

Areolae of the Placenta in the Antarctic Minke Whale (*Balaenoptera bonaerensis*)

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Abstract. In this study, we examined the existence and structure of areolae and the steroidogenesis of areolar trophoblast cells in the Antarctic minke whale placenta morphologically and immunohistochemically. Placentas were collected from the 15th, 16th and 18th Japanese Whale Research Program under Special Permit in the Antarctic (JARPA) and 1st JARPA II organized by the Institute of Cetacean Research in Tokyo, Japan. The opening and cavity of fetal areolae formed by taller columnar trophoblast cells (areolar trophoblast cells) with long microvilli and a bright cytoplasm, as compared with the trophoblast cells of the chorionic villi interdigitating with the endometrial crypts, were recognized in observations of serial sections. The opening of the areolar cavity was hidden by chorionic villi with areolar trophoblast cells. Furthermore, a closed pouch-like structure lined by tall columnar cells similar to areolar trophoblast cells within the stroma of chorionic villi was noticed and continued to the areolar cavity, with the opening seen on serial sections. In a surface investigation of the chorion and endometrium by SEM, maternal (endometrial) areolae irregularly surrounded by endometrial folds were obvious. Moreover, we distinguished areolar trophoblast cells with long microvilli attached with many blebs from trophoblast cells. In our immunohistochemical observations, a steroidogenic enzyme, cytochrome P450 side chain cleavage enzyme (P450scc), was detected with strong immunoreactivity in trophoblast cells. However, areolar trophoblast cells showed weak or no immunoreactivity for P450scc.

Key words: Antarctic minke whale, Areola, *Balaenoptera bonaerensis*, Placenta, Steroidogenesis

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In a previous study, we examined the structure and steroidogenesis of the placenta in the Antarctic minke whale (*Balaenoptera bonaerensis*) and demonstrated that the Antarctic minke whale has a diffuse and epitheliochorial placenta without specialized trophoblast cells, such as binucleate cells, but with fetal and maternal capillaries indenting between the epithelial cells [1]. Moreover, we revealed that the trophoblast cells of the Antarctic minke whale placenta had a steroidogenic enzyme, cytochrome P450 side chain cleavage enzyme (P450scc), suggesting that trophoblast cells produce sex steroid

hormones and/or their precursors by steroidogenesis of cholesterol.

It is well known that the placenta of ungulates (e.g., horse, cow, pig and camel), strepsirrhine primates (e.g., lorises) and many carnivores (except for the cat, raccoon and hyena) forms a gap between the chorion and endometrium called an “areola”, into which the uterine glands enter [2–8]. The areolae accumulate secretions from the uterine glands, and then the trophoblast epithelial cells of the chorionic villi absorb them to transfer them to the fetal circulation. The areolae of the horse placenta are located in the opening area of the uterine glands among the microplacentomes. In cows, the areolae are in the chorion laeve among the placentomes. On the other hand, those of the pig are distinctly subdivided into maternal (endometrial) and fetal areolae [3–6]. In the Amazon river dolphin, boto (*Inia geoffrensis*) and the tucuxi (*Sotalia fluviatilis*), it has been reported that the placenta has areolar regions with chorionic villi lined by

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trophoblast epithelial cells with a tall columnar shape and apparent brush border [9].

In our previous study of the Antarctic minke whale placenta, areolae showing contact with the space between the chorion and endometrium were not noted. However, a pouch-like structure with villous components that had no contact with the chorion-endometrium space was occasionally found within the stroma of chorionic villi [Fig. 2C in Ref. 1]. In the present study, therefore, we examined the Antarctic minke whale placenta histologically and microscopically in detail with particular reference to the existence and structure of areolae. Moreover, the steroidogenesis of the epithelial cells in the villus components of the pouch-like structure and trophoblast epithelial cells was analyzed immunohistochemically.

