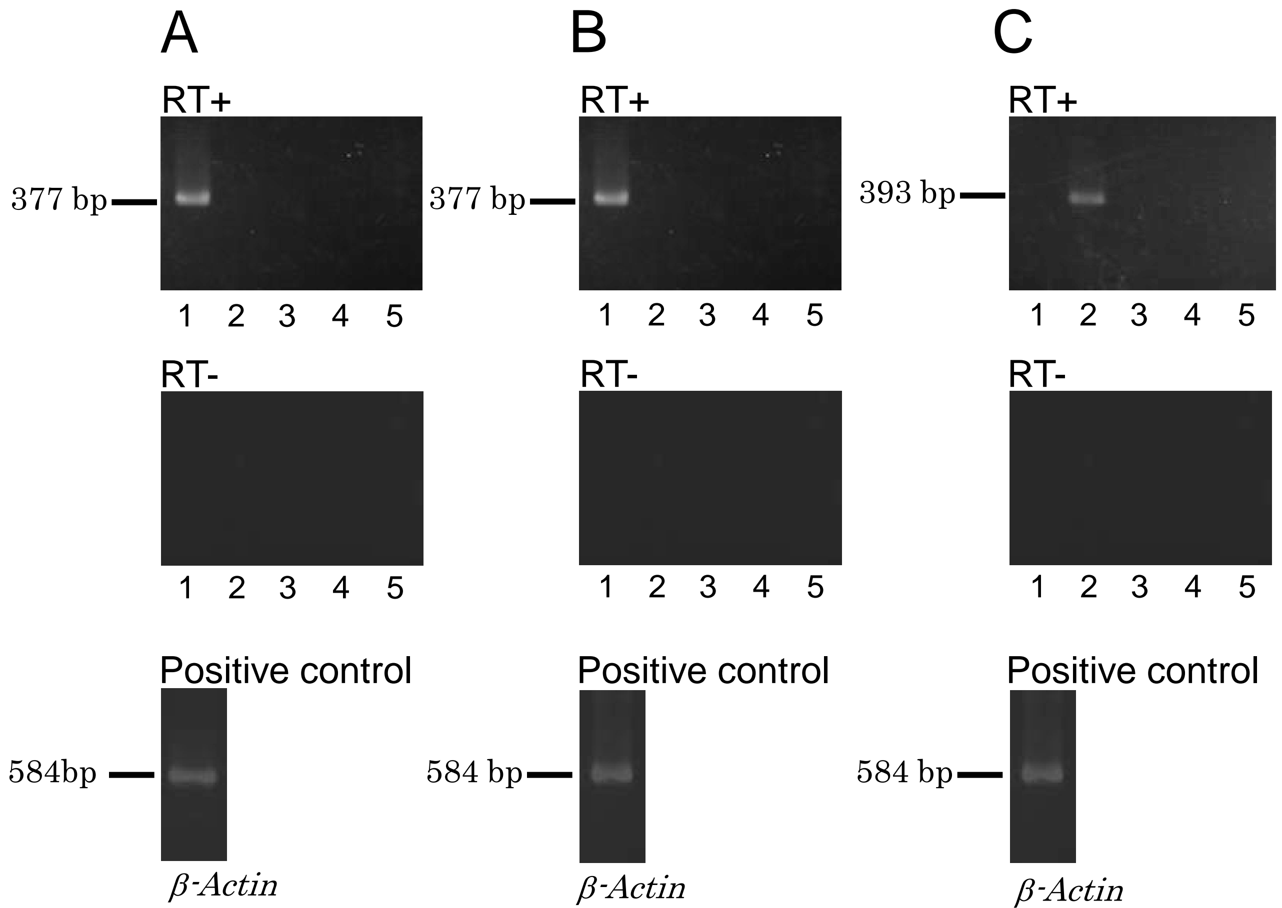
MC1R   YLHMFILAHVHsrrIMALH--------KsrrQATsmkGAITLTILLGVFVICWGPFFLHLILILICPTNP 271
MC2R   YVHMFLLARHHANRIASMP------GLHARQRQSGLRGALTLTILIGVFVACWAPFSLHLLISMICPENP 248
MC3R   YVHMFLLARLHVQRIAALPPAAAAAGNPAPRQRSCMEGAVTISILIGVFVCCWAPFFLHLILLVSCPHHP 276
MC4R   YVHMFLLARLHMKRIAALP------GNGPIWQAANMKGAITITILLGVFVVCWAPFFLHLILMISCPRNP 273
