

Spatiotemporal expression pattern of progranulin in embryo implantation and placenta formation suggests a role in cell proliferation, remodeling, and angiogenesis

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Abstract

Embryo implantation in the mink is preceded by a variable but obligate period of delay in development. Under the influence of progesterone and unknown luteal factors, the mink embryo implants 11–13 days following its exit from diapause. Recent work suggests that progranulin, a growth factor and secreted glycoprotein, is involved in trophoblast proliferation, placental development and endometrial differentiation in the mouse. Using the mink model of delayed implantation and endotheliochorial placentation, we examined the spatiotemporal distribution of progranulin in trophoblast and endometrium during pre- and early post-implantation gestation *in vivo*. A partial sequence of the mink progranulin gene was cloned and sequenced. Comparative sequence analysis revealed that exons 1 and 2 of mink progranulin share 86.6, 82.4, and 94.9% of nucleic acid sequence identity with the human, mouse, and dog sequences respectively, and indicated that the invariable residues of the cysteine-rich motifs of progranulin are well conserved in the mink sequence. By *in situ* hybridization, we show that mink progranulin transcript is present in the cytotrophoblast and in epithelial and stromal endometrial cells at the site of implantation and during early placental formation. Immunohistochemistry revealed the progranulin protein to be strongly expressed in endometrial luminal and glandular epithelium around the time of implantation. In the incipient labyrinth, progranulin expression is localized to cytotrophoblasts and fetal capillaries, as well as to the hypertrophied maternal endothelial cells. This study demonstrates that high levels of progranulin expression correspond to active cell proliferation, remodeling, and angiogenesis occurring during the establishment of the placenta in the mink.

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Introduction

Embryo implantation in the mink is preceded by an obligate delay in the development of variable length, occurring at the blastocyst stage (Hansson 1947, Enders 1952). This delay is terminated by lengthening of the photoperiod associated with the vernal equinox, which occurs in March in the Northern hemisphere (Enders 1952, DUBY & Travis 1972, Murphy & James 1974, Martinet *et al.* 1981, Sundqvist *et al.* 1988). The proximal event in termination of pre-implantation delay is the secretion of prolactin that reinitiates corpus luteum (CL) development and the uterine-priming progesterone secretion (Papke *et al.* 1980, Martinet *et al.* 1981, Murphy *et al.* 1981, Lopes *et al.* 2004). Resumption of embryo development in the mink is characterized by renewed nucleic acid and protein synthesis by trophoblast and ICM cells of the blastocyst, accompanied

by a logarithmic increase in blastocyst diameter (Desmarais *et al.* 2004). Under the influence of progesterone and unknown luteal factors, the mink embryo implants 11–13 days following its exit from diapause (Murphy *et al.* 1983, Desmarais *et al.* 2004) and post-implantation gestation requires an invariable 30 days (Hansson 1947, Murphy & James 1974).

As described in the ferret (*Mustela putorius*) and the spotted skunk (*Spilogale gracilis*), mustelid implantation is achieved by penetration of the endothelial epithelium by trophoblast cells in early stages of differentiation (Enders & Schlafke 1972, Enders & Mead 1996). Following trophoblast attachment in the ferret, the syncytiotrophoblast cells invade the endometrial tissue and the endometrial glands are progressively transformed into a maternal symplasma as the syncytiotrophoblast advances (Gulamhusein & Beck 1973, 1975).

During syncytiotrophoblast invasion, the cytotrophoblast proliferates to eventually form the chorionic villi (Gulamhusein & Beck 1973, 1975). In the mink, placenta is villous and endotheliochorial (Enders 1952, Krebs *et al.* 1997), less invasive than mouse or human hemochorial placenta, as the blood vessels of the mother become surrounded by trophoblast but are not destroyed (Steven 1975). In the labyrinth region of the placenta, extremely enlarged maternal endothelial cells can be observed (Jones *et al.* 1997, Backlin *et al.* 1998). This phenomenon also appears in other carnivores, but it is more pronounced in mink (Jones *et al.* 1997, Krebs *et al.* 1997, Backlin *et al.* 1998).

