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—Original Article—

Structure and Steroidogenesis of the Placenta in the Antarctic Minke Whale (*Balaenoptera bonaerensis*)

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Abstract. There are few reports describing the structure and function of the whale placenta with the advance of pregnancy. In this study, therefore, the placenta and nonpregnant uterus of the Antarctic minke whale were observed morphologically and immunohistochemically. Placentas and nonpregnant uteri were collected from the 15th, 16th and 18th Japanese Whale Research Programme with Special Permit in the Antarctic (JARPA) and 1st JARPA II organized by the Institute of Cetacean Research in Tokyo, Japan. In the macro- and microscopic observations, the placenta of the Antarctic minke whale was a diffuse and epitheliochorial placenta. The chorion was interdigitated to the endometrium by primary, secondary and tertiary villi, which contained no specialized trophoblast cells such as binucleate cells, and the interdigitation became complicated with the progress of gestation. Furthermore, fetal and maternal blood vessels indented deeply into the trophoblast cells and endometrial epithelium respectively with fetal growth. The minke whale placenta showed a fold-like shape as opposed to a finger-like shape. In both nonpregnant and pregnant uteri, many uterine glands were distributed. The uterine glands in the superficial layer of the pregnant endometrium had a wide lumen and large epithelial cells as compared with those in the deep layer. On the other hand, in the nonpregnant endometrium, the uterine glands had a narrower lumen and smaller epithelial cells than in the pregnant endometrium. In immunohistochemical detection, immunoreactivity for P450scc was detected in most trophoblast cells, but not in nonpregnant uteri, suggesting that trophoblast epithelial cells synthesized and secreted the sex steroid hormones and/or their precursors to maintain the pregnancy in the Antarctic minke whale.

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

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The Antarctic minke whale (*Balaenoptera bonaerensis*) belonging to the family Balaenopteridae (order Cetacea, suborder Mysticeti) is one of the baleen whales that lack teeth, inhabits polar to tropical water in the Southern hemisphere and has been taxonomically distinguished from the common minke whale (*Balaenoptera acutorostrata*) mainly distributed in temperate to polar water in the Northern hemisphere [1]. The Antarctic minke whale, which is likely a seasonal breeder without reproductive activity from early December to March in the southern Antarctic, breeds at low latitude in austral winter, and the pregnant females migrate to Antarctic feeding areas during austral spring to summer. The gestation period of the Antarctic minke whale is about 10–11 months, and pregnant females give birth around September after returning to low latitudes [2].

In female Antarctic minke whales, several studies on the reproductive organs have been reported. Maturation and fertilization *in vitro* of the follicular oocyte have been investigated [3–6]. Furthermore, Suzuki *et al.* [7] measured the concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) during the feeding season. Muranishi *et al.* [8] examined the preantral follicle number in the fetal ovary and the concentrations of sex hormones in the fetal heart, umbilical cord and maternal blood and suggested that steroidogenesis occurs in the minke whale placenta.

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The placenta of whales has been described as an epitheliochorial and diffuse placenta [9–14]. This kind of placenta is known as the placenta of the pig [15] and horse [16], but their detail appearance varies depending on the species. In the Antarctic minke whale placenta, the structural development with the progression of pregnancy and the existence of steroidogenesis have not yet been investigated. In this study, therefore, we examined the placental structure of the Antarctic minke whale histologically and by scanning electron microscopy and the immunolocalization of a steroidogenic enzyme, cytochrome P450 side chain cleavage enzyme (P450scc), to clarify the structural changes with the progress of gestation and the existence and site of steroidogenesis in the placenta.

Materials and Methods

Animals

Fifty-eight placentas (fetal length of 11.1–187.4 cm; estimated fetal age of 88–245 days, see below) and 12 nonpregnant uteri (5 mature and 7 immature, no corpus luteum and corpus albicans in each whale ovary) 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 Regulations 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, $W=0.059L^{2.676}$, W=fetal weight, L=fetal length) [17], 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) [2]; thus very early and late gestational stages were not examined in this study.

Histology and immunohistochemistry

Small pieces of tissue samples were randomly collected from the whole placenta and nonpregnant uterus, and immediately fixed in Bouin's fluid or 10% formalin. After 24 h, the samples were transferred to 70% ethanol, then dehydrated in 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 μm and placed on aminopropyltriethoxysilane-coated slides (S8226, Matsunami Glass Ind., Osaka, Japan). Deparaffinized sections were used for hematoxylin and eosin (HE) and were immunohistochemically stained using the avidin-biotin peroxidase complex (ABC) method [18]. The sections were treated by microwave in high pH target retrieval solution (1:10, S3307, DakoCytomation, 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 the endogenous peroxidase activity, and then incubated with normal goat serum (1:50, S-1000, Vector Laboratories, Inc., 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, Inc. 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) 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 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 graded series of ethanol. The specimens were then freeze-dried with t-butyl alcohol (Freeze-drying Device F-300, JEOL, Tokyo, Japan). The dried tissues were mounted on stubs and sputter coated with Pt (Magnetron Sputtering Device JUC-5000, JEOL). The samples were observed by SEM (JSM-6301F, JEOL) at an accelerating voltage of 1 or 5 kV.

