UNIVERSITÉ DE MONTRÉAL

IMPLANTATION IN THE MINK (*Mustela vison*): MORPHOLOGIC PROGRESSION OF TROPHOBLAST INVASION AND UTERINE GENE EXPRESSION

by

Jian Hua Song

Centre de recherche en reproduction animale (CRRA)
Faculté de médecine vétérinaire et Faculté de médecine

Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophiae Doctor (Ph.D.)
en sciences biomédecales

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DESCRIPTION MORPHOLOGIQUE DE LA PROGRESSION DE L'INVASION DU TROPHOBLASTE ET EXPRESSION DES GÈNES UTÉRINS PENDANT L'IMPLANTATION CHEZ LE VISON (*Mustela vison*):

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Cette thèse intitulée:

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présentée par

Jian Hua Song

a été évaluée par un jury composé des personnes suivantes:

Président du jury:	Dr. Alan K. Goff
Directeur de recherche:	Dr. Bruce D. Murphy
Membre du jury:	Dr. R.-Marc Pelletier
Examinateur externe:	Dr. Thomas G. Kennedy
Représentant du doyen:	Dr. André Dallaire

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Le processus d'implantation n'est pas complètement élucidé chez les mammifères. Chez le vison, où l'implantation connaît une diapause obligatoire, l'utérus commanderait la reprise du développement du blastocyste et son implantation subséquente. Or, les changements morphologiques qui accompagnent la fin de la diapause et l'expression de facteurs utérins durant les premiers stades de la gestation ne sont pas bien documentés. Cette étude porte donc sur l'aspect morphologique de la progression de la pénétration dans le trophoblaste et l'expression des gènes des facteurs utérins, le facteur inhibiteur de la leucémie leukemia inhibitory factor (LIF) et la cyclooxygénase-2 (COX-2).

Un des objectifs de cette étude était d'avoir recours à un grand nombre d'animaux dans l'espoir d'obtenir un portrait morphologique plus fidèle et détaillé de l'implantation et de la formation du placenta chez le vison particulièrement pendant les premiers stades de l'accolement au trophoblaste et de la pénétration de l'epithelium. Le jour 0 de l'implantation a été défini comme le moment où le blastocyste entouré de sa capsule atteint un diamètre de 2 mm et l'utérus a perdu ses cryptes endométriales. Après la rupture de la capsule embryonnaire, les couches du trophoblaste adhèrent à l'épithélium endométrial, principalement sur les faces latérales de la surface antimésométriale. Au jour 3 de l'implantation, le trophoblaste a supprimé l'épithélium et entre en contact direct avec les cellules stromales de l'endomètre. Les symplastes maternels commencent à se former à partir des cellules épithéliales glandulaires dès le jour 4 de l'implantation. Le placenta endothéliochorial, est constitué de 3 éléments: le labyrinthe materno-fœtal, le symplaste et les glandes maternelles, qui peuvent être observées aux jours 5 ou 6. Aux jours 11 ou 12 de l'implantation, soit lorsque le diamètre de l'utérus atteint 15 mm, le placenta est complet et fonctionnel.

Un autre objectif de cette étude était de cloner la séquence codante du LIF chez le vison et de déterminer la quantité de son ARNm dans

l'utérus pendant la diapause, l'implantation et après l'implantation. Nous avons établi que l'ADNc du LIF de vison comprenait 609 nucléotides encodant une protéine qui serait constituée de 203 acides aminés. L'homologie avec la souris, l'humain, le porc, la vache et le mouton serait respectivement. de 80.6, 90, 88.2, 87.6 et 86.8% au regard de la séquence codante et de 79.2, 90.1, 91, 90.1 et 85.4 % au regard de la séquence en acides aminés. Les mesures effectuées à l'aide de la réaction de polymérisation en chaîne indiquent que l'ARNm du LIF est exprimé dans l'utérus du vison tout juste avant l'implantation et pendant les 2 jours suivants, mais pas ultérieurement. La quantité d'ARNm de LIF était significativement plus élevée dans l'utérus pendant l'expansion embryonnaire qu'aux jours 1 et 2 de l'implantation. Cependant, étant donné la faible quantité présente chez le vison, les transcrits d'ARNm de LIF n'étaient pas détectables par transfert d'ARN (transfert de Northern)., Nous avons démontré par localisation immunohistochimique que le LIF est exprimé dans les glandes épithéliales utérines au moment de l'expansion embryonnaire et tout juste après l'implantation. La coïncidence de l'expression du LIF avec l'implantation chez cette espèce suggère que le LIF jouerait un rôle dans le processus d'implantation et pourrait être le signal maternel qui met fin à la diapause embryonnaire obligatoire.

L'objectif suivant était de cloner la séquence codante de la COX-2 chez le vison et de déterminer son expression dans l'utérus pendant la diapause et juste après l'implantation. Le cadre de lecture ouvert de la COX-2 du vison comprend 1 812 nucléotides encodant 604 acides aminés. L'homologie avec l'humain, la souris, le rat, le cochon d'Inde, le mouton et le lapin est respectivement de 86, 83, 83, 83, 85 et 84% pour les nucléotides et de 86, 87, 87, 85, 86 et 88 % pour les acides aminés. Les analyses de transfert d'ARN (transfert de Northern) ont révélé l'existence d'un transcrit de 4.2 kb pour la COX-2 dans l'utérus et la surrénale. Des analyses de RT-PCR semi-quantitatives ont montré l'absence d'ARNm de COX-2 pendant la diapause. La quantité d'ARNm de COX-2 atteint un pic

lors de la pénétration du trophoblaste à l'endomèttrium, soit des jours 3 à 5 de l'implantation, pour ensuite diminuer graduellement jusqu'au jour 9 et disparaître par la suite. L'immunohistochimie a révélé que la COX-2 était présente dans l'épithélium utérin, le stroma et le col des glandes endométriales aux sites d'implantation. L'expression de la COX-2 dans l'endomètre semble être induite par l'embryon et pourrait jouer un rôle dans l'implantation et la placentation chez le vison.

Cette étude menée avec un grand nombre d'animaux a permis d'établir un portrait précis des changements morphologiques pendant l'invasion du trophoblaste et la placentation. Grâce à des techniques de biologie moléculaire, nous avons démontré que l'expression du LIF et de la COX-2 est liée dans le temps au processus d'implantation chez le vison. La disparité entre les patrons d'expression génique suggère que l'expression transitoire du LIF pourrait jouer un rôle dans la reprise du développement alors que la COX-2 serait associée à la formation du placenta. Il faudra maintenant voir si le LIF peut induire la production de signaux capables de sensibiliser l'épithélium utérin en vue de l'implantation et, subséquemment, l'expression de facteurs utérins, dont la COX-2, qui facilite la pénétration du trophoblaste à l'endomèttrium.

ABSTRACT

The process of implantation is not completely understood in mammals, including the mink, a species with obligate delayed implantation. The uterus is believed to control the renewal of development of blastocyst and its consequent implantation in this species. The morphological changes which accompany the termination of diapause and expression of uterine factors during this early stage of gestation have not been well documented. Therefore, the progression of trophoblast invasion and gene expression of the uterine factors, leukemia inhibitory factor (LIF) and cyclooxygenase-2 (COX-2) were the subject of this investigation.

One of the aims of this study was to use a large number of animals in hopes to bracketing implantation and placenta formation in the mink, particularly during the early stages of trophoblast adhesion and invasion, to provide a better morphological description of this event than currently exists. Day 0 of implantation was defined as the time when the blastocyst, enclosed in its capsule, has expanded to a diameter at 2 mm and the uterus has lost its endometrial crypts. After breaching the embryonic capsule, plaques of trophoblast adhere to the endometrial epithelium, principally on the lateral aspects of the antimesometrial surface. At Day 3 postimplantation, the trophoblast has eliminated the epithelium and abuts directly on endometrial stromal cells. Maternal symplasmae begin to form from the glandular epithelial cells as early as Day 4 after implantation. The endotheliochorial placenta, comprised of three zones (the maternal-fetal labyrinth, the symplasma and the maternal glands) can be seen at Days 5-6. A completed, functional placenta appears to be present by Days 11-12 after implantation when the diameter of the uterine swelling is at 15 mm.

Another objective of this study was to clone the LIF coding sequence in the mink and determine the abundance of its mRNA in the uterus through embryonic diapause, implantation and early postimplantation. It

was determined that mink LIF cDNA contains 609 nt encoding a deduced protein of 203 amino acids. The homologies are 80.6, 90, 88.2, 87.6, and 86.8 % in coding sequence and 79.2, 90.1, 91, 90.1 and 85.4 % in amino acid sequence with mouse, human, pig, cow, and sheep respectively. Quantitation by polymerase chain reaction amplification indicated that LIF mRNA is expressed in mink uterus just prior to implantation and during the first two days after implantation, but not during diapause or later postimplantation pregnancy. The abundance of LIF mRNA was significantly higher in the uterus at the embryo expansion stage than at Days 1-2 postimplantation. However, the low abundance of LIF mRNA transcripts rendered it undetectable by Northern blot analysis in mink. By immunohistochemical localization it was shown that LIF protein is present in the uterine epithelial glands at the time of embryo expansion and during early postimplantation. The coincidence of LIF expression with implantation in this species suggests that LIF is involved in the implantation process, and may be a maternal signal which terminates obligate embryonic diapause.

The next objective of this study was to clone the COX-2 coding sequence in the mink and determine its expression in the uterus through diapause and early postimplantation gestation. The open reading frame of mink COX-2 contains 1812 nucleotides (nt) encoding 604 amino acids. The homologies are 86, 83, 83, 83, 85, and 84% in nt; and 86, 87, 87, 85, 86 and 88% in amino acids with human, mouse, rat, guinea pig, sheep and rabbit, respectively. Northern analysis revealed a transcript of 4.2 kb for COX-2 in mink uterus and adrenal gland. Semi-quantitative RT-PCR showed that COX-2 mRNA is not present during diapause. The abundance of COX-2 mRNA reached its maxima associated with trophoblast invasion, at 3-5 days of postimplantation, gradually decreased through day 9, and was not present thereafter. By immunohistochemistry, COX-2 was detected in uterine epithelium, stroma and necks of endometrial glands at the sites of implantation. COX-2 expression appears to be induced in the

endometrium by the embryo and may play a role in implantation and placentation in mink.

This study has a provided precise description of the morphological changes during trophoblast invasion and placentation based on a large number of animals. By employing molecular biological techniques, it has been shown that expression of both LIF and COX-2 are temporally related to the process of implantation in mink. Different patterns of gene expression suggest that transient LIF expression may play a role in the termination of delayed implantation, while COX-2 may be associated with formation of the placenta. Whether LIF induces embryonic signals to sensitize the uterine epithelium for implantation and induces the expression of uterine factors, including COX-2, which facilitates trophoblast invasion, merits further investigation.

AVANT-PROPOS (PREFACE)

Cette thèse comprend une introduction générale, qui renferme l'hypothèse de départ, une revue de littérature générale; trois articles comprenant chacun une introduction, une section *Matériel et méthodes*, des résultats, une discussion et des références; une discussion générale ainsi qu'une conclusion générale. On retrouvera en annexe le détail de la méthodologie, qui n'apparaît pas dans les articles.

This thesis composes a general introduction which indicates the hypothesis of the study, a general literature review; three publishable articles, each of which contains specific introduction, materials and methods, results, discussion and references; a general discussion and general conclusion. An appendix, which provides methodological details not present in the article chapters, is also a part of this thesis.

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I have not forgotten that Dr. Rodney A. Mead has spent time, in person and via electronic mail, in discussion of the role of LIF in implantation in the mustelids, including his recent results on the western spotted skunk, and for advice on the immunohistochemical aspects of this investigation. I would like to thank Drs. Goff and Smith for their time

and advice, and explanation during my studies in the past few years. All of the above give me the feeling that I am in a scientific family, it is warm, peaceful, and enjoyable. I also have the impression that successful scientists are always willing to share their expertise and to discuss their ideas.

This study was carried out at Centre de recherche en reproduction animale (CRRA), Faculté de médecine vétérinaire et Faculté de médecine. I would like to thank all members of the CRRA, for their friendship and kindness during the course of my study. Particularly, I am grateful to the other members of our laboratory Mira Dobias-Goff, Deborah A. Douglas, Nazario Pescador, Genevieve Moreau, Derek Boerboom, and Jianmin Liu. Not only was their help invaluable for the completion of this work but their friendship also made it fun.

Finally I would like to express my heart-felt gratitude to my wife, Taoyan Men and my sons. Without their support and love I never would have been able to finish.

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ABBREVIATIONS

µg	microgram
µl	microliter
aa	amino acid
BRL	Buffalo Rat Liver
cDNA	complementary deoxyribonucleic acid
CL	corpus luteum
COX	cyclooxygenase (COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2)
CSF	colony-stimulating factor
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
E_2	estradiol
ECM	extracellular matrix
EDTA	sodium ethyenediaminetetra acetic acid
EGF	epidermal growth factor
GITC	guanidinium isothiocyanate
GM-CSF	granulocytes/macrophages colony stimulating factor
h (hrs)	hour (hours)
IFN-τ	interferon-tau
ILs	interleukins (IL-1, IL-6, IL-11)
kb	kilobase
kDa	kilo Dalton
LIF	leukemia inhibitory factor
LIFR	leukemia inhibitory factor receptor
M	molar
mRNA	messenger ribonucleic acid
nt	nucleotides
P_4	progesterone

PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
pc	post coitum
PGs	prostaglandins (including PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$ and PGI_2, prostacyclin)
PGHS	PGH synthase
Prl	prolactin
Prl-R	prolactin receptor
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TIMPs	tissue inhibitors of metrix metalloproteinases
uPA	urokinase plasminogen activator

1.0 INTRODUCTION

Embryo implantation is a complex but fundamental phenomenon of reproduction in mammals. It is a highly orchestrated process that integrates morphological, physiological, endocrinological, and immunological events (Weitlauf, 1994). The earliest microscopically identifiable indication in many species is an increase in endometrial vascular permeability at implantation sites (Psychoyos, 1973a), which is followed by attachment, adhesion and trophoblast invasion (Enders, 1972; Psychoyos, 1973a). Invasion is an exquisitely controlled process during which the trophoblast gradually implants into the underlying stroma (Wegner and Carson, 1994). Formation of a definitive placenta completes implantation and supports further embryo development to the end of the pregnancy. Implantation mechanisms differ greatly among species, and this variability results in many different types of placentation (Weitlauf, 1994). The nature of implantation and placentation are not entirely understood in any species of carnivore, and there is a particular lack of information in the mink (*Mustela vison*, Figure 1-1) . As with many species of mustelids, there is an obligate delay of implantation resulting in an extension in gestation period (Mead, 1981; 1989). Mink displays a period of embryonic diapause which normal persists from one to three weeks (Enders, 1952; Hansson, 1947). The period of diapause, however, is relatively shorter in the mink than in other mustelids with an obligate delayed implantation (Mead, 1981; 1989). There, however, are also several mustelids have displayed the developmental progression is not interrupted by diapause. For instance, the ferret (*Mustela putorius*), implantation occurs on Day 12 after mating and the total length of gestation is 42-43 days (Enders and Schlafke, 1972). The process of implantation has been described in some detail in the ferret (Enders and Schlafke, 1972) and the western spotted skunk (*Spilogale putorius latifrons*; Mead 1981; Enders and Mead 1996). In the mink, evidence

Figure 1-1. Photograph of Pastel mink (*Mustela vison*). The mink is a small carnivore belonging to the family Mustelidae. This species has been extensively raised in captivity since the beginning of this century for its fur. This animal has also been used as a research model due to its interesting biology, particularly in reproduction, photoperiodic effects on seasonality and furring cycles. Among the reproductive characteristics are seasonality, induced ovulation and obligate delayed implantation.

indicates that the control of delayed implantation resides in the uterus (Chang, 1968; Daniel Jr., 1971; Martinet et al., 1981b; Moreau et al., 1995). However, there is no information on the gene regulation and expression during implantation in either the uterus or the blastocyst in this species.

Although observations of uterine and placental morphology and postimplantation development have been reported in the mink (Bychkova, 1971; Enders, 1957; Hansson, 1947; Song et al., 1995), there are no comprehensive investigation, especially during the time of implantation, trophoblast cell invasion and placenta formation. The first objective of this study was to provide a more precise description of implantation and placenta formation in the mink than currently exists.

