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—Original Article—

Structure and functions of the placenta in common minke (*Balaenoptera acutorostrata*), Bryde's (*B. brydei*) and sei (*B. borealis*) whales

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Abstract. The structure and functions of placentas were examined in 3 species of rorqual whales, common minke (*Balaenoptera acutorostrata*), Bryde's (*B. brydei*) and sei (*B. borealis*) whales, with the aim of confirming the structural characteristics of the chorion, including the presence of the areolar part, and clarifying steroidogenic activities and fetomaternal interactions in the placentas of these whales. Placentas were collected from the second phase of the Japanese Whale Research Program under Special Permit in the North Pacific (JARPN II). Histological and ultrastructural examinations revealed that these whale placentas were epitheliochorial placentas with the interdigitation of chorionic villi lined by monolayer uninucleate cells (trophoblast cells) and endometrial crypts as well as folded placentation by fold-like chorionic villi. Moreover, well-developed pouch-like areolae were observed in the placentas, and active absorption was suggested in the chorionic epithelial cells of the areolar part (areolar trophoblast cells). Berlin blue staining showed the presence of ferric ions (Fe^{3+}) in the uterine glandular epithelial cells and within the stroma of chorionic villi in the areolar part. An immunohistochemical examination revealed tartrate-resistant acid phosphatase (TRAP; known as uteroferrin in uteri) in the cytoplasm of glandular cells and areolar trophoblast cells. This result suggested that, in cetaceans, uteroferrin is used to supply iron to the fetus. Furthermore, immunoreactivity for P450scc and P450arom was detected in trophoblast cells, but not in areolar trophoblast cells, suggesting that trophoblast cells synthesize estrogen in whale placentas. Therefore, we herein immunohistochemically revealed the localization of aromatase and uteroferrin in cetacean placentas during pregnancy for the first time.
Key words: Areola, Aromatase, Placenta, Uteroferrin, Whale

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The rorquals of baleen whales belong to the family Balaenopteridae (including two genera, *Balaenoptera* and *Megaptera*), suborder Mysticeti, order Cetacea. Mead and Brownell Jr. [1] classified rorquals into 7 species, Antarctic minke (*B. bonaerensis*), blue (*B. musculus*), Bryde's (*B. edeni*), common minke (*B. acutorostrata*), fin (*B. physalus*), sei (*B. borealis*) and humpback (*M. novaeangliae*) whales, and included Omura's and Eden's whales in the Bryde's whale group. However, previous studies phylogenetically and taxonomically reclassified the rorquals of the Bryde's whale group into 3 independent species, Omura's (*B. omurai*), Eden's (*B. edeni*) and Bryde's (*B. brydei*) whales [2, 3].

The placentas of cetaceans have been examined previously [4–13]. However, detailed and fetal age-dependent studies on the structure and functions of placentas in baleen whales have not been conducted, except for two studies of the placentas of the Antarctic minke whale [14, 15]. In those two studies, the gross anatomy of the placenta of the Antarctic minke whale revealed a diffuse placenta, while histologically, an epitheliochorial placenta with complex interdigitation between the chorionic villi, which were lined by monolayer mononucleated cells (trophoblast cells) and endometrial crypts, had fetal areolae enclosed by tall trophoblast cells with long microvilli (areolar trophoblast cells). Furthermore, active steroidogenesis was detected in trophoblast cells, but less active or no steroidogenesis at all was detected in areolar trophoblast cells. However, the sex steroid hormones secreted from trophoblast cells and functions of areolar trophoblast cells and uterine glands in the Antarctic minke

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whale have not yet been elucidated in detail. Therefore, we herein investigated the basic structure and functions of the placenta in 3 rorqual whale species, common minke, Bryde's and sei whales.