Results

Morphology

Fetomaternal interface: The placenta of the Antarctic minke whale was classified as a diffuse placenta according to the distribution of chorionic villi (Fig. 1). Histologically, the placenta was an epitheliochorial placenta characterized by close contact between the fetal trophoblast (trophectoderm) and maternal endometrial epithelium (Fig. 2). The chorion was interdigitated to the endometrium by chorionic villi branching complicatedly with the advance of pregnancy (Figs. 2 and 3).

In early gestation, the interdigitation consisted of simple fetal primary villi and their corresponding maternal endometrial crypts, which are ditches among endometrial villi. Both villi branched into secondary and tertiary villi and continued to become longer and thinner with the pregnancy progression. Consequently, the interdigitation became more complicated during mid gestation with more branched chorionic villi (Figs. 2–4).

The trophoblast consisted of cuboidal or columnar cells in early gestation, and their cytoplasm became larger with the progress of gestation. The trophoblast cells were mostly columnar throughout mid gestation. On the other hand, the endometrial epithelium consisted of squamous or cuboidal cells in the early stage, and then both the nuclei and cytoplasm of these cells became flatter as fetal age progressed (Fig. 4).

Blood capillary indentation was observed on both the fetal trophoblast cells and maternal endometrial epithelium during the mid stage of pregnancy (Figs. 4 and 5). Specialized trophoblast cells, such as binucleate cells or giant multinucleate cells in ruminants [19] and camels [20], were not observed in the Antarctic minke whale during the pregnancy period examined in this study (Fig. 4).

The surfaces of the chorion and endometrium were observed

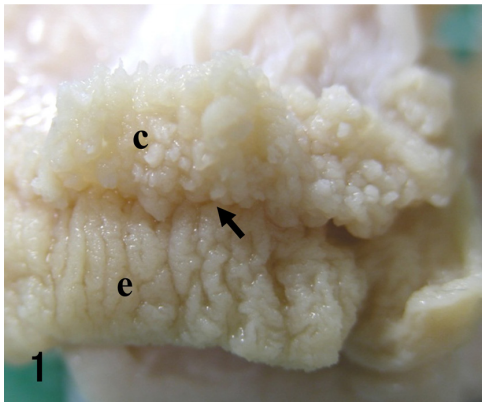

Fig. 1. Placenta of the Antarctic minke whale on day 153 of gestation (fetal length, L79.9 cm). The fetal side (chorion) of the placenta is picked up. The upper side is the chorion (c), and the bottom is the maternal side (endometrium) (e). The chorionic villi were interdigitated to the crypts of the endometrium. Interdigitation point (arrow).

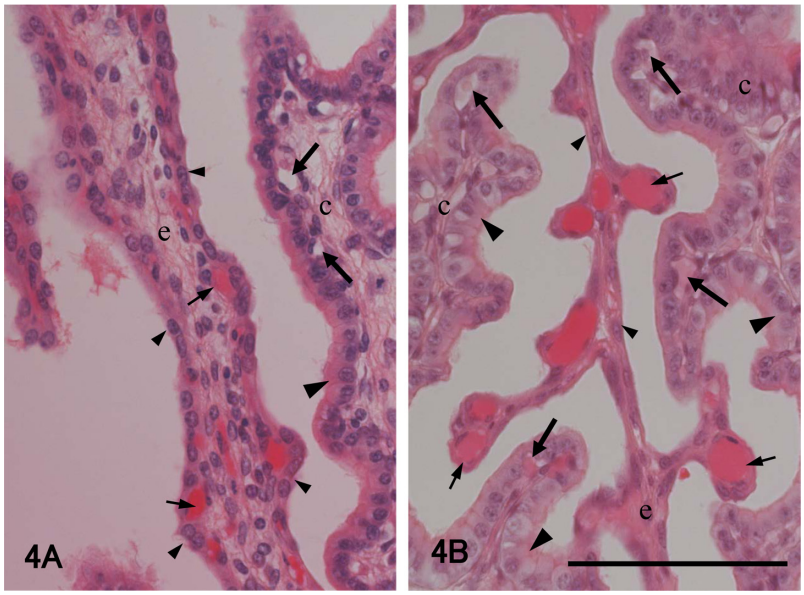

Fig. 4. Blood capillary indentation in the Antarctic minke whale placenta. A: Placenta on day 100 of gestation (L22.9 cm). Trophoblast cells were cuboidal or columnar. Endometrial epithelial cells were squamous or cuboidal. B: Placenta on day 219 of gestation (L155.6 cm). Trophoblast cells were mostly columnar. Endometrial epithelial cells were squamous. Note the deepening indentation of blood capillaries into both trophoblast cells and the endometrial epithelium and absence of specialized trophoblast cells. Trophoblast cells (large arrowheads), endometrial epithelial cells (small arrowheads), fetal blood capillaries (large arrows), maternal blood capillaries (small arrows), chorionic villi (c), endometrium (e). Both figures are at the same magnification. Bar=100 μm.

