2. Materials and methods

2.1. Study patients

Between January 2002 and October 2005, a total of 328 couples consisting of Japanese women and Caucasian men delivered infants at our hospital. There were 332 infants (324 singletons and four sets of twins). We selected the 324 singleton subjects, because twin pregnancy is one of the risk factors for pre-eclampsia. As a control, 36,829 singleton pregnant women were selected from the 2003 database of the Japan Society for Obstetrics and Gynecology. In this database, the nationalities of patients or husbands were not recorded, but the vast majority of cases (more than 98%) were couples consisting of Japanese women and Japanese men in Japan. Therefore, we used this database as the control.

Gestational hypertension was diagnosed by the following definitions: blood pressure (BP) levels >140/90 mmHg after 20 weeks of gestation. Gestational hypertension with proteinuria as indicated by a single albumin reading at least 30 mg/dl (a dipstick reading of 1+) after 20 weeks of gestation was diagnosed as pre-eclampsia. Statistical analysis was performed by Student's *t*-test.

3. Results

Table 1 summarizes the characteristics of couples consisting of Japanese women and Caucasian men, and the controls. There were no significant differences in age, parity, gestational weeks at delivery, maternal body weight at delivery, and treatment for sterility between the test couples and controls.

We observed gestational hypertension in 2.16% of couples consisting of Japanese women and Caucasian men, and in 3.84% of controls (Table 2). The frequencies of gestational hypertension among nulliparous and muliparous test couples were the same as that among controls. The relative risks of the incidence of gestational hypertension among test samples were 0.68 in nulliparous, 0.27 in multiparous, and 0.56 in total couples. The 95% confidence intervals (CI) were 0.3–1.55 in nulliparous, 0.04–1.91 in multiparous, and 0.27–1.19 in total couples, respectively. The frequency of pre-eclampsia among test couples (1.54%) was slightly lower compared to that among controls (2.67%) (Table 2), although there was

Table 1
Characteristics of couples consisting of Japanese women and Caucasian men and controls

	Couples consisting of Japanese women and Caucasian men ($n=324$)	Control ($n=36{,}829$)	p-Value
Age (years)	32.2 ± 4.3	31.8 ± 4.1	0.743
Primipara	199 (61.4%)	21816 (59.2%)	0.426
Maternal body weight at delivery (kg)	62.8 ± 6.9	62.1 ± 7.2	0.825
Neonatal body weight (g)	3133.0 ± 519.6	3026.2 ± 502.5	0.693
Therapy for sterility	17 (5.2 %)	1593 (4.3%)	0.417

The data were shown as mean ± S.D.

Table 2
Frequency of gestational hypertention and pre-eclampsia of couples consisting of Japanese women and Caucasian men and controls

	Couples consisting of Japanese women and Caucasian men ($n = 324$)	Control ($n = 36,829$)	p-Value	RR (95% CI)
Gestational hypertension				
Nulliparous	6/199 (3.02%)	961/21816 (4.41%)	0.341	0.68 (0.3–1.55)
Multiparous	1/125 (0.8%)	452/15013 (3.01%)	0.149	0.27 (0.04–1.91)
Total	7/324 (2.16%)	1413/36829 (3.84%)	0.117	0.56 (0.27–1.19)
Pre-eclampsia				
Nulliparous	4/199 (2.01%)	681/21816 (3.12%)	0.369	0.64 (0.24–1.74)
Multiparous	1/125 (0.8%)	303/15013 (2.02%)	0.334	0.40 (0.06–2.85)
Total	5/324 (1.54%)	984/36829 (2.67%)	0.209	0.58 (0.24–1.4)

RR, relative risk; CI, confidence interval.

no significant difference between the two groups. We observed pre-eclampsia in 3.12% of nulliparous women and 2.02% of multiparous women in the control group. The frequencies of pre-eclampsia among nulliparous women and multiparous women in the test group were 2.01% and 0.8%, respectively. There were no significant differences in the frequency of pre-eclampsia between couples consisting of Japanese women and Caucasian men, and the control group (Table 2). The relative risks and 95% CI of the incidence of pre-eclampsia among test samples were 0.64 (0.24–1.74) in nulliparous, 0.40 (0.06–2.85) in multiparous, and 0.58 (0.24–1.40) in total couples.

4. Discussion

The report by Hiby et al. (2004) has had significant impact on consideration of the pathophysiology of pre-eclampsia. They pointed out that the combination of maternal KIR-AA genotype and fetal HLA-C2 genes is at increased risk for pre-eclampsia, and showed that different human populations have a reciprocal relationship between KIR-AA frequency and HLA-C2 frequency. Their hypothesis is attractive, but further studies are needed for verification. As one method of proving their hypothesis, an epidemiological study could be valid. A population with a high frequency of KIR-AA genotype and a low frequency of HLA-C2 would result in a low frequency (3–5%) of pre-eclampsia but, if women from a population with a high frequency of HLA-AA marry men from a population with a high frequency of HLA-C2, the prevalence of pre-eclampsia should be increased. The Japanese population has the highest frequency of KIR-AA (~60%) (Yawata et al., 2002; Hiby et al., 2004), while, Caucasian populations have a 3.5 times higher frequency of HLA-C2 genotype than Japanese. If hypothesis of Hiby et al. (2004) is correct, the prevalence rate of pre-eclampsia among such couples should show three- to four-fold increase.

However, our data have now shown that the incidence of pre-eclampsia among couples consisting of Japanese women and Caucasian men did not differ significantly from that

among couples consisting of Japanese women and Japanese men. In this cohort, considering the nulliparae as pre-clampsia is often a disease of first pregnancies, the prevalence of gestational hypertensive disorders of pregnancy among nulliparae was 5.03% in cases versus 7.53% in controls. The relative risk and 95% CI of pre-eclampsia among the test group were 0.64 (0.24–1.74) in nulliparous, 0.40 (0.06–2.85) in multiparous, and 0.58 (0.24–1.4) in total couples. They did not reach to three- to four-fold increase in prevalence which was calculated by the hypothesis of Hiby et al. (2004). Thus, our epidemiological study does not support that hypothesis, although we did not investigate individual KIR and HLA-C genotypes. Robillard et al. (1994) reported that the duration of sexual cohabitation effects the risk of gestational hypertension. Unfortunately, we could not obtain the information about the length of sexual cohabitation. The present findings suggest that further investigations of maternal KIR genotype and fetal HLA-C genotype, or duration of sexual cohabitation, are needed to confirm this intriguing hypothesisin pre-eclampsia.

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Rembrandt's Bathsheba, possible lactation mastitis following unsuccessful pregnancy

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Summary Deformity of the left breast and axilla observed in Rembrandt's famous painting "Bathsheba at her toilet" (1654, Louvre Paris) has been discussed by several researchers. Proposed diagnoses were breast cancer and abscess due to tuberculosis. The present article reviews previous articles written concerning the left breast abnormalities of Bathsheba and carefully examines other works of Rembrandt modeled by Hendrickje and painted around 1654. Previous diagnosis of breast cancer and tuberculous mastitis is less probable. Because Hendrickje survived for more then 9 years after the painting and in other works modeled by Hendrickje shows no signs of cachexia or permanent changes in the left breast. The most likely diagnosis of the left breast deformity of Bathsheba is a sequela of lactation mastitis abscess following miscarriage or premature childbirth without breast feeding.

A case study and proposed diagnoses

Rembrandt van Rijn (1606–1669) born in Leiden was one of the most famous and leading representatives of the Dutch School of painting and was especially talented in the use of light and shadow [1]. He left correct paintings of medical scenes known as "Anatomy lesson by Dr. Nicholaes Tulp" (1632, The Hague, Mauritshuis museum) and "The anatomy lesson of Dr. Joan Dejiman" (1656, Amsterdam Historisch Museum).

Other than medical paintings, he recorded clinical findings of basal cell carcinoma in "Man in oriental costume" (1632, New York metropolitan Museum) [2], signs of ageing including brow and eyelid ptosis, rosacea and temporal arteritis in his self-portrait (1659, National Gallery of Art, Washington, DC) [3].

In 1983 Braithwaiste and Shugg suggested that Rembrandt's famous painting of "Bathsheba at her toilet" (1654, Louvre Paris) showed clinical signs of advanced left breast carcinoma based upon skin discolouration, distortion, axillary fullness and peau d'orange appearance [4]. The breast cancer hypothesis was presented independently by Dymarskii in 1984 [5].

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It is generally accepted that Bathsheba was painted in 1654 modeled by Hendrickje Stoffels. In 1654, Hendrickje was 28 years old. She was Rembrandt's de facto wife as of 1649 and had a pregnancy in 1652. She became pregnant again and delivered their daughter Cornelia in 1654 October.

No records on health of Hendrickje until her premature death on 21 Jul 1663 (37 years old).

