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RESEARCH PAPER

Differential mouse-strain specific expression of Junctional Adhesion Molecule (JAM)-B in placental structures

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ABSTRACT

The junctional adhesion molecule (JAM)-B, a member of the immunoglobulin superfamily, is involved in stabilization of interendothelial cell-cell contacts, formation of vascular tubes, homeostasis of stem cell niches and promotion of leukocyte adhesion and transmigration. In the human placenta, JAM-B protein is abundant and mRNA transcripts are enriched in first-trimester extravillous trophoblast in comparison to the villous trophoblast. We here aimed to elucidate the yet unexplored spatio-temporal expression of JAM-B in the mouse placenta. We investigated and semi-quantified JAM-B protein expression by immunohistochemistry in early post-implantation si tes and in mid- to late gestation placentae of various murine mating combinations. Surprisingly, the endothelium of the placental labyrinth was devoid of JAM-B expression. JAM-B was mainly present in spongiotrophoblast cells of the junctional zone, as well as in the fetal vessels of the chorionic plate, the umbilical cord and in maternal myometrial smooth muscle. We observed a strain-specific placental increase of JAM-B protein expression from mid- to late gestation in Balb/c-mated C57BL/6 females, which was absent in DBA/2J-mated Balb/c females. Due to the essential role of progesterone during gestation, we further assessed a possible modulation of JAM-B in mid-gestational placentae deficient in the progesterone receptor ($Pgr^{-/-}$) and observed an increased expression of JAM-B in $Pgr^{-/-}$ placentae, compared to $Pgr^{+/+}$ tissue samples. We propose that JAM-B is an as yet underappreciated trophoblast lineage-specific protein, which is modulated via the progesterone receptor and shows unique strain-specific kinetics. Future work is needed to elucidate its possible contribution to placental processes necessary to ensuring its integrity, ultimately facilitating placental development and fetal growth.

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Introduction

Junctional adhesion molecules (JAM) belong to the immunoglobulin (Ig) superfamily characterized by the presence of extracellular immunoglobulin-like domains (reviewed in ref. 1). The most investigated members of this family are JAM-A, -B, and –C, which were previously referred to as JAM-1, −2 and −3, respectively.[2] JAM-B extracellular Ig domains can bind either homophilically or heterophilically to JAM-C[3,4] and to integrin $\alpha1\beta4$ (also known as very late activation antigen (VLA)-4).[5] JAM-B is mainly known to stabilize endothelial tight junctions by forming clusters at intercellular contacts which bind JAM-B or –C.[6-10] Through the binding to its ligands in leukocytes, JAM-B promotes cell transendothelial migration[5,6,9,11] and has even been described to play a role in leukocyte rolling and adhesion.[12,13] Further, JAM-B is involved in pro-angiogenic processes and vascular lumen formation.[10,14,15] JAM-B is susceptible to up-regulation in inflammatory or tumoral processes.[12,16,17] JAM-B expression has been mostly investigated in human endothelium tissue obtained e.g. from the aorta,[15] skin,[18] tumors,[12] tonsils[4] and other lymphatic endothelium,[7] as well as in human umbilical vascular endothelial cells (HUVECs).[6,11,13] JAM-B$^+$ mouse endothelial tissues studied so far were e.g. derived from aorta,[14] skin,[17] spleen,[4] lymph nodes,[4] and high endothelial venules (HEV)[19] of lymph nodes. In addition, JAM-B has also been observed in other organs and tissues from both, humans and mice, including the heart,[11,20,21] lung,[11,20] and lymph nodes.[8,21,22] Moreover, human brain

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and small intestine,[11] and mouse liver and kidney[8] were found to express JAM-B. Interestingly, mouse embryonic, haematopoietic, and neural stem cells[23] also express JAM-B. Furthermore, it is present on stromal cells in the murine bone marrow where it maintains homeostasis of stem cell niches and regulates stem cell homing and mobilization in a JAM-C dependent manner.[24,25] In the human brain, JAM-B aberrant expression in glioma may support tumor cell invasion.[26] In epithelium of seminiferous tubules in the testis, it tightly controls spermatogenic processes.[12,27-29]

Interestingly, one of the main sites of JAM-B expression is the human placenta.[8,11,12,21] However, to date, only very little is known about placental JAM-B expression.[30] This is in part due to the limited access to human placental tissue. To overcome these limitations, mouse pregnancies have been validated as suitable models for translational studies, despite remarkable species differences. As in humans, the mouse develops a hemochorial placenta which mediates feto-maternal interactions by providing the fetus with nutrients and oxygen.[31,32] Murine placentation is initiated on gestational day (gd) 6.[33,34] Early during placentation, trophoblast giant cells invade into the decidualizing endometrium and form the outer layer of the placenta, which is in direct contact with maternal decidual tissue. Commencing on gd 8, trophoblast cells of the ectoplacental cone differentiate to give rise to the junctional zone, which bridges tissues of maternal and fetal origin and contains spongiotrophoblast, glycogen trophoblast cells and different types of giant trophoblast cells (TGCs).[35] The chorionic ectoderm undergoes extensive folding and differentiation until gd 10 forming the multinucleated syncytiotrophoblast, which, together with the mesodermally-derived fetal vasculature, comprises the labyrinth[33] with its maternal blood sinuses and fetal capillaries intertwined in close vicinity. Placental circulation starts on gd 9 and the placenta grows until gd 16,[34] accompanied by continuing branching of the labyrinthine vasculature until late pregnancy to maximize the surface for feto-maternal exchange.

The development of a functional placenta involves cell to cell interactions, migration, and invasion as well as vascularization; processes in which we hypothesize JAM-B may play a role. In mouse pregnancies, the early (gd 3.5) and late (gd 4.5) blastocyst stages of the mouse embryo have been found to be JAM-B positive, which is furthermore highly expressed in the luminal uterine epithelium on gd 3–5.[36] Thus, JAM-B could mediate blastocyst attachment to the receptive uterus on gd 4.[36] Further, the pro-gestational hormone progesterone has been described to up-regulate JAM-B expression in the early implantation site.[36] Progesterone regulates implantation and trophoblast invasion (reviewed in ref. 37), ensures uterine myometrial quiescence until parturition[38] and induces maternal tolerance toward the semi-allogeneic fetus by skewing the immune response toward a pregnancy-protective Th2-phenotype.[39,40] Reduced levels of progesterone have been associated with fetal loss in humans and mice.[41,42]

Taken together, insights on JAM-B expression in mice during early post-implantation and placentation and its potential modulation is still largely missing. Given the importance of JAM-B with regard to cell migration and angiogenesis in a number of settings, we here aimed to close this gap of knowledge by investigating the yet unexplored temporal and spatial distribution of JAM-B protein expression in the murine placentae derived from various mating combinations of wild type and Progesterone receptor gene (Pgr) knockout mice.