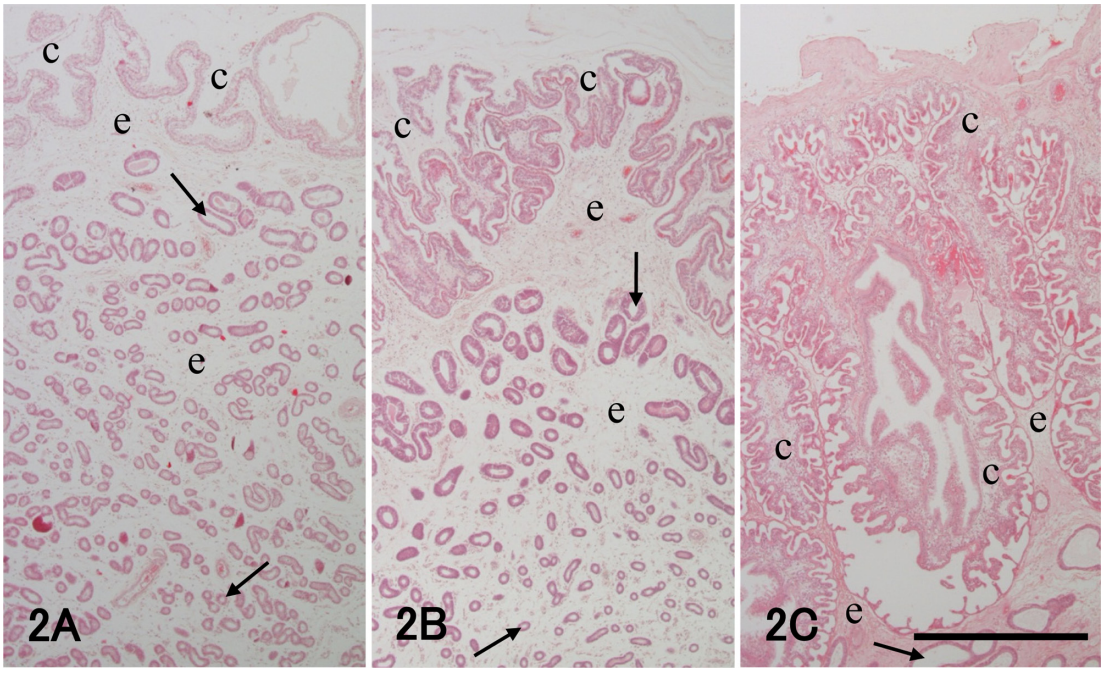

Fig. 2. Histological observation of the Antarctic minke whale placenta. A: Placenta on day 101 of gestation (L23.6 cm). The chorion was interdigitated to the endometrium by simple primary chorionic villi. B: Placenta on day 122 of gestation (L45.2 cm). Chorionic primary villi began to branch into secondary and tertiary villi. C: Placenta on day 221 of gestation (L158.6 cm). The interdigitation of the chorion and endometrium became more complicated. Chorion (c), endometrium (e), uterine glands (arrows). All figures are at the same magnification. Bar=1 mm.