In 2000, Bourne proposed on alternative diagnosis of an infective process such as tuberculous mastitis or less likely chronic lactational breast abscess [6]. He discussed that if the body of model was Hendrickje, she could hardly have lived 9 years with advanced breast cancer without any effective treatment. Possibly she had a chronic inflammatory condition, either tuberculous mastitis and less likely lactational breast abscess.

Other works by Rembrandt modeled by Hendrickje

We can see other works by Rembrandt modeled by Hendrickje. Rembrandt left several oil paintings modeled by Hendrickje around 1654, including "Hendrickje Stoffels" (1655, Paris Musee Louvre) "Woman in a Doorway" (1656, Berlin Gemeldegalerie) and famous "Hendrickje bathing" (1655, London National Gallery). No paintings show the naked left breast of the model. But we can see a healthy slightly obese woman at 25–30 years, and observe no remarkable signs of cachexia or chronic consumption.

Further, we can see several female nude etchings (1658, New York, Pierpont Morgan Library) and a precise drawing "Hendrickje in the Artist's Studio" (1654, Oxford, Ashmolean museum). We can observe frontal view of the left breast in 3 etchings in New York and left posterior view in Oxford drawing. Neither etchings nor drawing show deformities or distortions such as dimpling or peau d' orange appearance and axillary lymph node swelling.

Discussion

Taken together, if the model of Bathsheba was Hendrickje and she suffered from left breast disease at the time of the painting, it must be a benign and possibly reversible change. Because Hendrickje delivered Rembrandt's daughter Cornelia in 1654 and survived for 9 more years after the delivery and painting of Bathsheba. If the left mammary changes were caused by breast cancer she would not have survived so long. As Bourne pointed out, the mean survival of breast cancer patients was 2–3 years before the application of modern surgical treatment, radiation and/or chemotherapy.

Diagnosis of tuberculous abscess looks less probable. Because breast involvement of tuberculosis is a rather rare complication. If the model had suffered from tuberculosis, she must show some signs of chronic consumption and hardly had a chance becoming pregnant. Further, untreated tuberculous abscesses persist for several years and result in permanent deformity of the breast. As we cannot see any changes in the Oxford drawing of 1654 and in the New York etchings of 1658, we need to abandon the possible diagnosis of tuberculous abscess and benign breast tumours including fibroma.

We present as a possible alternative, chronic lactational breast abscess and mastopathy. Even in the 21st century Japan and other developed countries, lactating women are often experience mastitis. However they can be effectively treated by the oral administration of antibiotics. In serious cases, they also can be treated by bromocriptin administration that inhibits lactation.

It is possible that lactating women in the mid 17th century had a higher prevalence of mastitis and subsequent breast abscess. In the acute phase of mastitis, redness and swelling are remarkable but they become less observable in the chronic phase and after abscess formation. Hendrickje delivered her second child Cornelia in October of 1654, she had a chance of breast-feeding and subsequent infection. However, if Bathsheba was painted in 1654, birth of Cornelia looks less probable as the cause of chronic breast abscess formation. Thus, we consider it was painted early 1654, before the conception of Cornelia or during the first trimester of pregnancy. As Hendrickje had been reported to become pregnant in 1652, she had the opportunity for lactation and related inflammation. The outcome of Hendrickje's first pregnancy is not known. As there are no record concerning Rembrandt's children except for Titus (borne in 1641, the only living son between Rembrandt's first wife Saskia) and Cornelia, Hendrickje's pregnancy in 1652 possibly resulted in miscarriage or neonatal death due to premature delivery.

Our hypothesis explains why Bathsheva shows no evidence of abdominal striae, which can often be observed in multiparous women. Premature labour or miscarriage even in the first trimester of pregnancy never leave abdominal striae but can induce lactation and lactation without feeding is a major

cause of subsequent breast milk retention and inflammation.

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Molecular cloning and histological localization of LH-like substances in a bottlenose dolphin (*Tursiops truncatus*) placenta

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Abstract

All mammals exhibit pituitary-specific expression of LH and FSH, whereas placental expression of gonadotropins has been reported only in primates and equids. Some cetaceans, such as dolphins, have a long gestational period and a sexual cycle of about 27 days almost comparable with that of humans. Histologically, dolphins have an epitheliochorial placentae that resembles placentas of Perissodactyla including horses. In the present study, we cloned cDNAs encoding gonadotropins and observed their immunohistochemical localization in the placenta of bottlenose dolphin. The cDNAs obtained encoded 120 amino acids for the α-subunit (including 96 amino acids of mature proteins), and 141 amino acids for the β-subunit (including 121 amino acids of mature proteins). The sequence of the α-subunit was similar to that in the pig (Artiodactyla) pituitary glycoprotein hormone [96.7% homology at amino acids (aa) level], and the sequence of the β-subunit was similar to that of luteinizing hormone (LH) in the pig [94.3% homology at aa level] and white rhinoceros (Perissodactyla) [93.3% homology at aa level]. Of interest, dolphin LHβ lacks carboxyl-terminal-peptides (CTP). This fact suggests that CTP are not essential for placental expression of gonadotropin in dolphins. Immunohistochemical observations employing anti-ovine LHβ antibody revealed positive staining in the villositycal tissue. Our observations suggest placental expression of gonadotropin homologues in cetaceans and possible evolutionary conservation of placentae-derived hormonal control of ovarian functions during pregnancy.

Keywords: Cetaceans; cDNA cloning; Chorionic gonadotropin; Luteinizing hormone; Immunohistochemistry; *Tursiops truncatus*; Placenta; Carboxyl-terminal-peptides

1. Introduction

The primary function of placentae is to exchange oxygen for carbon dioxide and nutrients for wastes between fetuses and mothers, and they also produce multiple hormones and bioactive substances. In viviparous mammals, progesterone, produced in the corpora lutea and placenta, is one of the key molecules responsible for maintaining a successful pregnancy. In rodents, gonadotropins produced in the pituitary gland have sole roles for maintenance of pregnancy, while in primate species, including humans, chorionic gonadotropin (CG) production from trophoblasts begins soon after implantation and stimulates ovarian progesterone production during early pregnancy. Whether of pituitary or placental origin, gonadotropins are evolutionarily conserved and share common molecular structures.

Human gonadotropins including luteinizing hormone (LH), follicle stimulating hormone (FSH), and chorionic gonadotropin (CG) belong to the glycoprotein hormone family and contain a common α-subunit and hormone-specific β-subunits. In various mammalian species, the DNA sequences of glycoprotein hormones were evolutionarily conserved while the placental expression of CG has thus far only been reported in primates and equids.

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Cetaceans, including dolphins, have long gestational periods (about 10–16 months) and sexual cycles of around 27 days, similar to those in primates, though their endocrine backgrounds of reproduction have not been well investigated. Hobson and Wide (1986) studied the bioactivity and immuno-reactivity of chorionic gonadotropin in animal placentae, including those of dolphins and humans, and found high levels of free CGβ activity in term dolphin placentae, in contrast to the high level of α-subunit activity in human placentae. Unfortunately, a complete molecular analysis was not conducted in their study.

Morphologically, dolphins have epitheliochorial placentae resembling horse placentae as well as those of cows and pigs. Though recent studies suggest linage relationships between Cetaceans and Artiodactyls (Ursing and Arnason, 1998, Gatesy et al., 1996), there are no published data suggesting placental expression of gonadotropin genes in 3 subgroups of Artiodactyls namely, Suiformes (pigs, peccaries, and hippopotamuses), Ruminantia (bovids, deer, tragilids and giraffes) and or Tylopoda (camels). Thus we performed molecular cloning of gonadotropins in a dolphin placenta as well as in a pituitary gland obtained from the same species.

2. Materials and methods

2.1. Tissue sampling

A placenta was obtained from a captive healthy female bottlenose dolphin (*Tursiops truncates*, named Azami, age unknown) after normal vaginal delivery on 11 Oct 2002 at Enoshima aquarium, Kanagawa prefecture. The placenta was incised into small pieces for morphological and molecular analyses. Frozen samples were kept at −80 °C for later RNA extraction.

The pituitary tissue was obtained from another wild female bottlenose dolphin captured in the traditional drive fisheries at Taiji-wan, Wakayama prefecture on 3 December 2003, which was delivered by the Wakayama Taiji fishery cooperative union. Tissue samples were obtained by craniotomy soon after euthanasic sacrifice by cutting spinal cord and were kept at −30 °C in RNA later® (TaKaRa Biotechnology, Shiga, Japan) storage solution for later use.