Studies indicated that uterine factors, including the cytokines (Stewart et al., 1995; Simon et al., 1995; Stewart, 1994b; Sharkey, 1998), growth factors (Haimovici and Anderson, 1993; Kaye and Harvey, 1995; Taga, 1992) and prostaglandins (Kennedy, 1977; Kennedy et al., 1989; Mead et al., 1988), are involved in the process of implantation in many species. Among carnivores, the mink is a convenient model since it is commercially available and has a relatively shorter period of embryonic diapause and gestation than the other mustelids such as the western spotted skunk. The general hypothesis tested in this study is that uterine factors are involved in the termination of delayed implantation and initiation of implantation in the mink.

Leukemia inhibitory factor (LIF) is a cytokine that regulates the proliferation, differentiation and function of many cell types (Smith et al., 1992). LIF was originally identified on the basis of its ability to inhibit a mouse leukocyte cell line, but has subsequently been shown to have many other actions. LIF has been shown to have an essential role in gestation in mice since its deletion prevents implantation (Stewart et al., 1992). The coincidence of LIF expression and embryo implantation in other species including the rabbit (Yang et al., 1994), mouse (Bhatt et al., 1991) and human (Kojima et al., 1994) further suggests its role in embryo attachment

and invasion. Hirzel and Mead (1997) presented preliminary evidence indicates that LIF is expressed at the time of embryo activation in the western spotted skunk, suggesting a role for this cytokine in termination of embryonic diapause and initiation of implantation in carnivores. However, the nature of LIF in the mink is unknown. As specific objectives we sought to (1) determine whether LIF was present in the mink, and to investigate the identity of its cDNA sequence with other species, (2) to profile LIF expression in the uterus during diapause, embryo reactivation and postimplantation gestation, and (3) to localize LIF in the uterus throughout gestation.

Prostaglandins (PGs) have been shown to be essential for successful embryo implantation in a variety of species (Kennedy et al., 1989; Kennedy, 1987; Psychoyos et al., 1995a). Involvement of PGs in the process of implantation is still unclear. Nonetheless, there is evidence that PGs are involved not only in the initiation of the endometrial vascular changes, but also in the growth and differentiation of decidual cells in rodents (Kennedy, 1987). Further, transcripts for PG receptors have been found in mouse luminal endometrium coincident with the time of implantation (Lim and Dey, 1997). Cyclooxygenase catalyses the rat-limiting step in PG biosynthesis by conversion of arachidonic acid into PGH_2, which is then converted to different types of PGs by specific synthases (Smith et al., 1991) Cyclooxygenase-2 (COX-2), which is the regulated isoform of the enzyme (Williams and DuBois, 1996), is believed responsible for the rapid increase of local PG production (Crofford, 1997). Chakraborty et al. (1996) showed that COX-1, the constitutive form of the catalytic cyclooxygenase, is present in uterine luminal epithelium and subepithelial stromal cells prior to implantation in the mouse. COX-2, however, is locally expressed in the uterus in the region surrounding the implanting blastocyst only at the time of embryo attachment at day 4 persisting until early on day 5 of gestation (Chakraborty et al., 1996). Transgenic mice bearing a mutation that eliminates expression of the

COX-1 gene were capable of reproduction, in contrast, knockout of COX-2 interferes with ovulation, implantation and placentation (Lim et al., 1997). No role for PGs or COX-2 has yet been established to date in mustelid carnivores such as the mink. Thus, further objectives of this study were (1) to determine whether COX-2 was present in the mink, (2) to investigate COX-2 gene expression during implantation and postimplantation gestation in the uterus of this species, and (3) to establish the patterns of COX-1 and COX-2 protein expression in the mink uterus throughout early gestation by immunohistochemistry.

2.0 LITERATURE REVIEW

2.1 Delayed implantation/Embryonic diapause

2.1.1 Embryonic diapause

Embryonic diapause is described as a temporary arrest or retardation in the development of the embryo at any stage of embryogenesis. In contrast, delayed development is defined as embryonic development which becomes retarded after implantation, such as in bats, (Burns, 1981). There is, however, another type of delayed development known as delayed fertilization, a rare phenomenon found in a few species of bats (Renfree, 1982). Characteristically, spermatozoa from a mating in autumn remain viable in the oviduct near the tubo-uterine junction, until the spring when fertilization occurs and development promptly follows.

The term delayed implantation refers to embryonic development which is arrested at the blastocyst stage of embryogenesis before attachment of the embryo to the uterus. Although seven mammalian orders (Marsupialia, Insectivora, Chiroptera, Edentata, Carnivora, Rodentia and Artiodactyla) display an embryonic diapause (Renfree and Calaby, 1981; Mead, 1993), not all aspects of the process are the same among these different orders (reviewed by Mead, 1993). Two types of delayed implantation are recognized: facultative (lactational) delay and obligate delay of implantation.

Facultative delay of implantation has been observed in rodents and marsupials under certain natural conditions including lactation (Psychoyos, 1973a; Renfree, 1981; Mead, 1993). Population pressure induces the delay of implantation resulting in a reduction of reproduction in wild mouse species in their natural environment. In laboratory rodents, the delay can be experimentally induced by stress (Mayer, 1959) and by progesterone (P_4) treatment of the rats after ovariectomy (Nutting and Meyer, 1963). Termination of facultative delay can be achieved by removal of sucking young (Bindon, 1969; Bindon, 1970) or by hormone

treatments. Estrogen induces implantation in lactation delayed rats and mice (Abrahamsohn and Zorn, 1993; Gidley-Baird, 1981; Macdonald et al., 1967; Psychoyos, 1973b). During facultative diapause, metabolism of the embryos is depressed (Weitlauf, 1994) and cell division is believed to be arrested in the G1 phase of the cell cycle (Sherman and Barlow, 1972).

Obligate delayed implantation, the condition in which delay is present in every gestation, was first observed in the roe deer (*Capreolus capreolus*) by the German anatomist Zeigler in 1841 (cited from Aitken, 1981). Subsequently studies showed that the obligate delayed implantation occurs in at least one species of bat, armadillos and several species of carnivores (Mead, 1989; Renfree and Calaby, 1981). Obligate delayed implantation has been extensively investigated in the mink (Douglas et al., 1994; Enders, 1952; Enders and Enders, 1963; Hansson, 1947; Martinet et al., 1981b; Møller, 1973; Moreau et al., 1995; Murphy, 1974; Murphy et al., 1990; Pilbeam et al., 1979) and the western spotted skunk (Enders and Mead, 1996; Mead, 1968; 1981) during the past decades. Nevertheless, the factors which control obligate delay and subsequent implantation are not completely understood.

2.1.2 Control of delayed implantation

In lactational delay species, estrogen induces termination of diapause and initiates implantation (Psychoyos, 1973a). Its effects include induction of uterine permeability and the endometrial decidual cell reaction which are essential for implantation in rodents (Abrahamsohn and Zorn, 1993; Kennedy, 1977; Psychoyos, 1973a). Replacement of estrogen by epidermal growth factor (EGF) induced implantation in hypophysectomized rats (Johnson and Chatterjee, 1993a) suggesting that the effect of estrogen may be to induce local synthesis of this factor (Johnson and Chatterjee, 1993a; 1993b). Nevertheless EGF is not essential for the implantation process (Threadgill et al., 1995). Estrogen has also be shown to induce LIF production which, as noted above, plays an essential role in implantation in the mouse (Stewart et al., 1992). Together, the

results suggested that the role of estrogen in the initiation of implantation is potentially mediated by uterine growth factors or cytokines (Sharkey, 1998). Estrogen alone will not terminate diapause and induce implantation in carnivores (Hansson, 1947; Huang et al., 1993; Murphy et al., 1983). Luteal factors, and probably nonsteroidal hormones in the uterus are essential for implantation in the ferret (Huang et al., 1993; Mead and Neirinckx, 1989) and in the mink (Murphy et al., 1983).

In species with obligate delayed implantation such as the mink, the annual reproductive cycle is strongly influenced by photoperiod (Duby and Travis, 1972; Hansson, 1947; Martinet et al., 1981b; Murphy, 1974; Sundqvist and Bartke, 1988; Travis and Pilbeam, 1980). The seasonal cycle arises because synthesis and secretion of melatonin and prolactin (Prl) exhibit seasonal and circadian fluctuations in the circulation (Goldman et al., 1981; Harris and Murphy, 1981; Murphy et al., 1981; Curlewis, 1992). The mink pineal gland is the source of melatonin which is synthesized and secreted in the dark phase of the light cycle (Ravault et al., 1986; Tillet et al., 1989). Short photoperiod increases the frequency of pulsatile melatonin secretion (Martinet et al., 1981b; Papke et al., 1980) which inhibits the secretion of Prl (Curlewis, 1992) and down-regulates the uterine Prl receptor (Prl-R) (Rose et al., 1996). Photoperiod of the Spring equinox triggers termination of delayed implantation and initiates the process of implantation in mink (Duby and Travis, 1972; Martinet et al., 1981b; Sundqvist and Bartke, 1988). Administration of melatonin to female mink prevents the vernal rise in plasma Prl concentration (Murphy et al., 1990) and suppresses endogenous Prl secretion in male mink (Rose et al., 1985).

Prl is secreted by the pituitary gland as a luteotropin regulating ovarian luteal steroidogenesis during gestation in mink (Murphy et al., 1984; Murphy, 1985; Murphy *et al.*, 1993). The role of Prl in initiation of implantation in mink has been shown in several studies in which showed that treatment with Prl in vivo advanced implantation and

shortened gestation (Martinet et al., 1981a; Papke et al., 1980). Prl also induced termination of obligate diapause in vitro (Polejaeva et al., 1997). However, it is unknown if the Prl has a direct effect on the embryos. Increased serum Prl levels reactivate the corpus luteum (CL) and induce production of P_4 and luteal factors which are necessary for termination of embryo diapause and initiation of implantation (Martinet et al., 1981a; Murphy et al., 1981; Papke et al., 1980). There is a substantial binding of Prl to mink ovaries and uteri during delayed implantation (Rose et al., 1983; Rose and Oldfield, 1986). Douglas et al. (1998), however, showed that Prl-R mRNA levels were low in the mink ovary during embryonic diapause but increased during CL reactivation, i. e. embryonic preimplantation activation and early postimplantation gestation. Changes in the abundance of Prl-R mRNA closely parallel changes in serum Prl levels and Prl-R binding in that study (Douglas et al., 1998). Rose et al.(1996) reported that a high systemic ratio of P_4 to E_2 is essential for production of the uterine Prl-R in mink. Nevertheless, the role of Prl in the uterus is unknown.

Resumption of development of mink embryos in diapause occurs if they are transferred into the uterus of a pregnant ferret, a species which does not have an obligate delayed implantation (Chang, 1968). Conversely, the ferret embryos undergo developmental arrest when they are transferred into the mink uterus and display a typical delay of implantation (Chang, 1968). Mink embryos in diapause can expand and escape from the zona pellucida/capsule when they are co-cultured with uterine cells in vitro (Moreau et al., 1995; Moreau et al., 1996; Polejaeva et al., 1997). Stress of experimental handling during diapause also increases the period of delay of implantation in mink (Daniel Jr., 1971).

Recent studies in other species suggested that the uterine cytokines, including LIF, play essential roles in the process of implantation (Bhatt et al., 1991; Stewart et al., 1992). These factors may also be important in terminating embryonic diapause and initiating implantation in

carnivores, including the mink. A beneficial effect of additional LIF on embryo survival has been recently observed in vitro (Moreau, Song, Smith and Murphy, unpublished observations). In addition, the Buffalo Rat Liver (BRL) cell line which has been shown to produce LIF (Funston et al., 1997; Smith and Hooper, 1987a) enhances survival of mink embryo (Moreau et al., 1995). Together these data argue for the validity of the hypothesis that the maternal organism regulates embryonic diapause and implantation in the mink (Murphy, 1992). Further investigations on the mechanisms for initiation of implantation are necessary.

2.2 Mechanisms of embryo implantation

2.2.1 Initiation of implantation

Implantation is a continuum, the beginning of which is usually defined as when the blastocyst is in apposition to the epithelium of endometrium and the end corresponds the formation of the definitive placenta. In carnivores, implantation involves three steps: apposition, adhesion and intrusion (Enders and Schlafke, 1969; Enders and Schlafke, 1972; Leiser and Koob, 1993; Schlafke and Enders, 1975). Early studies defined implantation as beginning when the blastocyst was no longer free to move about within the uterus (Enders, 1972). Histological studies suggest that changes which mark the initiation of implantation can be observed in the endometrium earlier. Around the time of implantation in many species, there is an increase in capillary permeability in the endometrium at the site of the embryo implantation (Psychoyos, 1973a; Rogers et al., 1982; Takemori et al., 1984; Weitlauf, 1994). In the mink, there are no morphological studies to demonstrate the sequence of events during implantation. The status of the CL and circulating P_4 levels have been used to indicate initiation of implantation. Approximately 6-7 days after the increase in maternal plasma P_4 level (over 8 ng/ml), implantation is initiated (Allais and Martinet 1978; Møller, 1973; Murphy and Moger, 1977; Stoufflet et al., 1989). However, due to variability

between individual animals, this criterion is not precise enough to predict the time of implantation.

2.2.2 Synchronous development and uterine receptivity

One of the essential steps for successful implantation is synchronized development of the blastocyst which is capable of implanting and the endometrium which is prepared to receive it (Paria et al., 1993b; Psychoyos, 1973a). Embryo transfer studies have provided ample evidence that the establishment of pregnancy depends upon a close synchrony between the developmental stage of the embryo and the corresponding developmental stage of the recipient uterus. Embryos produced by in vitro fertilization can be easily cultured to the blastocyst stage in simple media in several species, suggesting that the maternal environment is not necessary for preimplantation embryo development. On the other hand, the presence of the preimplantation embryo is clearly not necessary for priming the maternal environment, because cleavage stage embryos and blastocysts can be successfully transferred to a pseudopregnant, hormone prepared uterus in rodents (Cross et al., 1994). Although the development of embryo and uterine endometrium can be independent, absence of proper synchrony between embryo and endometrial condition results in failure of implantation (Lea and Clark, 1991).

In cattle, the highest pregnancy rate (91%) was achieved with precise synchrony of donor and recipients whereas recipients that were ±1 day out of synchrony with donor animals displayed a dramatic decline in the pregnancy rate (52-57%, cited from Zavy, 1994). Although numerous studies have provided evidence that asynchrony between the mother and embryo will result in embryonic death, there is not much information which addresses the factors which regulate this synchrony.

The mammalian uterus undergoes a series morphological and functional changes when implantation occurs. These changes provide a condition in which the uterine milieu supports the attachment of the

embryo and implantation, referred to as receptivity (Wegner and Carson, 1994; Weitlauf, 1994). Embryo transfer studies in rodents have revealed that the receptive state is transient and is followed by a period in which the uterus not only fails to support embryo attachment, but is also hostile to non-implanted embryos (Psychoyos, 1986; Wegner and Carson, 1994; Yoshinaga, 1988). Mouse blastocysts transferred to the endometrial cavity on day 1-4 of pregnancy do not implant until Day 5, while those transferred on Day 5 implant immediately. When blastocysts are transferred on Day 6, they do not survive and are expelled within a few hours (Psychoyos, 1993). These findings suggested that there is a defined period or window during which embryos can implant (Psychoyos, 1993; Yoshinaga, 1988). Three uterine states have been defined during the implantation process: pre-receptive, receptive and refractory (Wegner and Carson, 1994). The receptive state, however, varies in time among species. It is days 4.5-6 of gestation in mice; days 7-9 in humans (cited from Cross et al., 1994), days 11-12 in the marmoset monkeys (Webley and Hearn, 1991), day 12.5 in the cat (Leiser, 1982) and the ferret (Lawn and Chiquoine 1965). In delayed implantation species, the blastocysts remain in diapause for an extended period until the receptive condition of the uterus is attained as in the mink (Hansson, 1947; Murphy and James, 1974; Stoufflet et al., 1989) and lactating rodents (Psychoyos, 1976; Weichert, 1940).