Materials and Methods

Animals

Four placentas (fetal length of 51.6–141.0 cm) and 3 uteri of common minke whales, 6 placentas (fetal length of 95.5–211.4 cm) and 10 uteri of Bryde's whales, and 11 placentas (fetal length of 14.0–181.0 cm) and 10 uteri of sei whales were used in this study. These samples were collected from the second phase of the Japanese Whale Research Program under Special Permit in the North Pacific (JARPN II) organized by the Institute of Cetacean Research (Tokyo, Japan) in 2009 and 2010. In order to reduce the time until death, special attention was paid to all sampled whales. Explosive harpoons were primarily used to kill whales according to Schedule III of the International Convention for the Regulations of Whaling. After removing the fetuses from pregnant uteri, the total length of each fetus was measured.

Histology and immunohistochemistry

Tissues were randomly collected from placentas and uteri and promptly fixed by Bouin's fluid or phosphate buffered 10% formalin (pH 7.4). Samples were embedded in paraffin (Paraplast Plus®, Kendall, Mansfield, MA, USA) by the usual method, cut serially at a thickness of 4 μm and placed on aminopropyltriethoxysilane-coated or FRONTIER-coated slides (S9226 or FRC-01; Matsunami Glass Ind, Osaka, Japan). Deparaffinized sections were stained with hematoxylin and eosin (HE), Berlin blue, periodic acid-Schiff (PAS) or Martius scarlet blue (MSB) and immunohistochemically stained using the avidin-biotin peroxidase complex (ABC) method [16]. Sections were treated by microwaving in high pH target retrieval solution (1:10, S3307; DakoCytomation, Carpinteria, CA, USA) for 20 min to retrieve antigens, immersed in methanol containing 0.3% H_2O_2 for 10 min at room temperature (RT) to prevent endogenous peroxidase activity and then incubated with normal goat serum (1:50, S-1000; Vector Laboratories, Burlingame, CA, USA) for 30 min at RT to block nonspecific staining. Sections were incubated overnight with the polyclonal anti-rat cytochrome P450 side chain cleavage enzyme (P450scc) raised in the rabbit (1:200, AB1244; Chemicon International, Temecula, CA, USA), monoclonal anti-human cytochrome P450 aromatase (P450arom) raised in the mouse (1:100, MCA2077; Serotec, Oxford, UK) or polyclonal anti-human tartrate-resistant acid phosphatase (TRAP; also called type 5 acid phosphatase, ACP5) raised in the rabbit in order to detect uteroferrin (1:100, CN6911; ProSci, Poway, CA, USA) at 4 C in a moisture chamber. The primary antibodies were diluted with phosphate-buffered saline with 0.5% Triton X-100 (PBS-Triton) or *Can Get Signal*® immunostain, solution B (NKB-601; Toyobo, Osaka, Japan). After being incubated with the primary antibody, biotinylated anti-rabbit IgG raised in the goat (1:200, BA-1000; Vector Laboratories) or anti-mouse IgG raised in the goat (1:200, BA-9200; Vector Laboratories), diluted with PBS-Triton or *Can Get Signal*® immunostain, solution B, was applied for 30 min at RT, and the sections were then incubated with ABC reagent for 30 min (PK-6100; Vectastain *Elite* ABC kit, Vector Laboratories) at RT. The binding sites were visualized with Tris-HCl buffer containing 0.02% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.006% H_2O_2. After being incubated, the sections were washed with phosphate buffered saline (PBS), dehydrated in a graded series of ethanol, cleared in xylene, coverslipped and observed under a conventional light microscope. The negative control sections were treated with the omission of each primary antibody.

Scanning electron microscopy (SEM)

Small pieces of the samples fixed in phosphate buffered 10% formalin were washed in PBS, postfixed in 1% osmium tetroxide in PBS and dehydrated in a graded series of ethanol for scanning electron microscopy. The specimens were then freeze-dried with t-butyl alcohol in a freeze drier (ES-2030; Hitachi High-Technologies, Tokyo, Japan). The dried tissues were mounted on stubs and sputter coated with Pt using an ion sputter (E-1045; Hitachi High-Technologies). The samples were then observed by SEM (S-3400N; Hitachi High-Technologies) at an accelerating voltage of 5.0 kV.