Results

JAM-B is expressed at the feto-maternal interface of mice in mid- to late pregnancy

The spatial and temporal expression of JAM-B in the fully developed mouse placenta was evaluated immunohistochemically in specimens obtained from Balb/c-mated C57BL/6 females (M Balb/c × F C57BL/6) on gd 13.5, 14.5 and 16.5, which corresponds to mid- to late pregnancy in mice. We observed that JAM-B was highly expressed in myometrial circular and longitudinal smooth muscle cells (Figs. 1A-C and 1E) and in smooth muscle cells of a small portion of medium-size blood vessel walls in the non-decidualized endometrium and myometrium. No JAM-B expression was detectable in decidual vessels. In the placenta, weak cytoplasmic JAM-B expression could be detected in a small number of parietal trophoblast giant cells lining the junctional zone in close contact with maternal uterine tissue. Remarkably, in the junctional zone, JAM-B was prominently present among spongiotrophoblast cells, while glycogen trophoblast cells and spiral artery-associated trophoblast giant cells showed no expression (Figs. 1A and 1B, Fig. 2C). Surprisingly, we could not detect JAM-B in the placental labyrinth, indicating that neither fetal endothelial cells, nor the syncytiotrophoblast bilayer forming the maternal blood sinuses expressed this adhesion molecule. However, JAM-B was expressed in vessels of the chorionic plate during late gestation (Fig. 3B) as well as in the umbilical cord vessel walls (Figs. 1D and 1F). Here, JAM-B could be observed in the muscular layer surrounding the vessels, whereas we could not confirm a distinct expression in the thin endothelial layer of these vasculatures. Figure 6A displays a schematic overview of JAM-B expression sites in murine utero-placental units.

As significant differences in the expression of adhesion molecules can be observed among inbred mouse

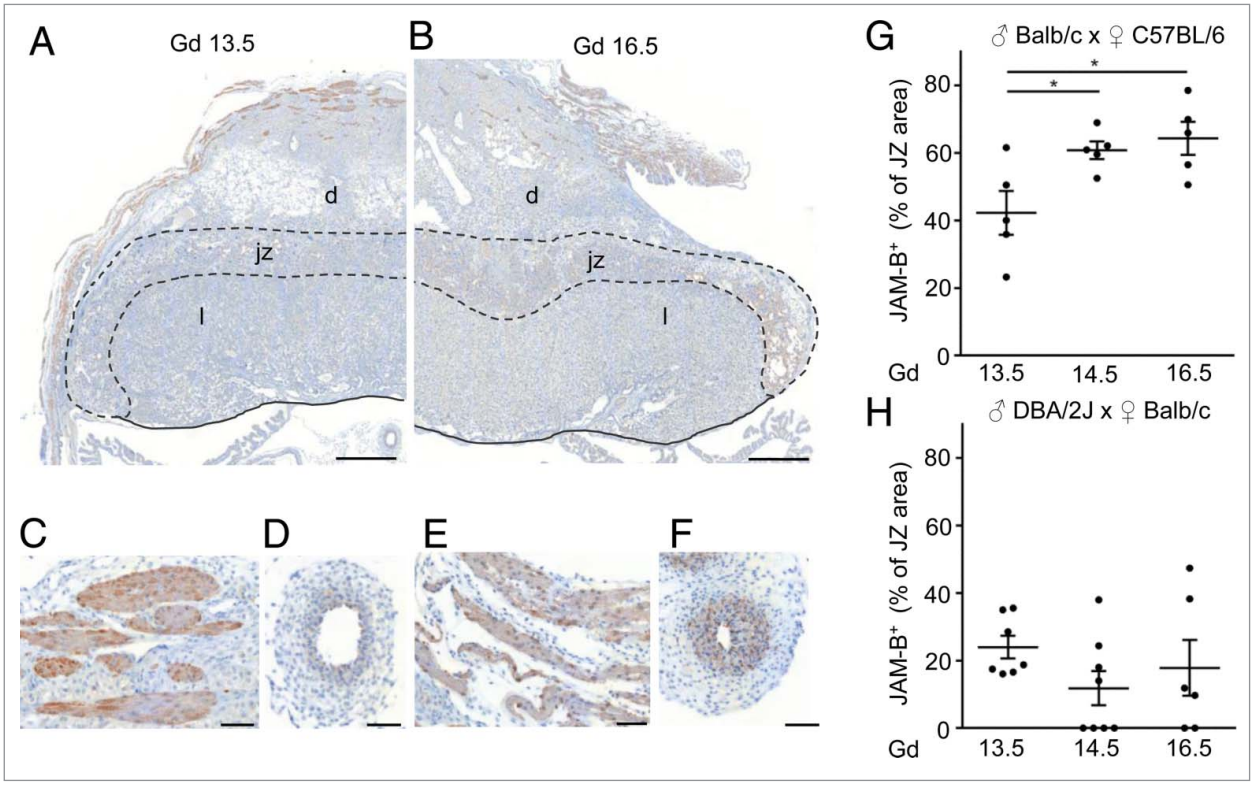

Figure 1. Spatio-temporal JAM-B protein expression in the junctional zone increases from mid- to late gestation in M Balb/c × F C57BL/6 matings. Representative mid-sagittal sections of gd 13.5 (A) and 16.5 (B) placental tissue from M Balb/c × F C57BL/6 matings. JAM-B positive area appears brown. Myometrial smooth muscle cells, both circular and longitudinal, might constitutively express JAM-B on gd 13.5 (C) and 16.5 (E). JAM-B expression in the fetal umbilical cord vessels increases from gd 13.5 (D) to 16.5 (F) at the time that the vascular wall thickens. (G) Percentage of JAM-B$^+$ spongiotrophoblast in the total area of junctional zone (JZ) increases significantly in M Balb/c × F C57BL/6 matings from gd 13.5 to 14.5 and 16.5. (h) Percentage of JAM-B$^+$ spongiotrophoblast in the total JZ area is lower in M DBA/2J × F Balb/c matings when compared to M Balb/c × F C57BL/6 matings and is not altered with increasing gestational age. gd: gestational day, d: decidua, jz: junctional zone, l: labyrinth. Scale bars in A, B = 500 μm; C-F = 50 μm. (G-H) bars represent the mean ± SEM. $^*p \leq 0.05$ as assessed by T-test.

strains,[43,44] we analyzed JAM-B expression in utero-placental units obtained from DBA/2J-mated Balb/c females (M DBA/2J × F Balb/c) on gd 13.5, 14.5 and 16.5. Despite a generally lower intensity of JAM-B expression in placentae obtained from M DBA/2J × F Balb/c pregnancies, we observed the same pattern of JAM-B localization as in the placentae from M Balb/c × F C57BL/6 matings.

Altogether, across the analyzed gestational days, the localization of JAM-B remained unaffected, while variations in the degree of positivity occurred especially in the spongiotrophoblast cells of the junctional zone and in the chorionic vessels.

Expression of JAM-B in the placental junctional zone is modulated during development

We next aimed to characterize the temporal changes that appeared to take place in JAM-B expression in the spongiotrophoblast cells by analyzing the surface area expressing JAM-B and its expression intensity.

In a first step, we quantified the expression as the percentage of the JAM-B positive area within the total area of the junctional zone. While JAM-B was expressed by the spongiotrophoblast, we observed that not all spongiotrophoblast cells were positive for JAM-B and that its area of expression increased significantly over time from gd 13.5 to gd 14.5 and 16.5 in M Balb/c × F C57BL/6 matings (Fig. 1G). Along these gestational ages, the total area of the junctional zone also increased (Table 1). In comparison, in gd 13.5 placentae from M DBA/2J × F Balb/c, a smaller proportion of the junctional zone was JAM-B$^+$, which remained unaltered over the subsequent gd analyzed (Fig. 1H). Similarly to placentae from M Balb/c × F C57BL/6, the junctional zone increased in size over gestation in M DBA/2J × F Balb/c matings, showing a significant increase in the area from gd 13.5 to 14.5