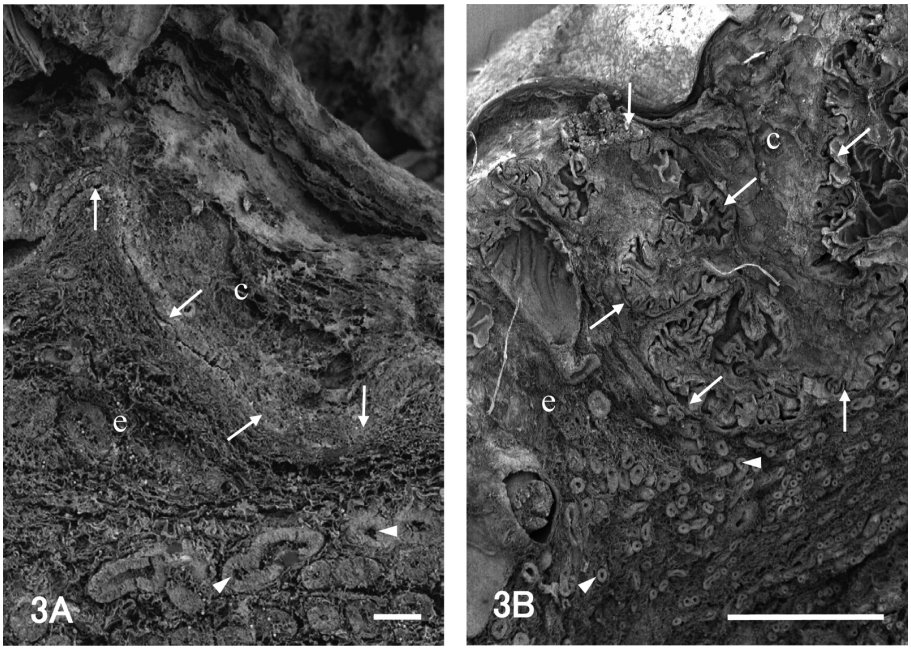

Fig. 3. Cross-sectional view of the Antarctic minke whale placenta by SEM. A: Placenta on day 101 of gestation (L24.0 cm). The interdigitation was simple. Bar=100 μm. B: Placenta on day 173 of gestation (L102.1 cm). The interdigitation became complicated compared with A. Bar=1 mm. The border between the fetal and maternal placenta (arrows), uterine glands (arrowheads), chorion (c), endometrium (e).

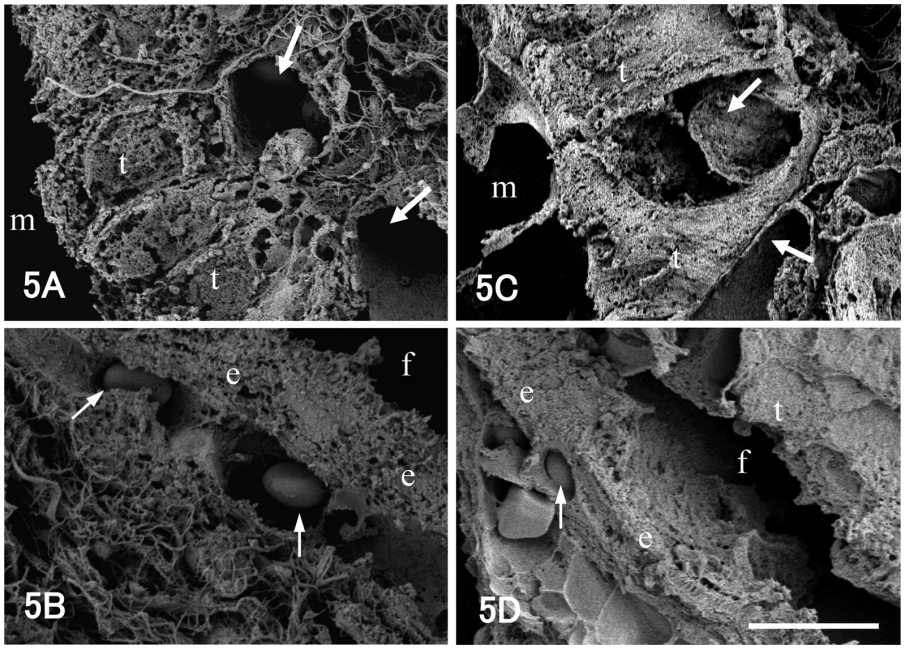

Fig. 5. Cross sectional view of the Antarctic minke whale placenta by SEM. A: Trophoblast on day 101 of gestation (L24.0 cm). Fetal blood capillaries only compressed trophoblast cells. B: Endometrium on day 101 of gestation. Maternal blood capillaries only compressed endometrial epithelial cells. C: Trophoblast on day 243 of gestation (L185.3 cm). Fetal blood capillaries invaded trophoblast cells. D: Endometrium on day 243 of gestation. Maternal blood capillaries invaded endometrial epithelial cells. Fetal blood capillaries (large arrows), maternal blood capillaries (small arrows), trophoblast (t), endometrial epithelial cell (e), fetal side (f), maternal side (m). All figures are at the same magnification. Bar=10 μm.

sterically by SEM. As with light microscope observations, there were chorionic villi and endometrial crypts on the surface of chorion and endometrium, respectively (Figs. 6 and 7). In early gestation, the chorionic villi were simple and irregular in shape and showed a fold-like shape (folded placentation) as opposed to the finger-like shape (villous placentation) in the human [21] and horse [16]. Then they became more complicated and compacted as fetal age progressed but did not branch finger-like villi, keeping the fold-like feature (Fig. 6). On the other hand, the endometrial villi were thick and simple in shape in early stage and then became thinner and more complicated with the progress of gestation (Fig. 7).