Results

Histology

Each placenta of the 3 whale species examined was an epitheliochorial placenta histologically (Fig. 1). No histological differences were observed between the 3 species. Chorionic villi lined by monolayer uninucleate cells (trophoblast cells) and endometrial crypts were complexly interdigitated, and these interdigitations became more complex with fetal growth (Fig. 2). The trophoblast cells of chorionic villi were cuboidal or columnar in shape and had short microvilli on the apical area (Figs. 3 and 4B). Specialized trophoblast cells such as binucleate trophoblast giant cells were not detected in the chorionic epithelium (trophoblasts). On the other hand, the endometrial epithelium consisted of cuboidal or squamous cells (Fig. 3). In a sei whale placenta with a fetal length of 14.0 cm, which was considered the earliest gestation period in this study, the epithelium was lined by cuboidal epithelial cells only (Fig. 3B).

Many areolae with a pouch-like structure projected by several villous chorionic folds were observed in the placentas of all 3 species (Fig. 4A). The chorionic epithelial cells of the areolar part (areolar trophoblast cells) were composed of taller columnar cells with longer microvilli than those of the part without areolae (trophoblast cells) (Fig. 4B). PAS-positive staining was detected in the fetal areolar pouch cavity and the cytoplasm of areolar trophoblast cells (Fig. 5A and C), and it was also found in the lumen of the uterine glands. Moreover, areolar trophoblast cells possessed substances stained by MSB (Fig. 5B and D). Areolar trophoblast cells with granules stained by PAS and/or MSB showed various large columnar cells (e.g., balloon- and goblet-like), on which longer microvilli were not recognizable (Figs. 4B and 5C, D). Berlin blue-positive staining was detected from the area under areolar trophoblast cells over the stroma of chorionic villi in the areolar part (Fig. 6A) and in the cytoplasm of uterine glandular epithelial cells (Fig. 6B).

Uterine glands, as indicated by a simple coiled branched tubular gland, were widely distributed throughout the endometrial stroma of nonpregnant and pregnant uteri. Significant differences were observed between nonpregnant and pregnant uteri. The uterine glands

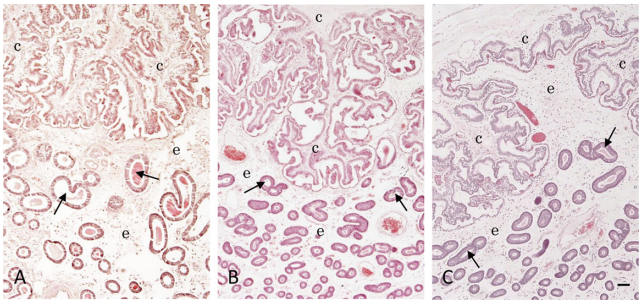

Fig. 1. Histological examination of placentas of common minke, Bryde's and sei whales. A: Common minke whale (fetal length, L 141.0 cm). B: Bryde's whale (L 158.6 cm). C: Sei whale (L 144.2 cm). The placenta of each species was an epitheliochorial placenta. c: Chorionic villi. e: Endometrium. Arrows indicate uterine glands. All images are at the same magnification. Bar = 100 μm. HE staining.