2.2.3 Apposition and adhesion

The physical contact between embryo and endometrium is an obligate event in the initiation of implantation. The nature of communication between embryo and epithelium is not well understood (Enders, 1972; Weitlauf, 1994). In rodents, the blastocysts which have hatched from their zona pellucida interact with epithelium of the endometrium. Trophoblast cells of the embryo begin a rapid phase of growth (Renfree, 1982) resulting in an apposition of their cells with epithelial cells of the endometrium. In many species, the apposition is characterized by increasing contact between the surface of the trophoblast

and uterine epithelium, leading to their interdigitation (Weitlauf, 1994). Implantation proceeds into the next stage, adhesion, in which the surface of the trophoblastic and luminal epithelial cells have lost their microvilli and are parallel to each other, separated only by a distance of 20 nm as shown in rodents (Schlafke and Enders, 1975). A specialized submembranous filamentous network that supports the stable cell-cell binding between these cells has been observed in mice (Denker, 1993). Closure of the lumen and interdigitation of the microvilli have been referred to as the attachment reaction in rodents (Hedlund and Nilsson, 1971; Nelsson, 1966). In mink, physical contact between blastocyst and endometrium results from expansion of the blastocyst rather than from the closure of the uterine lumen, as seen in the rabbits (Steven, 1975).

2.2.4 Decidual cell reaction

In many mammals, decidual transformation of the endometrium in response to the implanting embryo results in an increase in the size and weight of the uterus. In rodents, this growth is due not only to proliferation and differentiation of the endometrial stromal cells, but also to swelling of the tissue caused by localized vascular permeability and consequent tissue edema (Weitlauf, 1994). The decidual cell reaction is found in rodents, higher primates, insectivores (Renfree, 1982). Formation of the decidua is a conspicuous part of the process of implantation in that species.

Decidualization is a critical component of the maternal response to the embryo and results in a new tissue or "decidual organ" in a normal adults. The decidual cell reaction has been well established in rodents. Abrahamsohn and Zorn (1993) summarized the requirements for decidualization in rodents: (1) the endometrial stroma cells have to be well prepared by ovarian hormones; (2) it happens only around implantation sites; (3) similar decidual reaction can be induced by oil or air in the absence of embryos in pseudopregnant animals and (4) the induction of the decidual reaction is time-dependent. Decidual cells

consist of three transformed stromal cell types which differ morphologically from each other, depending on their position in the uterus. These cells are large and have more cytoplasmic inclusion than do unstimulated stromal cells (Renfree, 1982; Weitlauf, 1994). The decidual cells are typically binucleate and polyploid (Sachs and Shelesnyak, 1955). The importance of decidual tissue appears to be that it limits trophoblast invasion, defines placenta formation (Enders et al.,1981; Sherman and Wudl, 1976) and secrets both peptides and steroid hormones (Weitlauf, 1994). It has been suggested that the decidual cells provide nutrients for early embryo development (Abrahamsohn and Zorn, 1993; Weitlauf, 1994). It is believed that the decidual cells play a role in controlling trophoblast invasion (Loke et al., 1995). Several reviews have concluded that endometrial growth factors (EGF), cytokines and PGs play an important role to decidualization and implantation in many species (Cross et al., 1994; Lala and Graham, 1990; Tabibzadeh and Babaknia 1995; Wegner and Carson, 1994). Although profound changes are seen in the endometrium during implantation, it would appear that decidual cells do not appear in most carnivores (Renfree, 1982). Nevertheless, Leiser and Koob (1993) have reported that stroma-derived decidual cells are found in the cat.

In mink, the decidual response has been regarded to epithelial and the homologue of rodent decidual cells are absent (Enders, 1957; Mossman, 1937). The endometrium responds to the trophoblast by the production of symplasmal masses and greatly enlarged cells, by enlargement of the maternal endothelial cells and their supporting matrix and by the production of secretory granules (Enders, 1957). Asimilar response is also found in the ferret (Gulamhusein and Beck, 1973; 1975). Whether the symplasma is functionally homologous in any way to rodent decidual cells is unknown.

Early studies suggested that a variety of stimuli induce the initiation of the decidual cell reaction, including histamine (Shelesnyak,

1952), CO_2 (Torbit and Weitlauf, 1974), and actinomycin D (Finn and Bredl, 1973; Finn and Bredl, 1977). It is now widely accepted that PGs have a role in decidualization in rodents and primates (Kennedy, 1986; Kennedy et al., 1989). Recent studies showed that cytokines, including LIF, are also involved in the initiation of decidualization in several species (Bhatt et al., 1991; Kojima et al., 1994; Chard, 1995; Sawai et al., 1997; Sharkey, 1998). Further investigation of decidualization like changes in mink is necessary and appears to be important for understanding the mechanism of implantation.

2.2.5 Trophoblast invasion and placentation

In most species, blastocyst escapes from the zona pellucida and adheres to the epithelium of the endometrium in the attachment phase of the implantation process. Thereafter trophoblast cells, at least in species with invasive placenta such as the rodents and humans, penetrate the maternal endometrium, intrude through the epithelium and into the underlying stroma (Enders and Schlafke, 1969; Schlafke and Enders, 1975; Steven, 1975). This involves the breakdown of the normal cell relationships in the endometrium, resulting in direct apposition of the embryonic trophoblast with maternal connective tissue, capillaries and the maternal bloodstream (Steven, 1975). In non-invasive species, such as the pig, trophoblast cells do not invade the maternal endometrium. Instead, the trophoblast cells differentiate rapidly and attach to a large area of the epithelium of the endometrium (Steven, 1975; Renfree, 1982). In all species, poor invasiveness would result in inadequate feto-maternal exchange, whereas excessive invasiveness may result in pathological destruction of the uterus.

Different types of trophoblast invasion result in different placentation among species and they are the basis for classification of the definitive placentas (Steven, 1975). There are several critiria for placental

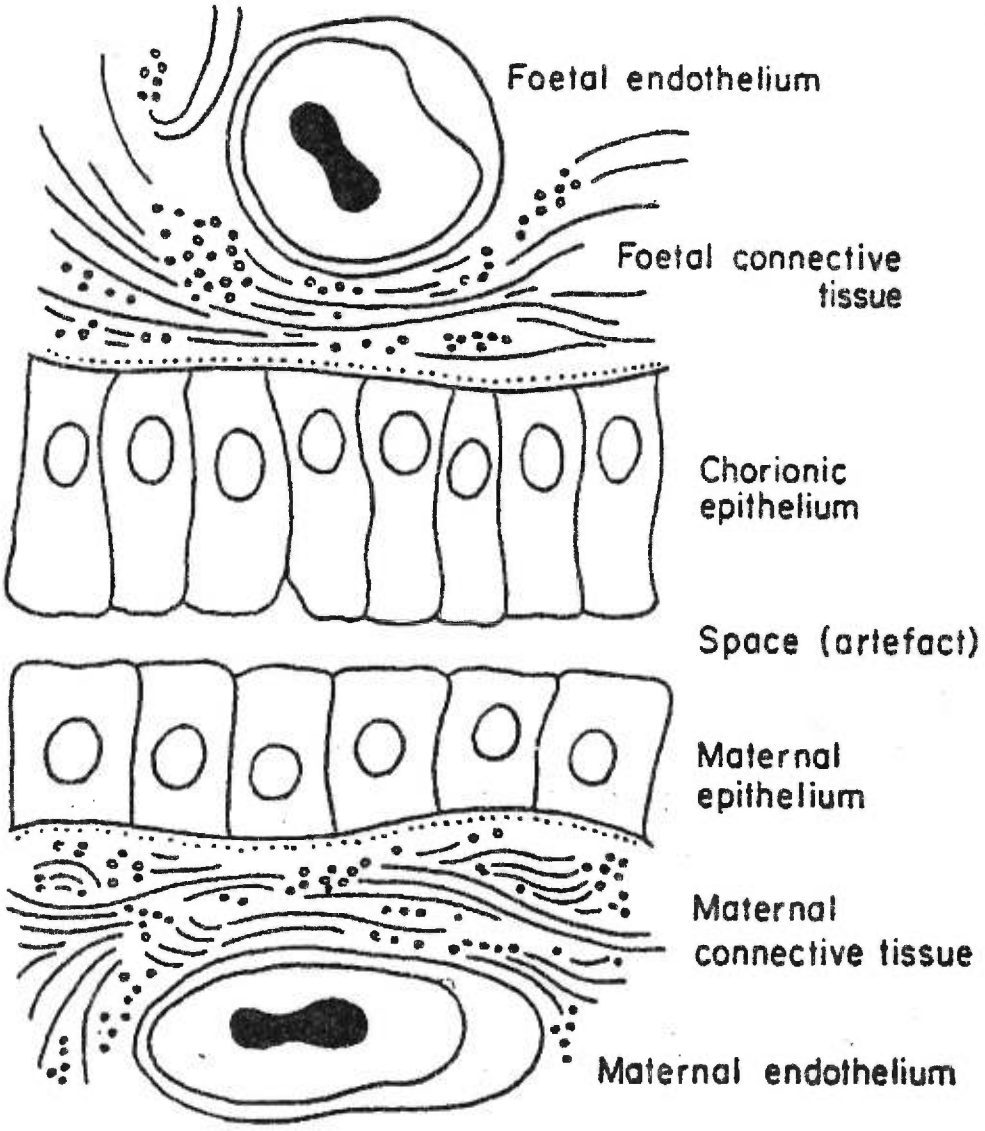
Foetal endothelium
Foetal connective tissue
Chorionic epithelium
Space (artefact)
Maternal epithelium
Maternal connective tissue
Maternal endothelium

Figure 2-1. Diagram of the basic concept of Grosser structural classification of placentas showing potential separation of the maternal and fetal capillaries (taken from Steven, 1975).

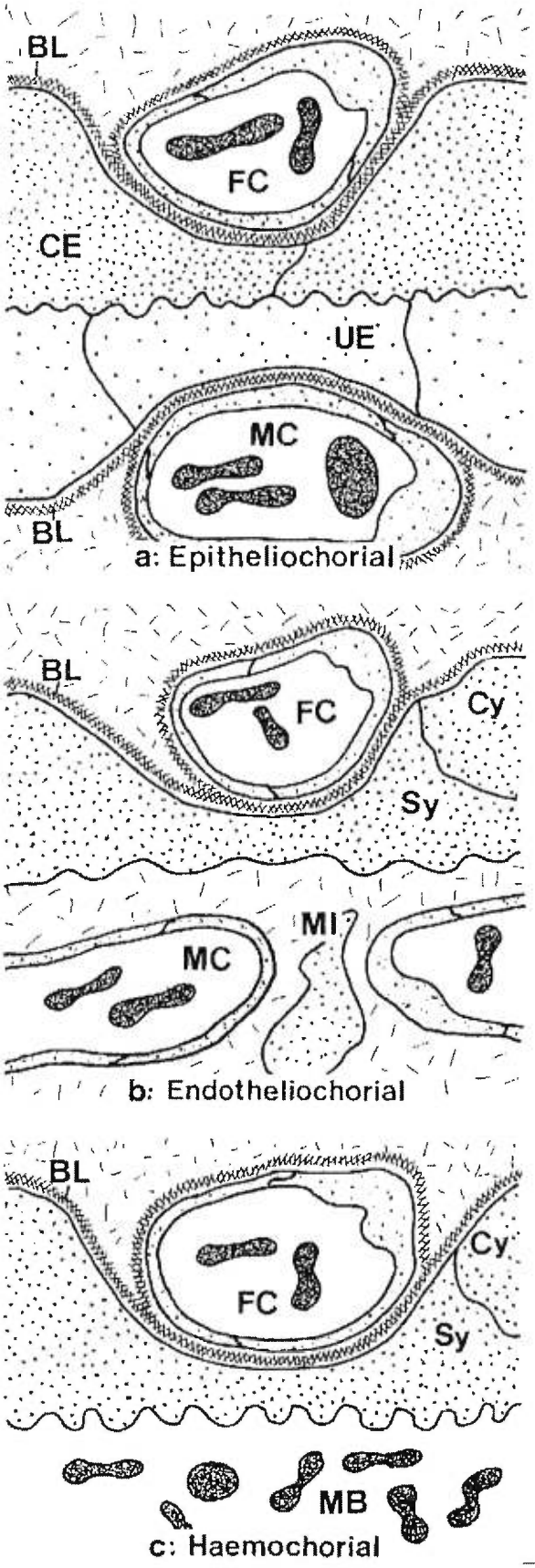
BL
FC
CE
UE
MC
BL
a: Epitheliochorial
BL
FC
Cy
Sy
MI
MC
b: Endotheliochorial
BL
FC
Cy
Sy
MB
c: Haemochorial

Figure 2-2. Placental types of materno-fetal barrier according to the Grosser classification. MC, maternal capillary; MB, maternal blood; FC, fetal capillary; BL basal lamina; CE, chorionic epithelium (trophoblast); Sy, cytotrophoblast; UE, Uterine epithelium; and MI maternal interstitium. Fetal components are comprised under the name chorion. Maternal components are reduced until, in the hemochorial situation, chorion comes into direct contact with maternal blood (taken from Kaufmann and Burton, 1994).

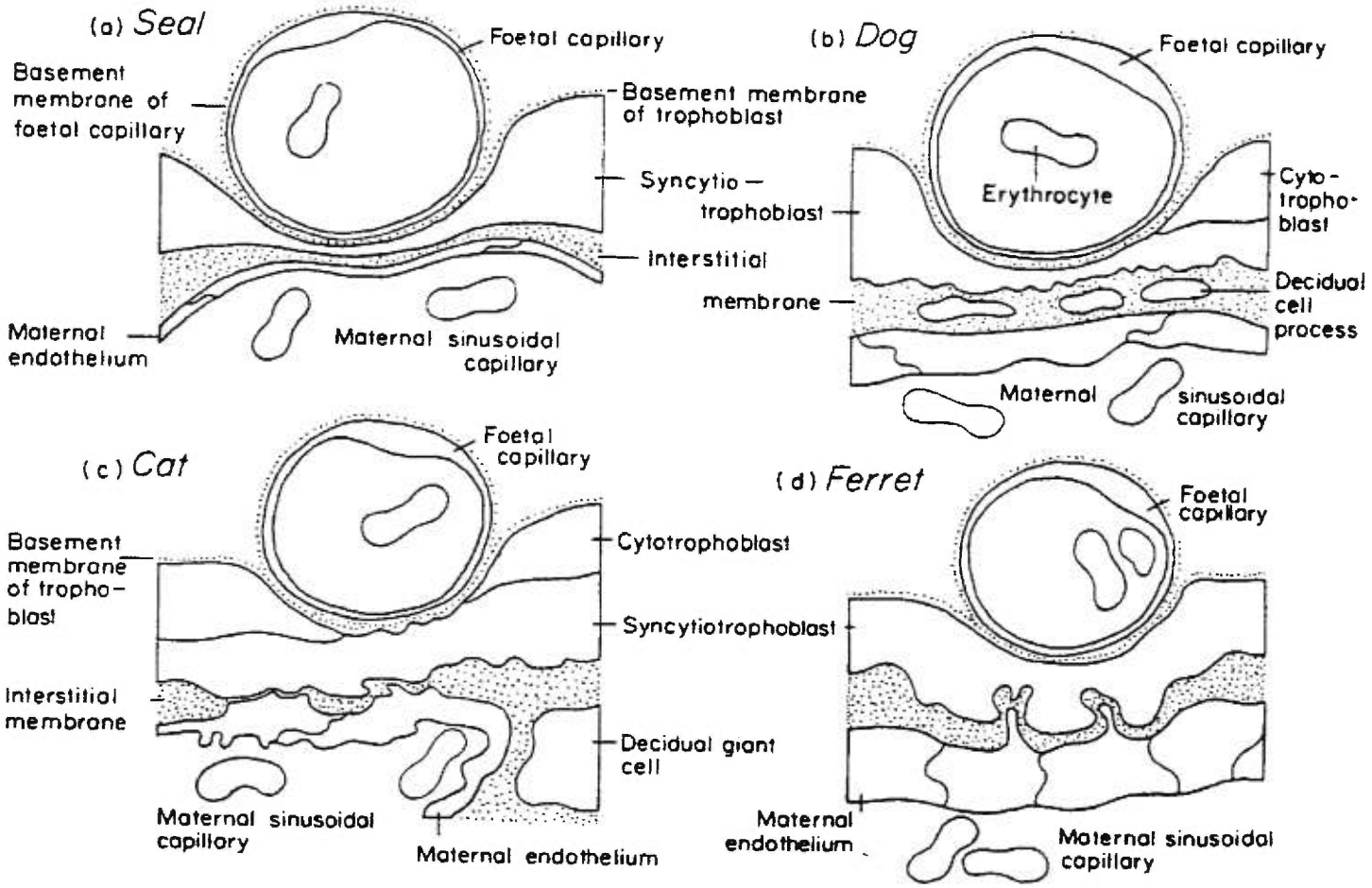
(a) Seal
Foetal capillary
Basement membrane of foetal capillary
Basement membrane of trophoblast
Syncytio-trophoblast
Interstitial membrane
Maternal endothelium
Maternal sinusoidal capillary
(b) Dog
Foetal capillary
Erythrocyte
Cyto-trophoblast
Decidual cell process
Maternal membrane
Maternal sinusoidal capillary
(c) Cat
Foetal capillary
Basement membrane of trophoblast
Cytotrophoblast
Syncytiotrophoblast
Interstitial membrane
Decidual giant cell
Maternal sinusoidal capillary
Maternal endothelium
(d) Ferret
Foetal capillary
Maternal endothelium
Maternal sinusoidal capillary

Figure 2-3 The endotheliochorial placenta. Variations in structure within the same general category (taken from Steven, 1975)

classification (Steven, 1975). Among these, Grosser's histological classification has been mostly used (Steven, 1975; Kaufmann and Burton, 1994). This system is based on the number of layers of tissue, which appears to separate fetal from maternal blood-streams under light microscope (Figure 2-1; Steven 1975). In general, such a classification sets out three categories of placentas: hemochorial, epitheliochorial and endotheliochorial (Figure 2-2; Kaufmann and Burton, 1994). There remain, however, differences in structure of individual placenta within the three categories (Steven, 1975). In rodents and humans, trophoblast tissue erodes maternal blood vessels, so that it becomes directly exposed to the maternal blood in the placental sinusoids, forming the most invasive hemochorial placenta (Kaufmann and Burton, 1994). At the other extreme, pigs and related species display an epitheliochorial placenta in which both the uterine epithelium and the chorionic trophoblast (trophectoderm) remain is in close apposition throughout pregnancy (King, 1993).