Recent work suggests that progranulin, a secreted glycoprotein and a growth factor, is involved in mouse trophoblast proliferation (Diaz-Cueto *et al.* 2000, Qin *et al.* 2005), placental development, and endometrial decidualization (Daniel *et al.* 2003). Progranulin, also known as granulin–epithelin precursor (Zanocco-Marani *et al.* 1999), proepithelin (Plowman *et al.* 1992), PC cell-derived growth factor (Zhou *et al.* 1993), or acrogranin (Baba *et al.* 1993), is a 66–88 kDa protein (Zhou *et al.* 1993) comprising 7.5 granulin/epithelin consensus sequences, each of these consisting of a conserved 12-cysteine motif (Bhandari *et al.* 1992). The granulin/epithelin domains of progranulin are unique and therefore progranulin cannot be classified into any known growth factor families, although a distant common origin with epidermal growth factor (EGF)/transforming growth factor- α (TGF α) family of growth factors has been suggested (Hrabal *et al.* 1996, Bateman & Bennett 1998). Progranulin expression is found across virtually all tissues in mammals (Baba *et al.* 1993a, 1993b, Bhandari *et al.* 1993, Daniel *et al.* 2000). Abundant transcript expression is found in rapidly dividing epithelia, i.e., keratinocytes or enterocytes from the deep crypt of the digestive tract (Daniel *et al.* 2000), suggesting a role in epithelial proliferation. Progranulin has also been associated with tumor progression and invasiveness in adrenocortical carcinomas (He *et al.* 2002), glioblastomas (Liau *et al.* 2000), renal carcinomas (Donald *et al.* 2001), and ovarian as well as breast cancers (Jones *et al.* 2003, Tangkeangsirisin *et al.* 2004, Tangkeangsirisin & Serrero 2004). Moreover, mutations in the progranulin gene were found to cause neurodegenerative frontotemporal dementia, suggesting roles for progranulin in the maintenance of healthy neurons (Baker *et al.* 2006, Cruts *et al.* 2006).

Progranulin transcripts have been detected in rodent oocytes, spermatocytes, and spermatogonia (Daniel *et al.* 2000, Diaz-Cueto *et al.* 2000, Suzuki & Nishihara 2002) and have been found in the embryo throughout pre-implantation development (Diaz-Cueto *et al.* 2000, Qin *et al.* 2005). After fertilization, the levels of progranulin mRNA slowly decline until the four-cell stage embryo in the mouse and rise again in the eight-cell stage embryo (Diaz-Cueto *et al.* 2000). Progranulin

can promote mouse blastocyst formation by enhancing trophoblast proliferation, blastocyst cavitation, and expansion (Diaz-Cueto *et al.* 2000, Qin *et al.* 2005). Progranulin also stimulates hatching, adhesion, and outgrowth of the mouse blastocyst *in vitro* (Qin *et al.* 2005). In the post-adhesion blastocyst, progranulin mRNA becomes more abundant than in the pre-adhesion blastocyst (Qin *et al.* 2003). Furthermore, the progranulin expression in the mouse is strongly induced in the primary giant cells of the placenta and in the decidual cells of the maternal endometrium (Daniel *et al.* 2003).

In vitro studies conducted on mouse blastocysts suggest that progranulin acts as a key player in embryo implantation and placental development, but there is a lack of direct confirmation in the literature. We therefore employed the mink model of delayed implantation and endotheliochorial placentation to investigate the expression of progranulin in trophoblast and endometrium during embryo implantation *in vivo*.

Results

Cloning and sequence analysis of mink progranulin partial cDNA

A partial sequence (214 bp) of the mink progranulin gene was cloned and sequenced, and sequence homology between the mink and three other mammalian species was determined. In Fig. 1, the mink progranulin sequence is presented as well as the comparison among four mammalian progranulin sequences. This analysis revealed that mink progranulin exons 1 and 2 share 86.6% of nucleic acid sequence identity with the human, 82.4% with the mouse, and 94.9% with the dog, a closely related carnivore species. The partial amino acid sequence was deduced from the cDNA and compared with human, dog, and mouse sequences (Fig. 2). Exons 1 and 2 encode for two hemidomains of highly conserved granulin/epithelin motifs, the C-terminal region of paraganulin and the N-terminal region of Granulin G (Bhandari *et al.* 1992). As expected, the mink sequence contained all the conserved cysteine residues of the cysteine-rich motifs found in human, dog, and mouse sequences, as well as the other invariant serine, proline, and glycine residues (Fig. 2, Bhandari *et al.* 1992).

Progranulin mRNA is expressed in mink endometrial and trophoblastic tissues around the time of implantation

To investigate the spatiotemporal pattern of progranulin mRNA expression in the endometrial and trophoblastic tissues around the time of implantation in the mink, we employed *in situ* hybridization. Nine days after

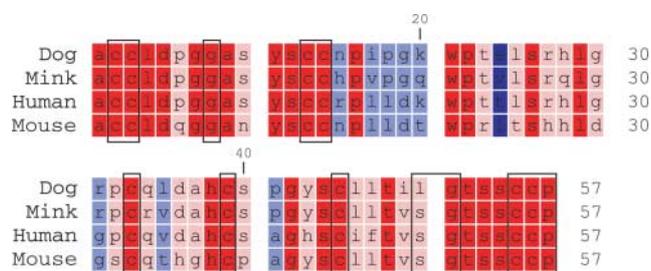


Figure 2 Alignment and comparison of the mouse, human, dog, and mink deduced partial amino acid sequences. The single-letter code identifies the amino acids. Perfect (100%) homology is indicated in red, residues occurring three times are indicated in pink, residues occurring two times are colored in light blue, and four different residues are indicated in dark blue. Amino acids 1 to 30 correspond to the C-terminal fragment of paraganulin, and amino acids 31 to 57 to the N-terminal fragment of granulin G. The highly conserved residues found in the cystein-rich motifs of progranulin sequence are delineated by boxes. It is noteworthy that all the boxed residues are well conserved in the four species analyzed, including the mink.