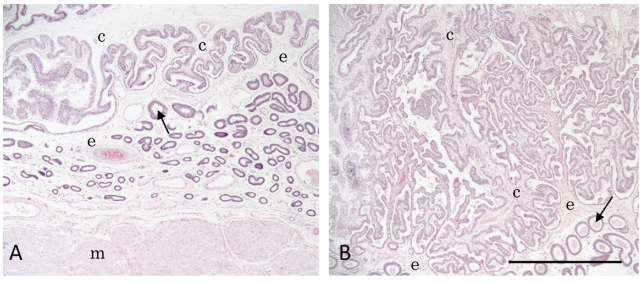

Fig. 2. Change in interdigitation between chorionic villi and endometrial crypts in the Bryde's whale. A: L 95.5 cm. B: L 137.9 cm. Interdigitations became more complex with the progression of pregnancy. c: Chorionic villi. e: Endometrium. m: Myometrium. Arrows indicate uterine glands. The two images are at the same magnification. Bar = 1 mm. HE staining.

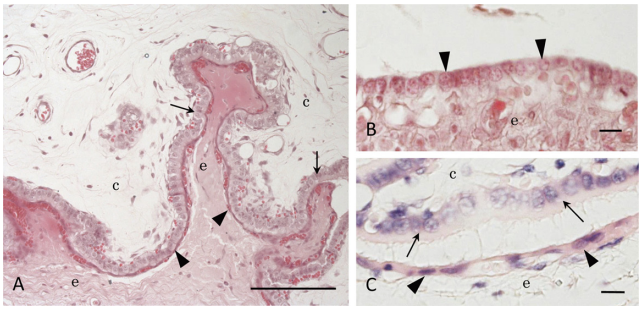

Fig. 3. Trophoblast cells and endometrial epithelial cells in the sei whale placenta. A: L 89.3 cm. B: L 14.0 cm. C: L 144.2 cm. All endometrial epithelial cells were cuboidal in shape in B. Cuboidal or columnar trophoblast cells covered the surface of chorionic villi, and the endometrial epithelium consisted of squamous cells in A and C. c: Chorionic villi. e: Endometrium. Arrows indicate trophoblast cells. Arrowheads indicate endometrial epithelial cells. Bar = 100 μm (A), 10 μm (B, C). HE staining.

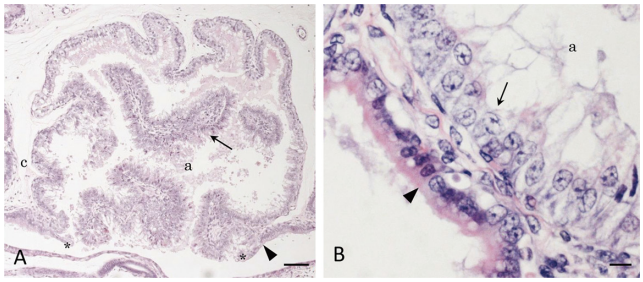

Fig. 4. Areola of a Bryde's whale placenta. A: L 95.5 cm. B: L 23.0 cm. Areolar trophoblast cells (arrows) were taller columnar epithelial cells and possessed longer microvilli than trophoblast cells (arrowheads). a: Areola. c: Chorionic villi. * Transitional part between trophoblast cells and areolar trophoblast cells. HE staining. Bar = 100 μm (A), 10 μm (B).