A less invasive, endotheliochorial placenta was noted in several carnivores such as in domestic cat, ferret, dog, the western spotted skunk and the mink (Enders, 1957; Lawn and Chiquoine, 1965; Steven, 1975; Sinha and Mead, 1976). In these species, blood vessels of the mother become surrounded by trophoblast but are not penetrated, such as shown in Figure 2-3 (Steven, 1975). The uterine epithelium is, however, completely eroded at the zone of attachment and invasion. Detailed descriptions on placentation have been made in dog (Anderson, 1969), cat (Leiser, 1982; Leiser and Koob, 1993) and ferret (Enders and Schlafke, 1972; Gulamhusein and Beck, 1973; 1975; Lawn and Chiquoine 1965). Mink placentation has only been superficially described as a zonary placenta as the ferret and dog (Enders, 1957; Kaufmann and Burton, 1994), but it is more complete zonary than that of the ferret (Enders, 1957). The chorionic villi or lamellae of the placental labyrinth are aggregations of the placental tissue which encircles the equatorial region of the chorionic

sac (Bychkova, 1971; Enders, 1957). There is, however, lack of information on the formation of the placenta during early stage of implantation in the mink.

2.2.6 Steroid hormones and implantation

It has been known that steroid hormones, estradiol (E_2) and P_4 are involved in the process of implantation in many species (Psychoyos, 1973a; Weitlauf, 1994). Levels of these hormones generally rise during the pre-receptive stage and P_4 is maintained if successful implantation occurs. The pre-receptive stage of the uterus and blastocyst can be maintained and implantation can be hormonally delayed for an extended period in rodents (Abrahamsohn and Zorn, 1993; Psychoyos, 1973a; Weitlauf, 1994). In some species, estrogens produced by the blastocyst are considered to serve as a signal for its presence to the uterus (Bazer et al., 1986; Bazer, 1992). Early studies have shown that estrogen induces implantation in P_4 maintained or lactational delayed rodent (Paria et al., 1993a; Psychoyos, 1973a; Wegner and Carson, 1994). Although estrogens function to induce implantation in rodents, they are also served as anti-implantation agents in humans. High doses of both estrogens and P_4 have been successfully used as contraceptive agents in human and rat (reviewed by Psychoyos et al., 1995). These observations suggested that dose and ratios of steroid hormones are essential for the regulation of the implantation process.

E_2 induces an increased endometrial vascular permeability, which coincides with the initial attachment reaction between blastocyst and uterus at the site of implantation in mice (Enders, 1972; Paria et al., 1993a; Psychoyos, 1973a). Further studies showed that E_2 induces hypertrophic, but not hyperplastic changes in the uterus. These hypertrophic changes included increases in wet weight, glucose metabolism and nutrient transport. These changes are essential to the embryo implantation in the species studied. In carnivores, however, neither administration of E_2 nor P_4 nor combination of these two steroids in doses employed can

successfully induce implantation (Hansson, 1947; Cochrane and Shackelford, 1962; Murphy et al., 1983).

Morphological and histochemical evidences suggest that the mink uterus undergoes changes in the nature or rate of secretion during embryo diapause, activation development and periimplantation phases of gestation (Enders et al., 1963; Murphy and James, 1974). These changes are the results of the influence of ovarian steroid hormones in other species studied (Weitlauf, 1994), presumably this is same in the mink. Implantation follows an increased P_4 level in circulation in the mink (Møller, 1973; Murphy and Moger, 1977; Stoufflet et al., 1989). Due to variation of implantation, the estrogen pattern related to implantation has not been precisely determined in this species although many investigators have attempted to measure the changes of E_2 in the circulation at periimplantation and throughout gestation (Pilbeam et al., 1979; Lagerkvist et al., 1992; Stoufflet et al., 1989). Whether mink blastocysts produce estrogen prior to implantation is unknown.

2.2.7 The role of endometrium in implantation

Cowell (1969) suggested that the endometrium possesses the ability to regulate trophoblast invasion. Subsequent studies have further confirmed this conclusion. Trophoblast transferred to ectopic sites such as beneath the kidney capsule (Zeilmaker and Timmermans, 1969), the testes (Bland and Donovan, 1965; James et al., 1972), the spleen (Bland and Donovan, 1965), the liver (Gordeev, 1980), the cycling nonpregnant and pseudopregnant uteri revealed that the extent and duration of invasion were greater in extra-uterine sites and nonpregnant uteri than in pseudopregnant and pregnant decidualized uteri (Belaisch-Allart and Frydman, 1986; Lala and Graham, 1990).

Various architectural, cellular and molecular events in endometrium are coordinated with the implantation window and thus may be the essential elements in endometrial receptivity. For example, growth factors and cytokines secreted by the endometrium (Bhatt et al.,

1991; Papke and Dey, 1990; Tabibzadeh and Babaknia 1995) and other endometrial proteins (Boomsma and Verhage, 1987; Julian et al., 1994) are potentially involved in implantation. On the other hand, some endometrial changes seem to occur only in response to the blastocysts. For instance, the permeability of the subepithelial capillaries is significantly increased in the endometrium surrounding the blastocyst (Weitlauf, 1994). This change occurs in all species studied so far and is universally considered as a response of endometrium to as yet unidentified signals from the blastocyst (Psychoyos, 1993; Weitlauf, 1994). Blastocyst-associated proteinase and glycosidases are certainly necessary for trophoblast attachment, penetration, and lysis of the uterine matrix (Lala and Graham, 1990; Weitlauf, 1994). The signals and the molecular pathways that underlie this controlling mechanism are unknown.

2.2.8 Degradation enzymes in implantation

Trophoblast cells of the blastocyst possess the capacity of invasion into the maternal uterine endometrial stroma and decidua. Invasion is facilitated via proteinases, such as matrix metalloproteinases (MMPs) expressed by trophoblast cells in human and rodent (Lala and Graham 1990; Strickland and Richards 1992; Abrahamsohn and Zorn, 1996; Das et al., 1997) and balanced by their tissue inhibitors (TIMPs) in the maternal decidua (Das et al., 1997; Leco et al., 1996). In the mouse, Das et al. (1997) showed that expression of MMP-9 and TIMP-3 corresponds with decidual reaction in the endometrium at sites of implantation and trophoblast cell invasion. A balance between MMPs and TIMPs may therefore regulate trophoblast invasion.

Plasminogen activators (PAs), particularly the urokinase-type PA (uPA), have been implicated in tissue remodeling because of their role in regulating extracellular matrix (ECM) turnover (cited in Wang et al., 1996). The rat endometrial stromal cells taken from uteri which have been sensitized for the decidual cell reaction, secrete uPA during in vitro decidualization and uPA mRNA was detected in vivo in decidual cells by

in situ hybridization (Wang et al., 1996). The uPA activity is controlled by many natural inhibitors, including plasminogen activator inhibitors (PAIs, Balsi et al., 1987). PAI-1 mRNA level increases in the uterus at the time of trophoblast invasion (on day 7 of pregnancy) and reaches the highest level on day 15 at decidual formation in rats (Wang et al., 1996). These results indicated that PAs and their inhibitors may play a role in regulation of MMPs during invasion. Kennedy et al. (1998), however, have suggested that PA activity is not a marker for decidualization. Indeed, the precise regulation and function of ECMs and PAs in implantation is not clear and merits further investigation.

2.3 Leukemia inhibitory factor (LIF) in implantation

2.3.1 Cytokines

Cytokines are a group of less than 100 kDa regulatory proteins secreted by cells of the immune system and act nonenzymatically in picomolar to nanomolar concentration (Abbas et al., 1994). Molecules generally accepted as cytokines are colony-stimulating factors (CSF), interleukins (IL-1, 2, 3, ...), tumor necrosis factor (TNF), transforming growth factors (TGF), LIF and interferons (IFNs) (Chard, 1995). The distinction between molecules described as cytokines and those described as growth factors is particularly blurred. One characteristic of this group of proteins is autocrine and paracrine action (Abbas et al., 1994). In a few cases, they may act in an endocrine fashion, binding to target cells in distant parts of the body (Abbas et al., 1994). The cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of various cells and by regulating the secretion of antibodies or other cytokines (Abbas et al., 1994). In addition to their role in immunomodulation, cytokines also function in maternal recognition of pregnancy, control of implantation and regulation of endocrine function of the placenta (Mathialagan and Roberts, 1994). Recent studies have shown that cytokines are expressed in a variety of non-immune cell types, and have a

wide range of activities in physiological and pathological processes as regulator of cells of many diverse lineages, including in the reproductive system (Chaouat et al., 1995; Mathialagan and Roberts, 1994; Robertson et al., 1994; Simon et al., 1995). The tissues involved in the implantation process (trophoblast and decidua) are extraordinarily active biosynthetically, producing a wide range of potential effector molecules (Edwards, 1994).

The target cell for a particular cytokine is determined by the presence of specific membrane receptors (Ihle et al., 1994). Cytokines binding to receptors elicits biochemical changes responsible for signal transduction and results in an altered pattern of gene expression in the target cells. In general, the cytokines receptors exhibit a high affinity for their ligands with dissociation constants ranging from 10^{-10} to 10^{-12} M (Abbas et al., 1994).

The role of cytokines is sometimes difficult to determined. Genetic knockouts show that the total absence of a particular cytokine may be associated with only relatively minor abnormalities in the adult animal. Other problems of interpretation are related to the source of cytokines. Purified natural granulocyte-macrophage colony-stimulating factor (GM-CSF) can stimulate trophoblast differentiation (reviewed by Robertson et al., 1994), whereas recombinant GM-SCF has no effect (Lea and Clark, 1993). Interferon-τ (IFN-τ), trophoblast origin, inhibites PGs (Danet-Desnoyers et al., 1994; Salamonsen et al., 1989) and COX-2 production (Godkin et al., 1991; recited from Teixeira et al., 1997), whereas Asselin et al. (1997b, and c) showed that administration of an elevated dose of IFN-τ up-regulates COX-2 expression and PG production. These results suggested a different explanation of regulation of COX-2 expression and PG production. Xiao et al. (1998), however, reported that the upregulating effect of IFN-τ on the COX-2 expression and PG production may be due to

the presence of the endotoxin (LPS) which is co-expressed by bacteria during the production of recombinant IFN-τ.

2.3.2 Leukemia inhibitory factor (LIF)

LIF is a 45-56 kDa glycoprotein produced by a number of different cell types, including Krebs ascites, Ehrlich ascites, various fibroblast, BRL cells, antigen-stimulated human alloreactive T lymphocytes and mitogen-stimulated human spleen cells (Gough and Williams, 1989). LIF has pleiotropic activities on a variety of cell types in vitro, including embryonic stem cells (Smith et al., 1988), primordial germ cells (Matsui et al., 1991), myeloid cell lines (Hilton et al., 1988), adipocytes (Mori et al., 1988), hepatocytes (Baumann and Wong, 1989) and peripheral neurones (Yamamori et al., 1989). Recent studies suggested that LIF is also involved in embryo implantation in several species, including human, mice, pig and western spotted skunk (Hirzel and Mead, 1997; Stewart, 1994a; Stewart, 1994b). The nucleotide sequence for LIF has been determined in several species, including the human (Moreau et al., 1988), mouse (Gearing et al., 1987; Gearing and King, 1988), pig and sheep (Willson et al., 1992), and cow (Kato et al., 1996). There is 79% amino acid (aa) sequence identity between the murine and human proteins, with a considerable number of conservative substitutions (Stahl et al., 1990). Human, murine and bovine LIF appear to be encoded by a unique gene located at chromosome 22q12 in man (Sutherland et al., 1989), 11A1-A2 in the mouse (Kola et al., 1990) and on chromosome 17 in cattle (Piedrahita et al., 1997). The LIF transcript in the mouse is about 4.2 kb in length, with a large (3.2 kb) 3'-untranslated region (Gearing and King, 1988; Moreau et al., 1988).

LIF has significant homology to IL-6, ciliary neurotropic factor (CNTF), IL-11 and cardiotrophin-1 (Bazan, 1991; Pennica et al., 1995). These molecules all have protein gp130 in common as part of the signal transduction complex (Gearing *et al.*, 1991; Pennica et al., 1995), which probably accounts for much of their redundancy (Sharkey, 1998). The

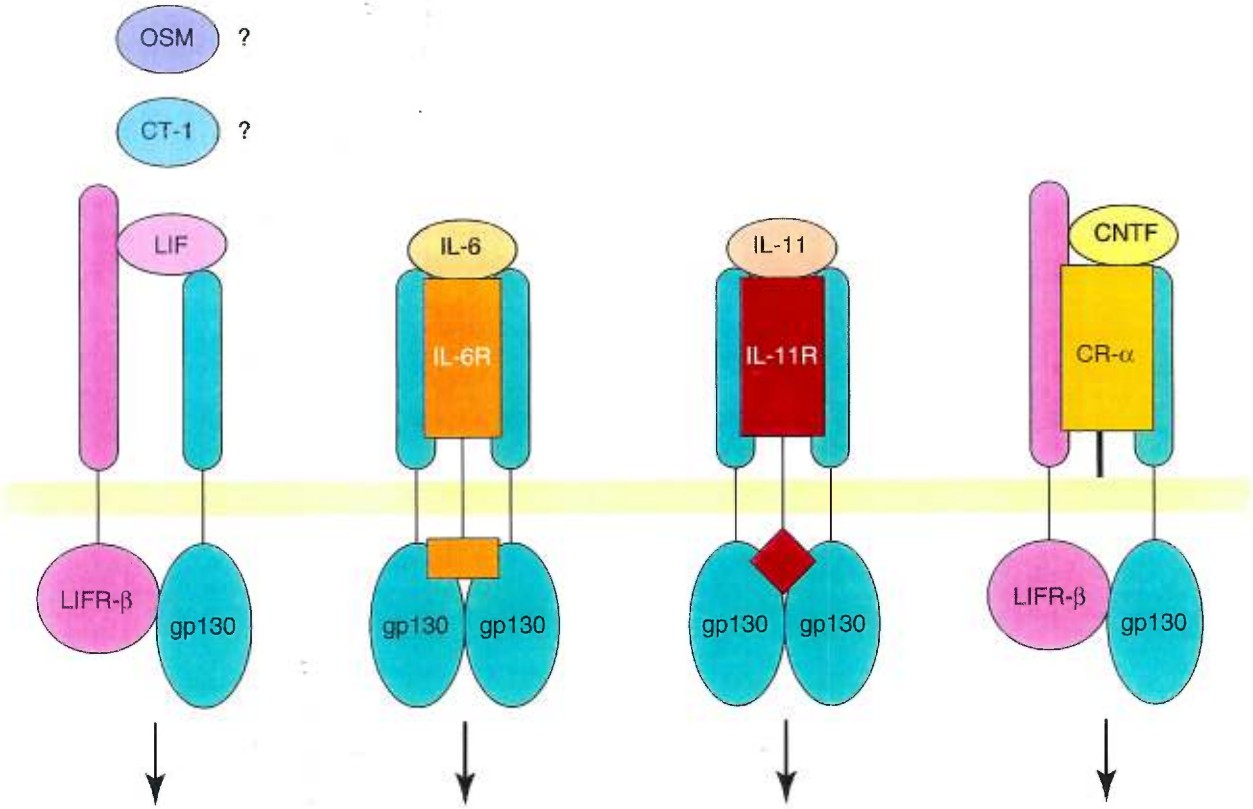
OSM
?
CT-1
?
LIF
LIFR-β
gp130
IL-6
IL-6R
gp130
gp130
IL-11
IL-11R
gp130
gp130
CNTF
CR-α
LIFR-β
gp130

Figure 2-4. Subunit structure of the receptor complex of the IL-6 family of ligands (taken from Sharkey 1998). Binding of LIF, IL-6, IL-11 and CNTF is mediated through ligand-specific receptor subunits, which then trigger association of the other receptor components required for signaling.

action of LIF is mediated by binding to two types of cell surface receptors, low affinity (LIFR-β) and high affinity (Hilton et al., 1988; 1991). The low affinity receptor, LIFR-β binds only LIF and subsequently binds with gp130, which transforms LIFR-β into a high-affinity form, and activates signal transduction (Figure 2-4). The gp130, by itself, does not bind LIF (Davis et al., 1993; Gearing et al., 1991). The LIF ligand-receptor system has been particularly well studied since the effects of LIF on the preimplantation embryos of several species are known and knockout mice deleted for all the components (LIF, LIFR-β and gp130) have been produced (Stewart et al., 1992; Ware et al., 1995; Yoshida et al., 1996).