2.2. Amplification of cDNA encoding placental and pituitary gonadotropins

RNA was extracted from the tissue sampled by TRIzol® Regent (Gibco BRL, Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Single-strand cDNA was prepared using Ready-To-Go You-Prime® First-Strand Beads (Amersham Biosciences, Tokyo, Japan) using the vect-dT (Amersham Biosciences, Tokyo, Japan) adapter primer which were designed to anneal adapter sequences in the unknown 3′region, according to the manufacturer's instructions. To clone dolphin gonadotropins with unknown molecular homologies to those of other published animals, we used the degenerative PCR technique employing multiple primers. RT-PCR for synthesizing and amplifying of cDNA was carried out under the best conditions after several trials. Briefly, to amplify evolutionarily conserved sequences in the gonadotropin α-subunit genes, the primers were designed based on published α-subunit mRNA for rat LH (*Rattus norvegicus*, Godine et al., 1982), horse LH/CG (*Equus caballus*, Min et al., 1994), marmoset CG (*Callithrix jacchus*, Simula et al., 1995), and deer FSH (*Cervus nippon*, unpublished data).

For the β-subunit of LH, we referred to the published cDNA sequences for the giant panda (*Ailuropoda melanoleuca*, Liao et al., 2003), cow (*Bos taurus*; Maurer, 1985), white rhinoceros (*Ceratotherium simum*; Sherman et al., 1997), dog (*Canis familiaris*; Wolf et al., 1987), horse (*Equus caballus*; Sherman et al., 1992), donkey (*Equus asinus*; Chopineau et al., 1995), zebra (*Equus burchelli*; Chopineau et al., 1999), cat (*Felis catus*; unpublished data), and siberian tiger (*Panthera tigris altaica*; Crichton et al., 2003). The forward degenerate primers were designed after published mRNA sequences of mammalian hormones including the common α-subunit of CG, LH, and FSH, and the LH β-subunit. Adapter primers were designed to anneal adapter sequences in the unknown 3′region.

PCR was performed in an iCyclar™ (Bio-Rad Laboratories, Tokyo, Japan) in 2XGC buffer I supplemented with 5 units/μl TaKaRa LA Taq® polymerase, 2.5 mM of each dNTP Mixture (TaKaRa Biotechnology, Shiga, Japan), 10 pmol of degenerate primers, and 3.5 ng/2 μl of cDNA. The PCR condition was as follows: 35 cycle at 94 °C for 30 s, 55 °C for 30 s, and 72 °C 1 min 30 s. PCR products were separated by electrophoresis in 2% agarose gel and purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Woods Hollow Road Madison, WI, USA).

2.3. DNA sequencing and analysis

The amplified fragments were subcloned into pGEM-T vector using the pGEM-T vector Easy Vector System (Promega,

Table 1
Oligonucleotide primers used for PCR amplification of the glycoprotein cDNAs

Primer	Sequence
α-subunit	
alpha FW1 (A1)	5′ ATG GAT TAC TAC AGA ARA YAT GCA 3′
alpha FW2 (A2)	5′ CAT TCC YTT CCT GAT GGA GAG TTT A 3′
β-subunit	
beta FW1 (B1)	5′ ATG GAG ATG YTC CAG GGR CT 3′
beta FW2 (B2)	5′ ATC AAC GCC ACY CTG GCC GCT GAG AA 3′
adapter primer	
AP-1	5′ GTA ATA CGA CTC ACT ATA GGG C 3′
AP-2	5′ CTA TAG GGC ACG CGT GGT 3′

The forward common α-subunit primers (A1, A2) were designed based on the GenBank V01253: rat, GenBank AY066018: sika deer, GenBank U04446: common marmoset, and GenBank AB000200: horse glycoprotein hormone α-subunit cDNA sequences. The forward β-subunit primers (B1, B2) were designed based on GenBank AF448455: giant panda LHβ, GenBank M10077: bovine LHβ, GenBank U72659: white rhinoceros LHβ, GenBank Y00518: dog LHβ, GenBank S41704: horse LH/CGβ, GenBank X80116: donkey LHβ, GenBank Y16265: zebra LHβ, GenBank AF095716: cat LHβ, and GenBank AF354938: amur tiger LHβ cDNA sequences. AP were the adapter primers. R: A+G, Y: C+T.

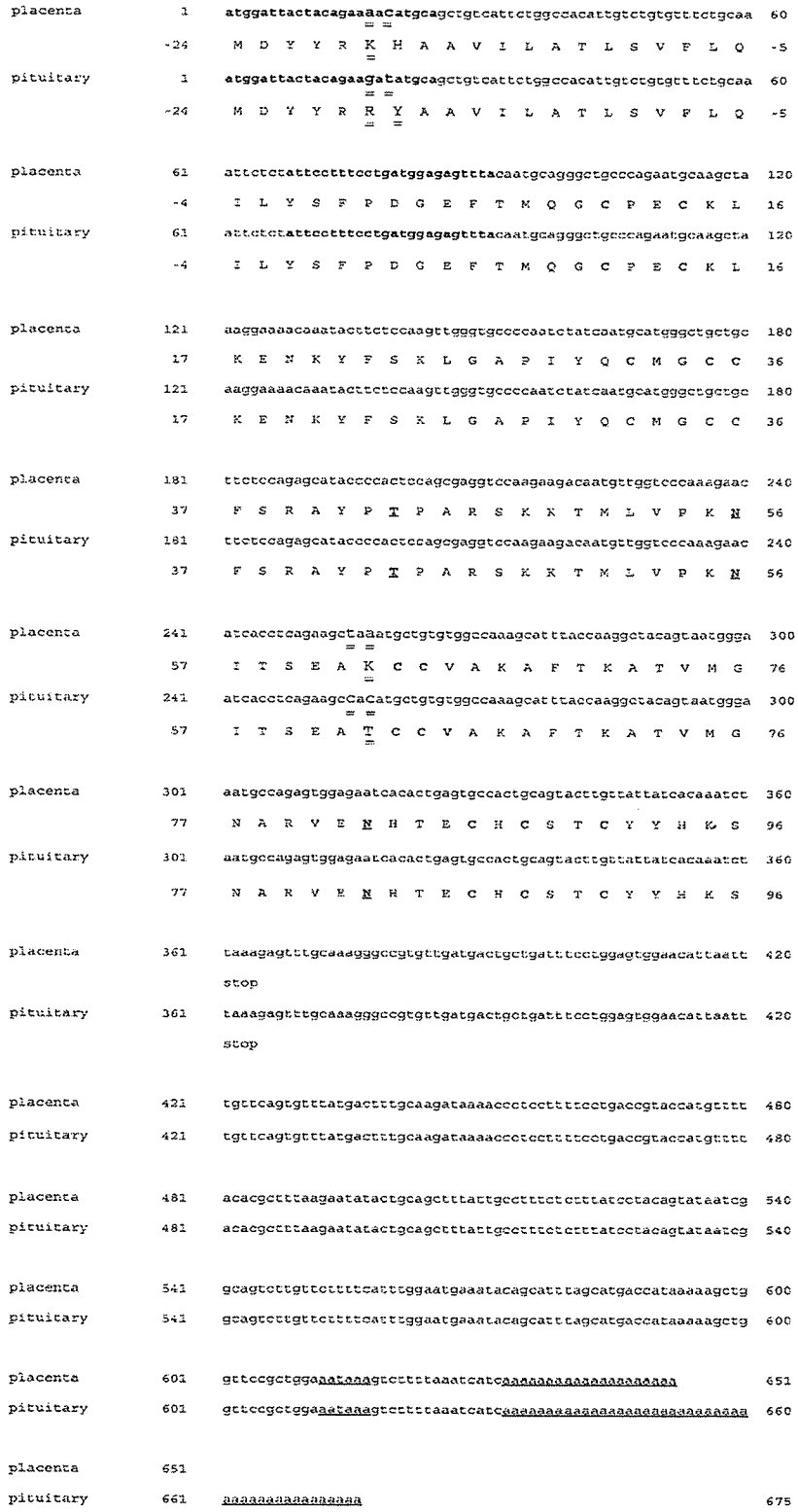

Fig. 1. cDNA nucleotide and deduced amino acid sequences of the bottlenose dolphin common glycoprotein α-subunit. The forward custom primers were designed from nucleotide positions 1 to 14(A1) and 68 to 91(A2). The primers sequences are shown in bold. The polyadenylation signal and poly(A)$^+$ tail are underlined. The free α-subunit is proposed to be the putative *O*-glycosylation site and the putative *N*-glycosylation sites are bolded and underlined. The Proposed proper subunit folding involves 10 cysteines, shown in bold. The nucleotide and amino acids residues showed a difference between the placenta and pituitary (double line).

Madison, USA) using the blunt-ended cloning method according to the manufacturer's manual. Vectors were transformed into competent cells (*E. coli* DH5α). Recombinant clones were selected using blue/white screening on X-gal/IPTG/ampicillin 2XYT plates. Then, positive clones for the α- and β-subunits were determined using PCR and enzymatic digestion with *Eco*RI. In order to avoid PCR and/or sequences errors, we tried sub-cloning with 2 or more bacterial colonies.