MC5R   YSHMFMLARSHVKRIAALP------GYNSIHQRAsmkAAVTLTILLGIFIVCWAPFFLHLILMISCPRNL 274
                                                       TM6

MC1R   YCKCYFSHFNLFLILIICNSLIDPLIYAYRSQELRKtlkEMIFCSWLFAM                 -     321
MC2R   YCECYRSLFQLHVLLLVSHAVIEPAIYAFRSTELRNtykKVFLCSASRIFKECV          (40%)    302
MC3R   LCLCYMSHFTTYLVLIMCNSVIDPLIYACRSLEMRKtfkEILCCFGCQPPL             (49%)    327
MC4R   YCICFMSHFNMYLILIMCNSVIDPLIYAFRSQEMRKtfkEICCCWYGLASLCV           (53%)    326
MC5R   YCMCFMSHFNMYLILIMCNSVIDPLIYAFRSQEMRKtlkEIICCYslrNVFGMSR         (51%)    329
              TM7
```

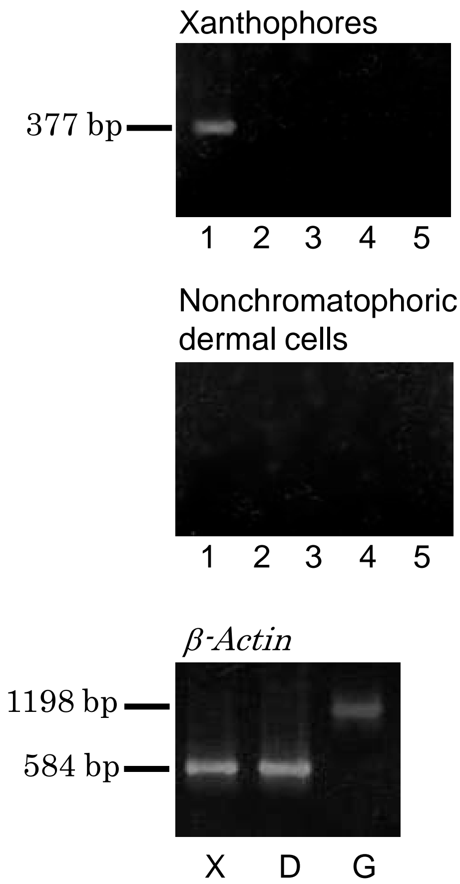
Kobayashi et al. Fig. 2

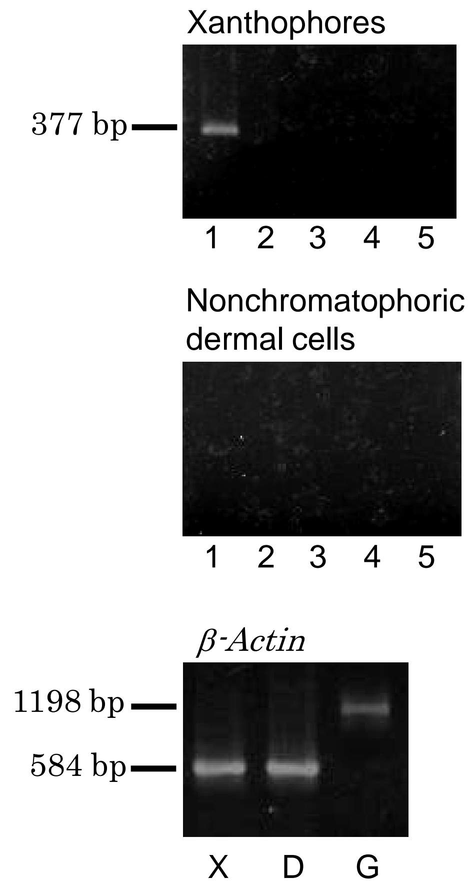


Kobayashi et al. Fig. 3

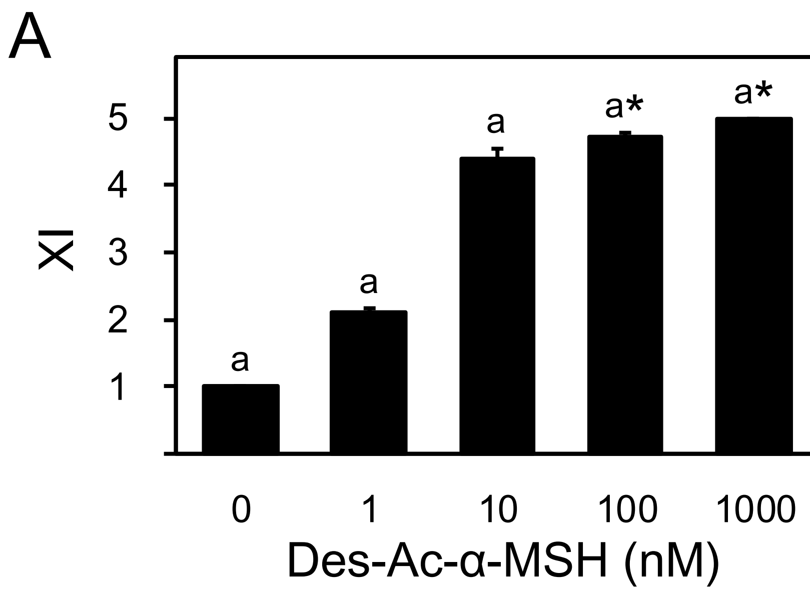


