The Journal of Veterinary Medical Science

FULL PAPER

Wildlife Science

Localization of peptide hormones in the placentas of Bryde's (*Balaenoptera brydei*), sei (*B. borealis*), and common minke (*B. acutorostrata*) whales

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ABSTRACT. In this study, we examined the morphological features of the placentas from 3 species of rorqual whales (Balaenopteridae), namely Bryde's (*Balaenoptera brydei*), sei (*B. borealis*), and common minke (*B. acutorostrata*) whales, and verified the secretion of 2 placental-specific peptide hormones, placental lactogen (PL) and chorionic gonadotropin (CG). The placentas were collected in the second phase of the Japanese Whale Research Program under a special permit in the North Pacific (JARPN II) between 2009 and 2010. For all three species of rorqual whales, as the fetus grew, the interdigitation between the maternal endometrial folds and chorionic villi became more complicated, and many blood capillaries of chorionic villi and endometrium became larger and infiltrated the trophoblast cells and endometrial epithelial cells, respectively. In the immunohistochemical examination, the trophoblast cells (except for areolar trophoblast cells) showed immunoreactivities for the PL and luteinizing hormone (LH) antibodies, and this phenomenon was similar in the placentas of all 3 rorqual whale species. Our results suggest that PL and LH-like CG play roles in regulating pregnancy in the placenta of cetacean.

KEYWORDS: chorionic gonadotropin, placenta, placental lactogen, rorqual whale, trophoblast cells

J Vet Med Sci
86(4): 451–457, 2024
doi: 10.1292/jvms.23-0439

Received: 19 October 2023
Accepted: 12 February 2024
Advanced Epub:
28 February 2024

Cetaceans are aquatic mammals that have completely shifted their habitat from land to water during evolution. The molecular biological analysis suggests that cetaceans belong to Cetartiodactyla, which combines the order Artiodactyla and the order Cetacea, and hippopotamuses are the most closely related to cetaceans [9, 13, 35]. Based on recent reports by McGowen *et al.* [12] and Rosel *et al.* [19], the extant cetaceans are classified into 4 families, 6 genera, and 15 species of baleen whales (Mysticeti), and 10 families, 34 genera, and 75 species of toothed whales (Odontoceti). In histological studies of cetaceans, most organ samples are obtained inadvertently after the animal becomes stranded, and much of the tissues are damaged and unsuitable for histological observation. Therefore, the histological information on cetaceans is lacking compared with other mammals.

Cetaceans have diffuse and epitheliochorial placentas, devoid of specialized trophoblast cells such as binucleate cells or giant multinucleate cells [1, 5, 10, 11, 22, 23, 30, 31]. The placenta of cetaceans has an areola that forms a gap between the chorion and the endometrium, and the trophoblast cells in the areolar region (areolar trophoblast cells) absorb secretions from the uterine glands that have accumulated in the areola [21]. Moreover, the supply of iron from the maternal organ is necessary for fetal growth, and uteroferrin may be involved in the transport of iron from the mother to the fetus in the placenta [11]. The placenta is also one of the endocrine organs that secrete hormones to regulate pregnancy. In our previous study, we showed that trophoblast cells, except for areolar trophoblast cells, synthesized sex steroid hormones in 4 species of rorqual whales (Balaenopteridae): Antarctic minke (*Balaenoptera bonaerensis*), Bryde's (*B. brydei*), sei (*B. borealis*), and common minke (*B. acutorostrata*) whales [11, 22]. However, little is known about the secretion of peptide hormones in the placentas of cetaceans. The presence and localization of hormones and growth factors secreted from the placenta vary depending on the animal species, and analyses of these factors are essential for comprehensive understanding of placental function in each animal.

In this study, we focused on peptide hormones secreted specifically from the placenta: placental lactogen (PL) and chorionic

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gonadotropin (CG) as the next step in the analysis of placental hormones following the analysis of steroid hormones [11, 22]. PL has been found in primates, rodents, and the Ruminantia and Tylopoda, which are suborders of the Cetartiodactyla [6, 7, 15, 29, 32, 34], but there have been no reports on the verification of PL in cetaceans. The expression of CG has also been observed in primates including humans (hCG) and equids (eCG), and the presence of luteinizing hormone (LH)-like CG has been reported in the placenta of cetaceans, namely bottlenose dolphins (*Tursiops truncatus*) [28].