Materials and Methods

Animals

Nine placentas (fetal length of 46.1–187.4 cm; estimated fetal age of 123–245 days, see below) of Antarctic minke whales were collected in the 15th (2001/2002), 16th (2002/2003) and 18th (2004/2005) Japanese Whale Research Program under Special Permit in the Antarctic (JARPA) and 1st (2005/2006) JARPA II (the Second Phase of the JARPA) organized by the Institute of Cetacean Research in Tokyo, Japan. Special attention to reduction of the time to death was given for all the sampled whales. According to Schedule III of the International Convention for the Regulation of Whaling, explosive harpoons were used for all whales as the primary killing method. The fetal age was calculated using the equation of Kato and Miyashita [$t_0 = W^{1/3}/0.243 + 74$, t_0 = estimated age (day), $W = 0.059L^{2.676}$, W = fetal weight (g), L = fetal length (cm)] [10], and the resulting number was rounded off and considered to be the fetal age. The fetal length at birth was presumed to be about 290 cm (day 330 of gestation) [10]; thus, very early and late gestational stages were not included in this study.

Histology and immunohistochemistry

Small pieces of tissue samples were randomly collected from the whole placenta and immediately fixed in Bouin's fluid or 10% formalin. After 24 h, the samples were transferred to 70% ethanol, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin (Paraplast Plus; Kendall, Mansfield, MA, USA). Tissue samples were cut serially at a thickness of 4 μ m and placed on aminopropyltriethoxysilane-coated slides (S8226; Matsunami Glass, Osaka, Japan). Deparaffinized sections were used for hematoxylin and eosin (HE) staining and were immunohistochemically stained using the avidin-biotin peroxidase complex (ABC) method [11]. For immunohistochemistry, the sections were treated by microwave in high pH target retrieval solution (1:10, S3307; DakoCytomation, Inc., Carpinteria, CA, USA) for 20 min to retrieve antigenicity. The sections were immersed in methanol containing 0.3% H_2O_2 for 10 min at room temperature (RT) to block 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 prevent nonspecific staining. Then the sections were incubated overnight with a polyclonal anti-rat P450scc antibody raised in the rabbit (1:200, AB1244; Chemicon International, Temecula, CA,

USA) at 4 C in a moisture chamber. After incubation with primary antibody, biotinylated anti-rabbit IgG raised in the goat (1:200, BA-1000; Vector Laboratories, Inc.) was applied for 30 min, and then the sections were incubated with ABC reagent for 30 min (1:2, PK-6100, Vectastain *Elite* ABC Kit; Vector Laboratories). The binding sites were visualized with Tris-HCl buffer (pH 7.4) containing 0.02% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.006% H_2O_2 . After incubation, the sections were washed with 0.01 M phosphate-buffered saline (PBS, pH 7.4), 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 normal rabbit serum instead of primary antibody and omission of the primary antibody.

Scanning electron microscopy (SEM)