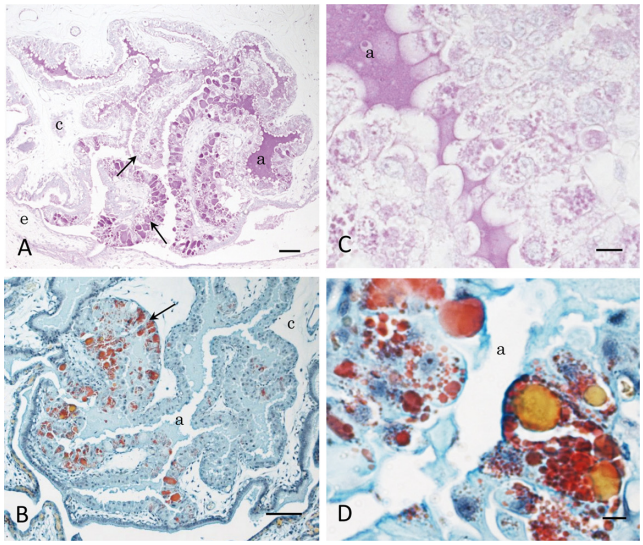

Fig. 5. Areola of the sei whale (L 144.2 cm). A, C: PAS staining. B, D: MSB staining. C and D are magnified sections of A and B, respectively. Substances stained by PAS or MSB were detected in the cytoplasm of areolar trophoblast cells. a: Areola. c: Chorionic villi. e: Endometrium. Arrows indicate areolar trophoblast cells. Bar = 100 μm (A, B), 10 μm (C, D).

of pregnant uteri had a wider lumen and larger epithelial cells with a nucleus arranged in the basal part compared with the nonpregnant uteri (Fig. 7). In each uterus, the uterine glands of the superficial endometrial layer were larger in size than those of the deep layer.

Immunohistochemistry

In all placental samples, immunoreactivities for P450scc and P450arom were detected in trophoblast cells, but not in areolar trophoblast cells (Figs. 8 and 9). Furthermore, P450scc and P450arom immunoreactivities were never observed in the maternal placentas (Figs. 8 and 9) and nonpregnant uteri.

TRAP immunoreactivity was detected in the cytoplasm of glandular cells in uterine glands and areolar trophoblast cells and in the areolar cavity (Fig. 10).

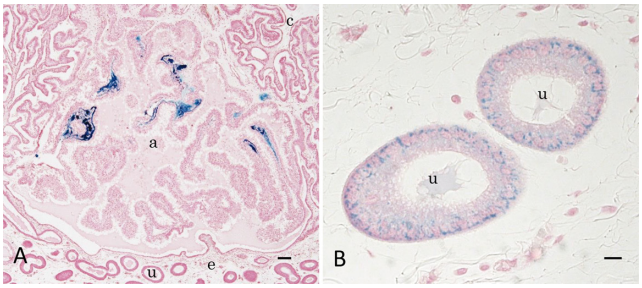

Fig. 6. Berlin blue staining of whale placentas. A: Areola of the Bryde's whale (L 137.9 cm). Berlin blue staining was detected within the stroma of chorionic villi in the areolar part. B: Uterine glands of the sei whale (L 181.0 cm). Staining was detected in the cytoplasm of uterine glandular epithelial cells. a: Areola. c: Chorionic villi. e: Endometrium. u: Uterine glands. Bar = 100 µm (A), 10 µm (B).

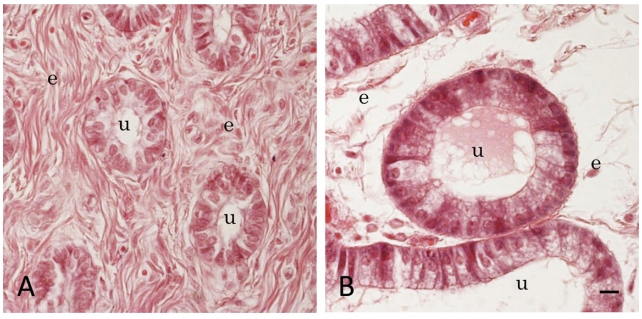

Fig. 7. Uterine glands of the sei whale uterus. A: Nonpregnant uterus. B: pregnant uterus (L 158.8 cm). The uterine glands of pregnant uteri had a wider lumen and larger epithelial cells than nonpregnant uteri. e: Endometrium. u: Uterine glands. The two images are at the same magnification. Bar = 10 µm. HE staining.