Herein, we examined the presence and distribution of PL and LH-like CG in the placentas of 3 species of rorqual whales (Bryde's, sei, and common minke whales) to clarify the hormone secretory potential in the placentas of cetaceans. Furthermore, we investigated the morphological features of the placentas in detail.

MATERIALS AND METHODS

Animals

The placentas of 8 Bryde's (fetal length of 23.0–211.4 cm), 14 sei (fetal length of 59.0–203.4 cm), and 4 common minke (fetal length of 51.6–141.0 cm) whales were used in this study. The samples were collected in the second phase of the Japanese Whale Research Program under a special permit in the North Pacific (JARPN II) organized by the Institute of Cetacean Research (Tokyo, Japan) between 2009 and 2010. Special attention was given to all whales sampled during the program, reducing the time to death. Explosive harpoons were used for all whales as the primary killing method in accordance with Schedule III of the International Convention for the Regulation of Whaling.

Histology and immunohistochemistry

Small pieces of tissue were randomly collected from the placentas and immediately fixed in Bouin's fluid or 10% phosphate-buffered formalin (pH 7.4) for at least 24 hr. The samples were transferred to 70% ethanol, then dehydrated in a graded series of ethanol, cleared in xylene, and embedded in paraffin (Paraplast Plus, Kendall, Mansfield, MA, USA). The tissue blocks were cut into 4 µm-thick sections and mounted onto slides (511617, Muto Pure Chemicals Co., Ltd., Tokyo, Japan). After deparaffinization and rehydration with xylene and ethanol, the sections were used for hematoxylin and eosin (HE) and immunohistochemical staining using ImmPRESS reagent (Vector Laboratories, Inc., Burlingame, CA, USA).

In the immunohistochemical staining, the sections were heated in target retrieval solution (1:10, pH 9, S1699; DakoCytomation, Inc., Carpinteria, CA, USA) using a microwave for 15 or 20 min to retrieve the antigen, and then immersed in methanol containing 0.3% H_2O_2 for 10 min at room temperature (RT) to block the endogenous peroxidase activity. The sections were then incubated with normal horse serum provided in an ImmPRESS kit for 30 min at RT to block nonspecific staining and incubated for 18 hr with a polyclonal anti-human PL antibody raised in the rabbit (CSH2, 16326-1-AP, 1:100, Proteintech Group, Inc., Rosemont, IL, USA) or anti-ovine Beta LH antibody raised in the rabbit (AFP697071P, 1:1,000, NHPP, Torrance, Ca, USA) at 4°C in a moisture chamber. After incubation with the primary antibody, the sections were incubated with horse anti-rabbit IgG provided in an ImmPRESS kit for 30 min. The binding sites were visualized using Tris-HCl buffer (pH 7.4) containing 0.02% 3,3′-diaminobenzidine hydrochloride (DAB) and 0.006% H_2O_2. The staining with DAB was stopped by washing the samples with 0.01 M phosphate-buffered saline (PBS, pH 7.4). The sections were counterstained with hematoxylin for nuclei, dehydrated in a graded series of ethanol, cleared in xylene, cover-slipped, and observed using an optical microscope. The negative control sections omitted the primary antibody.

RESULTS

Histology

The 3 species of rorqual whales have diffuse and epitheliochorial placentas, in which the maternal endometrial crypts and chorionic villi (trophoblast cells) were complicatedly interdigitated (Fig. 1). The endometrial epithelium consisted of monolayer squamous or cuboidal cells. In contrast, the chorionic villi were mostly lined with monolayer cuboidal or columnar cells and had no specialized trophoblast cells such as binucleate cells and giant multinucleate cells (Fig. 1). As the fetus grew, the interdigitation between the fetal chorionic villi and the maternal endometrial folds became complicated because they were more branched and elongated (Fig. 1A). Furthermore, as the pregnancy progressed, blood capillaries of the chorionic villi and those of the endometrial lamina propria located under the epithelium became larger in diameter and infiltrated the trophoblast cells and endometrial epithelial cells, respectively (Fig. 1B and 1C).