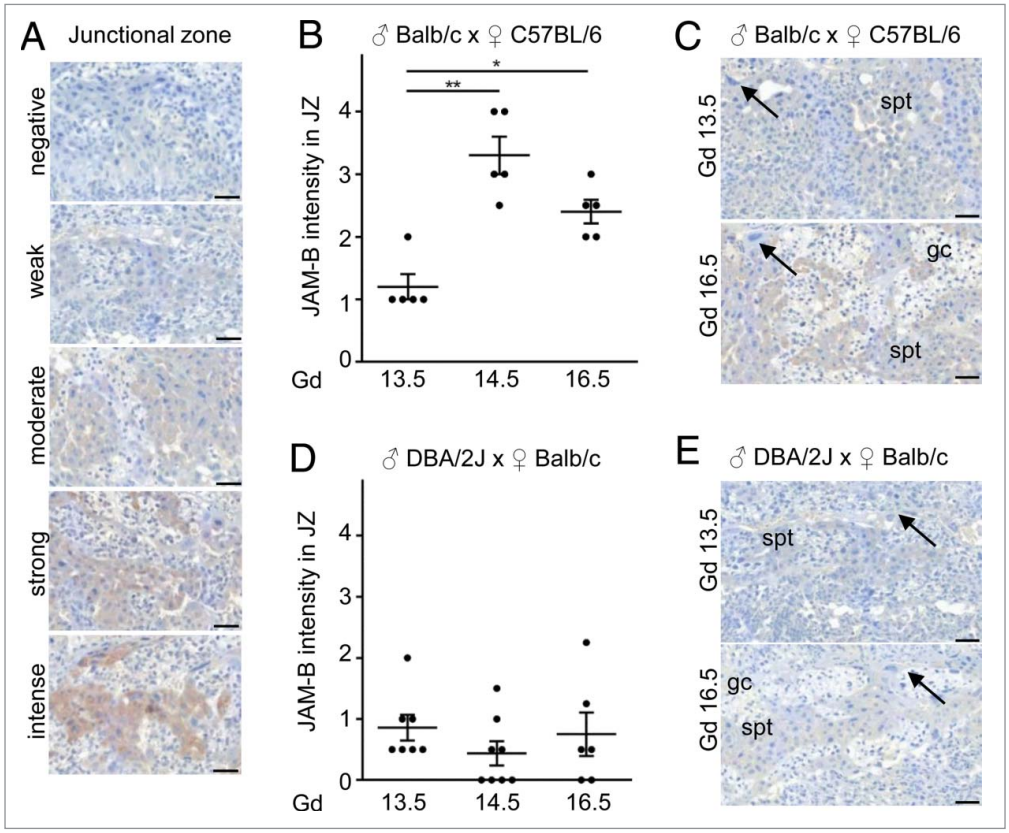

Figure 2. Intensity of JAM-B protein expression in the junctional zone increases from mid- to late gestation M Balb/c × F C57BL/6 matings (A) Representative JAM-B intensity staining scores in the junctional zone. Negative: 0, weak: 1, moderate: 2, strong: 3, intense: 4. (B) Intensity of JAM-B spongiotrophoblast expression in the junctional zone (JZ) increases significantly in M Balb/c × F C57BL/6 matings from gd 13.5 to 14.5 and 16.5. (D) Intensity of JAM-B spongiotrophoblast in the JZ area is not modified over gestational age in M DBA/2J × F Balb/c matings. Representative stainings of JAM-B intensity from gd 13.5 and 16.5 in M Balb/c × F C57BL/6 matings (C) and M DBA/2J × F Balb/c matings (e). gc: glycogen trophoblast cells, gd: gestational day, spt: spongiotrophoblast, arrows point to parietal trophoblast giant cells. Scale bars = 50 μm. (B,D) bars represent the mean ± SEM. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$ as assessed by Mann-Whitney-U-test.

(Table 1). In M DBA/2J × F Balb/c placentae, the size of the junctional zone was smaller compared to placentae from M Balb/c × F C57BL/6 at the same gd.

Next, we semi-quantified the amount of JAM-B protein expression by scoring the staining intensity of JAM-B^{+} areas in the spongiotrophoblast as shown in Figure 1A. Here, spongiotrophoblast cells in M Balb/c × F C57BL/6 placentae showed a significantly more intense JAM-B expression on gd 14.5 and 16.5, compared to gd 13.5 (Figs. 2B and 2C). In M DBA/2J × F Balb/c pregnancies, placentae showed a JAM-B expression intensity similar to M Balb/c × F C57BL/6 placentae on gd 13.5. However, opposed to placentae from M Balb/c × F C57BL/6, JAM-B expression remained unchanged over the later gestational days (Figs. 2C and 2E).

With regard to litter sizes, fetal loss rates or fetal growth curves, no difference could be observed in the DBA/2J–mated Balb/c females and Balb/c-mated C57BL6/J females (data not shown), despite an increased area and higher intensity of JAM-B expression during mid to late gestation in C57B6/J females mated to Balb/c males, compared to JAM-B expression in placentae from Balb/c × DBA/2J matings.

Noteworthy, we observed that the intensity of JAM-B staining in the smooth muscle cells of the uterine myometrium was significantly lower in M DBA/2J × F Balb/c than in M Balb/c × F C57BL/6 pregnant uteri, indicating that the strain differences in JAM-B expression were also extending into other structures of the utero-placenta unit. In both mating combinations, the myometrial smooth muscle cells intensity of JAM-B varied but remained stable over time, suggestive of a possibly constitutive expression in these cells (Fig. S1).

Expression of JAM-B in the chorionic vessels is modulated during development

We next semi-quantified the JAM-B protein expression intensity in chorionic vessels on gd 13.5, 14.5 and 16.5 in both mating combinations and observed a higher JAM-B

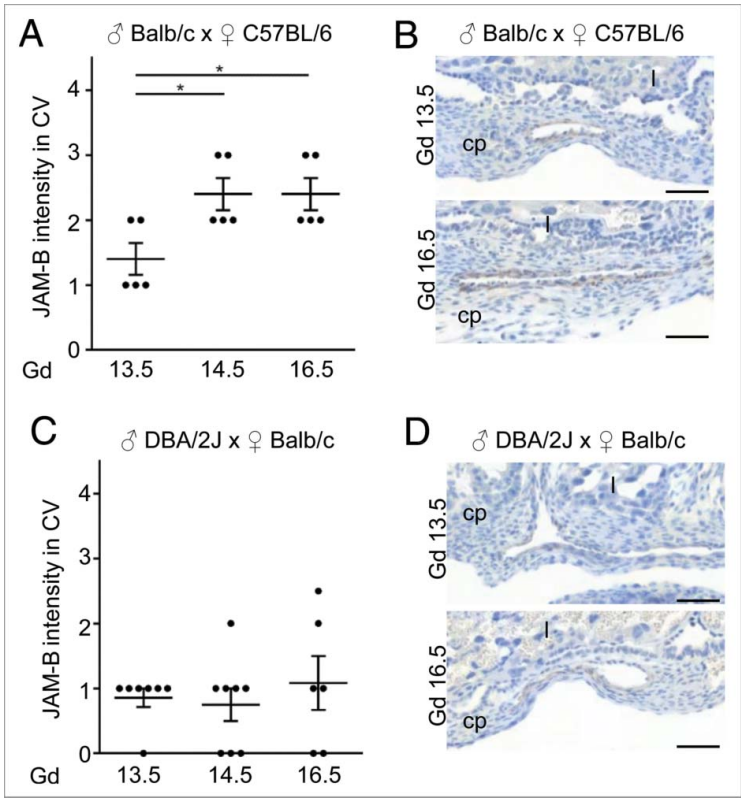

Figure 3. Intensity of JAM-B protein expression in the chorionic plate vessels increases from mid- to late gestation in M Balb/c × F C57BL/6 matings. (A) Intensity of JAM-B expression in chorionic plate vessels (CV) increases significantly in M Balb/c × F C57BL/6 matings from gd 13.5 to 14.5 and 16.5. (C) Intensity of JAM-B expression in the chorionic plate vessels is not modified over gestational age in M DBA/2J × F Balb/c matings. Representative stainings of JAM-B intensity from gd 13.5 and 16.5 in M Balb/c × F C57BL/6 matings (B) and M DBA/2J × F Balb/c matings (D). Staining score key as shown in Fig. 2A was applied. cp: chorionic plate, gd: gestational day, l: labyrinth. Scale bars = 50 μm. (A) bars represent the mean ± SEM. $^{*}p \leq 0.05$ as assessed by Mann-Whitney-U-test.

expression in samples obtained from M Balb/c × F C57BL/6 pregnancies on gd 14.5 and 16.5, compared to gd 13.5 (Figs. 3A and 3B). In contrast, JAM-B expression in chorionic vessels of placental tissue derived from M DBA/2J × F Balb/c matings remained weak at all 3 time points assessed (Figs. 3C and 3D).