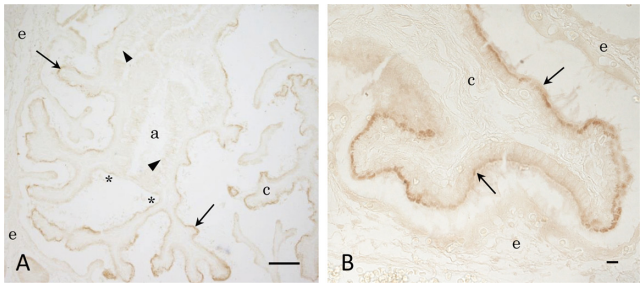

Fig. 8. Immunostaining of P450scc in the whale placenta. A: Areolar part of chorionic villi in the Bryde's whale placenta (L 158.6 cm). B: Enlargement of the fetomaternal attachment area in the sei whale placenta (L 144.7 cm). P450scc immunoreactivity was detected in trophoblast cells, but not in areolar trophoblast cells. a: Areola. c: Chorionic villi. e: Endometrium. Arrows indicate trophoblast cells. Arrowheads indicate areolar trophoblast cells. * Transitional part between trophoblast cells and areolar trophoblast cells. Bar = 100 µm (A), 10 µm (B).

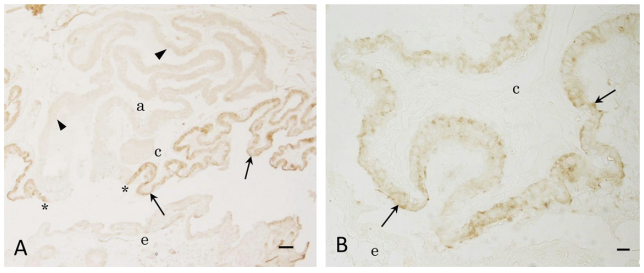

Fig. 9. Immunostaining of P450arom in the sei whale placenta (L 144.2 cm). A: Areolar part of chorionic villi. B: Enlargement of the fetomaternal attachment area. P450arom immunoreactivity was detected in trophoblast cells, but not in areolar trophoblast cells. a: Areola. c: Chorionic villi. e: Endometrium. Arrows indicate trophoblast cells. Arrowheads indicate areolar trophoblast cells. * Transitional part between trophoblast cells and areolar trophoblast cells. Bar = 100 µm (A), 10 µm (B).

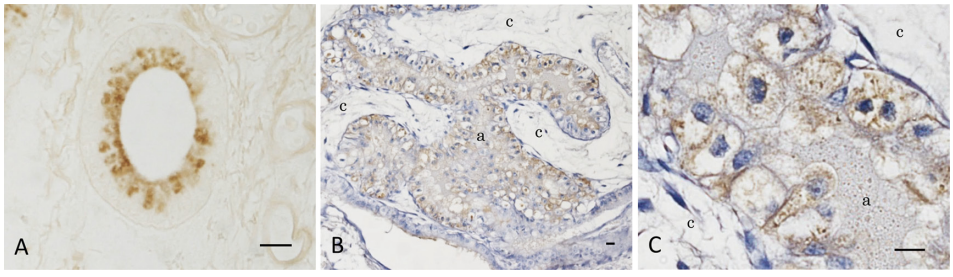

Fig. 10. Immunostaining of TRAP in the sei whale placenta. A: Uterine gland (L 144.2 cm). B, C: Areolar part of chorionic villi (L 89.3 cm). C is a magnified section of B. TRAP immunoreactivity was detected in the uterine glandular epithelial cells, areolar trophoblast cells and areolar cavity. a: Areola. c: Chorionic villi. Bar = 10 µm.

SEM observations

The surfaces of the chorion and endometrium were observed by SEM (Fig. 11). In all whale species, chorionic villi showed a fold-like feature (folded placentation), and a thin endometrial fold was observed in the maternal placenta.