In all of the placentas examined, areolae were observed, presenting as dome or pouch-like structures on the fetal side (Fig. 2A-1 and 2B-1), and the areolar trophoblast cells were identified as taller columnar trophoblast cells with bright cytoplasm (Fig. 2A-2 and 2B-2). The chorion lined with areolar trophoblast cells formed simple pouch-like areolae with unbranching in short fetal stage (Fig. 2A) and became deeply invaginated into the stroma of the chorion and developed multiple branches into the interior with fetal growth (Fig. 2B). The transition from typical trophoblast cells to areolar trophoblast cells was distinguishable in the histological observations (asterisks in Fig. 2A-2 and 2B-2).

Immunohistochemistry

Immunoreactivities for PL and LH were detected in the typical trophoblast cells in the placentas from all 3 species of rorqual whales (Figs. 3 and 4), and the localization of PL was found in the apical area of the cytoplasm (Fig. 3). On the other hand, LH immunoreactivities were confirmed throughout the cytoplasm (Fig. 4). In the areolar trophoblast cells and endometrium, no

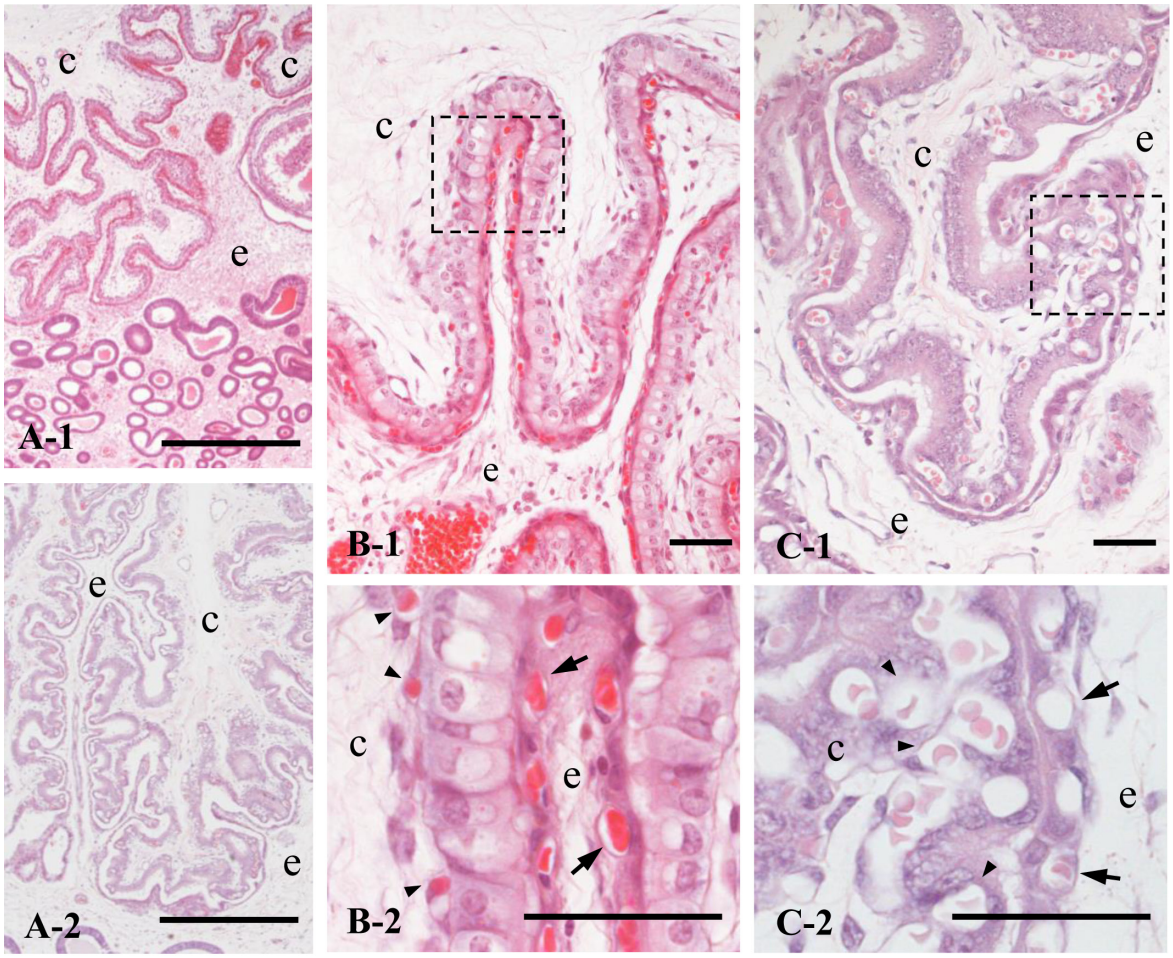

Fig. 1. Histological observation of the placenta in Bryde's whales. **A-1** and **B**: fetal length (L) 53.6 cm. **A-2** and **C**: L 211.4 cm. The interdigitation between the maternal endometrial epithelium and the fetal chorionic villi was simple in early gestation (**A-1**). As the fetus grew, this interdigitation became more complicated (**A-2**). As the fetus grew, blood capillaries of the chorionic villus (arrowheads) and endometrium (arrows) became larger and infiltrated the trophoblast cells and between endometrial epithelial cells, respectively (**B** and **C**). **B-2** and **C-2** are enlarged views of dotted squares in **B-1** and **C-1**, respectively. c, chorionic villi; e, endometrium. Bar=500 µm (A), Bar=50 µm (B and C). HE staining.

immunoreactivity for PL or LH was identified (Figs. 3B–D and 4). Immunostaining of PL and LH did not reveal any differences related to fetal length and species.

DISCUSSION