Endometrium: The endometrium consisted of the epithelium, uterine glands and connective tissue stroma, and many uterine glands, which indicate a simple coiled branched tuber gland, were distributed throughout the endometrial stroma of both the pregnant and nonpregnant whales. The stroma thickened during gestation, and the density of the uterine glands became lower (Fig. 8). In nonpregnant whales, the uterine glands in the superficial layer were slightly larger than those in the deep layer (Fig. 9A and B), and no significant structural differences were detected between the uterine glands of the mature and immature nonpregnant whales examined in this study.

In pregnant whales, the uterine glands were different in appearance between the superficial endometrial layer positioned near fetal trophoblast and the deep layer positioned near the myometrium (Fig. 9C and D). In early gestation, the glands in the superficial layer became much larger and had a wider lumen than those in the nonpregnant placenta, whose epithelial cells were columnar and contained rich cytoplasm and a nucleus located in each base (Fig. 9A and C). They did not change with the stage of pregnancy (Fig. 9C and E). The glands in the deep layer were small in size and had a narrow lumen, whose epithelial cells were cuboidal or columnar cells and contained scanty cytoplasm in early gestation, although they were slightly developed as compared with those in nonpregnant whales (Fig. 9B and D). During mid gestation, they became slightly larger and had wider lumens than those in the early stage (Fig. 9D and F). However, the glands in the deep layer were much smaller than those in the superficial layer (Fig. 9E and F).

Immunohistochemistry

In all samples of placenta, immunoreactivity for P450scc was detected only in trophoblast cells; immunoreactivity was not seen in the nonpregnant uterus. Strong P450scc immunoreactivity was observed in the supranuclear region of the cells, which was contact with the maternal endometrial epithelium. The distribution of P450scc immunoreactive cells and the staining intensity for P450scc did not show significant changes during the pregnancy period examined in this study (Fig. 10).

Discussion

This study reports for the first time morphological changes with gestational stages and the localization of steroidogenic enzyme in the Antarctic minke whale placenta. In this study, it was noted that the placenta of the Antarctic minke whale was a diffuse and epitheliochorial placenta, corresponding to other cetacean placentas [9–14]. The interdigitation between the chorion and endometrium became complex with the progress of gestation in the Antarctic minke whale, as seen in other animals with an epitheliochorial placenta [15, 16, 20, 22–25]. This not only establishes firm fetomaternal attachment but also induces increase in the surface area of the fetomaternal interface. Since one of the important functions of the placenta is the exchange of nutrients, oxygen and carbon dioxide between mother and fetus, the complex interdigitation seems to provide an extensive surface for the exchange, depending on the increase in consumption of nutrients and oxygen and the emission of waste products and carbon dioxide with fetal development.

Trophoblast and endometrial epithelial cells changed in shape with the progress of gestation. The cytoplasm of trophoblast cells became large, suggesting an increase in trophoblast functions, for example, synthesis, secretion and absorption of molecules. In the pig, it has been reported that the trophectoderm cells at the base of chorionic villi are tall, columnar and specialized for histotroph absorption [26–28]. In contrast, the endometrial epithelial cells became flatter, which seemed to bring maternal blood capillaries closer to fetal tissue. Furthermore, maternal and fetal blood vessels indented deeply into each epithelium, as seen in previous reports [15, 27–30]. These morphological changes probably decrease the distance between each vessel, and the increasing number of indenting vessels might result in a much quantity of blood flowing near the fetomaternal interface, supporting fetal growth by efficient molecular exchange.

The appearance of the chorionic surface with fold-like villi in the Antarctic minke whale was different from those with finger-like villi in the human [21], horse [16, 31] and cow [32]. This morphological characteristic in the Antarctic minke whale has also been seen in the pig [27] and alpaca [33]. Moreover, folded placentation has been reported also in other cetaceans [14, 28]. In pig and alpaca placentas, the chorionic villi continue to complexify and retain a fold-like shape until term; thus, the minke whale placenta also might maintain a fold-like shape up to late pregnancy. The placenta from late pregnancy will be examined in the future to reveal the structural dynamics of the placental surface.

In the ruminant and camelid placentas, binucleate (occasionally trinucleate) or giant multinucleate cells were observed in the chorionic villi [19, 20, 28, 33]; however, neither type of cells was observed in the chorionic villi of the pig [15, 26–28]. In this study, these specialized trophoblast cells were not detected in the Antarctic minke whale placenta. In previous reports, specialized trophoblast cells were also not observed in other cetacean placentas [9, 10, 12, 13]. Therefore, the absence of specialized trophoblast cells might be characteristic of the cetacean placenta.

Uterine glands synthesize and secrete a complex array of proteins and related substances termed histotroph [34–37]. Samuel *et al.* [38] showed that uterine glands produced secretions throughout gestation and suggested that the secretions were very important for the well-being of the fetus in the horse. For animals with an epitheliochorial placenta, uterofferin, an iron-containing glycoprotein, and histotroph in the secretions of uterine glands seem important as resources of iron and nutrition for the fetus, respectively [25, 39, 40].