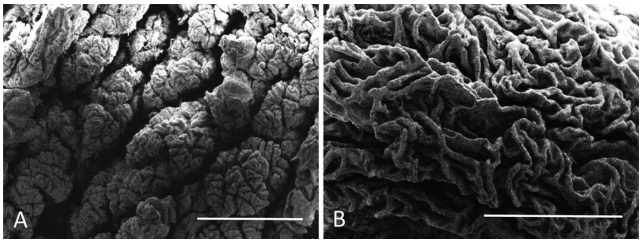

Fig. 11. SEM examination of the Bryde's whale placenta (L 173.8 cm). A: Surface of the chorion. B: Surface of the endometrium. Chorionic villi showed a fold-like feature, and a thin endometrial fold was observed in the endometrium. Bar = 1 mm.

Discussion

We herein demonstrated that each of the placentas of the 3 species of rorqual whale examined was a diffuse and epitheliochorial placenta and had well-developed pouch-like areolae, which is consistent with recent findings on the placenta of the Antarctic minke whale, a rorqual [14, 15]. SEM examinations revealed that the placentas of the 3 rorquals exhibited folded placentation. In the present immunohistochemical analyses, P450scc- and P450arom-positive reactivities were detected for the first time in the trophoblast cells of each whale's placenta. P450scc converts cholesterol into pregnenolone in the first step of steroidogenesis, and P450arom is an essential enzyme for converting androgen to estrogen by aromatization. The presence of these enzymes indicated that estrogen was synthesized through the conversion of cholesterol in the trophoblast cells of these whale placentas. Estrogen is an important sex steroid hormone that, like progesterone, maintains pregnancy in most placental mammals. In the bovine placenta, binucleate trophoblast giant cells are the main source of estrogen, not uninucleate trophoblast cells [17]. Although binucleate trophoblast giant cells were not detected in the placentas of the 3 species of rorqual whale examined, all uninucleate trophoblast cells, except those in the areolar part, showed strong immunoreactivity for aromatase. Therefore, trophoblast cells may be the main source of estrogen in rorqual placentas. Furthermore, a marked difference in steroidogenesis was observed between trophoblast cells and areolar trophoblast cells even though both are uninucleate trophoblast cells and have the same origin embryologically.

Previous studies reported that estrogen was secreted from the placentas of mammals with a long gestation period, such as horses, cows, sheep, goats, reindeer, monkeys and humans [18–24]; however, estrogen synthesis has not been detected in the placentas of giraffes, which also have a long gestation period [25]. The gestation periods of the common minke, Bryde's and sei whales are approximately 10, 12 and 10.5 months, respectively [26], and, thus, these rorqual whales may be considered mammals with long gestation periods. In mammals with short gestation periods, such as dogs, rats, guinea pigs and rabbits, estrogen was shown to be mainly secreted from the corpus luteum during pregnancy [18, 27]. However, placental synthesis of estrogen has been reported in the Japanese black bear, which has a gestation period of approximately 6–7 months and delayed implantation for several months [28]. Moreover, expression of P450arom was previously detected in the placenta of spotted hyenas (gestation period, 110 days) [29]. The placentas of cats (gestation period, approximately 62 days) were also found to produce estrogen [30]. These findings indicate that placental estrogen is also synthesized in some mammals with short gestation periods. Therefore, there appear to be no clear rules regarding the placental secretion of estrogen; however, placental estrogen is more commonly synthesized in mammals with long gestation periods. The expression of aromatase in the corpus luteum has been shown to increase in the late gestation stage in pregnant dogs and rats [27, 31]. A previous study reported that the fetal lengths of the common minke, Bryde's and sei whales at birth were approximately 2.6, 4.0 and 4.5 m, respectively [26]. Therefore, late gestation stages were not examined in all whale species in the present study. As a result, it would be interesting to investigate the dynamics of estrogen synthesis in the trophoblast cells of the placentas of each of these species.