In this study, the immunoreactivity for PL was detected in the trophoblast cells, except for the areolar trophoblast cells, in the placentas of 3 species of rorqual whales from the stage of short fetal length. This is the first report of PL expression in cetaceans. In the Cetartiodactyla, PL has been detected in the Tylopoda and Ruminantia, such as Bovidae, Cervidae, and Giraffidae, but was not expressed in the Suina or Tragulidae [6, 7, 15, 29, 32, 34]. Regarding other orders, PL has been confirmed in primates and rodents, but was not detected in insectivores, chiropteras, edentates, lagomorphs, carnivores, or perissodactyls [7]. In the Cetartiodactyla, PL has been studied mostly in the Bovidae such as cows and sheep. Bovine PL (bPL) was detected in the maternal circulation after day 60 of gestation (early gestation), and bPL levels increased gradually with the progression of gestation, rapidly peaking on days 200 to 220 (late gestation) [16, 17, 26]. The bPL level in fetal blood is much higher than that found in the maternal plasma, and although it decreased with the progression of gestation, the bPL level in fetal blood always remains higher than that in maternal circulation [2, 27]. In the ovine placenta, moreover, ovine PL stimulates hyperplasia and hypertrophy of the uterine glands and secretion of histotrophe (uterine milk) from the uterine glands [14, 24, 25]. Although this study cannot show whether PL acts on the mother or the fetus and what kind of effect it has, the apical localization of PL in the trophoblast cells may have the potential for secretion to the maternal side. In cetaceans, PL may be involved in the promotion of fetal growth by enhancing uterine gland development (indirect method) and/or by direct action on the fetus. In the future, the analysis of receptors for PL will be necessary to search for targets of PL.

Immunoreactivity for LH was also observed in the trophoblast cells, except for the areolar region, in the placentas of all 3 rorqual whale species. LH-like CG has been reported in the placenta of bottlenose dolphins obtained from afterbirth, which was