At the openings of the uterine glands, areolae, gaps between the chorion and endometrium, are formed that accumulate secretion from the uterine glands, which are absorbed by the trophoblast epithelial

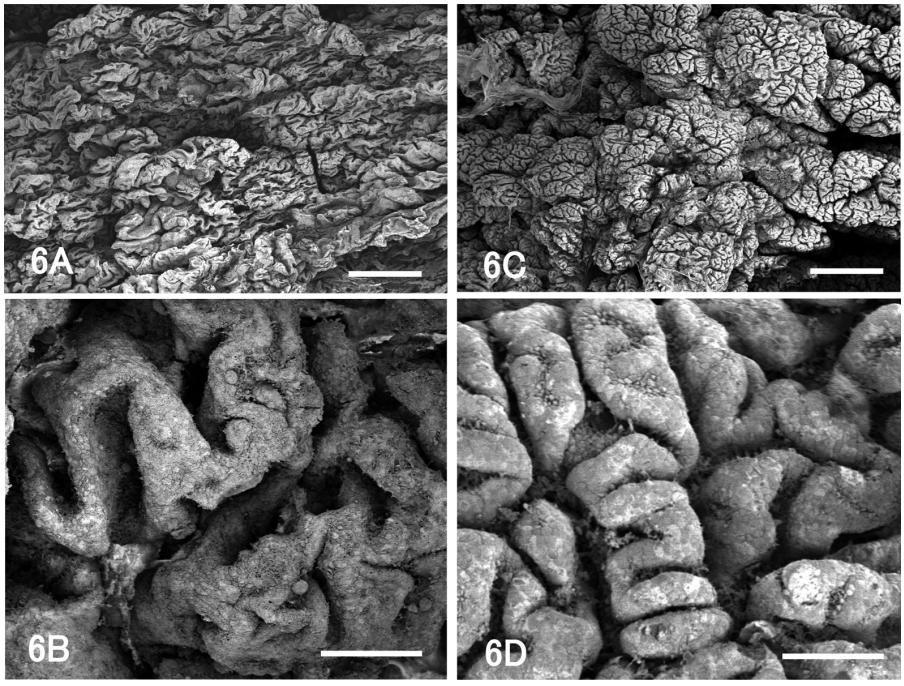

Fig. 6. Surface of the chorion of the Antarctic minke whale placenta by SEM. A , B: Placenta on day 98 of gestation (L20.7 cm). Chorionic villi were irregular in shape and simply folded. C, D: Placenta on day 245 of gestation (L187.4 cm). The fold of chorionic villi was complicated and compacted as compared with those of the placenta on day 98 of gestation. Bar= 1 mm (A, C), 100 μm (B, D).

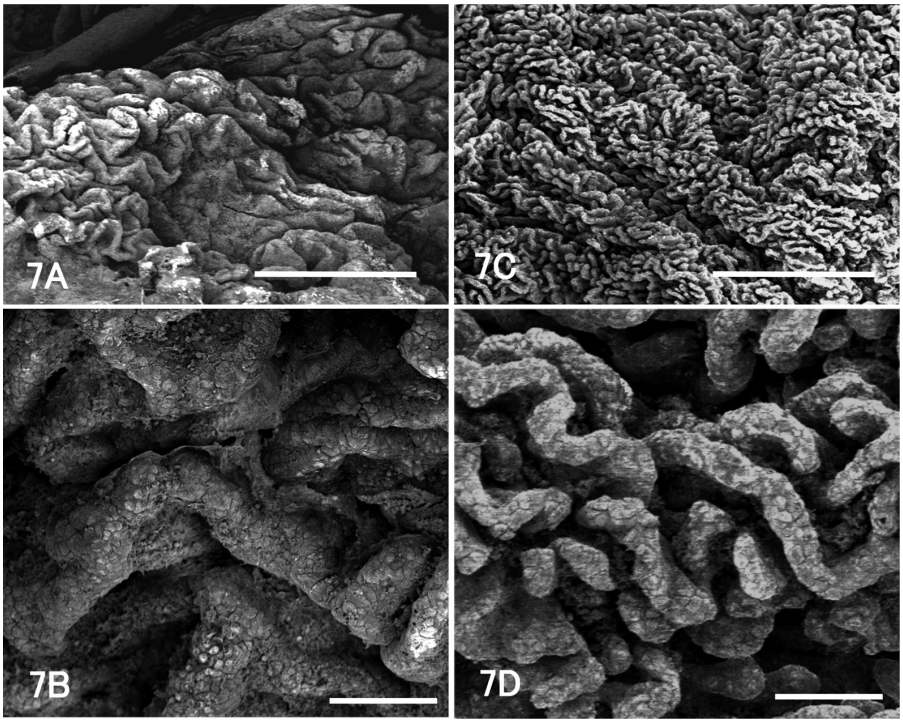

Fig. 7. Surface of the endometrium of the Antarctic minke whale placenta by SEM. A, B: Placenta on day 101 of gestation (L24.0 cm). Endometrial villi were thick and irregular in shape. C, D: Placenta on day 245 of gestation (L187.4 cm). Endometrial villi became thin. Bar= 1 mm (A, C), 100 μm (B, D).