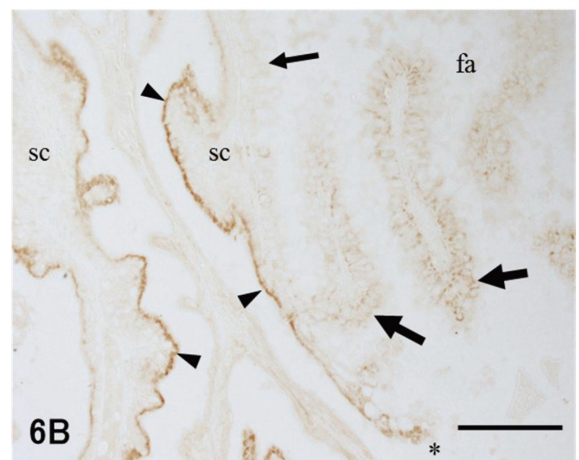
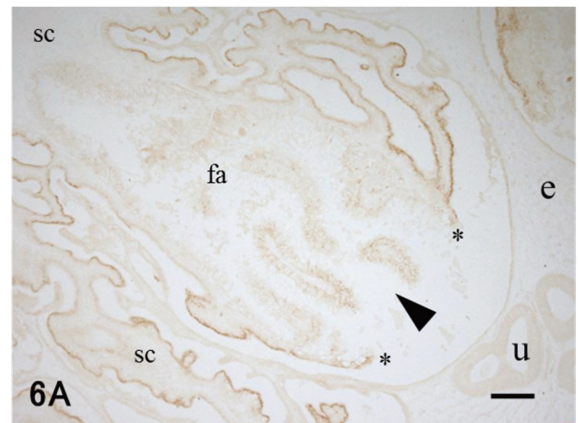
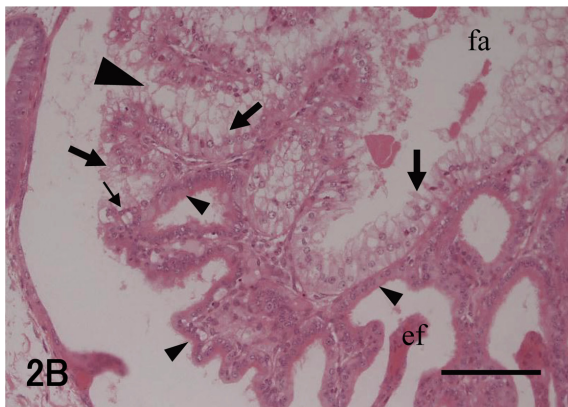
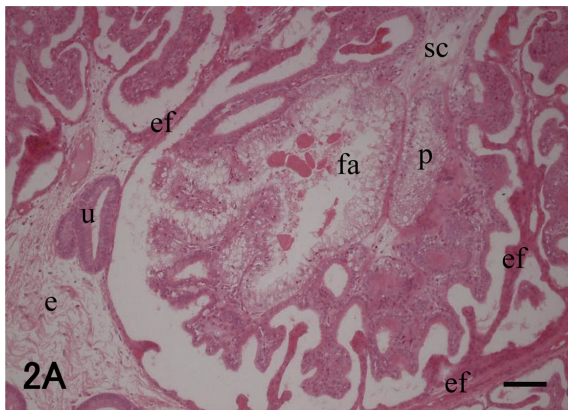
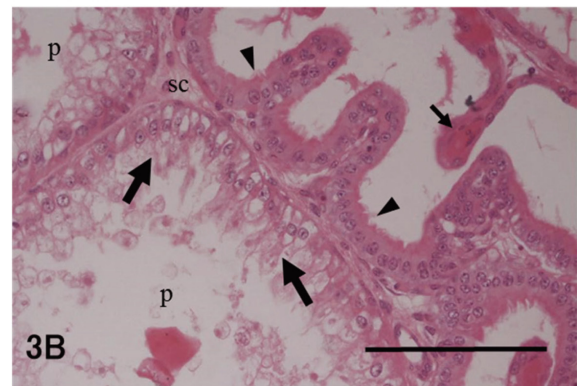
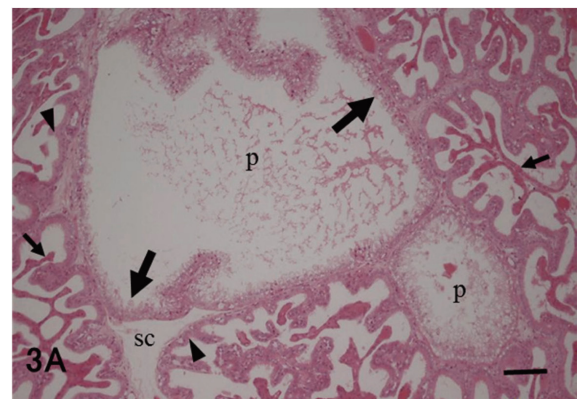
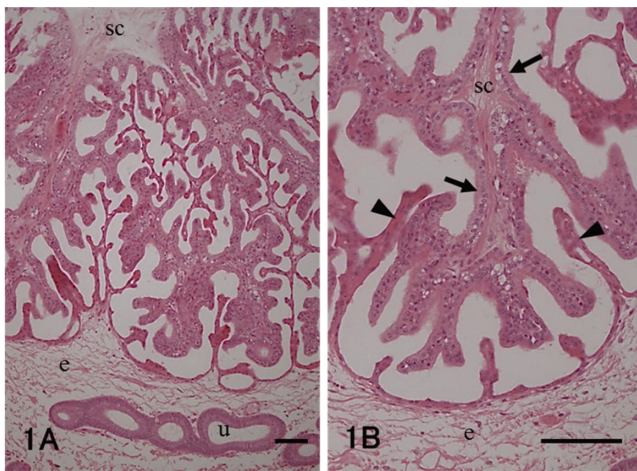
For scanning electron microscopy, small pieces of the samples fixed in 10% formalin were washed in PBS, postfixed in 1% osmium tetroxide in PBS and dehydrated in a graded series of ethanol. The specimens were then freeze-dried with t-butyl alcohol (JFD-300 Freeze-drying Device; JEOL, Tokyo, Japan). The dried tissues were mounted on stubs and sputter coated with Pt (JUC-5000 Magnetron Sputtering Device; JEOL). The samples were observed by SEM (JSM-6301F; JEOL) at an accelerating voltage of 1 or 5 kV.