Taken together, the altered temporal expression profiles we observed in spongiotrophoblast and chorionic vessels during mid- to late gestation allow to concluding that placental JAM-B follows a strain-specific pattern. The continuous increase from mid- to late gestation exclusively in M Balb/c × F C57BL/6 placentae is suggestive of a targeted modulation of JAM-B in this mating combination.

Progesterone receptor-deficient placentae show an increased JAM-B expression in the junctional zone

The observation that JAM-B expression is modulated during the course of mouse placental development in certain mating combinations raised the question if progesterone could be an upstream regulator of JAM-B, as JAM-B modulation during blastocyst hatching has been associated with progesterone-dependent pathways.[36] To assess this possible role of progesterone, female mice heterozygous for the deletion of the progesterone receptor gene ($Pgr^{+/-}$) in a mixed C57BL/6×129SvEv background were mated to $Pgr^{+/-}$ males in order to compare JAM-B

Table 1. Total junctional zone (JZ) areas[a].

		gestational day		
Mouse strain	mated to	13.5	14.5	16.5
C57BL/6 females	Balb/c	3.07 ± 0.22 (5)	3.18 ± 0.28 (5)	3.57 ± 0.38 (5)
Balb/c females	DBA/2J	1.94 ± 0.12 (7)	2.76 ± 0.18 (8)*	2.54 ± 0.35 (6)

[a] area in mm^2; mean ± SEM (n), * equals $p \leq 0.05$ vs. gestational day 13.5

expression in gd 13.5 placentae from $Pgr^{-/-}$ and $Pgr^{+/+}$ implantations. Here, we confirmed our evaluation on the staining intensity for JAM-B expression in the spongiotrophoblast cells of the junctional zone and in the chorionic vessels. The analyses revealed an increase in JAM-B protein expression intensity in the $Pgr^{-/-}$ placental spongiotrophoblast, compared to $Pgr^{+/+}$ placentae (Fig. 4A). This was also reflected by an increase in chorionic vessel staining intensity (Fig. 4B). Moreover, inline with our previous observations,[32] we observed a reduced fetal weight and a significantly decreased labyrinth/junctional zone ratio in $Pgr^{-/-}$ compared to $Pgr^{+/+}$ implantations as determined by histomorphometric analyses of Masson-Goldner stained placental tissue sections (Figs. 4C and 4D). These changes of the labyrinth/junctional zone ratio are due to a decreased labyrinth and an increased junctional zone size on gd 13.5, while the overall placental size remained unaffected. These results suggest that progesterone receptor-mediated signaling may act as an upstream suppressor of JAM-B expression.

JAM-B is expressed at the early post-implantation site

To investigate if JAM-B expression in the placenta has its origin in early extraembryonic structures, we investigated its expression in early post-implantation periods. We here studied M C57BL/6 × F C57BL/6 pregnancies, as our evidence supports a stronger modulation of JAM-B in the C57BL/6 background. Recently, JAM-B has been suggested to facilitate blastocyst attachment to the uterine lumen.[36] Our analysis revealed that JAM-B expression was present in gd 5.5 and 7.5 utero-placental units (Figs. 5A and 5B). Similarly to mid- to late pregnancy, prominent presence of JAM-B was observed in circular and longitudinal myometrial smooth muscle cells and in some of the blood vessels in the endometrium. In addition, weak JAM-B expression was observed in endometrial glands (Figs. 5E and 5F). On gd 5.5, positive staining was scarce in stromal cells of the endometrium and absent

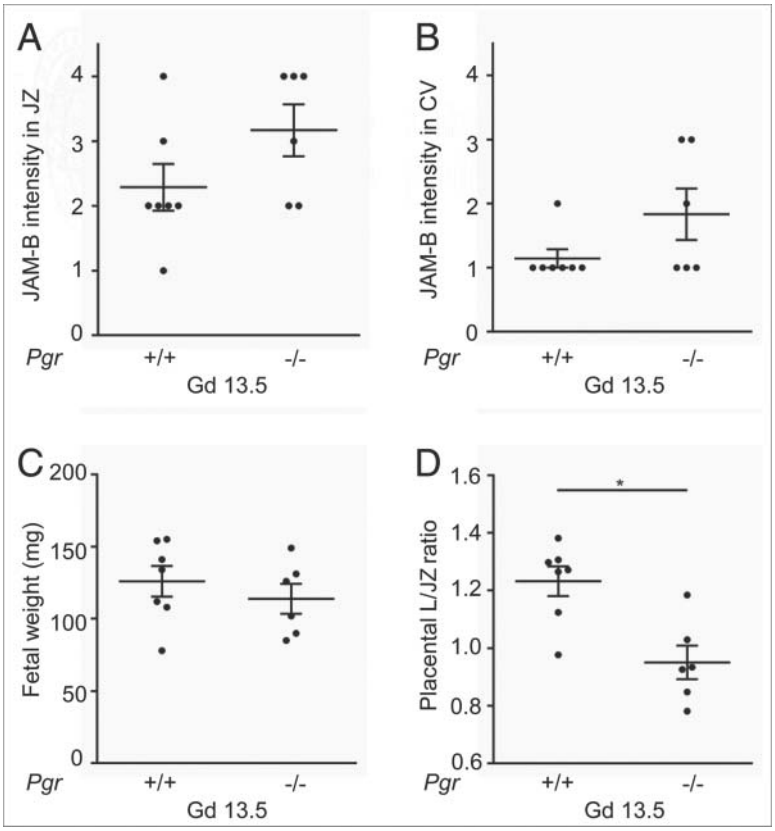

Figure 4. Intensity of JAM-B protein expression in the junctional zone and in chorionic plate vessels is increased in progesterone receptor-deficient placentae on gestational day 13.5 which is accompanied by a reduced L/Jz ratio. (A) Intensity of JAM-B spongiotrophoblast expression in the junctional zone (JZ) increases upon progesterone-receptor deficiency in placentae from $Pgr^{+/-} \times Pgr^{+/-}$ in comparison to wildtype placentae on gd 13.5. (B) Intensity of JAM-B expression in chorionic plate vessels (CV) is enhanced in the same placentae. (C) Fetal weight of progesterone receptor-deficient fetuses is lower than that of wild-type fetuses. (D) Placental labyrinth to junctional zone (L/JZ) ratio is significantly decreased in progesterone receptor-deficient placentae when compared to wildtype placentae. gd: gestation day. Bars represent the mean ± SEM. $^{*}p \leq 0.05$ as assessed by Mann-Whitney-U-test.