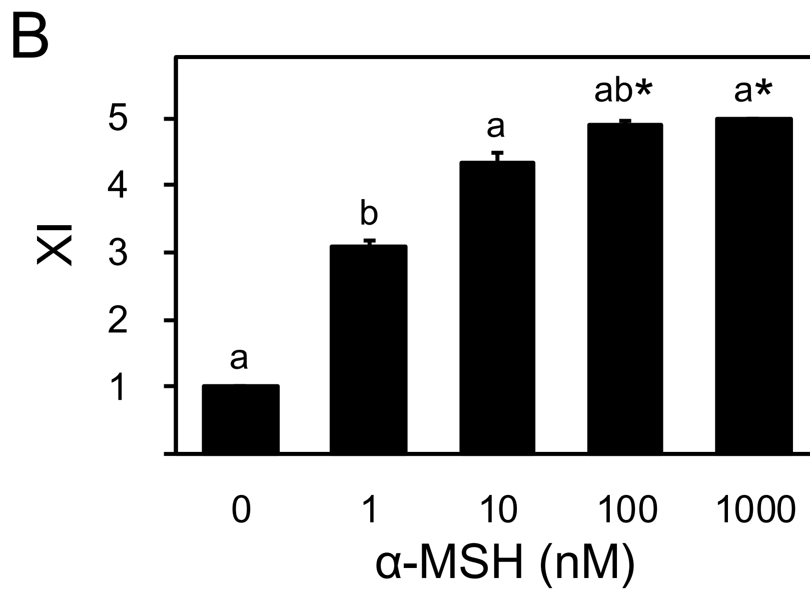
Kobayashi et al. Fig. 4

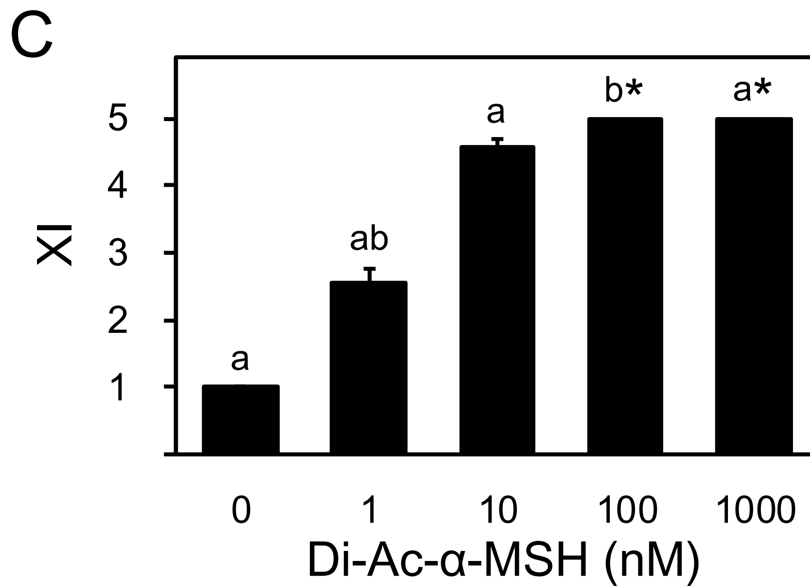




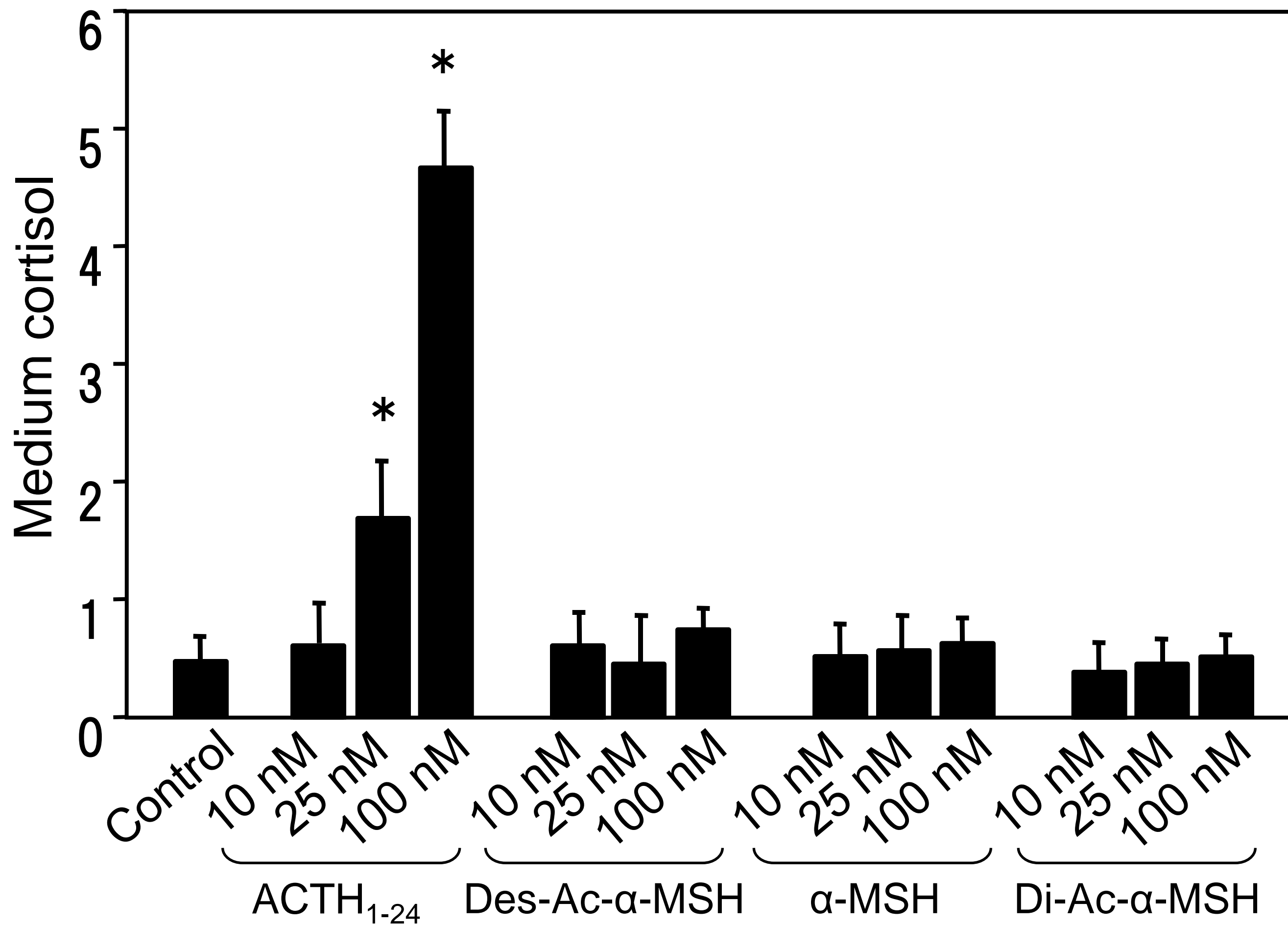
Kobayashi et al. Fig. 5







Kobayashi et al. Fig. 6



Kobayashi et al. Fig. 7

Table 1

Custom oligonucleotide primers used for PCR to amplify cDNA fragments of goldfish *Mc1r*, *2r*, and *3r*.