Positive clones were sequenced in both directions on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) using standard cycle conditions and the T7/SP6 promoter primer.

Nucleotide sequence data were compiled by using a computer software package: Sequence Editor Version 1.0.3 (Applied Biosystems, Tokyo, Japan). The sequence homology between the bottlenose dolphin and other published mammalians, obtained by the GenBank Database, were analyzed by the Blast (http://blast.genome.jp/) search program. Phylogenetic trees were calculated on the same program and displayed using Tree View version 1.5 software.

2.4. Immunohistochemistry

In order to localize expression of LH related cDNA in dolphin placentae, we examined placental tissues with immunohistochemical techniques. In the present study, we employed anti-ovine LHβ rabbit polyclonal antibody because the maximum homology was observed between ovine LHβ sequences (GenBank accession no. X52488) and newly sequences dolphin placenta derived LHβ cDNA. The placental tissue was fixed with Bouin fixation solution (15:5:1 of picric acid saturated solution in distilled water, 37% formaldehyde solution, glacial acetic acid) overnight. Then, after dehydration, the tissue was embedded with paraffin and cut into 5 μm sections on a microtome, which were then fixed onto slide glasses. Immunohistochemical staining was performed using LAB-SA Detection System (Histostain®-*Plus* Bulk Kit, ZYMED® Laboratories Inc., Invitrogen immunodetection, CA, USA) under the manufacturer's instruction with slight modifications. Briefly, deparaffinized sections were subjected to an antigen-retrieval step in TUF™ Target Unmasking Fluid (MONOSAN, Uden, The Netherlands) at 90 °C for 10 min. The sections were incubated with 0.6% H_2O_2 solution to inhibit endogenous peroxidase activity, and then washed in distilled water for 10 min. The sections were blocked with a solution of normal goat serum. After draining the blocking serum, the sections were incubated with the primary antibody [Rabbit Anti-ovine LH β subunit serum: code name HAC-OV27(β)-01RBP85 (Laboratory of Biosignal Sciences Inst. for Molecular and Cellular Regulation, Gunma University)], diluted to 1:500 in phosphate-buffered saline (PBS) at 4 °C overnight after standing at room temperature for 30 min. After three washes in distilled water and through PBS, the slides were incubated with biotinylated anti-

Table 2
GenBank accession numbers employed as the sources of vertebrate glycoprotein hormones

(Class) Order	Common name	Species	Genbank accession no.		
			α-subunit	β-subunit	
				LHβ	CGβ
(Mammalia)					
Marsupialia	Brush-tailed possum	*Trichosurus vulpecula*	AF017447	AF017448	
	Red kangaroo	*Macropus rufus*	AF017449	AF017450	
Cetartiodactyla	Pig	*Sus scrofa*	D00768	D00579	
	Bovine	*Bos taurus*	X00050	M10077	
	Sheep	*Ovis aries*	X16977	X52488	
Perissodactyla	Donkey	*Equus asinus*	X85170	X80116	
	Plains zebra	*Equus burchelli*	Y16326	Y16265	
	Horse	*Equus caballus*	AB000200	S41704 (LH/CHβ)	
	White rhinoceros	*Ceratotherium simun*	nonpublished	U72659	
Carnivora	Dog	*Canis familiaris*	AF160250	Y00518	
	Cat	*Felis silvestris catus*	AY972823	AF095716	
Rodentia	Mouse	*Mus musculus*	J00643	Y10418	
	Golden hamster	*Mesocricetus auratus*	AF307148	AY353074	
Primates	Human	*Homo sapiens*	V00518	X00264	J00117
	Common marmoset	*Callithrix jacchus*	U04446	nonpublished	U04447
	Crab eating macaque	*Macaca fascicularis*	AY026358	AJ781396	Y026359
(Aves)					
Galliformes	Common turkey	*Meleagris gallopavo*	M33698	L35519	
	Common quail	*Coturnix coturnix*	S70833	S70834	
(Osteichthyes)					
Anguilliformes	European eel	*Anquilla anquilla*	X61038	X61039	
Siluriformes	African catfish	*Clarias gariepinus*	X97760	X97761	
Salmoniformes	Chum salmon	*Oncorhynchus keta*	M27653	M27154	

The common α-subunit, LHβ, and CGβ sequences were used for amino acid alignment sequencing and phylogenetic analysis in the present study.

rabbit secondary antibody (included in the detection kit) at room temperature for 30 min. Then, the sections were washed three times in distilled water and treated with the streptavidin-HRP solution at room temperature for 15 min. After three washes in distilled water and through PBS, the sections were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, WAKO, Osaka, Japan) solution for 2 min.

3. Results

3.1. Cloning of the gonadotropin common α-subunit cDNA

We amplified one major fragment from the dolphin placenta and pituitary cDNA library by the first PCR using the A1 and AP-1 primers, followed by nested PCR using the A2 and AP-2 primers (Table 1). We obtained 651 and 675 base pair (bp) fragments as candidates for the putative dolphin gonadotropin α-subunit (Fig. 1). The amino acid sequence deduced from these fragments showed higher homologies to mammalian glycoprotein hormones (Table 3). The end of the signal peptide and the beginning of the mature protein amino acid sequence was designated as the − 1/+ 1 boundary (Table 2). When a signal sequence of 24 amino acids was located, the proposed mature α-subunit of dolphin gonadotropin started with cysteine and consisted of 96 amino acids. The free α subunit is proposed to be *O*-glycosylated at Thr-43. A putative *N*-linked glycosylation site was located at Asn-56 and Asn-82 from the N-terminus of the predicted mature peptide. Fig. 2 shows the alignment of amino acid sequences of the dolphin gonadotropin α-subunit and those of other mammals. All of the 10 cysteines (form 5 disulfide bonds), the putative *O*-linked glycosylation site, and the putative two *N*-linked glycosylation sites of the dolphin gonadotropin α-subunit were completely conserved among the mammals.

We cloned gonadotropin α-subunit-like sequences from cDNA libraries prepared from placental and pituitary tissues, and observed nucleotide substitutions in 17 (A/G), 19 (C/T), 255 (T/C), and 257 (A/C). The deduced amino acid sequences differed between the placenta and pituitary gland, as follows: AA −19 (Lys−>Arg),−18 (His−>Tyr), and 62 (Lys−>Thr).