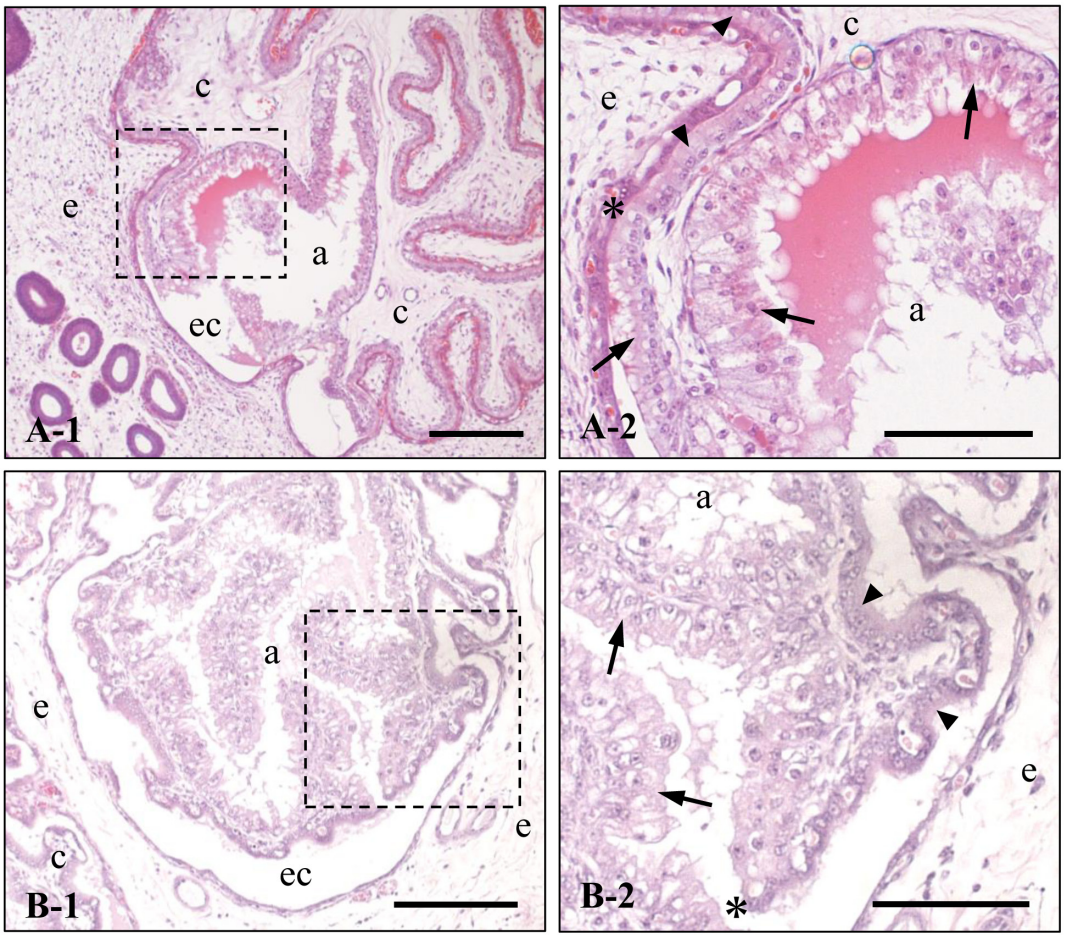

Fig. 2. Areola in the Bryde's whale. **A**: fetal length (L) 53.6 cm. **B**: L 211.4 cm. **A-2** and **B-2** are enlarged views of dotted squares in **A-1** and **B-1**, respectively. Areolar trophoblast cells were characterized by taller columnar trophoblast cells with a bright cytoplasm and long microvilli. The chorion became developed multiple branches with fetal growth. a, areola; c, chorionic villi; e, endometrium; ec, endometrial crypt; *, transition part from typical trophoblast cells to areolar trophoblast cells; arrow, areolar trophoblast cell; arrowhead, trophoblast cell. Bar=200 µm (A-1 and B-1), Bar=100 µm (A-2 and B-2). HE staining.

immunohistochemically stained using an ovine LH antibody, and the CG structure was genetically similar to that of the pituitary LH [28]. In the present study, the expression of LH was immunohistochemically observed from the stage of short fetal length, suggesting that LH-like CG is secreted persistently from early pregnancy to parturition. In the placenta of some primates and equids, CG is secreted from the trophoblast cells and plays a role in maintaining progesterone secretion during early pregnancy [4, 18]. In cetaceans, CG may also be involved in the maintenance of pregnancy in early development. In humans and horses, the major role of CG is similar, regulating the secretion of progesterone from the corpus luteum, but the structures of their CGβ subunits are different [3, 4]. In humans, hCG is secreted immediately after implantation and promotes progesterone production for 3–4 weeks during pregnancy. The secretion of hCG reaches a peak around 10 weeks of pregnancy, and then the secretion of progesterone shifts completely from the corpus luteum in the ovaries to the placenta; however, hCG continues to be produced at low levels until parturition [3]. In horses, eCG production begins at 6 weeks of pregnancy, which is much later than the onset of hCG production, maintains pregnancy until shifting to progesterone production in the placenta by stimulating the primary corpus luteum, and induces the formation of the secondary corpus luteum [4].