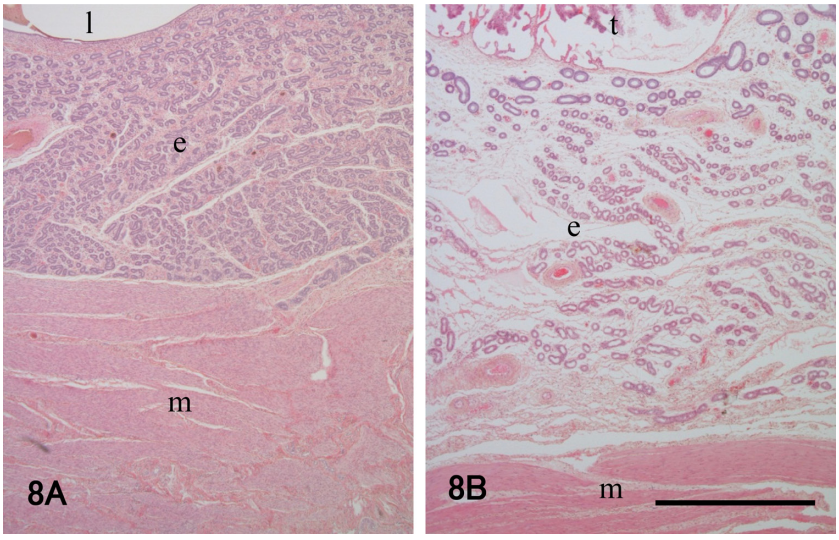

Fig. 8. Endometrium of the Antarctic minke whale. A: Nonpregnant uterus. The endometrium was thin, and uterine glands were densely distributed. B. Placenta on day 157 of gestation (L83.3 cm). The endometrium was thickened, and the density of the uterine glands was low. Lumen of uterus (l), myometrium (m), trophoblast (t), endometrium (e). Both figures are at the same magnification. Bar= 1 mm.

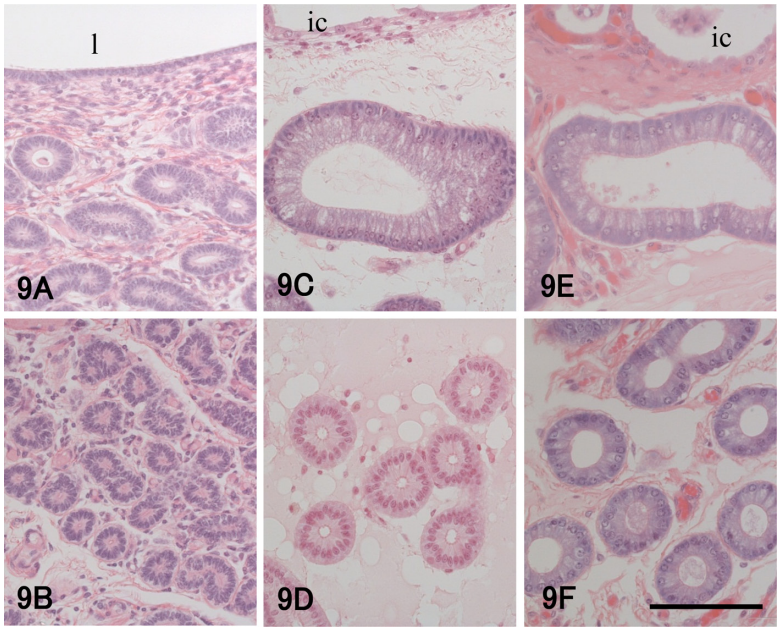

Fig. 9. Uterine glands of the Antarctic minke whale. A: Superficial endometrial layer of the nonpregnant uterus. B: Deep endometrial layer of the nonpregnant uterus. C: Superficial endometrial layer of the placenta on day 100 of gestation (L22.9 cm). D: Deep endometrial layer of the placenta on day 100 of gestation. E: Superficial endometrial layer of the placenta on day 238 of gestation (L179.6 cm). F: Deep endometrial layer of the placenta on day 238 of gestation. Note the remarkable difference in appearance between the superficial and deep layers with gestation. Lumen of uterus (l), interdigitated point by chorionic villi (ic). All figures are at the same magnification. Bar=100 μm.