3.2. Cloning of the gonadotropin β-subunit cDNA

We obtained 539 and 534 bp fragments as candidates for the putative dolphin gonadotropin β-subunit (Fig. 3), which we amplified from the dolphin placenta and pituitary cDNA library using B1, B2, AP-1, and AP-2 primers (Table 1). Surprisingly, the deduced amino acid sequence from these fragments showed higher homology to mammalian LHβ genes than to CGβ genes cloned in primates or horses (Tables 3 and 4). By comparing the sequence with that of other mammalian LHβ subunits, a signal peptide of 20 amino acids and a mature peptide of 121 amino acids were predicted for the dolphin gonadotropin β-subunit (Fig. 4). The end of the signal peptide and the beginning of the mature protein amino acid sequence were designated as the − 1/+ 1 boundary. A putative *N*-linked glycosylation site was found at

```
           -24        -10        1          10         20         30         40         50         60         70         80         90    96
                                               *  *                       *  **                            **                          *   *   *
                                                                                     ⇩          ⇩                                 ⇩
placenta   MDYYRKHAAV ILATLSVFLQ ILYSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEAKCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
pituitary  MDYYRRYAAV ILATLSVFLQ ILYSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
possum     MDYYRKYAAV ILATLSVFLH ILHSFPDGEF IMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYYHKS
kangaroo   MDYYRKYAAV ILATLSVFLH ILHSFPDGEF IMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYYHKS
pig        MDYYRKYAAV ILAILSVFLQ ILHSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
bovine     MDYYRKYAAV ILTILSLFLQ ILHSFPDGEF TMQGCPECKL KENKYFSKPD AAIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NVRVENHTEC HCSTCYYHKS
sheep      MDYYRKYAAA ILAILSLFLQ ILHSFPDGEF TMQGCPECKL KENKYFSKPD APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NVRVENHTEC HCSTCYYHKS
dog        MDCYRKYAAV ILAALSVFLH ILHSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYYHKS
cat        MDYYRKYAAV ILAILSVFLH ILHSFPDGEF TMQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NAKVENHTEC HCSTCYHHKI
mouse      MDYYRKYAAV ILVMLSMFLH ILHSLPDGDF IIQGCPECKL KENKYFSKLG APIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
hamster    MDYYRKYAAV ILVVLSMFLH ILHSLPDGDF MIQGCPECKL KENKYFSKLG APIYQCMGCC FSSAYPTPAR SKKTMLVPKN ITSEATCCVA KAFTKATVMG NARVENHTEC HCSTCYYHKS
donkey     MDYYRKHAAV ILATLSVFLH ILHSFPDGEF TTQDCPECKL KENKYFSKLG VPIYQCMGCC FSRAYPTPAR SKKTMLVPKN ITSEATCCVA KAFIRVTLMG NIRLENHTQC YCSTCYHHKI
zebra      MDYYRKHAAV ILATLSVFLH ILHSFPDGEF TTQDCPECKL KVNKYFSKLG VPIYQCMGCC FSRAYPTPAR SRKTMLVPKN ITSEATCCVA KAFIRVTLMG NIRLENHTQC YCSTCYHHKI
horse      MDYYRKHAAV ILATLSVFLH ILHSFPDGEF TTQDCPECKL RENKYFFKLG VPIYQCKGCC FSRAYPTPAR SRKTMLVPKN ITSESTCCVA KAFIRVTVMG NIKLENHTQC YCSTCYHHKI
human      MDYYRKYAAI FLVTLSVFLH VLHSAPD--- -VQDCPECTL QENPFFSQPG APILQCMGCC FSRAYPTPLR SKKTMLVQKN VTSESTCCVA KSYNRVTVMG GFKVENHTAC HCSTCYYHKS
marmoset   MDYYRKYAAI ILITLSVFLH ILHSLPDGEF TAEECPECKL KENKYFSRLG SPIYQCMGCC FSRAYPTPLR SQKTMLVPKN VTSESTCCVA KAYTKATVMG NIRVENHTEC HCSTCYHHKF
macaque    MDYYRKYAAV ILVTLSVFLH ILHSFPDGEF TMQDCPECKP RENKFFSKPG APIYQCMGCC FSRAYPTPLR SKKTMLVQKN VTSESTCCVA KSLTRVMVMG NVRVENHTQC HCSTCYYHKF
turkey     MDCYRKYAAV TLTILSVFLH LLHTFPDGEF LMQGCPECKL GENRFFSKPG APIYQCTGCC FSRAYPTPMR SKKTMLVPKN ITSEATCCVA KAFTKITLKD NVKIENHTDC HCSTCYYHKS
quail      MDCYRKYAAV TLTILSVFLH LLHTFPDGEF LMQGCPECKL GENRFFSKPG APIYQCTGCC FSRAYPTPMR SKKTMLVPKN ITSEATCCVA KAFTKITLKD NVKIENHTDC HCSTCYYHKS
e.eel      MMVCPGKPGA SLLMLSMLFH IIDSYPNNEM ARGGCDECRL QENKIFSKPS APIFQCVGCC FSRAYPTPLR SKKTMLVPKN ITSEATCCVA REVTRL---D NMKLENHTDC HCSTCYYHKF
a.catfish  MTLIPKYTGA TILLLCVLIE IGQLYPNNDF ---GCEECKL KENNIFSKPG APVYQCMGCC FSRAYPTPLR SKKTMLVPKN ITSEATCCVA KEVKRVIVND -VKLVNHTDC HCSTCYYHKF
salmon     ---------- -SLILSILLY MADSYQNSDM TNVGCEECKL KENKVFSNPG APVYQCTGCC FSRAYPTPLQ SKKAMLVPKN ITSEATCCVA KEGERVVV-D NIKLTNHTEC WCNTCYHHKS
                      *.:::  :    :          * **       *  * .  .  .; ** *** ** ***** ; *:*:*** ** ;***:.**** :    :          :: *** *   *.***;**
```

Fig. 2. Multiple sequence alignment of the glycoprotein hormone common α-subunit. The deduced amino acid sequences of the bottlenose dolphin glycoprotein hormone common α-subunit gene are aligned with these vertebrates. Gaps (−) are inserted to obtain maximum homology. Amino acid positions are indicated on the top. Ten conserved cysteines are denoted by "*", two putative *N*-linked glycosylation sites, and one *O*-linked glycosylation site by "⇓". Conserved and similarity residues are indicated by "*", "." or ":" under sequences.

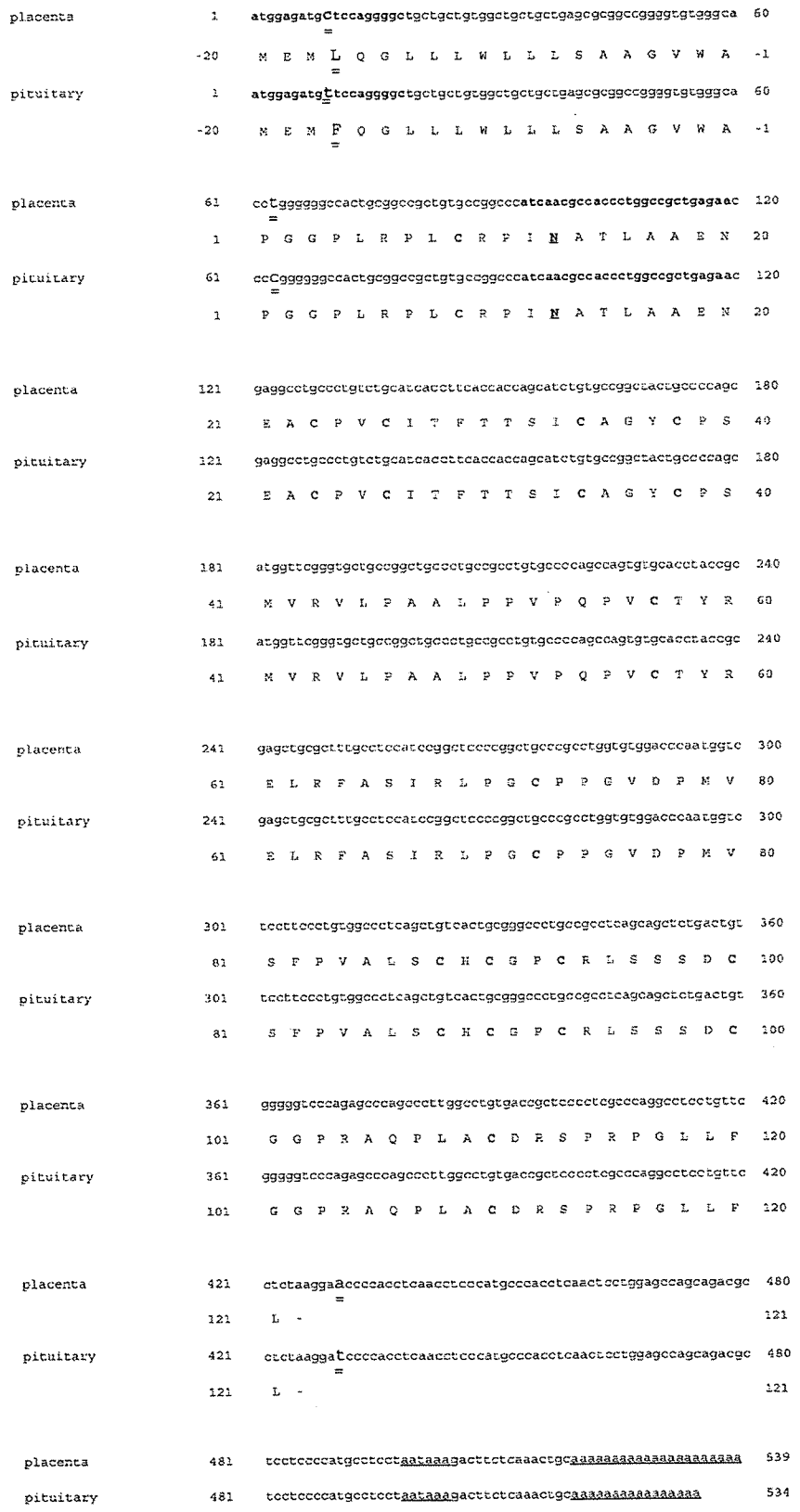

Fig. 3. cDNA nucleotide and deduced amino acid sequences of the bottlenose dolphin gonadotropin β-subunit. The forward custom primers were designed from nucleotide positions 1 to 20(B1) and 94 to 119(B2). The primers sequences are shown in bold. The polyadenylation signal and poly(A)$^+$ tail are underlined. The *N*-glycosylation sites are shown in bold and are underlined. The proposed proper folding involves 12 cysteines shown in bold. The nucleotide and amino acids residues showed that there was a difference between the placenta and pituitary (double line).

Table 3
Nucleotide similarities between the bottlenose dolphin placental cDNAs and those of other vertebrates

Common name	Bottlenose dolphin gonadotropin	Bottlenose dolphin gonadotropin
	α-subunit cDNA Homology (%)	β-subunit cDNA Homology (%)
Pituitary	99.4	99.4
Brush-tailed possum	75.8	68.3
Red kangaroo	76.6	69.9
Pig	NA	NA
Bovine	89.4	85.4
Sheep	89.6	88.0
Donkey	87.6	85.0
Plains zebra	87.3	86.1
Horse	85.4	68.8
White rhinoceros	NA	91.0
Dog	93.3	87.5
Cat	93.4	89.6
Mouse	73.3	85.8
Golden hamster	72.7	80.8
Human	83.1	LH: 67.8 CG: 77.4
Common marmoset	86.0	CG: 78.9
Crab eating macaque	87.9	LH: 84.5 CG: 81.8
Common turkey	66.6	52.8
Common quail	66.5	53.9
European eel	58.1	53.3
African catfish	58.7	58.1
Chum salmon	56.2	60.3

Similarities were calculated as the percent of identical nucleotides over the entire coding regions. Note: NA, cDNA data are not available.