implantation (day 0), the uterine glands undergo hypertrophy and elongation, while the trophoblast makes contact with the maternal epithelium. Progranulin protein is highly expressed in the trophoblast cells (Fig. 4d) as well as in the transformed epithelium of the glands, and in the stroma surrounding the glands (Fig. 4f). At this time, progranulin protein is also present in the intact basal glands. While progranulin expression is absent in the maternal blood vessels located near these basal glands, it is otherwise present in the blood vessels in the transformed gland area (Fig. 4h, arrowheads), indicating that progranulin is preferentially expressed in the newly formed maternal blood vessels. Together, these results suggest that progranulin is involved in implantation during trophoblast attachment, endometrial transformations, endometrial gland proliferation, and maternal angiogenesis.

As trophoblast invasion proceeds, the chorionic villi are formed in the incipient labyrinth of the placenta.

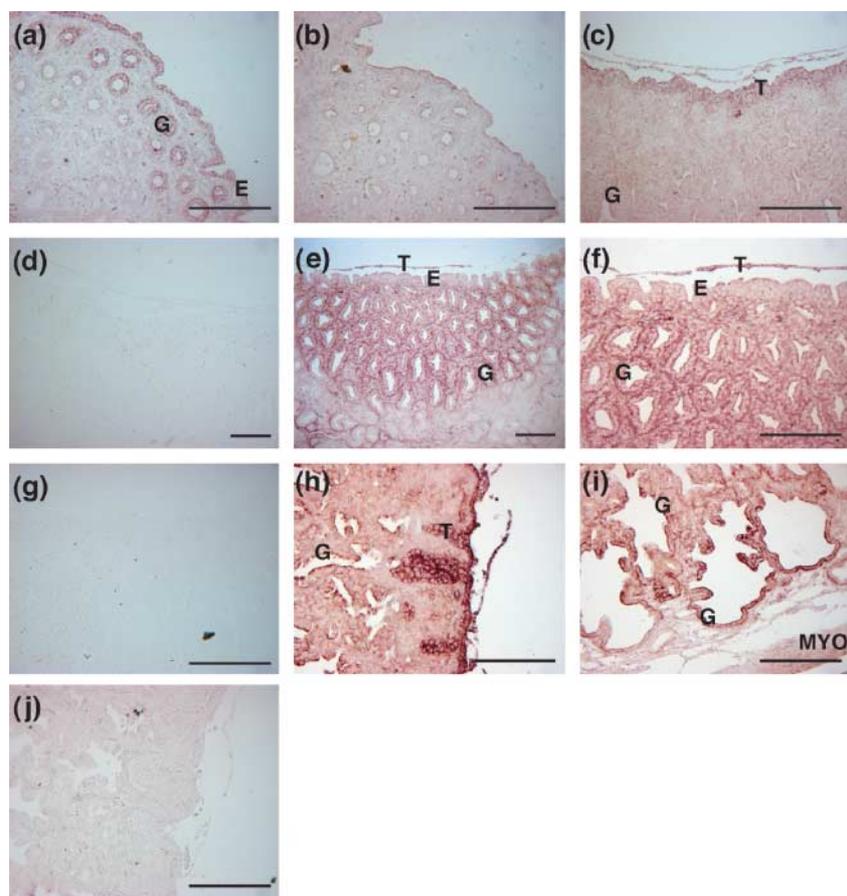


Figure 3 Progranulin mRNA is expressed in mink trophoblast and endometrium around the time of embryo implantation. *In situ* hybridization was performed with (a, c, e, f, h and i) antisense or (b, d, g and j) sense (control) probe for progranulin mRNA. (a and b) Prolactin-activated mink uterus, before implantation occurs. There is pronounced mRNA expression in luminal and glandular endometrial epithelium. (c and d) Early implanted stage uterus. Strong expression of progranulin transcript is observed in the trophoblast cells. (e–g) Early implanted stage uterus, during trophoblast apposition. (e) Progranulin transcript is strongly induced in glandular epithelium undergoing transformation, and (f) the cytoplasmic localization of progranulin transcript in the glandular epithelium and in the stroma can be appreciated at a higher magnification. (h–j) During villi formation in the incipient labyrinth, progranulin hybridization signal is most intense in cytotrophoblast cells of the embryo (h). There is also a positive signal in the transformed and basal endometrial glands (i). E, luminal epithelium; G, glandular epithelium; T, trophoblast; MYO, myometrium. Bars: 200 µm.