2.3.3 Role of LIF in implantation

The role of LIF in embry implantation was first observed in mouse (Bhatt et al., 1991). LIF is transiently expressed in the uterine glandular epithelium on day 4, prior to implantation. Analysis of LIF expression in pseudopregnant mice and in mice undergoing delayed implantation suggested that it is under maternal estrogen control (Bhatt et al., 1991). Further studies have shown that the failure of implantation in LIF deficient mice is due to failure of the decidual cell reaction (Stewart, 1994a; Stewart, 1994b). Thus, the essential role of LIF may be in sensitizing the mouse uterus to the decidual signal from the embryo, thereby initiating the process of implantation.

Studies on LIF in other species have shown, in general, a pattern similar to that seen in mice. In rabbits, LIF is expressed in the endometrial epithelium, myometrium and endometrial glands on day 5 of pregnancy and declines by days 6 and 7 (Yang et al., 1994). In pig and sheep, maximal expression of endometrial LIF occurs on days 11 and 16-20 of gestation, respectively (Anegon et al., 1994; Vogiagis et al., 1997) and trophoblast also is a site of production of LIF in sheep. In humans, LIF mRNA transcripts were first observed in endometrium on days 18-21 and persist throughout the remainder of the luteal phase (Arici et al., 1995; Charnock-John et al.,

1994), and in placental tissue (Kojima et al., 1994). A detailed study showed the human endometrial epithelial cell rather than stroma is the major site of LIF secretion (Chen et al., 1995; Vogiagis et al., 1996).

Beneficial effects of LIF on embryo development have been observed in vitro. Administration of LIF improves embryo viability and hatching in mouse and cow (Funston *et al.*, 1997; Lavranos et al., 1995). In vivo, excess LIF expression results in excessive bone formation, suppression of ovulation (Metclaf and Gearing, 1989) and formation of primitive ectoderm in mice (Shen and Leder, 1992). As noticed above, enhanced survival of mink embryos was observed in embryos co-cultured with BRL cells which secrete LIF (Moreau et al., 1996; Funston et al., 1997). However, infusion of human recombinant LIF into the uterus of the western spotted skunk did not terminate delayed implantation (Mead RA, 1998, personal communication).

The regulation of LIF by steroid hormones is species dependent. In mice, estrogen alone induces the highest LIF expression in the pseudopregnant uterus on Day 4 of pregnancy (Stewart, 1994b; Yang et al., 1996). In contrast, P_4 alone or E_2 and P_4 but not E_2 alone will induce LIF protein in the uterine epithelium and glands in unmated rabbits (Yang et al., 1996). In humans, estrogen up-regulates LIF secretion by decidual cells in vitro and this response can be blocked by protein kinase C (PKC) inhibitor, but not by other kinase inhibitors (Sawai et al., 1997). Vogiagis et al. (1997b) reported that treatment of ovariectomized ewes with estrogen or a combination of estrogen and P_4 reduces rather than increases LIF expression in the endometrium (Vogiagis et al., 1997). The role of steroid hormones in the regulation of LIF expression in the uterus is complicated and needs to be further investigated.

Evidence suggests that LIFR is expressed in trophoblast, in endometrial cells, or in both. LIFR mRNA was detected in the mouse peri-implantation embryo by RT-PCR (Harvey et al., 1995). Nichols et al. (1996) reported that the mRNA for LIF and its receptor (LIFR-β) were

undetectable in 1- or 2-cell embryos of mouse, but all were present by the blastocyst stage. LIF transcripts were localized in the differentiated trophectoderm, and were absent from the pluripotential inner cell mass. In contrast, LIFR mRNA was found in the inner cell mass but not in the trophectoderm (Nichols et al., 1993). Yang et al. (1995a; 1995b) also observed that LIFR and gp130 are expressed in the endometrium in mice and in rabbits around implantation sites. In humans, LIFR and gp130 mRNA expression were observed in blastocysts (Seifer et al., 1993; Sharkey, 1998) and luminal epithelium (Cullinan et al., 1996), whereas LIFR, gp130 and LIF binding activity are localized to luminal epithelium of the endometrium (Cullinan et al., 1996). These results suggest both the preimplantation embryo and the maternal endometrium are sites for the action of LIF. The LIF ligand can act in an autocrine and paracrine manner, but the mode of action may differ between species.

In transgenic mice with LIFR or gp130 receptor gene deficiency, embryos are able to develop successfully to the blastocyst stage and implant normally (reviewed by Sharkey, 1998). LIFR -/- mice develop successfully to term, although they exhibit numerous abnormalities, including placental defects (Ware et al., 1995). Yoshida et al. (1996) showed that given an involvement in signaling by several cytokines, disruption of the gp130 gene leads to a more severe by affected phenotype, with embryonic lethality between day 12.5 and day 18 of gestation.

Harvey et al. (1995) reported that both LIF and EGF stimulated the activity of uPA and MMP-9 in blastocyst outgrowths after 3 days of culture (day 7 of gestation), whereas LIF decreased synthesis of both proteinases. These results demonstrate that proteinase activity in early embryos can be regulated by growth factors and cytokines during the implantation process and, in particular, they demonstrate the possible involvement of LIF in priming the uterus for implantation and subsequently may be involved in the decidualization. Whether LIF plays a role in termination of delayed implantation is not clear. Hirzel and Mead (1997) reported

preliminary results which indicated that LIF is expressed in the uterus at the period of embryo reactivation in the western spotted skunk. This suggests that LIF may also be involved in implantation in carnivore species, particularly in termination of embryonic diapause and initiation of the implantation process. The mink presents an excellent model to study the role of LIF in the regulation of embryo reactivation and implantation in carnivores.

2.4 Prostaglandins and cyclooxygenase-2 in implantation

2.4.1 Characteristics of prostaglandins

Prostaglandins (PGs) are derived from arachidonic acid. The PGs were first discovered in the secretion of the prostate gland and were thought to regulate the activity of male reproductive tissues (Stenesh, 1989). Subsequent studies showed that PGs are made by, and function in, virtually all organs (Smith and Hooper, 1987). PGs are associated with inflammatory responses, wound healing, bone development, glomerular filtration and water-balance, and homeostasis (Smith et al., 1991).

PGs are a group of biologically active lipids that consists of C_{20} carboxylic acid with a unique structure (Figure 2-5): they have a central five-membered ring with two side chains with lengths of 7 and 8 carbons, respectively, attached to adjacent positions on the ring (Granstrom, 1981). Various oxygen-containing substituents determine the type of PGs and according mainly to the ring structures, the PGs are classified as the A, B, C, D.... types.

PGs are produced by every nucleated cell of the body and act locally in a paracrine or autocrine manner (Smith et al., 1991). Different types of PGs display varieties of physiological function or even have an opposite actions, mediated by specific receptors which may take multiple forms of a signal PG (Coleman et al., 1994). PGs do not appear to be essential for life because prolonged treatment with non-steroidal anti-inflammatory drugs (NSAIDs) do not result in death (Levy, 1997). Nevertheless, apart from

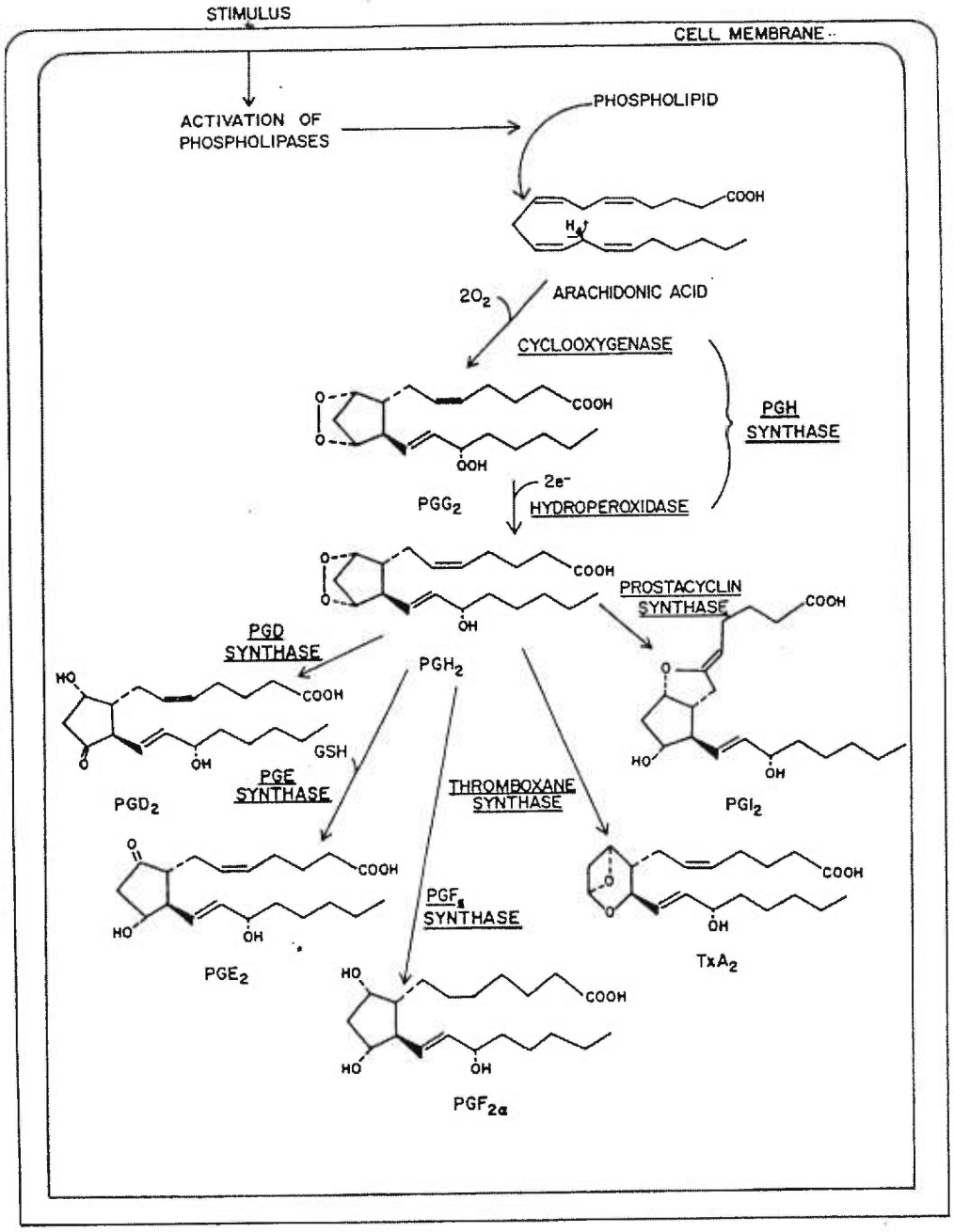
STIMULUS
CELL MEMBRANE
ACTIVATION OF PHOSPHOLIPASES
PHOSPHOLIPID
COOH
ARACHIDONIC ACID
$2O_2$
CYCLOOXYGENASE
PGH SYNTHASE
OOH
PGG_2
$2e^-$
HYDROPEROXIDASE
OH
PGH_2
PROSTACYCLIN SYNTHASE
PGD SYNTHASE
HO
GSH
PGE SYNTHASE
THROMBOXANE SYNTHASE
PGD_2
PGI_2
PGE_2
PGF_s SYNTHASE
TxA_2
$PGF_{2\alpha}$

Figure 2-5 Pathways for the formation of prostaglandins (taken from Smith et al. (1991).

sex hormones, PGs are probably the most important regulators involved in reproduction (Bygdeman, 1981; Weitlauf, 1994). Numerous studies have shown that PGs are involved in the processes of ovulation, luteolysis, and menstruation, induce an increase of uterine vascular permeability before implantation, induce the uterine immune response and regulate uterine contractility at the time of parturition.

The production of PGs in the ruminant uterus is known to be at least partially under steroid hormone control, and this control is via regulation of oxytocin and its receptors in the endometrium (Roberts et al., 1976; Xiao et al., 1998). The factors from both the conceptus and the endometrium are involved in the regulation of the synthesis of endometrial PGs (Bazer et al., 1986; Salamonsen and Findlay, 1990). Recent studies have shown that cytokines and growth factors, for example IFN-τ, are involved in the regulation of COX-2 expression and $PGF_{2\alpha}$ production in the endometrial cells (Xiao et al., 1998; Salamonsen et al., 1989).

The different types of PGs elicit different physiological responses. PGE_2, $PGF_{2\alpha}$, and $PGI_{2\alpha}$ (prostacyclin) are more relevant to reproduction. $PGF_{2\alpha}$, a vasoconstrictor, stimulates myometrial contractility and is responsible for luteolysis in most species (Niswender and Nett, 1988; Unezaki et al., 1996). PGE_2, a vasodilator, increases vascular permeability prior to implantation (Kennedy, 1983) and it locally modulates the immune system to prevent rejection of the conceptus in ruminants (Hansen et al., 1989). PGI_2 is a potent relaxing factor of smooth muscle cells of the uterus and the vascular system (Sinzinger et al., 1997).

2.4.2 Prostaglandins in implantation

PGs are involved in the process of blastocyst implantation in several species (Evans and Kennedy, 1978; Holmes and Gordashko, 1980; Kennedy, 1977; 1987; Labhsetwar, 1971; Lau et al., 1973). During pregnancy, the concentrations of PGs increase at implantation sites in several species

(Evans and Kennedy, 1978; Kennedy, 1977; Kennedy, 1994). In many species, both the embryo and the endometrium are able to produce from arachidonic acid (Figure 2-5; Dey et al., 1980; Poyser, 1981). These PGs are responsible for the increase in endometrial vascular permeability and subsequent decidualization (Kennedy, 1986; Kennedy, 1980; Kennedy, 1994). There is as yet no good evidence to indicate that blastocyst-produced PGs mediate the endometrial responses (Kennedy, 1994). Snabes and Harper (1984) suggested that the endometrium rather than the blastocyst is the major source of PGs involved in implantation and decidualization in rabbits. This hypothesis needs to be further investigated in other species.