Asn-13 from the N-terminus of the putative mature peptide. All of the 12 cysteines and the putative *N*-linked glycosylated site of the dolphin gonadotropin β-subunit were completely conserved among the mammals (Fig. 5). A phylogenic analysis showed that the dolphin placental and pituitary gonadotropin β-subunits belong to the cluster of mammalian LHβ genes (Fig. 6).

In the present study, we cloned LHβ-like sequences from cDNA libraries prepared with placental and pituitary tissues. As with LHβ, we observed two nucleotide substitutions, 9(C/T) and 63(T/C), in the coding region and a single substitution, 430 (A/T), in the untranslated region. The deduced amino acid sequence differed between the placenta and pituitary gland as follows: AA-17 (Leu−>Phe) (Table 4).

3.3. Statistical analysis

We tried PCR based cloning with at least 2–3 colonies. For placenta LHα, 3 of 3 colonies showed identical cDNA sequences. For placenta LHβ, 2 of 2 colonies showed identical cDNA sequences.

In pituitary LHα, 3 of 3 colonies showed identical cDNA sequences, while in pituitary LHβ the 1st and 2nd subclones showed 2 base pair differences. Then we tried to sequence 3 additional colonies and obtained identical cDNA sequences with the 1st one. Thus, we considered the difference was arisen from PCR or sequencing errors.

3.4. Immunohistochemistry

To determine the localization of the LH β-like subunit, we performed enzyme labeled immunohistochemistry on sections of a dolphin placenta. Anti-ovine LH β-subunit anti-sera, applied to a dolphin placenta, stained the outer syncytiotrophoblasts. No labeling was seen in the interior layer of cytotrophoblasts or fibrovascular connective tissue cores (Fig. 7−1,4). We observed negative staining replaced primary antibody with PBS (Fig. 7−3) and kidney (Fig. 7−6) and other tissues(data not shown).

4. Discussion

Glycoprotein hormones, such as follicle-stimulating hormones (FSHs), luteinizing hormones (LHs), thyroid-stimulating hormones (TSHs), and chorionic gonadotropin (CG), are key endocrine hormones secreted from the pituitary gonadotrophs and thyrotrophs and the placenta in primates. In primates, successful pregnancy depends on placental CG expression. CG is a glycoprotein hormone expressed in the human placenta that binds to LH/CG receptors on the corpus luteum and prevents its regression at menstruation. It also stimulates continued progesterone production which maintains the uterine lining in a specialized state that is receptive to implantation and placental development, and which regulates maternal immune responses. However, CG has not been found in other mammalian orders. Recent genomic analyses have shown that CGβ genes do not exist

Table 4
Deduced amino acid sequence similarities between bottlenose dolphin placental gonadotropins and other gonadotropins of other vertebrates

Common name	Bottlenose dolphin gonadotropin	Bottlenose dolphin gonadotropin
	α-subunit a.a. Homology (%)	β-subunit a.a. Homology (%)
Pituitary	97.5	99.3
Brush-tailed possum	95.0	73.4
Red kangaroo	95.0	75.4
Pig	96.7	94.3
Bovine	91.7	84.2
Sheep	92.5	84.4
Donkey	86.6	83.2
Plains zebra	85.7	84.7
Horse	83.2	81
White rhinoceros	NA	93.3
Dog	94.2	90.6
Cat	94.1	88.1
Mouse	90.8	96.5
Golden hamster	90.0	85.8
Human	70.8	LH: 67.9 CG: 72.3
Common marmoset	84.0	CG: 71.8
Crab eating macaque	82.4	LH: 75.9 CG: 77.9
Common turkey	79.2	46.4
Common quail	79.2	48.2
European eel	62.5	44.3
African catfish	60.5	42.1
Chum salmon	59.1	42.3

Similarities are expressed as the percent of identical amino acids over the total for the species.

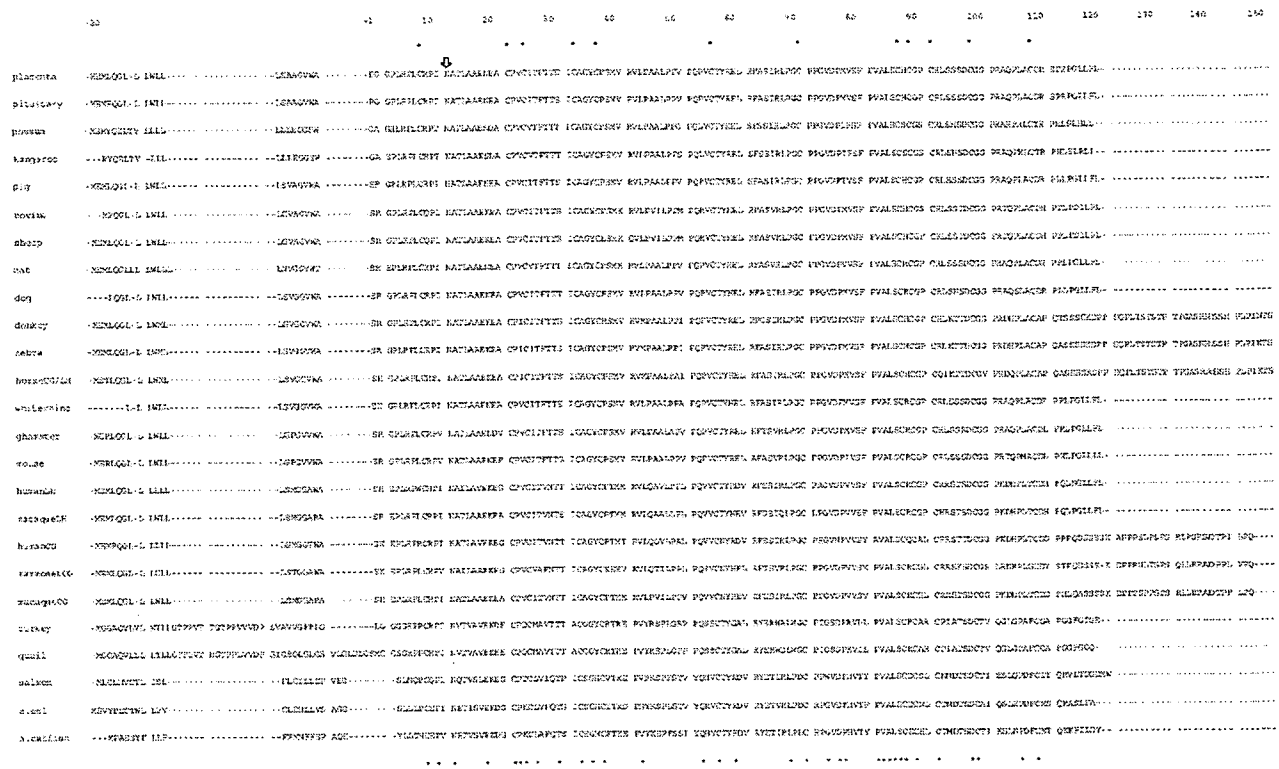

Fig. 4. Multiple sequence alignment of the gonadotropin β-subunit. The deduced amino acid sequences of the bottlenose dolphin gonadotropin β-subunit gene are aligned with these vertebrates. Gaps (−) are inserted to obtain maximum homology. Amino acid positions are indicated on the top. Residues identical to bottlenose dolphin gonadotropin β-subunit are presented in white letters. Twelve conserved cysteines are denoted by "*", and a putative *N*-linked glycosylation site by "Ψ". Conserved residues are indicated by "*" under the sequences.

in rats (Jameson et al., 1984; Tepper and Roberts, 1984; Carr and Chin, 1985), mice (Kumar and Matzuk, 1995), cows (Virgin et al., 1985), pigs (Ezashi et al., 1990), sheep (Brown et al., 1993), or the rhinoceros (Lund and Sherman, 1998). Though there is only one report, employing highly sensitive RT-PCR technique, suggesting mRNA expression of LH in rat placentae (Shinozaki et al., 1997), most researchers regard placental expression of gonadotropin is observed only in primates and equids. From comparative molecular analysis of cDNA structure, Maston and Ruvolo (2002) proposed its recent origin within primates. It is noteworthy that horses have evolved placental LH expression, which is functionally convergent upon the anthropoid CG, but with a different molecular basis (Sherman et al., 1992).