Areolae are gaps between the chorion and endometrium that are filled with essential substances for fetal development. They have been detected in many placental mammals that have epitheliochorial (e.g., horses, pigs, camels, lamas, hippopotamuses and Antarctic minke whales), synepitheliochorial (e.g., ruminants) or endotheliochorial placentas (e.g., many carnivores, except for cats, raccoons and hyenas) [15, 32–38]. On the other hand, mammals with hemochorial placentas generally do not have areolae [37]. Areolae are mainly formed at the opening of the uterine glands, and areolar trophoblast cells absorb nutrition secreted from maternal uterine glands, referred to as histotroph, and also take in cell debris [39–42]. In the present study, well-developed areolae with a large pouch-like structure and projection of several villous folds were detected in the placentas of all whale species examined, which was consistent with previous findings on the Antarctic minke whale placenta [15]. This result strongly suggests that, in whale species with an epitheliochorial placenta, absorption of histotroph secreted from the uterine glands may be carried out by areolar trophoblast cells. In areolae, areolar trophoblast cells with stained granules differed in shape from those without granules. Therefore, areolar trophoblast cells may largely change their shape as a result of the absorption of accumulating substances in areolae.

Iron is indispensable for fetal growth and is supplied by maternal tissues. In mammals with a hemochorial placenta, trophoblast cells generally absorb iron from circulating maternal blood by endocytosis of transferrin, a plasma iron-binding protein, bound by its receptors [42, 43]. On the other hand, most endotheliochorial placental mammals supply iron to developing fetuses by the uptake of erythrocytes in hemophagous areas of the trophoblast. In mammals with epitheliochorial or synepitheliochorial placentas, areolar trophoblast cells absorb uteroferrin, an iron-containing glycoprotein secreted from uterine glands by the stimulation of progesterone, as a source of iron [40, 42–47]. A recent study reported that APC5 (uteroferrin) may be expressed in the pregnant endometrium of all mammalian orders to perform common functions for pregnancy regardless of the placentation styles [48]. In sheep, goats, cattle and water buffalos, erythrocytes are also taken up from hemophagous areas of the placentome for iron supply [37, 42, 43, 47, 49]. In the present study, PAS-positive staining was detected in the cytoplasm of areolar trophoblast cells and in the lumen of uterine glands.

Furthermore, large and small granules stained red or yellow by MSB staining were detected in the cytoplasm of areolar trophoblast cells. The presence of granules stained by PAS and/or MSB suggests that glycoproteins and acidophilic substances may be absorbed by areolar trophoblast cells [50]. In this immunohistochemical study, TRAP immunoreactivity was observed in the cytoplasm of areolar trophoblast cells and glandular cells. TRAP is an acid phosphatase enzyme transcribed from the same gene as uteroferrin and has a very similar primary structure [45, 51]. Therefore, the present study is the first to immunohistochemically show the existence and localization of uteroferrin in cetacean placentas during pregnancy. Furthermore, the results of Berlin blue staining, which detects ferric ions (Fe^{3+}), were positive in the epithelial cells of uterine glands and within the stroma of chorionic villi only in the areolar part. Therefore, whale placentas may develop areolar-gland complexes for the absorption of histotroph such as uteroferrin, namely, iron from uterine glands and the ferric iron transport system in the fetal placenta. In the placentas of alpacas, acid phosphatase and PAS-positive reactions were detected in the cytoplasm of areolar trophoblast cells and the lumen of uterine glands, and deposition of ferric irons was observed at the fetoplacental capillaries surrounding areolae [36]. These findings are consistent with the present results for the rorqual placenta. However, in addition to the areolar region, a hemophagous region in which erythrocytes accumulate, which has been reported in sheep, goats, cattle and water buffalos [42, 43, 47, 49], was not found in the present study. Therefore, iron supply from uteroferrin was sufficient for the growth of rorqual fetuses.

In the present study, we revealed for the first time that the trophoblast cells of the placentas of the 3 species of rorqual whale expressed aromatase. This result indicates that whale placentas produce estrogen in trophoblast cells. We also confirmed the presence of uteroferrin for the first time in placentas during pregnancy. This result suggests that uteroferrin plays an important role in iron transfer in the areolar part of the whale placenta.

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