Decidual cell reaction and implantation can be blocked or delayed by inhibitors of PG synthesis and PG antagonists. This blockade can be partially overcome by exogenous PGs in mice (Lau et al., 1973), in rats (Yee and Kennedy, 1988) and in rabbits (Hoffman et al., 1978). Although both $PGF_{2\alpha}$ and PGE_2 are able to restore the endometrial responses after inhibition of PG production, PGE_2 is, in general, more effective (Kennedy, 1994). PGE_2 is expressed at peak levels on day 5 and remains significantly higher throughout days 6-7 in rabbits (Pakrasi, 1997) and at a time when an increase in vascular permeability was observed (Hoffman et al., 1978). Rankin et al. (1979) reported that tranylcypromine, an inhibitor of PGI_2 synthesis, inhibited decidualization in mice. PGI_2, however, has not been shown to override effects of inhibitors (Rankin et al., 1979).

Production of PGs by blastocysts from mice, rat, rabbits, sheep, and cows has been considered as an embryonic signal (see review by Psychoyos et al., 1995). PGE_2 is seen to be produced by early embryos in rabbits (Dey et al., 1980) and horses (Weber et al., 1991). One role of PGE_2 is to increase endometrial sensitivity to PGE_2 itself at the time of the implantation, as seen in rabbits (Fortier et al., 1989). In the mouse, mRNA transcripts for the PGE receptor, but not for $PGF_{2\alpha}$ receptor, are present in the

endometrium during implantation (Lim and Dey, 1997; Yang et al., 1997). Fortier et al. (1990) suggested that PGE_2 of embryonic origin may be the signal for the presence of the embryos, and induces local alteration in the secretion of growth factors and nutrients and increases vascular permeability at the time of implantation or recognition of pregnancy in rabbits.

In ruminants, in which $PGF_{2\alpha}$ of uterine origin is luteolytic, local recognition of the presence of a viable embryo is necessary to prevent the release of $PGF_{2\alpha}$ and ensure establishment of pregnancy (McCracken et al., 1984). Administration of $PGF_{2\alpha}$ terminates pregnancy in several species (Horton and Poyser, 1976). Production of IFN-τ by trophoblast during preimplantation period (Bazer, 1992; Roberts et al., 1992) serves as the embryonic signal to prevent the luteolytic process from occurring by inhibiting the production of $PGF_{2\alpha}$ in ruminants (Danet-Desnoyers et al., 1994; Salamonsen et al., 1989).

In carnivores, PGE_2 has been identified in medium of mink uterine cell lines co-cultured with mink embryos (Moreau G, Song JH and Murphy BD, unpublished observation). However, it is not clear that PGE_2 is produce by embryo or by uterine cells. In ferret, the block of PG synthesis at preimplantation caused a delay in implantation (Mead et al., 1988). It has not been conclusively shown that PGs function via alteration of uterine vascular permeability in that study. Tsutsui et al. (1989) showed that administration of $PGF_{2\alpha}$ at early stage of pregnancy resulted in failure of implantation in bitches. However, this failure of implantation appears mostly to be caused by regression of the CL and inadequate P_4 production.

Zhang et al. (1996a) reported that PGE_2 regulates the in vitro secretion of uPA, at least partially by regulating the steady-state level of uPA mRNA. These results may account for the role of PGE_2 in the endometrial decidual cell reaction (Kennedy et al., 1989). PGs may

potentially regulate the balance of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in decidualization and trophoblast invasion. Although there is no typical decidual cell reaction in the mink, instead, the maternal symplasma may play a role in control of trophoblast invasion. Whether PGs are involved in the formation of symplasma and trophoblast invasion in carnivores requires more comprehensive investigation.

2.4.3 Biology of cyclooxygenase

COX, also known as PG endoperoxide synthase (PGHS), is the first rate-limiting enzyme in the biosynthetic pathway of PGs. It catalyzes the reaction whereby arachidonic acid is oxidized to endoperoxide which is then converted to PGs, prostacyclin, and thromboxanes (Williams and DuBois, 1996, Figure 2-5). Recent studies have established the presence of two distinct, but related isoforms of COX, referred as COX-1 and COX-2 (also known as PGHS-1 and PGHS-2, Williams and DuBois, 1996). These isoforms are encoded by two different genes (Fletcher et al., 1992; Kraemer et al., 1992). The primary structures of these two isoenzymes were shown to be about 60% identical, as determined from deduced aa sequences of complementary DNA (cDNA) in sheep (DeWitt and Smith, 1988; Merlie et al., 1987), in mice (DeWitt et al., 1990; Kujubu et al., 1991), and in humans (Funk et al., 1991; Hla and Neilson, 1992). Comparison of each COX isoform across species revealed a high degree of 90% of homology. Although the two isoforms are encoded by distinct genes located on different chromosomes (Funk et al., 1991; Xie et al., 1993), the major difference between the two isoforms is their different patterns of expression and regulation in mammalian cells. COX-1 is known as a constitutive enzyme and relatively unresponsive to stimuli (Williams and DuBois, 1996). The expression of COX-1 is associated with the endoplasmic reticulum (ER) in virtually every tissues, including the brain (Kawasaki et al., 1993), kidney and uterus (Wong and Richards, 1991), fibroblasts (Kujubu et al., 1991), platelets (Funk et al., 1991), endothelial

cells (Hla and Ncilson, 1992), and rat mesangial cells (Simonson et al., 1991). Due to its constitutive expression, it is considered a housekeeping gene involved in PG synthesis necessary for normal cellular processes. When the COX-1 gene is disrupted, mice survive, although they produce fewer live offspring than do wild-type mice (Langenbach et al., 1995).

In contrast, COX-2 clearly appears as an inflammation and/or mitogenic response gene; low or undetectable levels of COX-2 are dramatically induced during inflammation or after mitogenic stimuli. A variety of agonists, including growth factors (Sheng et al., 1997), cytokines (Huang et al., 1998; Pilbeam et al., 1997), endotoxins, gonadotropins, and GnRH have been shown to markedly induce the expression of COX-2 in fibroblasts (Kujubu et al., 1993), monocytes and macrophages (Hoff et al., 1993; Lee et al., 1993), endothelial cells (Habib et al., 1993), chondrocytes (Lyons-Giordano et al., 1993) and granulosa cells (Sirois and Richards, 1992; Wong and Richards, 1991; 1992). COX-2 is primarily associated with the nuclear envelope (cited by DuBois et al., 1998). Deficiency in COX-2 synthesis results in reproductive failure, which included failure of ovulation, fertilization, implantation and decidualization in mice (Lim et al., 1997). The two isoenzymes also differ in their susceptibility to glucocorticoids and nonsteroidal anti-inflammatory drugs (Levy, 1997).

2.4.4 Cyclooxygenase-2 in implantation

The role of COX-2 in reproduction, particularly in implantation has been observed in several species (Chakraborty et al., 1996; Charpigny et al., 1997b; Huang et al., 1998; Lim et al., 1997; Yang et al., 1997). Both implanted embryo and uterus are sites of COX-2 expression. Chakraborty et al. (1996) reported that COX-2 gene is expressed in the luminal epithelium and subepithelial stromal cells at the anti-mesometrial pole exclusively surrounding the mouse blastocyst at the time of attachment reaction on day 4. It persists through the morning of day 5, but it is not present in ovariectomized, steroid treated mice (Chakraborty et al., 1996). These results suggested that COX-2 expression requires the presence of

embryo in the mouse. In sheep, COX-2 is highly expressed in the trophoblast (Charpigny et al., 1997b) and in the uterine endometrium (Charpigny et al., 1997a) just prior to implantation. In humans, COX-2 was identified by immunohistochemistry in the uterine glandular epithelium throughout the menstrual cycle, in early pregnancy and in the decidual cells (Jones et al., 1997; O'Neill and Ford-Hutchinson, 1993). Together, the endometrial COX-2 expression may result in increased PG secretion from the pregnant uterus during early gestation in these species (Chakraborty et al., 1996; Charpigny et al., 1997b; Yang et al., 1997).

There is no direct evidence to show COX-2 expression in carnivores. In ferret, treatment of indomethacin reduced litter size but did not change uterine vascular permeability at the time of implantation (Mead et al., 1988). In the mink, we have observed that the presence of the mink embryos induces PGE_2 accumulation in the uterine stromal cell cultures (Moreau, Song, and Murphy, unpublished). This result suggested that the presence of mink embryo or an embryo released factor induce COX-2, resulting an accumulation of PGE_2 in the medium.

Regulation of COX-2 gene expression by several cytokines and growth factors has been shown. The expressions of IL-1β and TNFα in rat endometrial stromal cell line are associated with an increase in vitro $PGF_{2\alpha}$ release (Arslan and Zingg, 1996; Jacobs et al., 1994). Bany and Kennedy (1997) showed that EGF causes an increase in COX-1 and COX-2 gene expression in endometrial stromal cells isolated from the uteri of rats which have been sensitized for decidualization.

2.5 Characteristics of mink reproduction

2.5.1 Seasonality of reproduction

The annual reproductive cycle of the mink in the Northern Hemisphere comprises a long period of anestrus, which lasts from shortly after parturition in May through December. In January, after the winter solstice, the mink enters proestrus, a poorly defined period during which

the transition from anestrus to breeding competence is made. The breeding season is defined as the period of time over which the female will accept mating; this begins at the end of February and lasts through the third week of March (Enders, 1952; Hansson, 1947). Mating and ovulation can occur at any time and the mink can successfully conceive more than once in the breeding season.

2.5.2 Induced ovulation

The mink is an induced ovulator. Ovulation is stimulated by mating, specifically by the pressure of the baculum on the cervix (Enders, 1952; Hansson, 1947). Studies from other induced ovulators have shown that the neuroendocrine events triggered by mating are channeled through the hypothalamic-preoptic-pituitary-ovarian control system which operates under two conditions: (1) a steady state of basal (tonic) operation, which is responsible for follicular development; and (2) a transient phase, which leads to ovulation (Ramirez and Beyer, 1988). Variation exists between species in the pattern of mating behavior necessary to induce an ovulatory surge of LH as well as the timing of the LH peak and ovulation. Few studies on mating induced secretion of LH have been performed in the mink. It would nevertheless appear that cervical stimulation results in a release of LH within a few minutes of initiation (Murphy, 1988). In mink treated with gonadotropin releasing hormone (GnRH) early in the breeding season, elevations in LH levels were detected as soon as 15 minutes after the injection, with a peak level occurring at 45 minutes after the injection. Animals treated later in the breeding season displayed a slightly more prolonged response to the GnRH, but it uniformly induced ovulation at both times in gestation. Estimates of the time interval between mating and ovulation in mink have been reported to vary from 36 to 53 hrs post-coitum (Enders, 1952; Hansson, 1947). In other studies, by 48 hrs after mating virtually all mink have ovulated (Murphy, 1988; Douglas et al., 1994).

2.5.3 Delayed implantation

Mink gestation begins with fertilization of the oocytes in the oviduct (Hansson, 1947). The fertilized eggs develop to the blastocyst stage and move into uterus at 6-7 days after mating (Enders, 1952; Hansson, 1947). As soon as the blastocysts arrive in uterine lumen, their mitotic division ceases, and development is arrested for a period from 10 to 40 days or more, a period known as obligate delayed implantation or embryonic diapause (Enders, 1952; Hansson, 1947). The obligate delayed implantation in the mink is relatively shorter than in other carnivore species (Mead, 1993). In the western spotted skunk, the diapause has been shown to be as long as 200-220 days (Mead, 1968) while it lasts 220-260 days in the sable (Mead, 1989; Song and Tong, 1988)

2.5.4 Superfecundation and superfetation

Superfecundation and superfetation usually occur in mink due to induced ovulation and delayed implantation, respectively (Cavan, 1944; Enders, 1952; Hansson, 1947). Superfecundation is a result of fertilization ofthe oocytes from the same wave of follicles by the sperm from different males within two days. Superfetation takes place when a second mating fertilizes the second wave of follicles which have developed 6 days or more after the previous mating (Douglas et al., 1994; Shackelford, 1952). Usually 80-90 percent of offspring result from mating with the last male (Hansson, 1947).

2.5.5 Embryo development

Fertilization represents the beginning of life for a new individual. It comprises a series of steps beginning with sperm penetration of the egg coats, termed zona pellucida/capsule, followed by incorporation of the spermatozoon into the cytoplasm of the egg, and then the activation of the egg. In mammals, fertilization takes place in the oviducts at certain time after copulation depending on species. It occurs at 25-28 hours in the domestic cat (cited by Leiser and Koob, 1993). In the mink, fertilization takes place in the middle region of the oviduct within 12 hrs of ovulation

(Hansson, 1947; Murphy and Douglas, 1992), suggesting that the passage of the egg through the first half of the oviduct is rapid in the mink, similar to other species (cited by Hansson 1947).

Mink embryogenesis can be divided into four stages. The first is formation of blastocyst, a period that begins with fertilization and ends with the arrival of the blastocysts into uterine lumen. Cell cleavage of the fertilized ova occurs in the oviduct while they pass through the second half of oviduct down into the uterus. Fertilized embryos take 6-7 days to arrive into the uterine lumen (Hansson, 1947; Murphy and Douglas, 1992). Apparently, the passage of embryos takes place slowly after fertilization. The second stage is the obligate delayed implantation which begins with the blastocyst arrival into the uterus, ends with a renewal of development of the blastocysts. During delayed implantation, the development of blastocysts is arrested. The blastocysts are compacted and consist of 200-400 cells (Baevsky, 1963) and they are enclosed in zona pellucida. The diameter of the blastocysts ranges from 0.2 to 0.8 mm at this stage (Baevsky, 1963). There is a variation in delayed implantation which results in a variation of gestation length from 40 to 92 or more days in this species (Enders, 1952; Hansson, 1947). The third stage is the reactivation of development, a period between renewed mitosis of the blastocyst to implantation. It usually occurs 6-7 days after a marked increase of plasma P_4 level (Allais and Martinet 1978; Møller, 1973; Murphy and Moger, 1977; Pilbeam et al., 1979; Stoufflet et al., 1989). During the stage of reactivation, the diameter of the blastocyst increases from 0.9 to 2.0 mm (Stoufflet et al., 1989). Implantation usually occurs when the diameter of the blastocyst reaches 2.0 mm (Stoufflet et al., 1989). Finally, the fourth stage of gestation is postimplantation development, the period from initiation of implantation to parturition. This period is consistently by 30-31 days (Hansson, 1947; Murphy and James, 1974; Song et al., 1995).

3.0 ARTICLE ONE

THE MORPHOLOGICAL PROGRESSION OF TROPHOBLAST INVASION IN THE MINK *(Mustela vison)*

Jian H. Song and Bruce D. Murphy[1]

Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal;
3200 rue Sicotte, St-Hyacinthe, Québec, Canada J2S 7C6

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[1]Correspondence and reprint requests to B. D. Murphy, Fax (450) 778-8103
email: murphyb@medvet.umontreal.ca

3.2 ABSTRACT

The time of initiation of implantation is variable in species with obligatory delayed implantation, particularly in the mink. The uterine and embryonic changes associated with trophoblast invasion have not been well documented in this species. The aim of this study was to employ a large number of animals in hopes to describe implantation and placenta formation in the mink, with the particular goal of bracketing the early stages of trophoblast adhesion and invasion, to provide a better morphological description of this event than currently exists. Initiation of implantation begins when the blastocyst, enclosed in its capsule, has expanded to a diameter 2 mm and the the uterus has lost its endometrial crypts (Day 0 of implantation). After breaching the embryonic capsule, plaques of trophoblast adhere to the endometrial epithelium, principally on the lateral aspects of the antimesometrial surface. Day 3 postimplantation, the trophoblast has eliminated the epithelium and abuts directly on endometrial stromal cells. Maternal symplasma begin to form from the glandular epithelial cells as early as Day 4 after implantation. Formation of endotheliochorial placenta can been seen at Days 5-6; it is comprised of three zones, the maternal-fetal labyrinth, the symplasma and the maternal glands. A complete, functional placenta appears to be present by Days 11-12 after implantation when the diameter of the uterine swelling is 15 mm.