In the present study, we cloned LHβ-like substances in a dolphin placenta. To our knowledge, this is the second report to show evolutionary convergence of glycoprotein hormones among mammalian placentae. However, it is improbable that only horses and dolphins express LHβ in their placentae. The placentae from other mammalian animals, viviparous reptiles, and fishes are possible candidates for the expression glycoprotein hormones, since a recent article reported evolutionary conservation of glycoprotein hormones, with α- and β-subunits, not only in vertebrates but also in invertebrate animals (Hsu et al., 2002; Sudo et al., 2005; Park et al., 2005). LH and FSH genes were cloned in all classes of vertebrates including pisces (Chatterjee et al., 2005; Degani et al., 2003; Gen et al., 2000; Hassin et al., 1995; Hsieh et al., 2001; Hellqvist et al., 2004; Huggard-Nelson et al., 2002; Hurvitz et al., 2005; Jackson et al., 1999; Kim et al., 2005; Kitahara et al., 1988; Kumar and Trant 2004; Koide et al., 1992; Kwok et al., 2005; Li et al., 2005; Mateos et al., 2003; Parhar et al., 2003; Querat et al., 1990a,b, 2004; Rebers et al., 1997; Sekine et al., 1989; So et al., 2005; Vischer and Bogerd, 2003; Weltzien et al., 2003), amphibians (Komoike and Ishii, 2003; Saito et al., 2002), Reptiles (Aizawa and Ishii, 2003; Chien et al., 2005), birds (Ando and Ishii, 1994; Foster and Foster, 1991; Kawasaki et al., 2003; Kikuchi et al., 1998; You et al., 1995) as well as mammals (Bello et al., 1989; Brown et al., 1993; Chin et al., 1981, 1983; Chopineau and Stewart, 1996; Chopineau et al., 1999; Crawford et al., 1986; Crichton et al., 2003; D'Angelo-Bernard et al., 1990; Degani et al., 2003; Ezashi et al., 1990; Fiddes and Goodman, 1979, 1980; Fiddes and Talmadge, 1984; Godine et al., 1982; Harrison et al., 1998; Hirai et al., 1989; Jameson et al., 1984; Kato and Hirai, 1989; Kato et al., 1991; Koura et al., 2004; Liao et al., 2003; Lund and Sherman, 1998; Lovejoy et al., 1992; Maurer, 1985; Nilson et al., 1983; Schmidt et al., 1999; Sherman et al., 2001, 1997, 1992; Simula, 1995; Tepper and Roberts, 1984; Virgin et al., 1985; Wolf et al., 1987; Yang et al., 2000; Zanella et al., 1996). Thus we can expect to find expression of LH or related glycoprotein hormones in placenta other than primates, equids and dolphins.

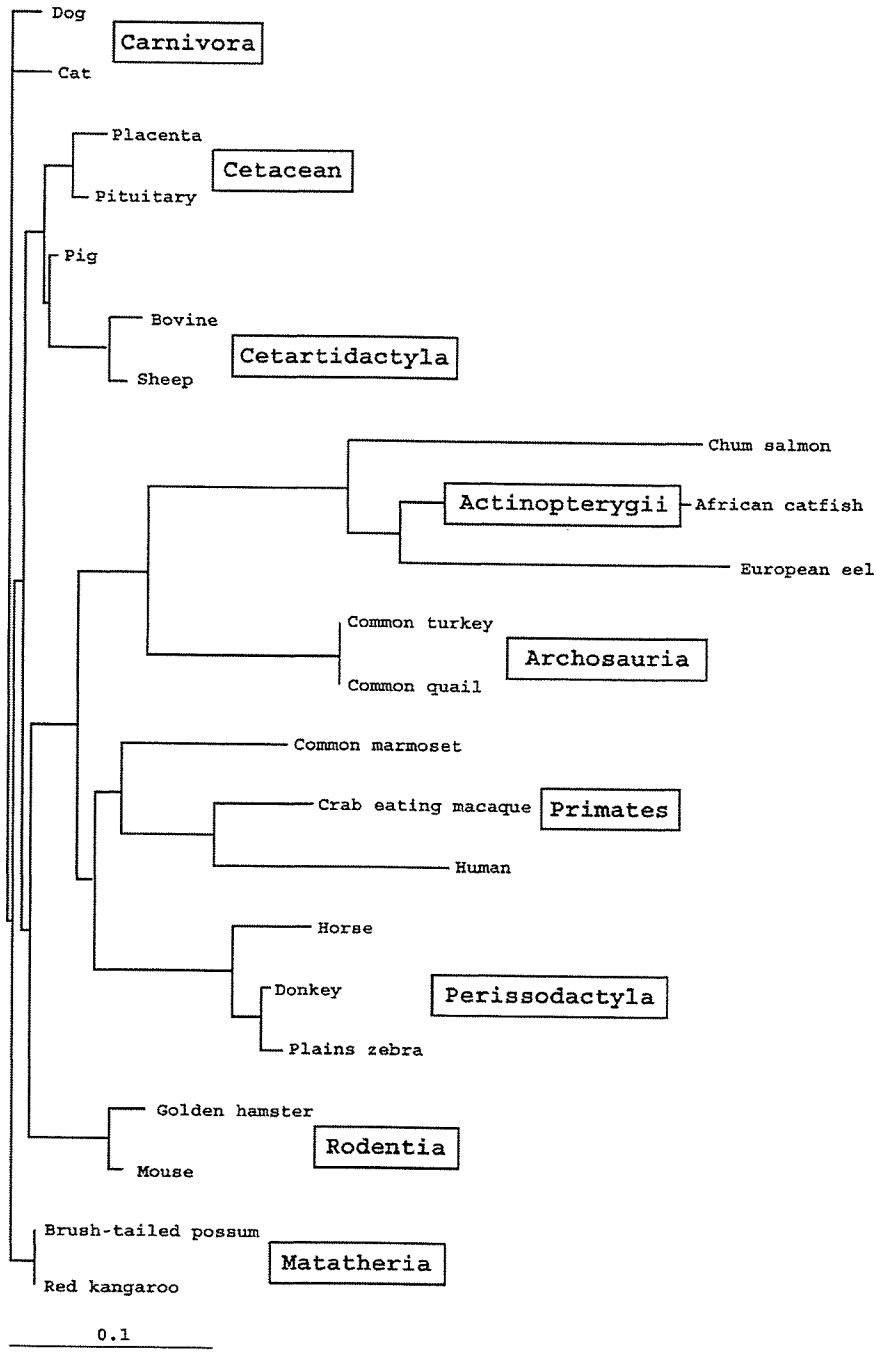

Fig. 5. Phylogenetic tree of the amino acid sequences of the vertebrate gonadotropin common α-subunits. The lengths of the horizontal lines indicate genetic distances. The sequences were made with reference to the same studies cited in Figs. 1 and 2.

Because one of the authors recently presented a hypothesis that viviparity is evolved from its evolutionary advantage on reproductive success in all vertebrate classes except for Avians (Hayakawa, 2006).

Of interest, we cloned the α-subunit (common to pituitary glycoprotein hormones) and LH β-subunit-like sequences from a dolphin placenta and found that the cDNA sequences showed several point mutations from pituitary cDNA. We propose two possible explanations. First, dolphins may have two or more genes coding the common α-subunit and LH β-subunits. In fact, primate genomes contain at least two LHβ analogues (LHβ and CGβ). However, we consider this explanation to be improbable because most mammalian species have only single common α-subunit gene and molecular homologies between dolphin placenta derived LHβ and pituitary LHβ and because the placenta-derived α-