Results

The placenta of the Antarctic minke whale histologically showed an epitheliochorial placenta with complex interdigitation between the chorionic villi lined by monolayer cells (trophoblast cells) and endometrial crypts and without specialized trophoblast cells (Fig. 1) [1]. In the chorionic villi, two different types of trophoblast cells could be recognized. Almost all epithelial parts of the chorionic villi were composed of cuboidal or columnar trophoblast cells with microvilli (Fig. 1). In our observations using serial sections, taller columnar trophoblast cells with long microvilli and a bright cytoplasm, continuing to trophoblast cells, were newly identified (Fig. 2). The chorionic villi with taller columnar trophoblast cells turned over deeply onto the fetal (allantois) side (Figs. 2, 4A and B, 5A), forming a pouch-like structure with several folds (Figs. 2, 4A and B). Therefore, it was judged that this pouch-like structure with an opening was the fetal areolae of the Antarctic minke whale placenta. The structure and distribution of the fetal areolae showed no significant differences among the fetal stages examined in this study.

Moreover, a pouch-like structure completely closed and lined by tall columnar cells with long microvilli and a bright cytoplasm, the same as trophoblast cells of fetal areolae (areolar trophoblast cells), was also noticed within the stroma of chorionic villi (Figs. 2A, 3, 4A and C), and the trophoblast cells and tall columnar cells that lined the closed pouch were arranged with their basal parts facing each other (Fig. 3). In our observations of serial sections, the tall columnar cells that lined the closed pouch-like structure continued to areolar trophoblast cells of the fetal areolae. So, it was demonstrated that the closed pouch-like structure was also the fetal areola, largely and complexly extended within the stroma of the chorionic villi and plate (Figs. 3 and 4).

In observations of the surface of the chorion and endometrium



by SEM, maternal (endometrial) areolae with no endometrial fold were clearly recognized (Fig. 5B). Areolar trophoblast cells with long microvilli and with many blebs could be distinguished from the trophoblast cells (Fig. 5A). Furthermore, the transitional region between the trophoblast cells and areolar trophoblast cells was recognized in SEM observations, and the slit-like grooves between the chorionic villi seemed to be the areolar opening, without a large opening as in pigs (Fig. 5A) [3–6].