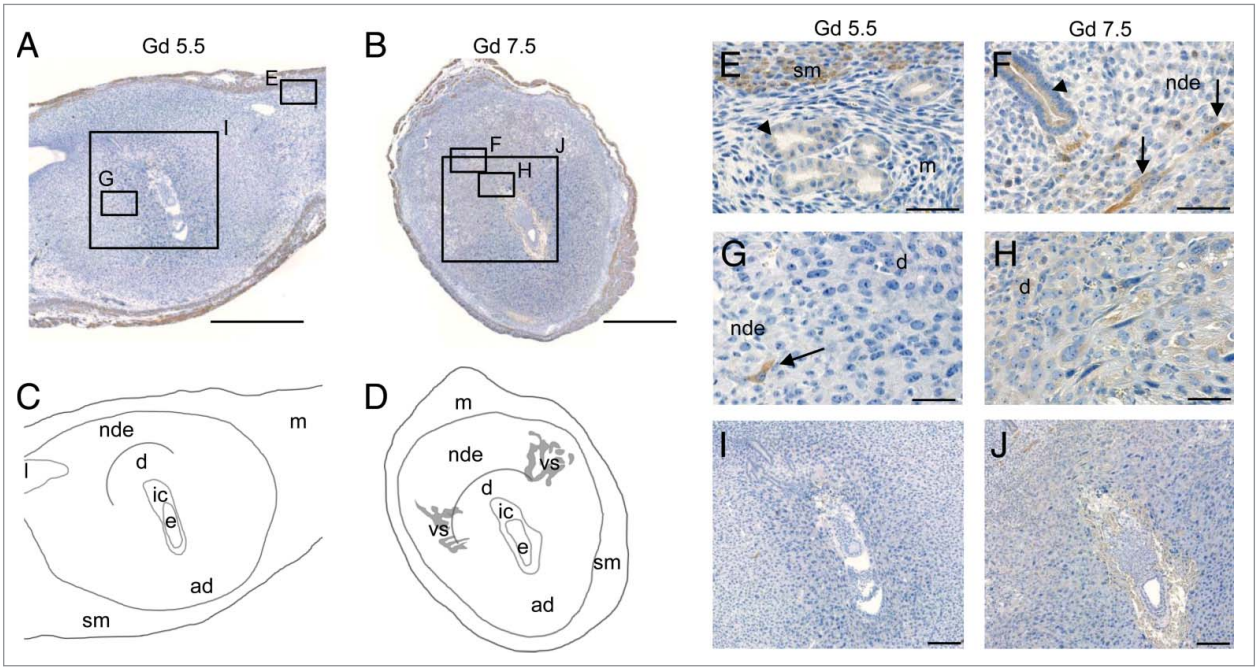

Figure 5. Spatial JAM-B protein expression in gd 5.5 and 7.5 implantation sites. (A) Transversal section of a gd 5.5 embryo (syngeneic mating of C57BL/6) stained for JAM-B expression (brown). (B) Sagittal section of a gd 7.5 embryo (syngeneic mating of C57BL/6). Schematical overview of major morphological areas of gd 5.5 (C) and gd 7.5 (D) embryo. Mesometrial glands in early post-implantation are expressing JAM-B (arrowheads) on gd 5.5 (E) and 7.5 (F). Myometrial smooth muscle cells (sm) are abundantly expressing JAM-B (E). Individual JAM-B^{+} cells are present in gd 7.5 non-decidualized endometrium (nde) (arrows) (F). (G, H) The primary decidua (d) consists of decidualized endometrial stroma distinguished by cells with larger polyploid nuclei in comparison to nde. Primary decidua is negative for JAM-B on gd 5.5 (G), while it is JAM-B^{+} on gd 7.5 (h). Few individual JAM-B^{+} cell are present in gd 5.5 nde (arrow) (G). The embryo itself and the implantation crypt (IC) on gd 5.5 (I) and 7.5 (J) are JAM-B^{neg}. This includes ic structures facing the primary decidua from which placental structures arise from gd 8 onwards. m: mesometrium, e: embryo, ad: anti-mesometrial decidua, l: uterine lumen, vs: lateral vascular sinuses, gd: gestational day. Scale bars in A, B = 500 μm; E-H = 50 μm; I, J = 100 μm.

in the zone of the primary decidua basalis (Fig. 5G). On gd 7.5, JAM-B was weakly expressed in decidualized cells localized in the primary decidua basalis, as well as in individual cells in the non-decidualized endometrium (Figs. 5F and 5H). Interestingly, this decidual expression was absent later in pregnancy, e.g. on gd 13.5 to 16.5, as described above. Remarkably, we could not detect JAM-B positivity in the extra-embryonic area, the site where trophoblast cells of ectoplacental cone and the early mesodermal chorion reside, on gd 5.5 and 7.5 (Figs. 5I and 5J). This was surprising, since these structures give rise to spongiotrophoblast cells and chorionic and umbilical cord vessels in mid- to late pregnancy, in which we detected JAM-B expression.

Discussion

We here aimed to elucidate the yet unexplored spatiotemporal distribution of JAM-B expression in the functional murine placenta including its strain-specific appearance. Surprisingly, the endothelium of the placental labyrinth was devoid of JAM-B expression in mid- to late pregnancy (gd 13.5–16.5). JAM-B was mainly present in spongiotrophoblast cells of the junctional zone, as well as in the fetal vessels of the chorionic plate, the umbilical cord and in maternal myometrial smooth muscle. We observed a strain-specific placental increase of JAM-B protein expression from mid- to late gestation in Balb/c-mated C57BL/6 females, which was absent in DBA/2J-mated Balb/c females. Further, its modulation in mid-gestational placentae deficient in the progesterone receptor was assessed. We observed an increased expression of JAM-B in $Pgr^{-/-}$ placentae, compared to $Pgr^{+/+}$ tissue samples, suggesting that progesterone receptor-mediated signaling may act as an upstream suppressor of JAM-B expression. Investigating the origin of mid- to late gestational JAM-B expression in early extraembryonic structures revealed the presence of JAM-B expression in the decidualizing endometrial stromal cells adjacent to the implantation site on gd 7.5. Remarkably, we could not detect JAM-B positivity in the extra-embryonic area on gd 5.5 and 7.5, the site where trophoblast cells of ectoplacental cone and the early mesodermal chorion reside, which will originate the JAM-B^{+} spongiotrophoblast cells and chorionic and umbilical cord vessels in mid- to late pregnancy.