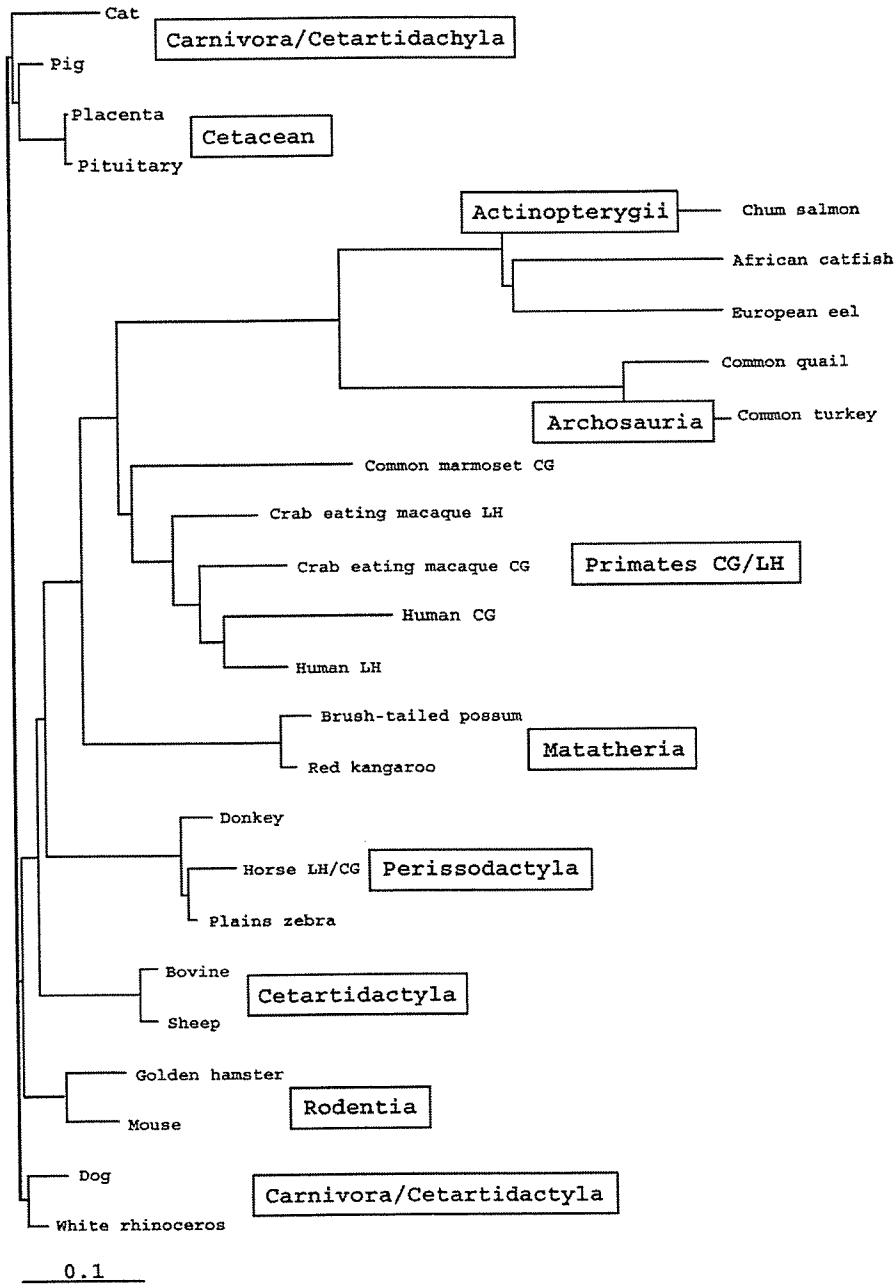

Fig. 6. Phylogenetic tree of the amino acid sequences of the vertebrate gonadotropin β-subunits. Genetic distances are indicated by the lengths of the horizontal lines. The sequences were made with reference to the same studies cited in Figs. 3 and 4.

subunit and pituitary α-subunit were highly homologous compared with hCGβ and human LHβ.

Second, these genetic differences were derived from a single nucleotide polymorphism (SNP) in bottlenose dolphins. Although most scientists today only recognize one species of bottlenose dolphin, the variations have been classified into at least three subspecies. Some physical characteristics vary so much among them that separate species classifications have been suggested. The captive Azami in Enoshima aquarium and the wild dolphin captured at Taiji may have had different genetic backgrounds. Due to the relative inaccessibility of wild dolphin placentae, it is difficult to establish cDNA cloning of LH genes both from placental and pituitary tissues simultaneously.

Recently, carboxyl terminal peptide (CTP) sequences of the chorionic gonadotropin (CG) beta subunit have been cloned in primates and horses genes. These sequences are considered to serve as an effective linker to enhance the secretion of the analogs compared to variants lacking the CTP (Nakav et al., 2006). However, as the gonadotropin

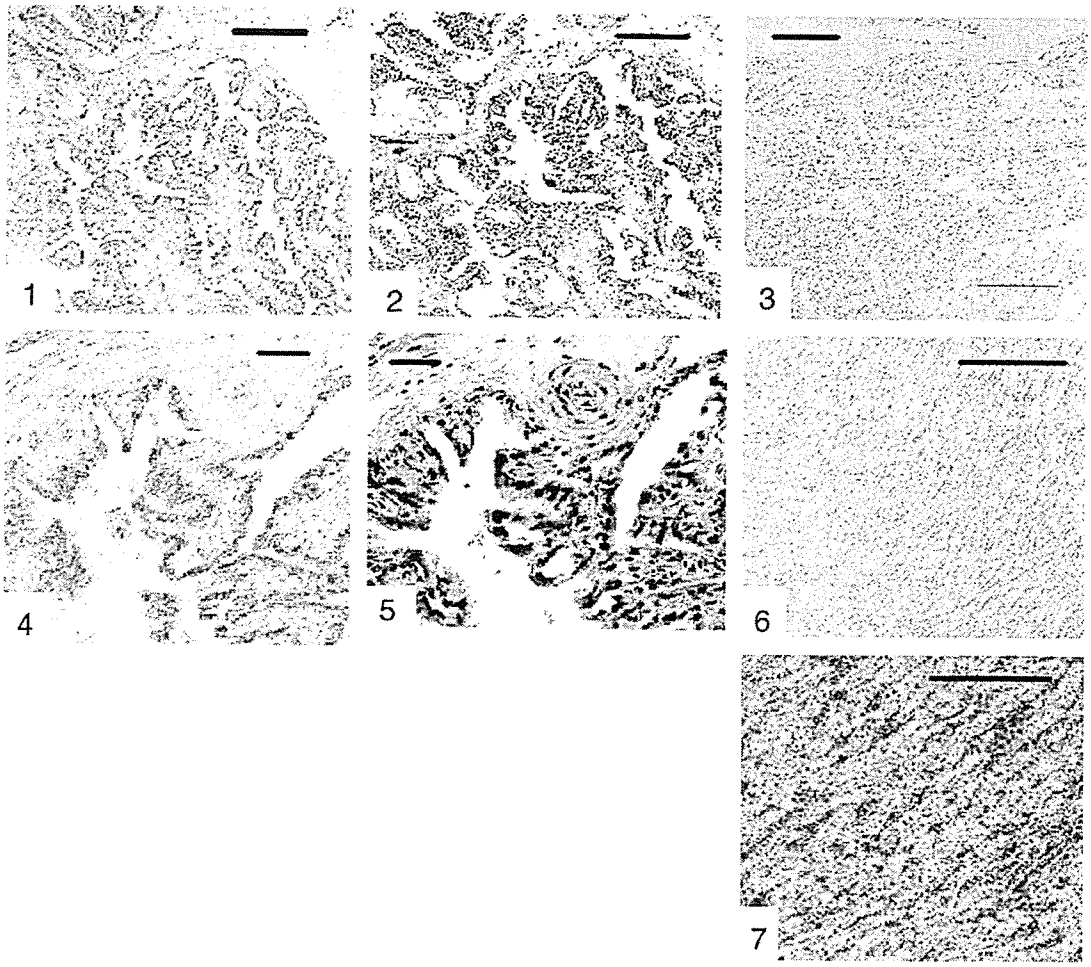

Fig. 7. Immunohistochemical distribution of the LHβ-subunit-like substance(s) in a dolphin placenta. 1, Low-power image of dolphin placenta showing the immunohistochemical staining treated with rabbit anti-ovine LH β-subunit serum. 2, Low-power image of hematoxyline/eosine staining of dolphin placenta. 3, The negative control set up by omitting the primary antibody. 4, High-power image of dolphin placenta showing the immunohistochemical staining treated with rabbit anti-ovine LH β-subunit serum. 5, High-power image of hematoxyline/eosine staining of dolphin placenta. 6, The negative control set up by kidney of dolphin. 7, Image of hematoxyline/eosine staining of dolphin kidney. Bars: 200 μm (1,2,3,6,7), 30 μm (4,5).

subunits of most non-primates, non-equid species lack a CTP domain suggesting it is not essential for endocrine function. Further, primate and equine CTP sequences were generated by read-through of consensus translation stop codons and frame-shift mutation (Fiddes and Talmadge, 1984; Sherman et al., 1992). In the present study, we searched CTP and/or related sequences in cDNA obtained from dolphin placenta and pituitary samples but failed to find out these sequences.

We identified the stop codon in 3′ terminus of LHβ genes in cDNA clones obtained from both placenta and pituitary gland. Taken together, though CTP is effective for endocrine functions, it is not essential for gonadotropin functions at least in dolphins. In other words, the primordial mammalian LHβ gene existed in the common ancestor of Perissodactyla, Artioductyls (putative ancestor of dolphins) and primates. CTP sequences evolved toties quoties by different genetic mechanisms by its virtue of endocrine efficiency.

In conclusion, we identified placental expression of LH-like substance(s) in a bottlenose dolphin for the first time in non-primate animals other than equids. Our findings suggest the evolutionary convergence of placenta-derived hormone(s) for the maintenance of pregnancy. Further studies are requested to better understand the genetic evolution of glycoprotein families in Cetaceans.

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