Strong immunoreactivity for P450scs was detected in the trophoblast cells (Fig. 6). However, areolar trophoblast cells showed a

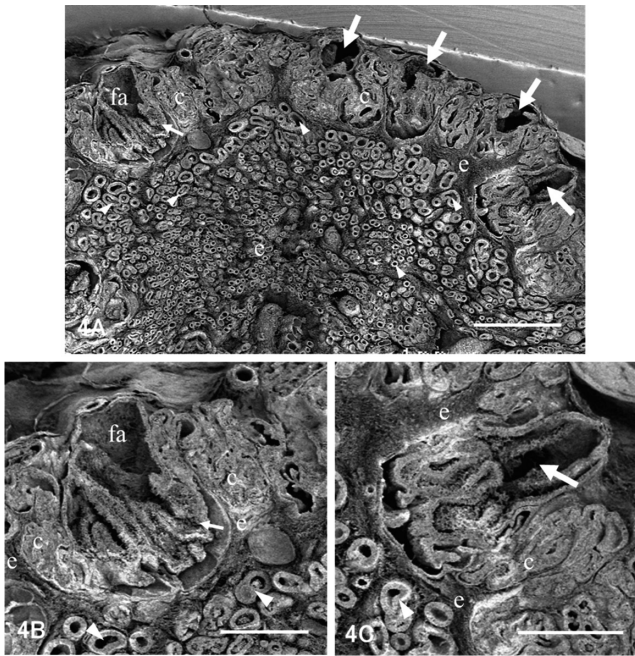


Fig. 4. Cross-sectional view of the Antarctic minke whale placenta by SEM. A-C: Placenta on day 123 of gestation (L 46.1cm). Closed pouch-like structure (large arrows), fetal areola (fa), opening of a fetal areola between villi (small arrows), uterine glands (arrowheads), chorionic villi (c), endometrium (e). Bar = 1 mm (A), 500 μ m (B, C).

weak positive reaction to P450scc at the apical part of the chorionic villi, and immunoreactivity disappeared toward the basal part (Fig. 6). The P450scc immunoreactivity of trophoblast cells and areolar trophoblast cells showed no significant differences among the fetal stages examined in this study.

Discussion

In this histological and electron-microscopic study of the Antarctic minke whale placenta, the structure of areolae and the steroidogenesis of areolar trophoblast cells were examined. The existence of areolae has been reported in dolphins, the boto and tucuxi [9]. However, the