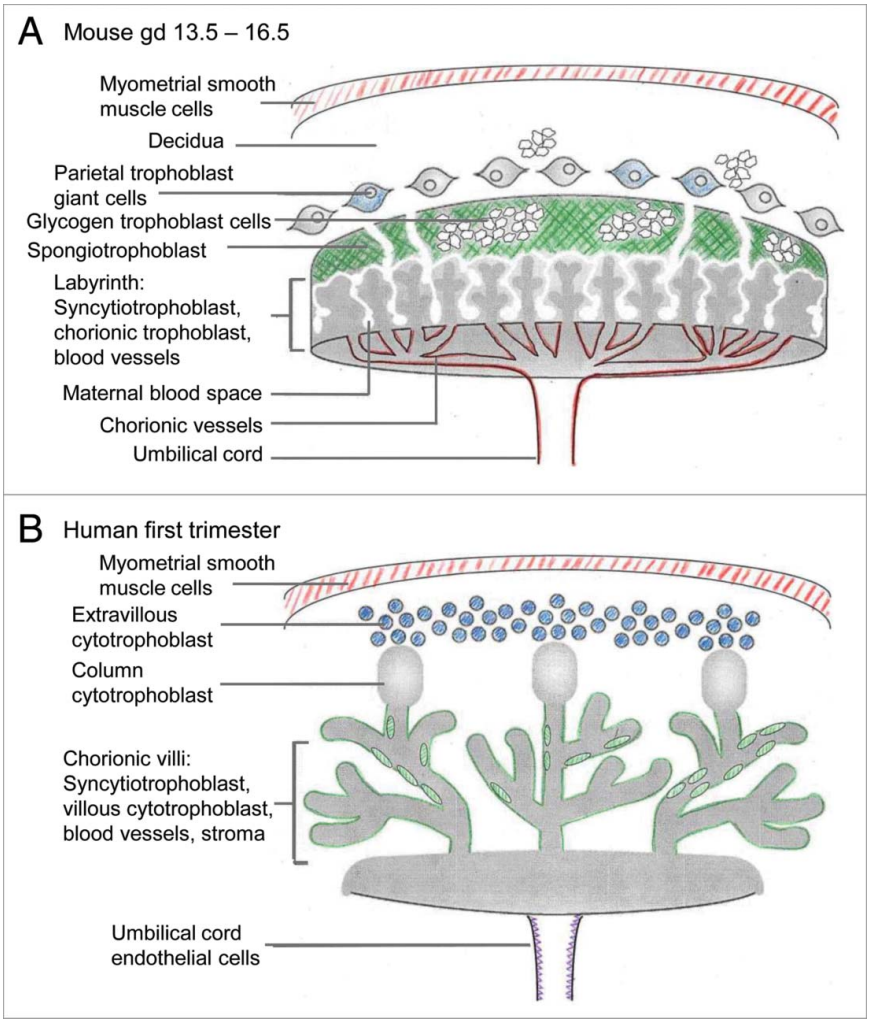

Figure 6. Comparative display of JAM-B expression in mouse and human utero-placental cells. (A) In the murine placenta from mid- to late gestation (gd 13.5–16.5), JAM-B is specifically expressed in the spongiotrophoblast (green), whereas it is scarcely present in parietal trophoblast giant cells (pTGC) (blue) and absent in glycogen trophoblast cells and other TGCs. Decidual stromal cells are negative, in contrast to JAM-B^{+} decidualizing endometrium on gd 7.5 (not shown). Chorionic plate vessels and umbilical cord show JAM-B expression (red). The labyrinthine endothelium and syncytiotrophoblast do not express JAM-B. (B) In human first trimester placenta, the extravillous cytotrophoblast, corresponding to murine pTGC and glycogen trophoblast cells, highly expresses JAM-B mRNA transcripts (blue). To a lesser extent, JAM-B is also present in the villous cytotrophoblast (green).[30] *Ex vivo* analyses of umbilical cord endothelial cells (HUVEC) revealed JAM-B positivity (purple).[11,13] Both mouse and human myometrial smooth muscle cells express JAM-B (red).[50] Presence or absence of JAM-B expression at additional gestational ages and in anatomical locations of the human placenta unmarked in this display is currently unknown. The figure is modified after ref. 31.

Previously, JAM-B has been shown to be predominantly expressed in endothelium.[11-13,16,17,19,21] In the placenta, endothelial tissue arises from allantoic mesoderm and is mainly found in labyrinthine fetal vessels and the placental chorionic vessels which continue into the umbilical cord.[33,45] Unexpectedly, the labyrinthine endothelial capillaries were devoid of JAM-B expression. Due to this absence in the labyrinth, the key site for placental transport, a direct role of JAM-B with regard to nutrient, hormone or antibody transport can likely be neglected. Still, the vasculature of the labyrinth is suspected not only to be the site for gas and nutrient exchange between mother and fetus, but is also the site where cell migration from mother to child and *vice versa* can occur, a phenomenon termed pregnancy associated microchimerism.[46,47] In other tissues, JAM-B can facilitate transendothelial migration,[9,17] whereas its absence in the placental labyrinth indicates that it may not be necessary for the transfer of microchimeric cells and other factors. Moreover, the absence of JAM-B may constitute a regulatory mechanism for limiting excessive cell transfer across the materno-fetal barrier. In vessels of the umbilical cord and the chorionic plate, JAM-B localized largely in the smooth muscle cells in the tunica media, while we could not confirm its

endothelial expression. This is in line with the observed weak surface JAM-B positivity on human umbilical vascular endothelial cells (HUVEC).[13] The observed lack of JAM-B in the single amuscular layer of fetal endothelium in the labyrinth[48] argues for JAM-B expression to be confined to smooth muscle cells of the chorionic and umbilical cord vessels. This is in agreement with the observations in endothelium of decidual blood vessels, which lose their smooth muscle cells during remodeling, where we could not detect JAM-B expression, while we observed JAM-B positivity in the tunica media of some myometrial vessels. Interestingly, similar to human uterine and other non-uterine smooth muscle cells,[49,50] we observed abundant JAM-B expression in murine myometrial smooth muscle cells. Here, JAM-B expression varied between the 2 different mouse strains analyzed. Still, in both strains, its expression remained stable over gestational age, suggesting a possibly constitutive expression in smooth muscle cells. A potential role of JAM-B in the myometrial smooth muscle cell syncytium which contains many gap junctions beyond the previously described function as interendothelial junctional molecule remains to be investigated. In this context, it is interesting to note that Jamb has been observed to synergize with Jamc in zebra fish myogenesis to mediate the fusion of muscle cell precursors.[51] This is the only evidence available to date supporting a role of JAM-B in muscle cells.

We further report the specific presence of JAM-B protein expression among spongiotrophoblast cells of the junctional zone. Their analogous structure in humans, the villous trophoblast also expresses JAM-B RNA transcripts[30] when investigated in first trimester pregnancies. In parallel with the increasing overall size of the junctional zone, the area fraction in which JAM-B was expressed increased over gestation from gd 13.5 to 16.5 in placentae from M Balb/c × F C57BL/6 matings. This could – at least in part – be due to the increase in spongiotrophoblast cell number and complexity that takes place until gd 16.[52,53] Interestingly, the higher JAM-B intensity in $Pgr^{-/-}$ placentae compared to $Pgr^{+/+}$ placentae coincides with the enlargement of the junctional zone surface area in $Pgr^{-/-}$ placentae,[32] suggesting that JAM-B may be involved in the growth of this placental zone.

In human extra-villous trophoblast (EVT), JAM-B was significantly higher transcribed than in the villous counterpart, advocating that EVT may utilize JAM-B for migration through decidual stroma cells.[30] The EVT is considered equivalent to the murine trophoblast giant cells and glycogen trophoblast cells of the junctional zone, as these cells invade the maternal spiral arteries and the decidua basalis, respectively.[54] Remarkably, we observe that JAM-B was only weakly expressed in individual parietal trophoblast giant cells and absent in other giant cell subtypes and glycogen trophoblast cells, which may be explained by the less invasive phenotype of the mouse placenta.

Interestingly, JAM-B expression was present in the decidualizing endometrial stromal cells adjacent to the implantation site during the early post-implantation period (gd 7.5), one day after the initiation of trophoblast invasion into the decidua.[34] This was in strong contrast to the observed JAM-B negativity in the decidua later in gestation (gd 13.5 - 16.5). We hypothesize that in early pregnancy decidual JAM-B may facilitate the migration of mouse trophoblast cells, in contrast to the human pregnancy, in which the invading EVT cells themselves express JAM-B. Still, we cannot exclude single invading cells of trophoblast origin to be present in the region of JAM-B^+ decidual stromal cells.

On gd 5.5 and 7.5, we did not detect JAM-B in the uterine lumen, indicating that the reported JAM-B expression in the receptive uterus on gd 3[36] has ceased, possibly reflecting the termination of the implantation window period.[34] The lack of JAM-B expression in the implantation crypt on gd 5.5 or 7.5 - where ectoplacental cone and chorio-allantoic ectoderm localize and later give rise to the JAM-B^+ spongiotrophoblast cells, chorionic and umbilical cord vessels - indicates that JAM-B expression is up-regulated once placentation is completed. Figure 6 displays the current knowledge on the up to now identified expression sites of JAM-B in murine and human placental cells and adjacent structures.