3.3 INTRODUCTION

In the mink (*Mustela vison*), gestation length is variable, ranging from as few as 42 to 90 or more days. In spite of mating dates that can vary from late February to late March, initiation of implantation is usually only observed after the vernal equinox in the North Hemisphere (Hansson, 1947; Murphy, 1982; Murphy and Douglas, 1992). It is known that fertilization takes place in the oviduct 12 hours after ovulation and that the embryos arrive in the uterine lumen at the blastocyst stage, 7-8 days post coitum (Hansson, 1947). When the blastocysts reach the uterus, their development is arrested and they remain in the uterine lumen, first in the cranial regions and later throughout the uterus. This occurs in every gestation in mink, and is therefore known as obligate delayed implantation. During diapause, the blastocysts remain encased in a glycoproteinaceous coat which is derived, at least in part, from the zona pellucida of the oocyte. The variation in gestation is due to the variation in the length of diapause, which can persist for a days to as much as 60 days or more under experimental conditions (Hansson, 1947; Enders, 1952, Murphy and James 1974). Postimplantation development is a consistent 30-31 days (Hansson, 1947; Murphy and James, 1974; Song et al., 1995). During diapause, the mitotic division of the blastocyst nearly ceases and growth is slow, any increase in size being the result of fluid uptake, rather than cell replication (Daniel, 1963). The resumption of blastocyst growth by implantation is related to reactivation of the corpus luteum and the consequent increases in progesterone secretion (Møller, 1973; Murphy and Moger, 1977; Pilbeam et al., 1979; Stoufflet et al., 1989). The plasma progesterone levels begin to rise 6-7 days before implantation, reach a peak at 20 days before parturition, then gradually decrease to basal levels (Allais and Martinet, 1978; Møller, 1973; Murphy and Moger 1977; Pilbeam et al., 1979; Stoufflet et al., 1989). The corpus luteum appears to be the sole source of progesterone, since removal of fetal-maternal complex by

hysterectomy did not affect the circulating progesterone profile (Song, 1994), and the enzymes necessary for steroid synthesis are not expressed in the fetal-maternal complex/placenta at any time during gestation in the mink (Song, 1994; Douglas et al., 1997). However, all attempts to induce implantation by administration of progesterone to ovariectomized mink (Hansson, 1947; Murphy et al., 1983) or to hasten it in intact females (Hammond, 1951; Cochrane and Shackelford, 1962) have failed. This suggests that other steroidal or non-steroidal luteal factors are required (Murphy et al., 1980; 1983) as shown in the ferret (Foresman and Mead, 1978) and the western spotted skunk (Huang et al., 1993; Mead et al., 1988).

The placenta of mink takes a zonary form, in that the chorionic villi or lamellae of the placental labyrinth are aggregated into a band of placental tissue which encircles the equatorial region of the chorionic sac. This form is also present in the dog and cat (Enders, 1957; Steven, 1975). The lamellar labyrinths are discontinuous mesometrially and are partially interrupted antimesometrially by a hematoma, which is also formed in the ferret (Lawn and Chiquoine, 1965). However, in the mink, the placental band is more complete than that in the ferret (Enders, 1957; Steven, 1975). Compared to the primates, the mink placenta is less invasive (Steven, 1975). In mink, the maternal capillaries become surrounded by trophoblast but are not penetrated, resulting in its classification as an endotheliochorial placenta (Steven, 1975).

Embryonic diapause and the attendant variability in the preimplantation interval have rendered it difficult to predict implantation and to establish the progression of events in the mink. The purpose of this study was to employ a larger number of animals in the hope of bracketing implantation and placenta formation in the mink, to provide a better morphological description of this event than currently exists.

3.4 MATERIALS AND METHODS

3.4.1 Animals

Primiparous Standard Dark variety female mink were maintained on a commercial farm (Visonnaire Richard, St-Damas, QC), under approved husbandry conditions. Breeding was performed by exposing the females to males every two days until mated during the breeding season, which begins in early March. Females were then remated to different males 7-9 days after the first mating according to accepted husbandry practice. All matings were confirmed by observation of sperm in vaginal smears. All animal treatment protocols were approved by the Faculté de médecine vétérinaire, Comité de déontologie, in accordance with the regulations of the Canadian Council of Animal Care.

3.4.2 Uterine sample collection and estimation of gestation stages

Uteri were collected from 3-5 pregnant mink randomly selected from the experimental population every 3 days, beginning at least 6 days after the final mating and continuing through the periods of embryonic diapause and implantation and into early postimplantation gestation. Animals were terminated by injection of euthanol (T-61, Hoechst, Regina, SK), the uteri were removed by midventral laparatomy and one side of the uterine horn from each animal was flushed by 5 ml of PBS. The embryos were collected and the diameters were measured by means of an ocular micrometer. The non-flushed uterine horn was collected for histological studies. The uteri containing blastocysts with diameter ranging between 0.2 to 0.8 mm were considered in diapause (Baevsky, 1963), and a range of 0.9-2.0 mm were considered in the peri-implantation phase (Stoufflet et al., 1989). In animals where implantation had occurred, uterine swellings indicative of implantation were measured by vernier caliper, and representative enlargements and adjacent tissue were collected and processed for histological evaluation. A visible implantation chamber, in the absence of evidence of trophoblastic invasion, was considered day 0 of implantation. Postimplantation age

was determined by swelling size and by embryonic characteristics, as previously described (Murphy and James 1974; Song et al., 1995).

3.4.3 Specimens collected

This report is based on 90 specimens derived from mink with embryos in diapause (n=5), activated embryos (n=5), Day 0 of implantation (n=3), Days 1 (n=2), 2 (n=3), 3-4 (n=4), 5-6 (n=4), 8-9 (n=3), 12 (n=2) and 13-15 (n=2) of postimplantation, respectively.

3.4.4 Tissue preparation

Uteri from delay, implantation chambers, swelling sites were collected for histological analysis. Tissues were fixed in Bouin's solution for 20-24 hours, dehydrated in ethanol, and embedded in paraffin. Six μm sections, three or more sections from each animal, were cut and the hematoxylin-eosin-safran staining procedure was performed.

3.5 RESULTS

3.5.1 Morphology of the uterus and embryo during delayed implantation

The mink blastocysts in diapause are readily flushed from the uterine lumen. They are enclosed in a capsule derived, at least in part, from the zona pellucida of the oocyte (Figure 3-1A). In sectioned embryos it is possible to discern both the trophoblast, which is comprised of discrete cells with elongated nuclei, and the inner cell mass with larger and more rounded nuclei. The trophoblast and inner cell mass could be easily distinguished in flushed blastocysts as well.

The lumen of the uterus during diapause displays prominent folds, resulting in formation of numerous crypts (Figure 3-2A). The epithelium which is tall columnar to palisade in form, overlies flattened stromal cells (Figure 3-2B). Highly convoluted uterine glands with basal regions are formed adjacent to vascularized layer of stroma, which is itself abutted against the myometrium. The endometrial stroma is interspersed among the glandular structures and numerous small blood vessels are present. The bases of the uterine glands are dilated and many contain a homogenous secretory product. The necks of the glands are confluent

with the uterine lumen at frequent intervals via gland outlets which have epithelium which is both continuous with and characteristic of the endometrial epithelium (Figure 3-2A and 3-2B). On the epithelial surface there appears to be a mucoid apical fringe.

3.5.2 The implantation chamber

During the expansion phase, the embryo enlarges as much as tenfold, embryos reach a diameter of 2.0 mm (Figure 3-1B). It is of interest that the zona derived capsule in these blastocysts is intact. Expansion engenderes morphological changes in the blastocyst, the most striking of which is the formation of trophoblastic plaques. While difficult to discern at the light microscopic level, it would appear that these structures are cytotrophoblastic, and that they have not yet become syncytical. Figure 3-3A is a photomicrograph of a plaque from an embryo in the process of expansion, in which basophilic nuclei with prominent nucleoli are present. Notable is the occurrence of a mitotic figure, indicating the division of the trophoblastic cells. During the later phase of expression, the trophoblastic epithelium becomes cuboidal to columnar at the sites of the plaque and displays a prominent brush border. The embryonic endoderm is first evident at these sites.

The uterus containing the expanded embryo comprises the implantation chamber (Figure 3-3B, defined as Day 0 of implantation). The folds have been eliminated on all but the region closest to the mesometrium. The endometrium is compressed on the antimesometrial side of the uterus and is considerably thinned relative to other regions of the uterus, at the expense of the layer of connective tissue that overlays the myometrium in the delay uterus. The uterine gland necks appear to be losing their convoluted aspect, as indicated by the presence of longer continuous openings into the epithelium in individual sections. There are remarkable and consistent changes in the basal regions of the glands. In addition to dilation, the glandular cells have enlarged to become tall columnar (Figure 3-3C). The nuclei of these cells have been displaced

from their parabasal location to the center of the cells, apical cytoplasm has become foamy, and there is a prominent brush border. Along with the presence of amorphous material in the dilated gland bases, these morphological changes are indicative of synthesis of a secretory product.

3.5.3 Apposition and adhesion

Day 1 of implantation has been defined as the day in which adhesion of embryo to the antimesometrial region of the implantation chamber occurs. The swelling diameter at this time is approximately 4.5 mm (Figure 3-4), and the antimesometral portion of the chamber is flattened (Figure 3-4A). The embryonic plaques, which will eventually invade the uterus, show extensive proliferation and lateral expansion (Figure 3-4B). In adjacent regions, the trophoblastic epithelium has become more columnar and is in direct apposition to the endometrial epithelium (Figure 3-4B and 3-4C). Numerous mitotic figures can be seen in the trophoblastic plaque, but none are in evidence in regions of the trophoblast other than the plaque. The embryonic capsule has disappeared at the sites of adhesion to the epithelium, but fragments could be observed in some specimens at regions adjacent to the trophoblastic plaque (Figure 3-4C). Its presence overlying of plaques of a blastocyst that is attached at other sites indicates that the process of capsule dissolution and attachment is not necessarily synchronous. Another change which accompanies the apposition phase is that the endodermal layer, which is squamous in form, has become evident just below the trophoblast and is present throughout the antimesometrial aspect of the blastocyst.

The embryonic characteristics of adhesion include interaction between apical membrane of trophoblast and the endometrial epithelium, an event which appears to occur exclusively in the areas between the openings of the uterine glands. There are a number of uterine changes present at this stage, including evidence of both hyperplasia and hypertrophy of the epithelium and gland neck cells (Fig. 3-4D). There are

numerous examples of nuclear hypertrophy in both of these cell types. The gland necks are straighter, and the gland bases show the same indication of secretory activity that was present in the implantation chambers. The stromal cells just below the epithelium appear less flattened. The presence of polymorphonuclear leucocytes in the stroma at basal regions of endometrium suggests their estravasation at the time of implantation.

3.5.4 Trophoblast invasion and elimination of the uterine epithelium

The earliest stage of invasion is derived from uterine swellings of approximately 5.5 mm in diameter (Day 2 postimplantation). In these samples, the trophoblastic plaque is intact and contains numerous mitotic figures. The formerly squamous regions of the trophoblast have become more cuboidal to have attached over a wide front. The endoderm is intact, and there is evidence of the first mitoses of the incipient mesodermal layer between the trophoblast and the endoderm. Fragments of the capsule can be found at regions where no attachment is present. At later stages of invasion in specimen, derived from uterine swellings of 6-7 mm diameter (Days 3-4 postimplantation), the proliferating trophoblast has replaced the endometrial epithelium and intrudes down the necks of the uterine glands (Figures 3-5, Day 2 and Figure 3-6, Day 3). At this time, organization of the embryo proper has advanced and the incipient neural tube is evident.

The uterine changes of early invasion include loss of cellular integrity of the epithelium, accompanied by apparent karyorrhexis of the nuclei at the surface in contact with the trophoblast. Gland necks have become much straighter and there is hyperplasia of the cells which line this section of the glands (Figure 3-5). The glandular fundi are less dilated, and their cells have lost the secretory morphology which were present at the embryo expansion and attachment phases of the implantation process. At later stages of invasion (Figure 3-6, Day 3) the epithelium is completely eliminated and the trophoblast abuts directly on stromal cells. The gland

necks become more elongated and dilated and the hyperplasia is evident in neck cells. The gland bases, however, are less dilated, and display no evidence of cell division.

3.5.5 Trophoblast intrusion and formation of the maternal symplasma

In uterine swellings of 6-7 mm diameter, representative of 3-4 days after implantation, the trophoblast has intruded into the endometrium by way of the necks of the uterine glands (Figure 3-7, Day 3). The invasive tissue is in the form of a villus which at its most invasive point, appears syncytial (Figure 3-8, Day 4) The remainder of the invasive trophoblast maintain the form of individual cells (Figure 3-7). There are cells within the lumen of the trophoblastic villus which have the characteristics of mesenchyme. The embryos present at the time of the early intrusion process have a prominent neural tube. At a later stage of gestation, represented by uterine swellings of 10 mm, and the embryo with a fetal rudiment, the trophoblastic morphology is similar, in that the definitive maternal-fetal labyrinth is not yet present. This indicates that the mature placenta has not formed by 6 days of postimplantation gestation.

The uterine response at the foci of invasion is the formation of the maternal symplasma, which is a syncytium derived from the cells of the gland necks (Figure 3-7 and 3-8). These structures are displaced ahead of the intruding trophoblast and mark the zone of transition between maternal and fetal tissues, and thus the limit of invasion. By the time that the trophoblast has intruded, the maternal glands have become elongated and grossly dilated from their base to their intersection with the maternal symplasma. The presence of mitotic figures demonstrates continued cell proliferation in the necks of the glands near their fundi. In regions of the uterus where no invasion has taken place, the glands are dilated from the base to the opening into the uterine lumen. Intact stromal cells are present at sites where there is trophoblast attachment, but not invasion, and no such stromal cells are readily identifiable at in the zone of invasion.

3.5.6 The maternal-fetal labyrinth and the definitive placenta

In later gestation, in uterine swellings of 10 mm or more (i.e. Day 6 or more), three zones are grossly evident in the placenta and uterus at the sites of attachment (Figures 3-9, Days 5-6 and Figure 3-10, Day 11). From the lumen to the myometrium these are: the maternal-fetal labyrinth, a junctional zone and the maternal gland zone. The labyrinth, which is derived from the invasive chorionic villi and maternal blood vessels, is separated from the allantoic cavity by the allantoic membrane. The embryonic component of the labyrinth is comprised of cytotrophoblast forming a three-dimensional network, and cytotrophoblast surrounding intact maternal capillaries (Figure 3-11, Day 10). In the developing labyrinth villar spaces fetal blood vessels, replete with nucleated erythrocytes can be found. These villar spaces display frequent, but apparently unconnected mesenchymal cells. Connective tissue is sparse in the extravillar trophoblast regions. In the latest placenta examined, representative of 12 days of postimplantation gestation, the terminal regions of the trophoblastic villi are dilated and abut directly on the maternal symplasma. The complete placenta has been observed at uterine swellings of 15 mm and an embryo with a crown-rump length of 4-5 mm. The embryo age was estimated as Day 11 of postimplantation (Figure 3-10).

At the junctional zone, the maternal symplasma persists in samples from later gestation, but the nuclei have become condensed and show signs of degeneration. In the latest samples examined, the nuclei are uniformly pyknotic and the cells are completely degenerated. At the interface between the symplasma and the intact glands, the nuclei of the glandular epithelial cells are enlarged, indicating a response to continued invasion of the trophoblast.

The maternal glands appear relatively straight. The cells of the necks and of the fundus are columnar and possess a brush border. As gestation proceeds, the maternal gland zone narrows, indicating that invasion has not been arrested. The glandular lumena are dilated,

particularly at the fundus. They contain detritus, comprised of amorphous matter and pyknotic cell nuclei, the same sort of material found in the placental hematoma.

3.6 DISCUSSION

Given the variability in implantation time resulting from embryonic diapause in the mink, acquisition of sufficient samples to study the progression of the trophoblast has been difficult. This is the first study to demonstrate the sequence of events which occur in the mink, and show that it has both similarity to and differences from the same process in other mammals.

3.6.1 Morphology of the blastocyst prior to implantation

The mink blastocyst of delayed implantation is surrounded by an acellular glycoprotein coat. This covering in mustelids is believed to be derived, at least in part, from the zona pellucida of the oocyte, and is therefore given the same name. Ultrastructural observations in the western spotted skunk (Enders and Mead, 1996) indicate that the acellular capsule that surrounds the embryo is trilaminar. Further, the difference in size between the oocyte and the expanded embryo, which may reach 2.0 mm, and yet be contained in the capsule, suggests that the glycoprotein coat could not be derived entirely from the zona pellucida. It is therefore necessary to invoke the deposition of glycoproteins during the process of blastocyst development in the formation of the capsule.