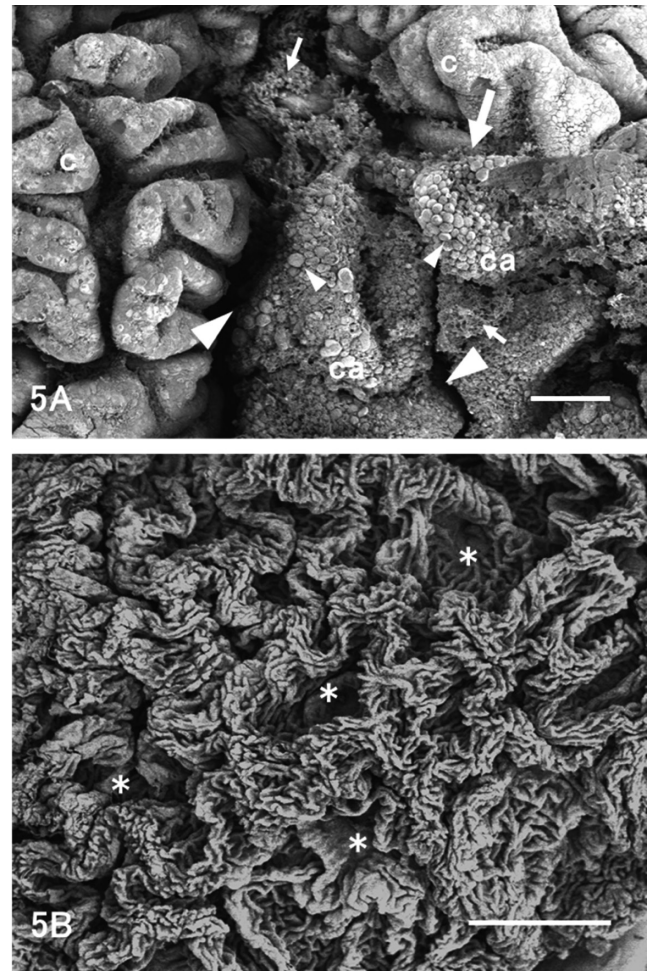


Fig. 5. Surface of the chorion (A) and endometrium (B) of the Antarctic minke whale placenta by SEM. A, B: Placenta on day 245 of gestation (L187.4 cm). Border between areolar trophoblast cells and trophoblast cells (large arrow), areolar trophoblast cells with long microvilli (small arrows), crevice between chorionic villi (large arrowheads), blebs attached to areolar trophoblast cells (small arrowheads), chorionic villi with the trophoblast cells (c), chorionic villi with areolar trophoblast cells (ca), maternal areolae (asterisk). Bar = 100 μ m (A), 1 mm (B).

- Fig. 1.** Interdigitation between the chorionic villi and endometrial crypts in the Antarctic minke whale placenta. A, B: Placenta on day 219 of gestation (fetal length, L155.6 cm). The chorionic villi were complicatedly interdigitated with the endometrial crypts. Trophoblast cells of chorionic villi (arrows), endometrial folds (arrowheads), stroma of chorionic villi (sc), endometrium (e), uterine glands (u). Bar = 100 μ m.
- Fig. 2.** Areola in the Antarctic minke whale placenta. A, B: Placenta on day 219 of gestation (L155.6 cm). B is a magnified section of A. The areola was detected by serial observation. Trophoblast cells (small arrowheads) continued to the areolar trophoblast cells with long microvilli (large arrows). Opening of a fetal areola between villi (large arrowhead), border between areolar trophoblast cells and trophoblast cells (small arrow), endometrial folds (ef), fetal areola (fa), closed pouch-like structure (p), stroma of chorionic villus (sc), endometrium (e), uterine gland (u). Bar = 100 μ m.
- Fig. 3.** Closed pouch-like structure within the stroma of chorionic villi. A, B: Placenta on day 219 of gestation (L155.6 cm). B is a magnified section of A. Tall columnar cells with long microvilli lining a closed pouch-like structure (large arrows), endometrial folds (small arrows), trophoblast cells of chorionic villi (arrowheads), closed pouch-like structure (p), stroma of chorionic villi (sc). Bar = 100 μ m.
- Fig. 6.** Immunostaining for P450scc in the areolar part of the Antarctic minke whale placenta. A, B: Placenta on day 219 of gestation (L155.6 cm). Areolar trophoblast cells with weak immunoreactivity (large arrows), areolar trophoblast cells with no immunoreactivity (small arrow), opening of a fetal areola between villi (large arrowhead), trophoblast cells with strong immunoreactivity (small arrowheads), border between areolar trophoblast cells and trophoblast cells (asterisk), fetal areola (fa), stroma of chorionic villi (sc), endometrium (e), uterine glands (u). Bar = 100 μ m.

entire detailed structure of areolae with other placental components has not been described, except for the features of areolar trophoblast cells and the distribution of fetal capillaries; the areolar trophoblast cells had a tall columnar shape with an apparent brush border and were supplied by sparser fetal capillaries than trophoblast cells. In this study, we distinguished the pouch-like structure (fetal areolae) to a great extent, and it complexly extended within the stroma of the chorionic villi and plate and opened at the tip of the chorionic villi with a slit-like opening. Enders and Carter [8] briefly mentioned regions composed of columnar trophoblast cells with mixed phagocytosis (heterophagous appearance) under the cetacean chorionic plate as a personal observation. It is thought that this structure might correspond to the pouch-like structure in the Antarctic minke whale placenta.