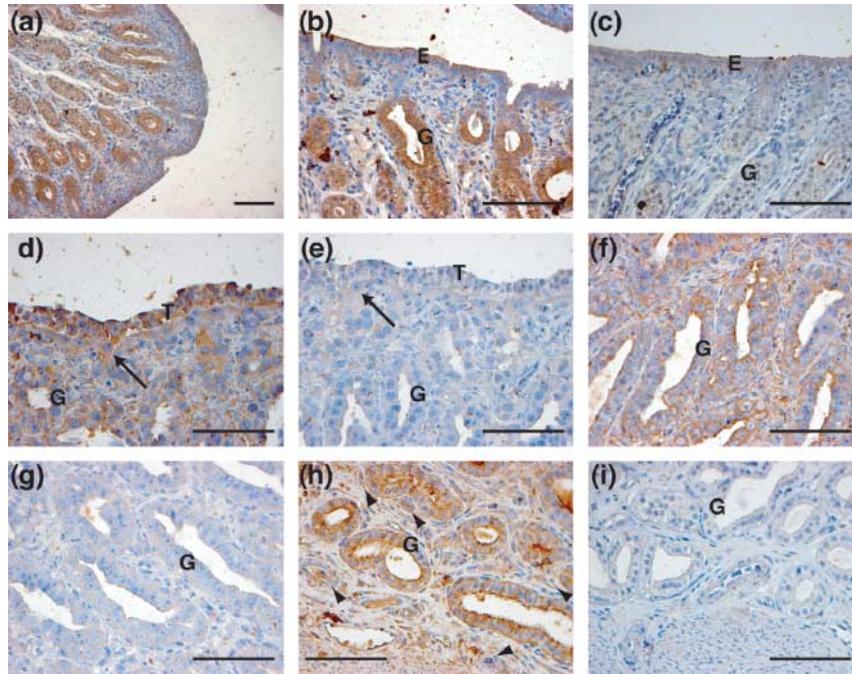


Figure 4 Progranulin protein is expressed in the trophoblast and endometrial cells in the mink pre-implantation and early implanted uterus. To detect progranulin protein by immunohistochemistry, (a, b, d, f and h) immune rabbit serum against human progranulin or (c, e, g and i) pre-immune rabbit serum (control) were employed. (a–c) In the prolactin-activated mink uterus, progranulin protein is present in the endometrial glands (a), and at higher magnification (b), we can detect the presence of progranulin protein in the cytoplasm of the luminal epithelium. (d, e, f, g, h and i) Early implantation sites. Progranulin protein was detected in the trophoblast, including the cytotrophoblast and the syncytiotrophoblast (d), in the transforming glandular epithelium and surrounding stroma (f), as well as in the intact basal glands and in the maternal blood vessels, at different intensities (h). E, Luminal epithelium; G, glandular epithelium; T, trophoblast; arrow, syncytiotrophoblast; arrowheads, maternal blood vessels. Bars: 100 μ m.

Progranulin protein remains elevated in the cytotrophoblast during the first days following implantation (days 1 and 2, Fig. 5a and b respectively). Some expression is also clearly discernable in the syncytiotrophoblast as it progresses into the maternal endometrium (Fig. 5a). Progranulin expression can be continuously observed in the transformed (Fig. 5d and g) and intact endometrial glands (Fig. 5c and g), and at a lower degree in the stroma (Fig. 5c and d). As above, the progranulin expression in the cytotrophoblast of the forming villi suggests a role for progranulin in cytotrophoblast proliferation.

Five days after implantation, in the early placenta, the four layers composing the mink endotheliochorial placenta (allantochorionic, labyrinth, junctional, and basal) are discernable. The labyrinth is composed of the chorionic villi, the fetal mesenchyme, and the fetal blood and maternal blood vessels, which are constituted of hypertrophied endothelial cells. Progranulin protein is present in high concentrations in the newly formed maternal hypertrophied endothelial cells (Fig. 6a and b). Furthermore, progranulin immunoreactivity is observed in the cytotrophoblast cells composing the chorionic villi and in the fetal capillaries of the mesenchymal core of the villi, but in the syncytiotrophoblast cells surrounding the cytotrophoblast, the signal is much weaker (Fig. 6a and b). In the elongated glands and the stromal cells from

the junctional zone, progranulin has become less expressed (Fig. 6d and e), while in the intact glands from the basal glandular zone, progranulin is still present (Fig. 6d and e). This pattern of expression for progranulin protein suggests that progranulin is involved in the establishment of the labyrinth and in maternal as well as fetal angiogenesis in the mink placenta.

Discussion

We used the mink model of endotheliochorial placentation to follow progranulin mRNA and protein expression pattern in pre- and early post-implantation events, and during early development of the placenta. This investigation provides the first evidence for the expression of progranulin in the trophoblast and the endometrium at the time of embryo implantation *in vivo*. Furthermore, the data obtained in this study suggest a new role for progranulin in the establishment of fetal villi and maternal and fetal vascular components in the endotheliochorial placenta of the mink.