On gd13.5, JAM-B expression increased in spongiotrophoblast and chorionic vessel walls from placentae unable to respond to progesterone-induced signaling due to progesterone receptor knock-out ($Pgr^{-/-}$) when compared to $Pgr^{+/+}$ controls. Progesterone plays an essential role for placental function and pregnancy outcome. Our group previously reported that $Pgr^{-/-}$ placentae exhibit an increased junctional zone in relation to the labyrinth and that these changes were associated with fetal growth restriction.[32] These observations were reflected in our groups of $Pgr^{-/-}$ placentae and $Pgr^{+/+}$ controls. Impaired progesterone signaling affects maternal immune tolerance to the fetal allograph and leads to local inflammation.[32,39,41,42,55] Our present results support progesterone as a negative upstream regulator of JAM-B in the placenta and suggest JAM-B could be involved in progesterone-mediated regulation of placental structure and fetal growth.

To identify potential downstream pathways by which progesterone receptor might affect JAM-B expression, we first searched for possible progesterone receptor–binding sites within the JAM-B promotor and gene sequence. We only observed the presence of an antisense sequence for the known progesterone response element

(PRE).[56] This does not exclude the possibility of other response elements within this region, as our search was restricted to the sequences identified to date. However, other molecules downstream of the progesterone receptor with an important function in the placenta might be able to influence JAM-B expression e.g. Galectin-1, which has been shown to be upregulated by progesterone.[40] A promising potential mediator of progesterone actions could be transforming growth factor (TGF)-β, an immunosuppressive mediator at the feto-maternal interface,[57,58] which can repress JAM-B transcription.[28,29] TGF-β mainly acts via activation of Smad proteins,[59,60] which, in turn, have been reported to suppress JAM-B gene transcription.[28] TGF-β also activates ERK1/2 and p54 JNK pathways leading to JAM-B post-transcriptional and post-translational degradation.[29] This hypothesis is supported by the fact that progesterone can induce TGF-β at the human utero-placental unit[61-63] and that Smads are expressed in trophoblast cells.[59,64,65] Another potential regulatory mechanism upstream of JAM-B could involve progesterone-mediated blocking of IL-1alpha signaling.[28,66]

From gd 13.5 to 16.5, JAM-B expression intensity increased in the spongiotrophoblast and chorionic vessel walls in placentae from M Balb/c × F C57BL/6 matings. These changes cannot be assigned to modulation by progesterone, as progesterone remains relatively stable in this gestational age.[38,67,68] In humans, TGF-β levels have been reported to decrease in the villous trophoblast with gestational age.[64] While this remains to be confirmed in murine trophoblast, a decrease of TGF-β availability during gestation could account for the observed increase in JAM-B. Additionally, upregulation of the TGF-β-inactivating proteoglycan Decorin after gd 11 in the spongiotrophoblast of C57BL/6 placentae[52] may reduce TGF-β availability.[69] Another potential regulatory mechanism upstream of JAM-B could involve an increased level of VEGF-A over gestation.[14,70]

While M Balb/c x F C57BL/6 placentae presented a progressive increase in JAM-B protein expression intensity as well as in the positive area per total junctional zone, no such regulation was found in M DBA/2J × F Balb/c tissues which overall depicted lower JAM-B levels. This strain-specific pattern of JAM-B expression did not originate from differential exposition to progesterone, as previous experiments indicated no differences between M Balb/c x F C57BL/6 and M DBA/2J x F Balb/c in maternal progesterone serum levels on gd 13.5 or on gd 16.5 (data not shown). JAM-B has been reported to be upregulated in inflamed tissues and stimulated by pro-inflammatory IL-1alpha or lack of TGF-β.[7,17,28] We hypothesize that the observed differences in JAM-B expression among mating combinations may result from the well-recognized immune polarization in these inbred mouse strains.[71-73] While BALB/c mice display a bias toward a Th2-prone immune response, C57BL/6 are inflammation-prone, both of which also manifests in the strain-specific innate immune responses.[74-76] In order to enhance maternal tolerance to the semi-allogeneic fetus at the feto-maternal interface, pregnancy is accompanied by a local suppression of inflammatory pathways, which may also present strain-specific traits. Unfortunately, to date there is no information available on strain-specific placental TGF-β pathways. Still, comparisons of utero-placental units from mating combinations resulting in either successful pregnancy or fetal rejection revealed strain-specific alterations in the expression of inflammatory markers and cell adhesion molecules.[43,44] More moderate differences are anticipated in placentae from M Balb/c x F C57BL/6 and M DBA/2J x F Balb/c matings, as these matings do not overtly reduce the success of implantation, placentation and fetal growth. However, JAM-B differential expression in pregnancy could contribute to confer differential susceptibility to inflammation and disease. For example, upon Toxoplasma gondii infection C57BL/6 dams experienced increased resorption rates compared to the Balb/c strain, which was due to a Th1/Th2 imbalance correlated to higher systemic TNF–α levels.[77]

Alternatively, differences in JAM-B expression might be epigenetically regulated, triggered e.g. by the paternal strain. Indeed, the pattern of genomic imprinting by DNA methylation depends on the background strain and the gene's parental origin.[78,79] The analysis of the JAM-B promotor and gene sequence (http://bioinformatics.org/sms2/cpg_islands.html) revealed the presence of a potential CpG island according to the definition published by Gardiner-Garden and Frommer.[80] In this regard, the JAM-B DNA sequence exhibits more than 50% frequency of G and C as well as an observed vs. expected CpG ratio higher than 0.6. This sequence starts in the JAM-B promoter where it extends for 320 nucleotides upstream of the transcription start site until approximately 470 nucleotides downstream, which includes exon 1 and a section of intron 1. This observation highly suggests that JAM-B can be subjected to epigenetic modifications, e.g. by methylation of CpG sites. However, the potential paternal contribution to the epigenetic modulation of JAM-B expression in the mouse placenta and if it could be differentially affected by a paternal Balb/c or DBA/2J mouse strain still needs to be elucidated. Hence, epigenetic modulation of JAM-B may explain the surprising observation that fetal weight was unaffected in C57B6/J females mated to Balb/c males, compared to Balb/c x DBA/2J matings despite the different expression of placental JAM-B.

Finally, the spongiotrophoblast-specific expression of JAM-B questions a functional implication. In the bone marrow, JAM-B has been shown to be expressed by stromal cells to mediate maintenance of stem cell niches.[24,25] At the blood-testis barrier, JAM-B was found to tightly regulate the passage and release of developing spermatozoa from the epithelium.[29] We hypothesize that JAM-B could act as an anchoring molecule ensuring proper localization of the different trophoblast giant cell subtypes and enabling migration of glycogen trophoblast cells across the spongiotrophoblast into the decidua after gd 13.[52,54] Both scenarios would require expression of counter-receptor JAM-C on the interacting cells. Thus, by stabilizing intercellular junctions and cell polarity,[6,7,9,10] JAM-B may ensure a dynamic integrity of the junctional zone, which is in need of constant re-structuring with progressing gestational age.[52] In this regard, we can only speculate that JAM-B could be involved in regulation of blood flow in spiral arteries and veins that cross the junctional zone and bring blood into and collect it from the labyrinthine circulation.[48] Thereby, JAM-B may have an indirect role in regulating blood supply to the placenta, in turn influencing placental transport.