In pigs, the structure of areolae has been investigated in detail and has maternal and fetal parts [3–6], and two types, regular and irregular areolae, were structurally distinguished, unlike other mammal placentas with areolae including the Antarctic minke whale [5, 6]. In regular areolae, the maternal areolae showed shallow cups around the openings of the uterine glands, and the endometrial fold or crypts spread radially, centering on these cups. The fetal areolae built up a dome-like structure, and the chorionic villi of the interareolar placenta radiated similarly in accordance with the endometrial crypts [5]. In irregular areolae, on the other hand, the fetal areolae were larger in size and less frequent, and were characterized by a few blunt processes [5, 6, 12]. In the dromedary (*Camelus dromedarius*), it has been reported that maternal and fetal areolae have an area surrounded by the demarcation rim of the endometrium and the areolar cavity roundly bordered by the rim with spindle-shaped trophoblast cells, respectively [7]. In the Antarctic minke whale, the maternal areolae could be distinguished as protean cups surrounded by the endometrial fold. However, the endometrial folds of the interareolar placenta were not arranged radially as in the pig placenta. In the pig and dromedary, the entrance of the fetal areolae into the areolar cavity was very large and distinct [3–5, 7]. However, the opening of the areolar cavity was not distinguishable in the Antarctic minke whale because chorionic villi with areolar trophoblast cells hid the opening. Therefore, the fetal areolae of the Antarctic minke whale tend to be recognized as a closed pouch-like structure within the stroma of chorionic villi in a section. In this study, we could identify the opening where trophoblast cells replaced areolar trophoblast cells by observation of serial sections. In the Antarctic minke whale, furthermore, slit-like grooves that could be the areolar opening were noticed by surface observation of the chorion using SEM.

It is well known that the areolae have important functions such as absorption of secretions from uterine glands (uterine milk or histotrophe), including uteroferrin, an iron-containing glycoprotein [5–8, 13–15], and uptake of cell debris [5, 8] and erythrocytes (hemophagous) [8, 16, 17]. In general, areolar trophoblast cells have a tall columnar shape with long microvilli at the apical free end, have many large phagosomes and droplets of different size and have rich cellular organelles in the cytoplasm as compared with trophoblast cells [7, 13]. In areolar trophoblast cells, many blebs (possibly uterine gland secretory granules) were attached to the apical surface with the microvilli [7, 13]. In the Antarctic minke whale placenta, areolar trophoblast cells had a tall columnar shape and had long microvilli and many blebs on the apical surface. It is therefore suggested that

areolar trophoblast cells of the Antarctic minke whale also have high ability for absorption of histotrophe secreted from the uterine glands. It is thought that the whale epitheliochorial placenta, which has a complicated structure with well-developed areolae, may have not only a function that allows gas exchange by trophoblast cells but also many other functions for fetal development performed by areolar trophoblast cells.

In the present immunohistochemical observations, trophoblast cells of the Antarctic minke whale placenta showed strong immunoreactivity for P450scc, in accordance with our previous study [1]. In areolar trophoblast cells, on the other hand, immunoreactivity for P450scc was weak in the apical part of the chorionic villi but was not detected in most areolar trophoblast cells. It may be assumed that trophoblast cells interdigitating with the endometrium become specialized for gas exchange with many fetal capillaries indenting the cells and also for the synthesis of hormones (e.g., sex steroid hormones such as estrogen and progesterone) and that areolar trophoblast cells function mainly for the absorption of secretions from uterine glands, although both types of trophoblast cells differentiate from the same origin, ectotrophoblasts in blastocysts. It is necessary for further understanding of areolar differentiation and functions to examine the distributional differences of many factors between these two cell types, trophoblast cells and areolar trophoblast cells.

In conclusion, the present results demonstrate that the Antarctic minke whale has fetal areolae with a small opening, largely extending within stroma of the chorionic villi, and that the maternal areolae are irregularly surrounded by the endometrial fold. Moreover, we revealed that areolar trophoblast cells showed weak or no immunoreactivity for P450scc, unlike other trophoblast cells, which show strong immunoreactivity.

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