Mink progranulin partial cDNA sequence

Herein, we report the partial cDNA and deduced amino acid sequences for mink progranulin. Comparative

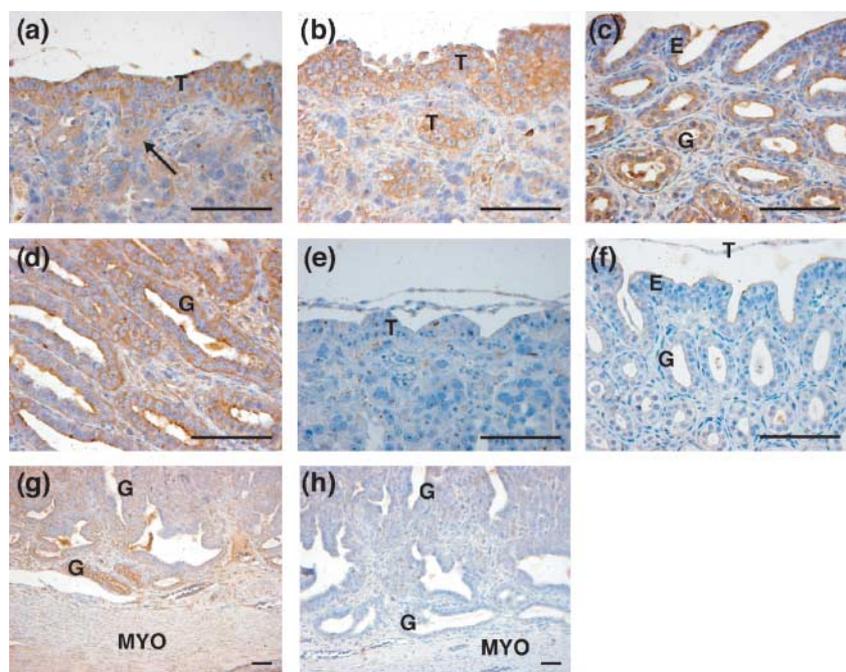


Figure 5 Progranulin protein localizes in the mink endometrium and trophoblast during the early post-implantation period. To detect progranulin protein by immunohistochemistry, either (a, b, c, d and g) rabbit serum against human progranulin or (e, f and h) pre-immune rabbit serum (control) were employed. (a and e) In the implantation site, 1 day after implantation, progranulin protein is detected in the cytotrophoblast and in the syncytiotrophoblast invading the maternal endometrium. (b, c, d, f, g and h) The implantation site, 2 days after implantation, when the trophoblast initiates the chorionic villi formation. An immunopositive signal indicating the presence of progranulin in the trophoblast (b), in the luminal epithelium, and in the glandular epithelium of rounded (c), or elongated (d) glands. The signal is also present in the intact basal glands at the implantation site (g) and in the stroma (d). E, luminal epithelium; G, glandular epithelium; T, trophoblast; MYO, myometrium; arrow, syncytiotrophoblast. Bars: 100 μ m.

analysis shows that exons 1 and 2 are highly conserved among mink, dogs, mice, and humans, and are present in both isoforms, granulin/epithelin precursors 1 and 2, identified in rodents (Plowman *et al.* 1992). Here, we show that the mink progranulin sequence for exons 1 and 2 share 86.6% of identity with the human sequence, 82.4% with the mouse sequence, and 94.9% with the dog sequence. Moreover, when the amino acid sequence alignment of mink, human, dog, and mouse partial progranulin protein was analyzed, it was seen that, in the mink partial sequence, all the cysteine residues were well conserved, as well as the serine, proline, and glycine residues shared by the 7.5 cysteine-rich motifs (Bhandari *et al.* 1992, Plowman *et al.* 1992). The presence of the well-conserved motifs of progranulin in the mink sequence suggests that the function of progranulin in mink parallels known functions in other species.

Progranulin and endometrial gland proliferation

During prolactin-mediated termination of embryonic diapause, progesterone is secreted by the corpus luteum and the consequent change in plasma progesterone concentration corresponds with a series of pre-implantation changes in the mink uterus (Moller 1973). We show progranulin mRNA and protein to be expressed in

the luminal and glandular endometrial epithelium following prolactin-mediated reactivation of the uterus and embryo. Moreover, the high expression of progranulin in the glandular epithelial cells during the peri-implantation transformation, and its lower expression in the transformed glands of the post-implantation period suggest that progranulin is involved in the implantation-related epithelial cell transformations. The sustained strong expression of progranulin protein in the intact basal glands during all the stages examined suggests that progranulin is important for normal secretory activity of the endometrial glands. While progranulin protein is expressed in the transformed endometrial glands at the site of trophoblast apposition, the cognate progranulin mRNA expression is not observed. This suggests that the secretory and synthetic functions of the transformed glands are altered at the implantation site, in comparison with the intact glands.