In this study, the examined peptide hormones were not detected in the areolar trophoblast cells. This localization was also observed for the steroid hormone synthase in the placentas of 4 species of rorqual whales [11, 22], suggesting that the areolar trophoblast cells are not involved in the secretion of steroid and peptide hormones in the placenta.

In our histological observation of the 3 rorqual whale placentas, as fetal and maternal capillaries infiltrated between the epithelial cells with fetal growth, the distance between the fetal and maternal capillaries became closer. This result is consistent with other reports for the epitheliochorial placenta [8, 20, 33]. This proximity of capillaries is thought to enable efficient transport of diffusible substances, such as oxygen, to the fetal capillaries in order to meet their increased demands with fetal growth [8, 20].

In our previous study, the basic areolar structure of same 3 species of rorqual whales has been reported [11]. In this study during the early stage of the fetal developmental process, we newly identified simple pouch-like areolae with unbranched chorion lined by

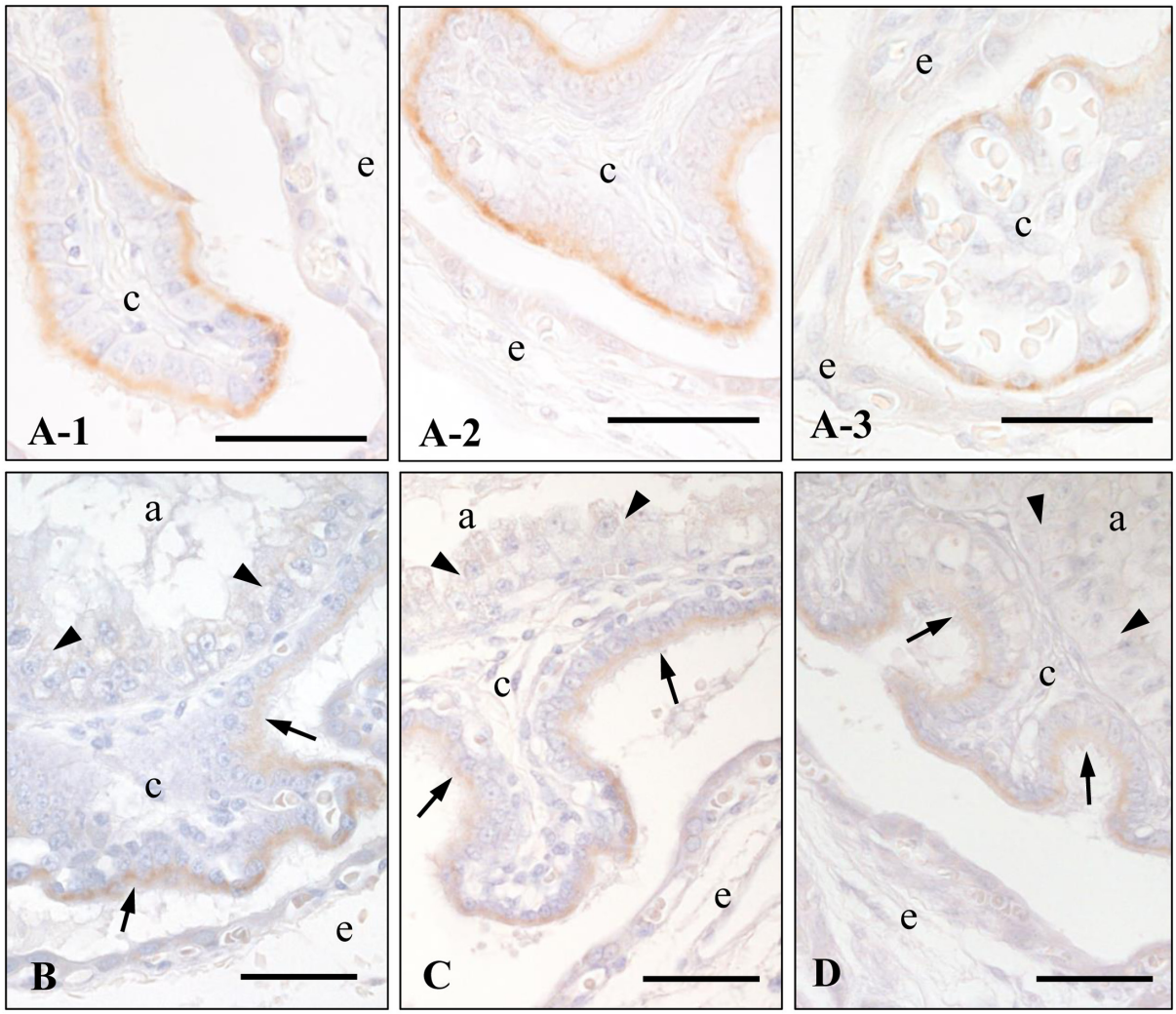

Fig. 3. Immunostaining of placental lactogen (PL) in the three rorqual whale placentas. **A** and **B**: Bryde's whale. **A-1**: fetal length (L) 23.0 cm. **A-2**: L 95.5 cm. **A-3** and **B**: L 211.4 cm. **C**: sei