Primer	Target	Nucleotide sequence
MC1-fw1	MC1R	5'-ATG (TC)TA CTG A(CA)G GA(GC) CAT GG-3'
MC1-rv1	MC1R	5'-(GT)GC TGA A(AG)T AGC ACT TGC AG-3'
GSP-MC1-fw1	MC1R	5'-CGT CAC GTT TTT TGG CTT GA-3'
GSP-MC1-rv1	MC1R	5'-GGA AAC GAC GGA ACT GCA TA-3'
GSP-MC1-fw2	MC1R	5'-GTC AAA GGT GTG CTG AAG GA-3'
GSP-MC1-rv2	MC1R	5'-CAA CGC AGA TGC TCC TTA AG-3'
MC2-fw1	MC2R	5'-AC(TA) GAC TGC GCT GAG GTC CA-3'
MC2-rv1	MC2R	5'-CAC ATG CAG AGT AGA GAG TC-3'
MC2-fw2	MC2R	5'-GTT GTT TAA AGA CGC CGG AC-3'
MC2-rv2	MC2R	5'-GAG TGA (AG)CG GTA GCA (TC)TC AC-3'
GSP-MC2-fw1	MC2R	5'-TTG ATT GGG GTG TTT GTG GC-3'
GSP-MC2-rv1	MC2R	5'-CTT GAT GTC GGC TAG GAT CA-3'
GSP-MC2-fw2	MC2R	5'-CCA GAC TCA TGT CTC TGA GA-3'
GSP-MC2-rv2	MC2R	5'-GTG AAG CAT GTA TTG CTG GG-3'
MC3-fw1	MC3R	5'-TAT GTG ACG AGG TCC (AC)(AG)A T(CT)C A-3'
MC3-rv1	MC3R	5'-AG(AG) A(CT)C AGG TAT GTG (GT)TG AA(AG)-3'
GSP-MC3-fw1	MC3R	5'-CCA CCT CAT TCT GCT GGT GT-3'
GSP-MC3-rv-1	MC3R	5'-CCA AGA TGA CGA GGA TGT TC-3'
GSP-MC3-fw2	MC3R	5'-CAG TCC ACC ATC TGA ATC AG-3'
GSP-MC3-rv2	MC3R	5'-ACC ACC ATG CTT TGG CAT CT-3'
UPM	MC1R, 2R, 3R	5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT -3'

Synthesis of primers was performed by Nihon Gene Research Lab. (Sendai, Japan).

Table 2

Custom oligonucleotide primers used for tissue distribution of goldfish *Mcrs*.

Primer	Target	Nucleotide sequence
MC1-TD -fw1	MC1R	5'-GCT TGT CAC GGC AAA GAT GT-3'
MC1-TD-rv1	MC1R	5'-TGG CTT GTC GGC GAC TCT TA-3'
MC2-TD -fw1	MC2R	5'-ACA CCT GAA CGG TCG TTT CG-3'
MC2-TD-rv1	MC2R	5'-CTC AAG CCA CTT TGT CTC TG-3'
MC3-TD -fw1	MC3R	5'-TGT CTG TTC TTC CCC ATC TC-3'
MC3-TD-rv1	MC3R	5'-GGC GAT TGT TTA GTA CAG CA-3'
MC4-TD -fw1	MC4R	5'-TGC CTC CGA AAC GGT AGT GA-3'
MC4-TD-rv1	MC4R	5'-GCT GAT AAG GCA GAT GAG AA-3'
MC5-TD -fw1	MC5R	5'-CTG TCA CTT TGG GCC ATC AG-3'
MC5-TD-rv1	MC5R	5'-TCT GAT GAA ATG GTC CTC CA-3'

Synthesis of primers was performed by Nihon Gene Research Lab. (Sendai, Japan).