Progranulin and cytotrophoblast proliferation

The present work followed the distribution of progranulin in the trophoblast on the day of implantation and through early post-implantation *in vivo*. Progranulin mRNA and protein expression are manifestly present in the mink cytotrophoblast during blastocyst adhesion and

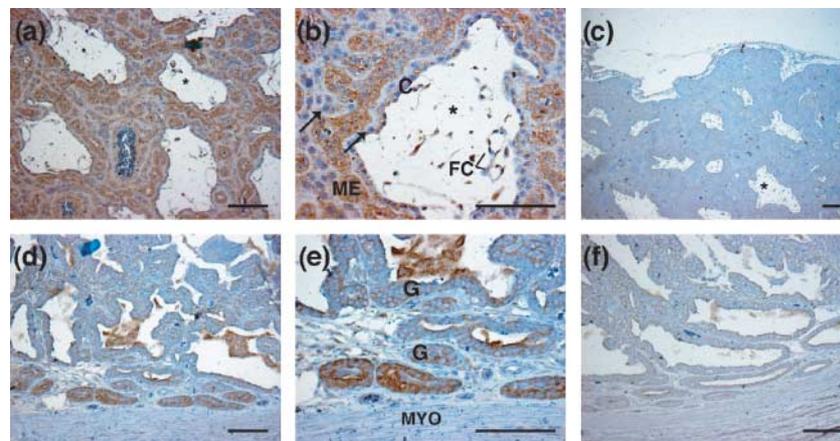


Figure 6 Progranulin protein is expressed in the trophoblast and the maternal hypertrophied endothelial cells in the early mink placenta. To detect progranulin protein by immunohistochemistry, (a, b, d and e) the immune rabbit serum against human progranulin or (c and f) the pre-immune rabbit serum (control) were employed. (a–c) Labyrinth of the early mink placenta (at day 5 following implantation). (a) High expression of progranulin protein is detected in many cell types of the labyrinth. (b) It is present in the cytotrophoblast and the syncytiotrophoblast, and highly expressed in the fetal capillaries of the mesenchymal core and in the maternal hypertrophied endothelial cells surrounding the fetal villi, as shown at higher magnification. (d–f) Glandular zone of the early mink placenta. The immunostaining is strongly positive in the intact basal glands, and somewhat weaker in the epithelium of the transformed glands (d and e). E, luminal epithelium; G, glandular epithelium; C, cytotrophoblast; MYO, myometrium; ME, maternal endothelial cells (maternal blood vessels); *Chorionic villi comprising the fetal mesenchyme and fetal capillaries (FC), the cytotrophoblast, and the syncytiotrophoblast; arrows, syncytiotrophoblast. Bars: 100 μ m.

syncytiotrophoblast penetration into the endometrial epithelium, and this is consistent with the high levels of progranulin transcripts found previously in the mouse post-adhesion blastocyst by cDNA microarray (Qin *et al.* 2003). Our data are also in accordance with the findings that progranulin promotes mouse trophoblast proliferation as well as blastocyst hatching, adhesion, and outgrowth *in vitro* (Diaz-Cueto *et al.* 2000, Qin *et al.* 2005). In mustelid species, the cytotrophoblast undergoes active cell division soon after implantation to achieve formation of the chorionic villi (Enders & Schlafke 1972). Here, we show that progranulin expression remains elevated throughout the chorionic villi development during early gestation in the mink. Thus, our results argue for a role for progranulin in cytotrophoblast proliferation and migration during formation of the chorionic villi throughout early implantation and placentation in the mink.

Progranulin and maternal endothelial cell proliferation

Winther *et al.* (1999) described the neovascularization of the mink placenta at different stages of development. During early gestation, maternal endothelial cells undergo extensive mitosis. At mid-gestation, the vessel walls are remodeling and periendothelial cells appear; and at late gestation, the mitogenic activity of these endothelial cells is decreased. According to our data, progranulin is differentially expressed in the endothelial cells of the maternal blood vessels of the early implanted uterus, with stronger expression in the blood vessels nearby the transformed endometrial glands. These vessels are expected to be the newly formed blood

vessels that will eventually irrigate the placental labyrinth. Our results reveal progranulin expression at high levels in the maternal hypertrophied endothelial cells of the incipient labyrinth. These results suggest that progranulin may play a role in angiogenesis, as has previously been reported in neovascularization during embryogenesis, tumorigenesis, and throughout the wound repair process (He & Bateman 2003). Progranulin is usually absent from normal adult endothelial cells, but during wound repair, its expression is strongly induced in the endothelial cells at the site of the lesion (He *et al.* 2003). Moreover, progranulin promotes tubule-like structure formation of endothelial cells *in vitro* (He *et al.* 2003) and enhances vascular endothelial growth factor (VEGF) expression in breast cancer cells (Tangkeangsirisin & Serrero 2004). Progranulin is also expressed in blood vessels surrounding invasive ovarian tumors (Jones *et al.* 2003) and during establishment of the microvasculature in the murine embryo (Daniel *et al.* 2003). We have previously shown that VEGF of embryo and stromal cell provenance to be strongly expressed during the implantation and early invasion in the mink (Lopes *et al.* 2003). Together, the morphological evidence for expression in tissues at a time when extensive revascularization and VEGF expression is taking place support a role for progranulin in neovascularization of the early placenta in the mink.