whale (L 144.2 cm). **D**: common minke whale (L 51.6 cm). Strong immunoreactivities for PL were detected in the apical area of trophoblast cells except for the areolar trophoblast cells in the placenta from early gestation. arrow, typical trophoblast cell; arrowhead, areolar trophoblast cell; a, areola; c, chorionic villi; e, endometrium. Bar=50 µm.

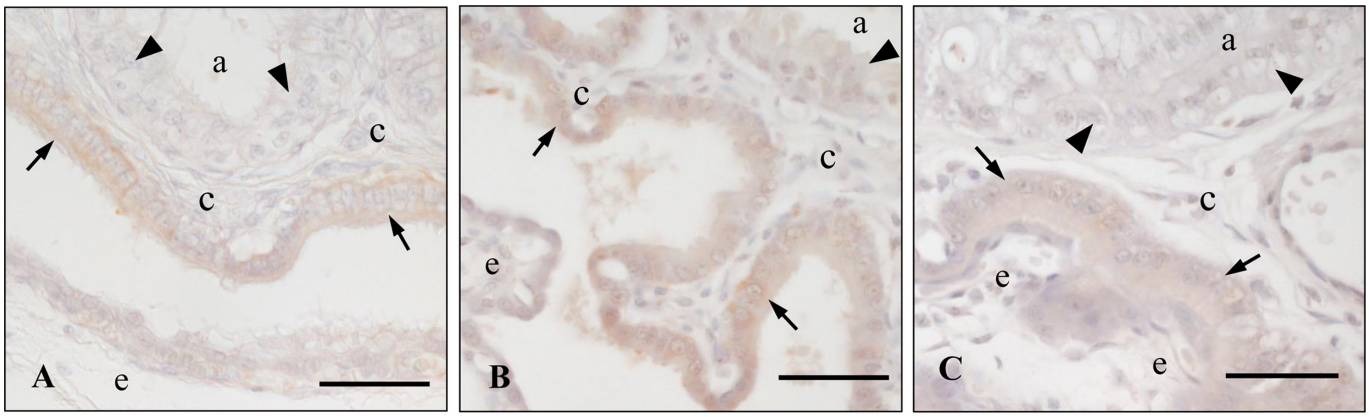

Fig. 4. Immunostaining of luteinizing hormone (LH) in the three rorqual whale placentas. **A**: Bryde's whale, fetal length (L) 95.5 cm. **B**: sei whale (L 144.2 cm). **C**: common minke whale (L 60.2 cm). Immunoreactivities for LH were detected in the typical trophoblast cells (arrows). On the other hand, LH was not expressed in the areolar trophoblast cells (arrowheads). a, areola; c, chorionic villi; e, endometrium. Bar=50 µm.

areolar trophoblast cells. It is thought that, therefore, the chorion lined by absorbent areolar trophoblast cells become more branched to accommodate increased nutritional demands with the progression of gestation.

In conclusion, we revealed PL and LH-like CG localization in the trophoblast cells secreted from the placentas of 3 rorqual whale species. This result provides insight into the regulatory mechanism of placental hormones in the pregnancy of cetaceans.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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