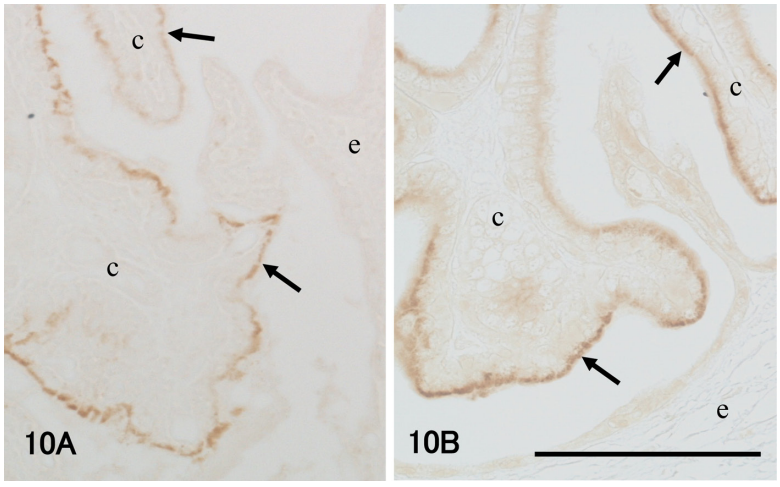

Fig. 10. Immunostaining of P450scc in the Antarctic minke whale placenta. A: Placenta on day 100 of gestation (L22.9 cm). B: Placenta on day 219 of gestation (L155.6 cm). P450scc immunoreactivity was detected in the trophoblast epithelium (arrows). Conspicuous changes with the stages of pregnancy were not observed. Chorion (c), endometrium (e). Both figures are at the same magnification. Bar=100 μm.

cells [38, 41]. In the Antarctic minke whales examined in this study, however, areolae were not noted in any of the samples.

Whale uterine glands may have similar functions to those in the animals with an epitheliochorial placenta. The glands in the superficial endometrial layer in pregnant whales are different from those in nonpregnant whales; they contained epithelial cells with rich cytoplasm and a quite wide lumen throughout the gestation period examined. These suggest that the uterine gland activity might already be intensified in the superficial layer in early gestation to supply uteroferrin and histotroph to the growing fetus. In sheep, cattle and pigs, the uterine glands are dramatically changed by extensive hyperplasia and subsequent hypertrophy during pregnancy, increasing the secretions for fetal development [37, 39, 42]. In alpacas, on the other hand, they apparently compensate for this by intense biosynthetic activity, although the density of the glands at the final stages of pregnancy was not high [33]. In pregnant minke whales, the density of the uterine glands became lower than that of the nonpregnant uterus. However, as seen in alpacas, the activity of the uterine glands might be intensified instead of the number of glands increasing.

Progesterone and estrogen are essential to maintenance of pregnancy in most mammals. The presence of P450scc in the trophoblast cells of the minke whale shows that sex steroid hormones are synthesized there. However, it remains to be seen whether progesterone or estrogen is secreted by the trophoblast cells because P450scc only converts cholesterol into pregnenolone. It is thought that estrogen is predominantly secreted in the placenta of mammals with a long gestation period (human, monkey, horse, cow, sheep and goat) [43]. Therefore, the trophoblast epithelial cell of the minke whale very likely synthesizes and secretes a large amount of estrogen, and high concentration of estradiol-17β in the fetal heart and umbilical cord blood might coincide with the secretion from epithelial cells [8]. In the humpback whale (*Megaptera novaeangliae*), estrone sulfate and progesterone were detected in homogenized placenta [44]. Moreover, it has been reported that progesterone and relaxin are also detected in placental extracts of the bottlenose dolphin (*Tursiops truncatus*) [45].

The cow and camel have specialized trophoblast epithelial cells [19, 20]. In the cow, binucleate cells produce estrogens at the fetal cotyledons in the placenta [19, 46, 47]. Furthermore, it has been reported that syncytiotrophoblasts in the Shiba goat [48] and giant cells in the camel [20] also have an ability to synthesize estrogen. In the cetacean placenta, no specialized trophoblast epithelial cells have been reported in previous studies [9, 10, 12, 13]. In this study, such specialized cells were also not observed, and most mononucleate trophoblast cells showed positive immunoreactivity for P450scc, suggesting that the mononucleate trophoblast epithelial cells synthesize sex steroid hormones and/or their precursors in the Antarctic minke whale and possibly also in other cetaceans. In further studies of the Antarctic minke whale placenta, investigation of the localization of other steroidogenic enzymes including cytochrome P450 aromatase, which converts testosterone to estradiol, is needed to identify the sex steroid hormones secreted from the placenta.

In conclusion, the results of the present observations demonstrate that the Antarctic minke whale placenta has a diffuse and epitheliochorial placenta with the chorion interdigitated to the endometrium by primary, secondary and tertiary villi, which have no specialized trophoblast cells and blood vessels deeply indented into the trophoblast cells and endometrial epithelium. Moreover, we could confirm steroidogenesis in the trophoblast cells of the minke whale placenta.

Acknowledgments

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