The regulation and the role of progranulin during implantation in the mink

Estrogen has previously been shown to stimulate the expression of progranulin in human breast cancer cells

(Lu & Serrero 1999), and progranulin was shown to mediate the mitogenic effects of estrogen in these cells (Lu & Serrero 2001), even in the absence of a typical estrogen response element on the promoter of human and mouse progranulin genes (Baba *et al.* 1993a, 1993b, Bhandari *et al.* 1996). In the rodent model displaying a facultative delay of implantation, estrogen, in combination with progesterone, is the signal that initiates blastocyst implantation (Yoshinaga & Adams 1966, Psychoyos 1973). Via the nuclear estrogenic receptor (ER), estrogens can induce the expression of many growth factors and their receptors in the mouse uterus during implantation by activating the membrane-trafficking pathway (Chen *et al.* 1999). Moreover, early estrogenic responses can be observed in the presence of catechol-estrogen or xenoestrogens in the mouse uterus, independently of ER α /ER β (Das *et al.* 1997a, 1997b), suggesting that the activation of nongenomic pathways can be responsible of an estrogenic response in the uterus, independently of nuclear ERs. The roles of estrogen in mink during implantation are not clear, but based on this evidence, it would be reasonable to speculate that estrogen or other estrogenic compounds are potential regulators of progranulin expression in the mink endometrium and trophoblast.

The pattern of progranulin expression and activity is, in many ways, similar to that of other growth factors in the blastocyst and the uterus. For example, around the time of embryo implantation in the mouse, several members of the EGF family of growth factors are upregulated in the uterus, including EGF itself, TGF α , HB-EGF, betacellulin, epiregulin, neuregulins, and amphiregulin (Das *et al.* 1997a, 1997b, Lim *et al.* 1998). The overlapping expression of several EGF family members in the endometrial epithelium around the time of implantation, and the absence of any defects in the implantation phenotype in targeted mice for EGF/TGF α /amphiregulin in combination suggests that these growth factors play compensatory roles in the uterus (reviewed by Dey *et al.* 2004). In addition, consistent with the effects of progranulin on mouse blastocysts (Diaz-Cueto *et al.* 2000, Qin *et al.* 2005), HB-EGF promotes development and hatching of human blastocysts *in vitro* (Martin *et al.* 1998). Together, these findings suggest that the role of progranulin is redundant with the roles exerted by other similar growth factors during mink implantation. In summary, this report demonstrates the spatiotemporal distribution of progranulin mRNA and protein for the first time at the time of embryo implantation and early placenta development *in vivo*. Progranulin expression is mainly associated with epithelial hypertrophy and proliferation in the endometrium, with cytotrophoblast proliferation and migration, and with maternal angiogenesis in the endotheliochorial placenta. Therefore, we suggest that high levels of progranulin expression indicate that this protein plays a role in cell proliferation, remodeling, and

angiogenesis which occur during the establishment of the placenta in the mink.

Materials and Methods

Animals

Primiparous and multiparous Pastel and Dark female ranch mink (*Mustela vison*) were purchased from Mr A Richard (St. Damase, QC, Canada) and were bred according to usual commercial farming procedures. The biological samples were collected during the normal breeding season in March. All procedures involving live animals were submitted to, and approved by, the Comité d'éthique de l'utilisation des animaux, Faculté de Médecine Vétérinaire, Université de Montréal, and were in accordance with the Canadian Council on Animal Care guidelines. To terminate pre-implantation delay, 1 mg/kg per day ovine prolactin (Sigma) was administered i.m. daily to the animals, for 11 days, after which implantation occurs (Desmarais *et al.* 2004). Uterine tissues and implantation sites were collected after 9 days of prolactin treatment, and 0, 1, 2, or 5 days after implantation in experimental groups representing the pre- and early post-implantation periods. The tissues were flash frozen in liquid nitrogen for RNA preservation, or fixed overnight with 4% (w/v) paraformaldehyde (PFA) in PBS (pH 7.4), rinsed three times overnight in PBS, and processed for paraffin embedding. Tissues were sectioned at 5 μ m for *in situ* hybridization or immunohistochemistry.

Cloning of mink partial cDNA sequence

Total RNA was isolated from mink uterine tissues including the implantation site, with the RNeasy mini kit (Qiagen). The isolated RNA was treated with RNase-free DNase 1 (Ambion, Austin, TX, USA) to remove DNA, and cognate cDNA produced by employing the Superscript II Reverse Transcriptase enzyme (Invitrogen), following the manufacturer's instructions. A PCR was conducted with canine-specific progranulin primers to amplify a mink progranulin partial cDNA sequence. The forward primer sequence is 5'-AGA TGG TCA GTT CTG CCC TGT G-3' and the reverse primer sequence is 5'-ACA CGA CAC GGC CTC TGG GAA-3'. For PCR, 1 U Taq DNA polymerase (Invitrogen) was used per μ g of DNA in a 20 μ l PCR, and the amplification was conducted in a PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA, USA), programmed for an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 3 min. The 214 bp PCR fragment was purified from an agarose gel using an extraction kit (Qiagen), ligated into a pDrive cloning vector (Qiagen) and the clones were amplified in an XL1-blue bacterial strain. The amplified plasmids were purified with a Qiaprep spin miniprep kit (Qiagen) and digested with EcoRI (GE Healthcare, Baie d'Urfé, QC, Canada) to confirm the presence of the insert. The purified plasmid was sequenced with an automated sequencer by the Services d'analyse et de synthèse SCF, Université Laval (Québec, QC, Canada), to confirm the identity of the PCR product. Multiple sequence alignment was conducted