Further, the role of JAM-B downstream signaling pathways has not yet been investigated in trophoblast cells. JAM-B's cytoplasmic tail, a PDZ domain, couples to polarity protein Par3[6] and Cdc42, a Rho GTPase,[15] thereby activating intracellular signaling pathways such as the MAP-Kinase cascade via ERK1/2 phosphorylation[14] or cell-motility associated c-Src kinase.[26] The potential of JAM-B to impact cytoplasmic and genomic signaling suggests its involvement in mediating inflammatory, motility and growth processes in the placenta.

In conclusion, we here report a strain-dependent spongiotrophoblast-specific JAM-B protein expression, which can be modulated over the course of gestation in the M Balb/c x F C57BL/6 mating combination. Moreover, we provide evidence that placental JAM-B protein expression is under the modulation of the progesterone receptor. We suggest JAM-B as an as yet underappreciated protein among trophoblast lineage-specific proteins, which are fundamental to the dynamic processes necessary to ensuring placental integrity and pregnancy maintenance, ultimately facilitating placental development and fetal growth.

Materials and methods

Mice

Eight-week-old C57BL/6 female, BALB/c female and DBA/2J male mice were purchased from Charles River Laboratories whereas CBy.SJL(B6)-Ptprca/J males in Balb/c background were purchased from Jackson Laboratory. C57BL/6 females were mated to CBy.SJL(B6)-Ptprca/J males (M Balb/c x F C57BL/6) and BALB/c female were mated to DBA/2J male mice (M DBA/2J x F Balb/c) in order to harvest placental tissues on gd 13.5, 14.5 and 16.5. Mice heterozygous for the deletion of the progesterone receptor ($Pgr^{+/-}$) on a C57BL6/129SvEv background[81] were mated ($Pgr^{+/-}$-mated $Pgr^{+/-}$ females) and sacrificed on gd 13.5. For tissue collection on gd 5.5 and 7.5, 8-week-old C57BL/6 female mice bred in the animal facility of University Medical Center Hamburg-Eppendorf were mated with C57BL/6 males. All mice were housed in the animal facility of the University Medical Center Hamburg-Eppendorf according to institutional guidelines in a 12-hour light/12-hour dark cycle with ad libitum access to food and water.

Tissue collection

Dams were anaesthetized by CO_2 inhalation and euthanized by cervical dislocation. Uterine (gd 5.5, 7.5) and placental tissues (gd 13.5, 14.5, 16.5) were collected and fixed in 4% paraformaldehyde in PBS for 24h and embedded in paraffin for subsequent immunohistochemistry.

Determination of Pgr genotypes by PCR

Genomic DNA from offspring of $Pgr^{+/-}$-mated $Pgr^{+/-}$ females was isolated using the DNeasy Kit (Qiagen) according to the manufacturer's instructions and genotyped by PCR using 2 sets of primers: Wildtype Primer Forward (20mer) 5′-AGCCACTCATAGGGAGGGAG-3′; Wildtype Primer Reverse (20mer) 5′-GTCGCCGTAAA GAGGGAACA-3′; Mutant Primer Forward (20mer) 5′-CAAGATGGATTGCACGCAGG-3′; Mutant Primer Reverse (20mer) 5′-TGATATTCGGCAAGCAGGCA-3′. The wildtype $Pgr^{+/+}$ and mutant $Pgr^{-/-}$ alleles generated 856bp and 580bp PCR products, respectively. Placental tissue from wildtype $Pgr^{+/+}$ and mutant homozygous deletion $Pgr^{-/-}$ offspring was used for subsequent analyses.

Immunohistochemistry of JAM-B in placental tissue

Paraffin-embedded placental tissues were cut transversally (gd 5.5), vertically (gd 7.5), or at the mid-sagittal plane (gd 13.5, 14.5, and 16.5) into histological sections of 4 μm using a microtome (Leica). Sections were deparaffined, rinsed in distilled water, and dehydrated twice in 96% ethanol before JAM-B staining. Prior to staining, heat mediated antigen retrieval was performed with Cell Conditioning Solution, CC1 buffer pH 8,5. Automated

slide staining was performed with Benchmark XT (Ventana Medical Systems, Inc.). Reaction with rabbit polyclonal anti-mouse JAM-B antibody (1:400; abcam, Cat. No.ab139645) was carried out for 32 min at room temperature in Reaction Buffer (Tris based buffer solution (pH 7.6 ± 0.2) (Ventana, Cat. No. 950-300)) and detected with anti-rabbit HRP-conjugated secondary antibody (Histofine, Cat. No.414142F) after quenching of endogenous peroxidase activity. The staining was visualized by ultraView Universal DAB Detection Kit (Ventana, Cat. No. 760-500). Sections were automatically counterstained with Hematoxylin (Ventana, Cat. No. 760-2021) und Bluing Reagent (Ventana, Cat. No. 760-2037) and mounted with Tissue Tek (Sakura, REF1408). JAM-B expression has been described for endothelial tissue.[4,11,14,15] Therefore, we used tissue specimen from mouse abdominal aorta as a positive control. Slides were scanned using NanoZoomer 2.0 HT (Hamamatsu Photonics K.K.) and analyzed using the software NanoZoomer Digital Pathology Image (Hamamatsu Photonics K.K.). JAM-B expression area in the junctional zone was encircled and its percentage of the whole junctional zone area was calculated. JAM-B expression intensity in the junctional zone was blindly scored as follows: (0) negative; (1) weak; (2) moderate; (3) strong; (4) intense. Mean of 2 visual fields, one in the central, a second in the peripheral junctional zone, was calculated. JAM-B expression in the chorionic plate vessel was scored equally in one visual field in the central chorionic plate.

Histomorphometric analysis of placental tissue

Prior to Masson-Goldner trichrome staining of placental paraffin sections to visualize the morphologically different areas of placental tissue on gd 13.5, sections were deparaffined and dehydrated twice in ethanol (96%). For Masson-Goldner trichrome staining, in brief, tissue sections were stepwise stained with Weigert's iron hematoxylin (Waldeck, Cat. No. 2E-032 and 2E-052), Goldner 1 (azophloxine solution (Sigma, Cat. No. P2395-25G and F8129-25G), Goldner 2 (phosphotungstic acid (Merck, Cat. No. 1.00532.0100), orange G (Roth, Cat. No. 0318.1) solution), and Goldner 3 (light-green SF solution (Merck, Cat. No. 1.15941.0025)). Finally, sections were dehydrated and mounted using Eukitt medium (O. Kindler). Image acquisition was performed using a slide scanner (Mirax Midi, Zeiss). Areas of junctional zone and labyrinth zones were quantified using the program Pannoramic Viewer (3DHISTECH) to calculate a ratio by dividing both values.

Statistical analysis

All statistical analyses were performed using SPSS Version 20 (SPSS, Inc.). For statistical analyses, a two-sided Student's T-test was used where normal distribution of independent data sets was given. Otherwise, the non-parametric Mann-Whitney-U test was employed. Level of significance was set to $p \leq 0.05$.

Study approval

Animal care and all experimental procedures were performed according to institutional guidelines and conform to requirements of the German Animal Welfare Act. Ethical approvals were obtained from the State Authority of Hamburg (Germany, approval numbers ORG_526, ORG_702, G10/067, G11/094).

Abbreviations

CV	chorionic plate vessel
gd	gestational day
JAM-B	Junctional Adhesion Molecule-B
JAM-C	Junctional Adhesion Molecule-C
JZ	Junctional Zone
Pgr	Progesterone receptor gene
pTGC	parietal Trophoblast Giant Cell
SpT	Spongiotrophoblast
TGF-β	Transforming Growth Factor-β
VLA-4	Very Late Activation Antigen-4.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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