with DNAsis v2.0 software (Hitachi Software Engineering, Yokohama, Japan), and the percentage of homology was calculated thereafter. Amino acid alignment was performed using the CLC Sequence Viewer software 4.6.1 (CLC bio A/S, Aarhus C, Denmark). The accession numbers of the sequences used for the alignments can be found in GenBank as follows: human NM_002087/NP_002078; dog XM_537620/XP_537620; and mouse NM_008175/NP_032201. The mink partial progranulin coding sequence and deduced amino acid sequence were entered in GenBank (accession number EF566010/ABQ_44207).

In situ hybridization

Human-specific digoxigenin-UTP-labeled progranulin RNA sense and antisense probes of 237 bp were generated as described (Daniel *et al.* 2000). *In situ* hybridization was carried out as in Daniel *et al.* (2000) with modifications. Paraffin sections were deparaffinized in Citrosolv (Fisher, Whitby, ON, Canada) and rehydrated in a graded series of ethanol. Tissues were post-fixed in 4% PFA (pH 7.4, Fisher) and washed with $0.5 \times$ SSC buffer. Permeabilization of the tissue was carried out by incubation with 0.2 M HCl for 5 min and followed by incubation with proteinase K (3.5 μ g/ml) in 100 mM Tris-HCl and 50 mM EDTA buffer (pH 8.0), at 37 °C for 15 min. Another post-fixation step with 4% PFA was conducted and slides were washed thoroughly with PBS and $0.5 \times$ SSC. The sections were then pre-hybridized for 3 h in hybridization solution ($5 \times$ SSC, $5 \times$ Denhardt's solution), 50% (v/v) deionized formamide (Bioshop, Burlington, ON, Canada), and 250 μ g/ml tRNA (Roche), and hybridized with 75 ng digoxigenin-UTP-labeled RNA probes for 18 h at 42 °C. After stringency washing with different concentrations of SSC at 50 °C, sections were blocked with 1% of Blocking Reagent (Roche) in 1 M maleic acid and 0.15 M NaCl buffer (pH 7.5), and were incubated with 750 mU/ml anti-digoxigenin-AP-conjugated antibody (Roche). The unbound antibody conjugate was washed off and sections were incubated with freshly prepared NBT-BCIP color substrate (Roche) in a 100 mM Tris and 100 mM NaCl solution (pH 9.5), until the desired signals were obtained. The reaction products were visualized with a brightfield Leica AS LMD microscope (Leica Microsystems Inc., Wetzlar, Germany). For each sample, two successive slides were incubated either with sense or antisense probe to monitor non-specific hybridization signal.

Immunohistochemistry

Immunohistochemical localization of mink progranulin protein was carried out using antiserum from a rabbit immunized against the full-length progranulin coding sequence, generated in house. The recombinant progranulin full-length fragment was expressed in a baculovirus expression system and purified to homogeneity before immunization. The resulting polyclonal antibody reacts with human, mouse, rat, and porcine progranulin in comparative immunohistochemical evaluation. It recognizes multiple individual 6 kDa granulin/epithelin proteolytic peptides, but only weakly. The sections were deparaffinized in Citrosolv (Fisher) and

rehydrated in a graded series of ethanol. Antigen retrieval was performed by boiling the slides in a 10 mM sodium citrate solution for 20 min in a microwave oven. Dako LSAB2 System-horseradish peroxidase (HRP) kits (DakoCytomation, Carpinteria, CA, USA) were employed following the manufacturer's recommended protocol. Briefly, endogenous peroxidases were blocked with 3% (v/v) peroxide solution for 30 min and sections were treated with 3% BSA and 0.1% (v/v) Tween-20 in PBS for 3 h at room temperature to block the non-specific binding, and thereafter, were incubated with rabbit-antiserum against human progranulin, diluted 1:100 in antibody diluent (DakoCytomation), in a humidified chamber, over night at 4 °C. The biotinylated secondary antibody, streptavidin-HRP, and diaminobenzadine (DAB) substrate were subsequently employed for signal visualization, according to the manufacturer's (DakoCytomation) recommended protocol. Between steps, sections were washed three times for 3 min. Finally, brown precipitate was observed at the antigen site and the sections were briefly counterstained with Harris modified hematoxylin (Fisher). Before mounting, sections were dehydrated in the series of ethanol. In the control slides, rabbit antiserum against progranulin incubation was replaced by a pre-immune rabbit serum incubation. The signals were visualized with a brightfield Leica AS LMD microscope (Leica Microsystems Inc).

Declaration of interest

The authors have no real or potential conflicts of interest to declare with entities related to the material being published.

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