The light microscopic descriptions in the present investigation indicate that the structure of the embryo in diapause concurs with observations in other mustelids prior to implantation (Enders and Schlafke, 1972; Sinha and Mead, 1976). The trophoblast appears to be made up of discrete squamous cells, and the endoderm is not yet present. In expanded blastocysts found in implantation chambers, the trophoblastic plaques are comprised of cells that have become cuboidal-columnar in nature. This configuration is consistent with homologous structures in the western spotted skunk (Enders and Mead, 1996; Sinha and Mead, 1976)

and the ferret (Enders and Schlafke, 1972). Syncytiotrophoblast, but not cytotrophoblast is believed to be terminally differentiated in mammals (MacCalman et al., 1996), and the former has therefore exited from the cell cycle. In the present study, mitosis was present in trophoblastic plaques from expanded blastocysts, indicating that these elements have retained the capacity to divide, and may not yet be syncytial in nature during the period that the embryo remains enclosed in the capsule.

3.6.2 The endometrium during embryonic diapause

The description of the epithelium and glands during delay in the present study conforms with previous observations in the mink (Given and Enders, 1989; Murphy and James, 1974). The corpus luteum of the delay phase of gestation secretes progesterone (Moller, 1973, Douglas et al., 1998). While the level of this hormone is low relative to postimplantation gestation, there appears to be sufficient steroid support to bring about uterine development and maintain glandular activity (Given and Enders, 1989). This is indicated by the apparent steroid-dependent changes to the epithelium, such as the change of the epithelium from cuboidal to columnar and palisade.

The first indication of impending implantation is the formation of a chamber in the uterine lumen, presumably as a result of expansion of the blastocyst, as is seen in the ferret (Enders and Schlafke 1972). The principal change observed in mink uterus is in hypertrophy of the endometrial and glandular epithelium, particularly in the region of the gland necks. A second alteration, increased glandular secretion, particularly in the fundal regions, can be inferred from the secretory morphology which appears at this time. As it is known that ovarian steroid levels are increasing during the periimplantation phase (Stoufflet et al., 1989), it is reasonable to implicate progesterone as a causative agent in these endometrial changes. However, these changes appear more pronounced at the sites of embryo apposition. It may therefore be that factors from the blastocyst, or secreted by the uterus in response to the

blastocyst, are equally responsible for periimplantation endometrial differentiation.

3.6.3 Adhesion and intrusion of the trophoblast

In this study we observed trophoblastic plaques that had penetrated the capsule and adhered to the endometrial epithelium coexisting with plaques that were yet enclosed. This indicates that breaching of the capsule by the trophoblast is not a synchronous event in the mink. This concurs with observations of mink blastocyst hatching *in vitro* where there appears to be focal thinning and breaching of the capsule at a single site (Moreau et al., 1995), and with observations of implantation in the ferret, where thinning of the capsule occurs on the lateral aspects of the implantation chamber (Enders and Schlafke 1972). It is believed that localized dissolution of the capsule, accompanied by pressure brought to bear by the swollen blastocyst results in its rupture (Enders and Schlafke 1972).

Rodents and humans undergo the much-studied decidual reaction, consisting of remodelling of stromal cells in the subepithelial region of the endometrium (reviewed by Abrahamsohn, 1993). This reaction can be induced in pseudopregnant rodents by physical stimuli such as scratching the endometrial surface of the endometrium or interlumenal oil administration (Parr and Parr, 1989). Enders (1957) alluded to a decidual response in the mink uterus, which he described as epithelial in nature. In the present study, no stroma-derived decidual cells could be identified in the mink placenta at any stage of gestation. This is in contrast to the domestic cat, where there appear to be fibroblast-derived homologues of rodent decidual cells present at sites of implantation (Leiser and Koob, 1993). Both maternal and fetal origins have been speculated for these cells, and their true progenitors remain uncertain (Leiser and Koob, 1993). Nevertheless, it is significant that they are few in number and only focally present in the cat placenta.

The absence of a classic decidual reaction and formation of decidual cells in mink has implications for the process of implantation. In rodents, the mode of penetration of the uterine epithelium is by displacement, which occurs across a broad front (Reviewed by Schlafke and Enders, 1975). The consequence is apoptic cell death of the epithelial cells followed by their phagocytosis by trophoblast cells (Parr and Parr 1989). This process is aided by the decidual reaction in two ways, first, decidual changes appear to free the epithelium from its basal lamina resulting in more ready displacement, and second, the decidual cells may cause death of the uterine epithelial cells (Parr and Parr 1989). In mustelid carnivores, the implantation process is described as intrusive (Schlafke and Enders 1975). The syncytiotrophoblast first adheres to the epithelium and then invades between cells, unaided by any obvious changes in either the basal membrane or underlying stroma.

3.6.4 Formation of the maternal symplasma

The present investigation indicates that the maternal symplasma is found at the limit of invasion of the trophoblast into the endometrium. This tissue is a syncytium derived from the hypertrophied cells of the gland necks that have been displaced by the trophoblast. The first changes indicative of symplasma formation is the aggregation of gland cell nuclei soon after the initiation of trophoblast intrusion. The nuclei of the symplasma are displaced away from the lumen of the mink uterus, becoming progressively more degenerate as gestation proceeds. They are nearly completely pyknotic or disintegrated in later specimens. Similar structures are seen in the ferret (Lawn and Chiquoine, 1965) and hyena placentae (Morton, 1957) but are less evident in the cat (Leiser and Koob, 1993). The function of the symplasmae, beyond their obvious role as a mechanism of elimination of the endometrium displaced by the invading trophoblast, is not known. In the cat (Leiser and Koob, 1993) and the ferret (Lawn and Chiquoine, 1965), trophoblastic invasion of the endometrium persists through gestation, resulting in progressive

reduction of the maternal component of the endometrium. In the mink and ferret, the symplasmae mark the interface between the trophoblastic front and the uterine glands and may play a role in limiting or slowing the rate of trophoblastic invasion.

3.6.5 The structure of the maternal-fetal labyrinth.

The endotheliochorial placenta of carnivores has been described as intermediate between the epitheliochorial placenta of the pig and the hemochorial placenta of rodents, primates, rabbits and many other species (Steven 1975, Lawn and Chiquoine, 1965). The region of exchange of nutrients, gases, metabolites etc. in carnivores is the maternal-fetal labyrinth, where maternal capillaries are surrounded by trophoblastic syncytium, which is in contact with the chorionic blood vessels. In the ferret, the synctiotrophoblast removes both glandular and stromal tissue, leaving only the maternal blood vessels intact in the mature placenta (Gulamhusein and Beck, 1975). In both the ferret and mink, there is hypertrophy of the maternal endothelial cells (Lawn and Chiquoine, 1965; Enders, 1957). In the ferret, the developing labyrinth can be seen within the first week after implantation (Gulamhusein and Beck, 1975). The labyrinth in mink in its lamellar configuration was first seen in specimens from uterine swellings of 6-8 mm, i.e. Days 5-6 after implantation, also within the first week after implantation occurred. The change in the placenta as gestation progressed was largely due to an increase in the width of the lamellar labyrinth, resulting from increased trophoblast invasion. This concurs with results in the cat (Leiser and Koob, 1993) and ferret (Lawn and Chiquoine, 1965).

In summary, we report morphological changes which accompany implantation in the mink, providing new information on the adhesion process, and on the formation of the symplasma and the maternal-fetal labyrinth. Embryo expansion and focal escape from the embryonic capsule results in the attachment and adhesion of trophoblast to epithelium. Trophoblast plaques contact and invade the uterine endometrium.

Maternal symplasma are formed as early as Day 4 of postimplantation gestation while the maternal-fetal labyrinth can be seen at Days 5-6. Although the formation of placenta was first observed at Days 5-6, a mature placenta was only seen at Day 11 and later on postimplantation gestation.

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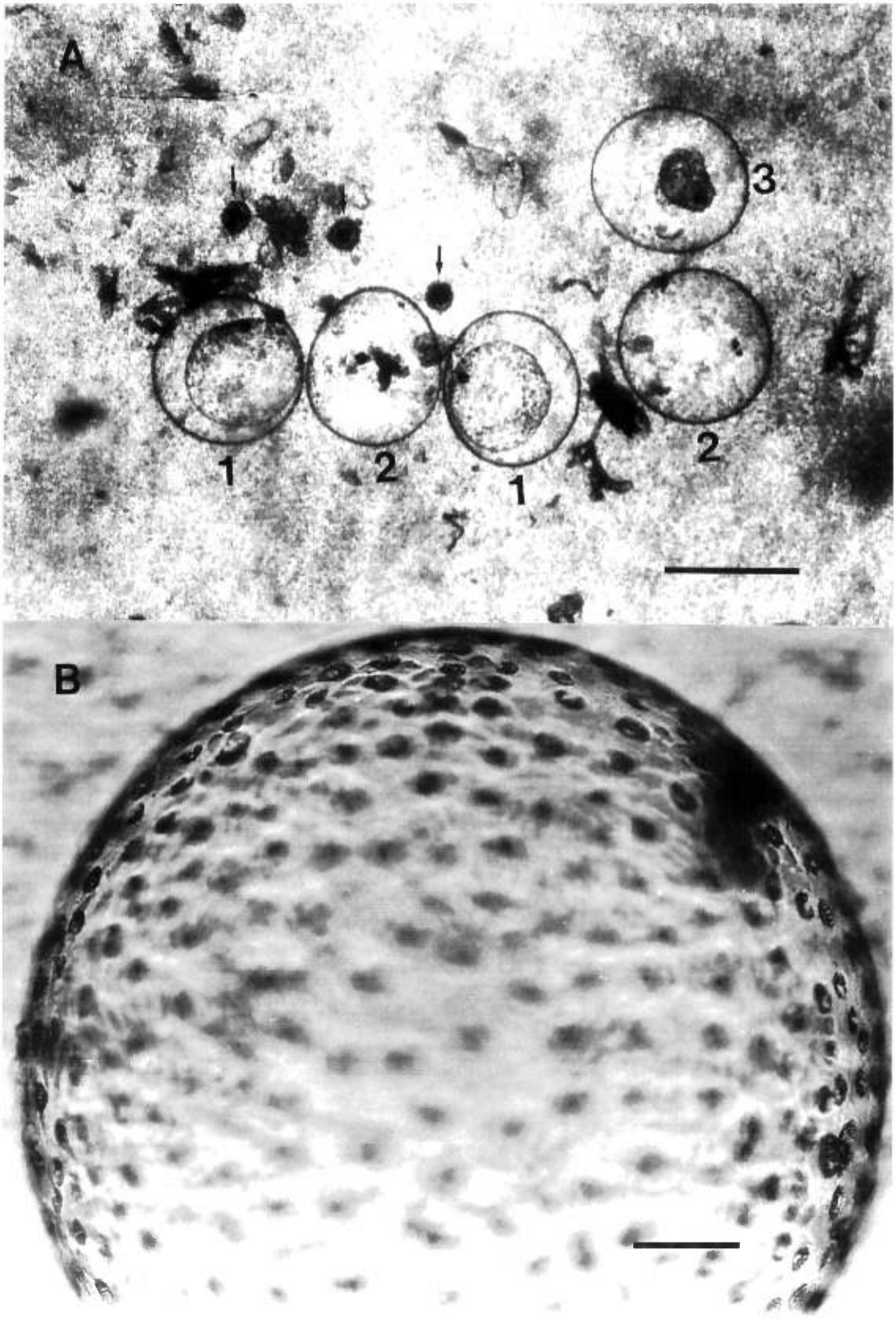
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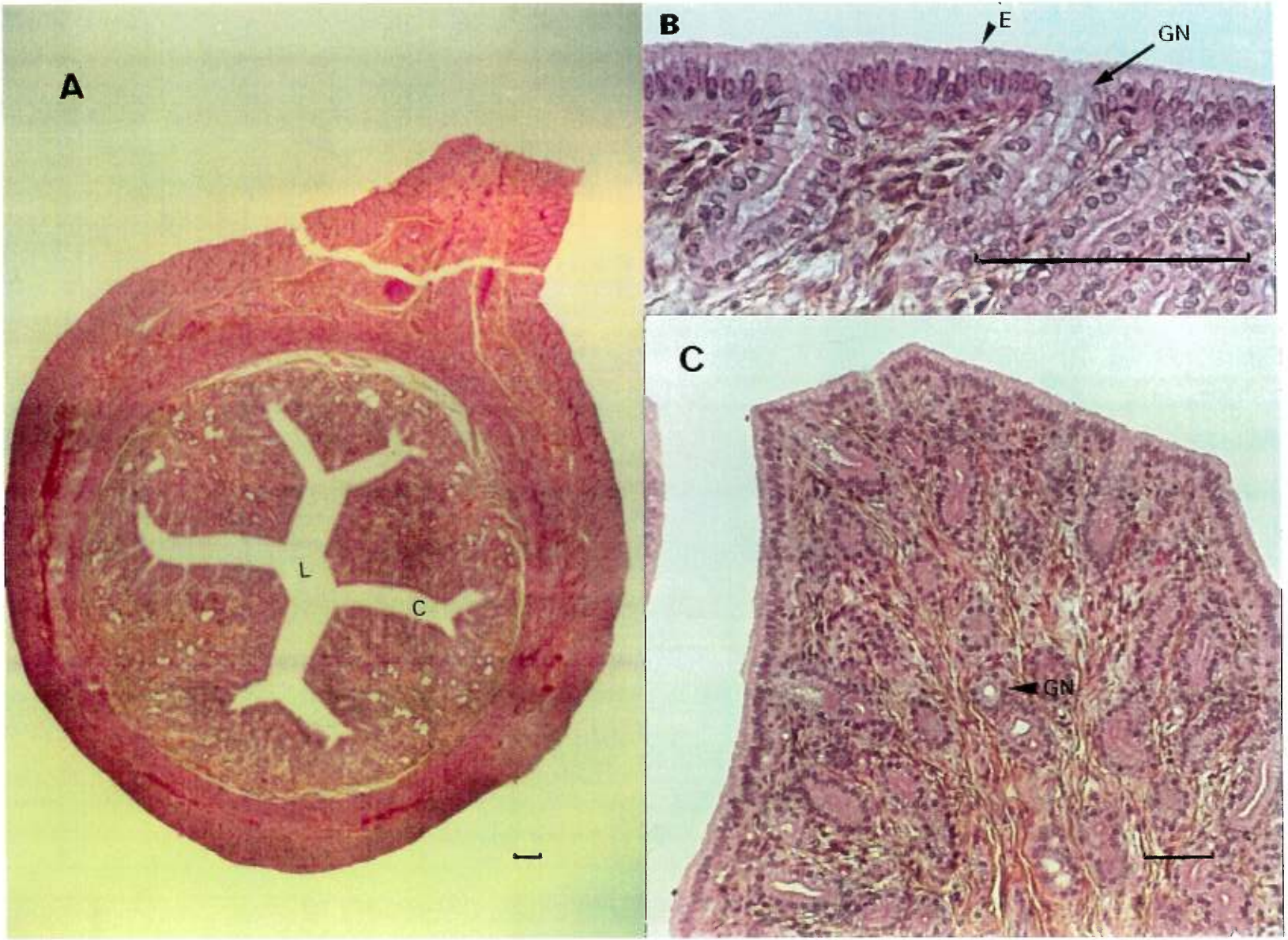
Figure 3-1. Blastocysts from mink after isolation from the uterus. 1A. Five blastocysts in the first hour after flushing, some of which have shrunk (designated 1 and 3), presumably from osmotic shock, and some which remain intact (designated 2). The arrows indicate unfertilized oocytes. 2B. Expanded blastocyst after 6 days in co-culture with BRL cells, showing a thinned capsule and monolayer of trophoblast. The horizontal bar is a measurement of 200 μm in 2A and 250 μm in 2B.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GN

Figure 3-2. The uterus of the mink during the period of delayed implantation. 2A. Cross section of the uterus showing lumen (L) and crypts (C) resulting from folding of the endometrium. 2B and 2C. The lumenal surface of the uterus showing palisade configuration of epithelial cells (E) and gland necks (GN) Figure 2C demonstrates the convolution of the gland necks because a continuous glandular lumen is not evident. The bar represents 10 μm.

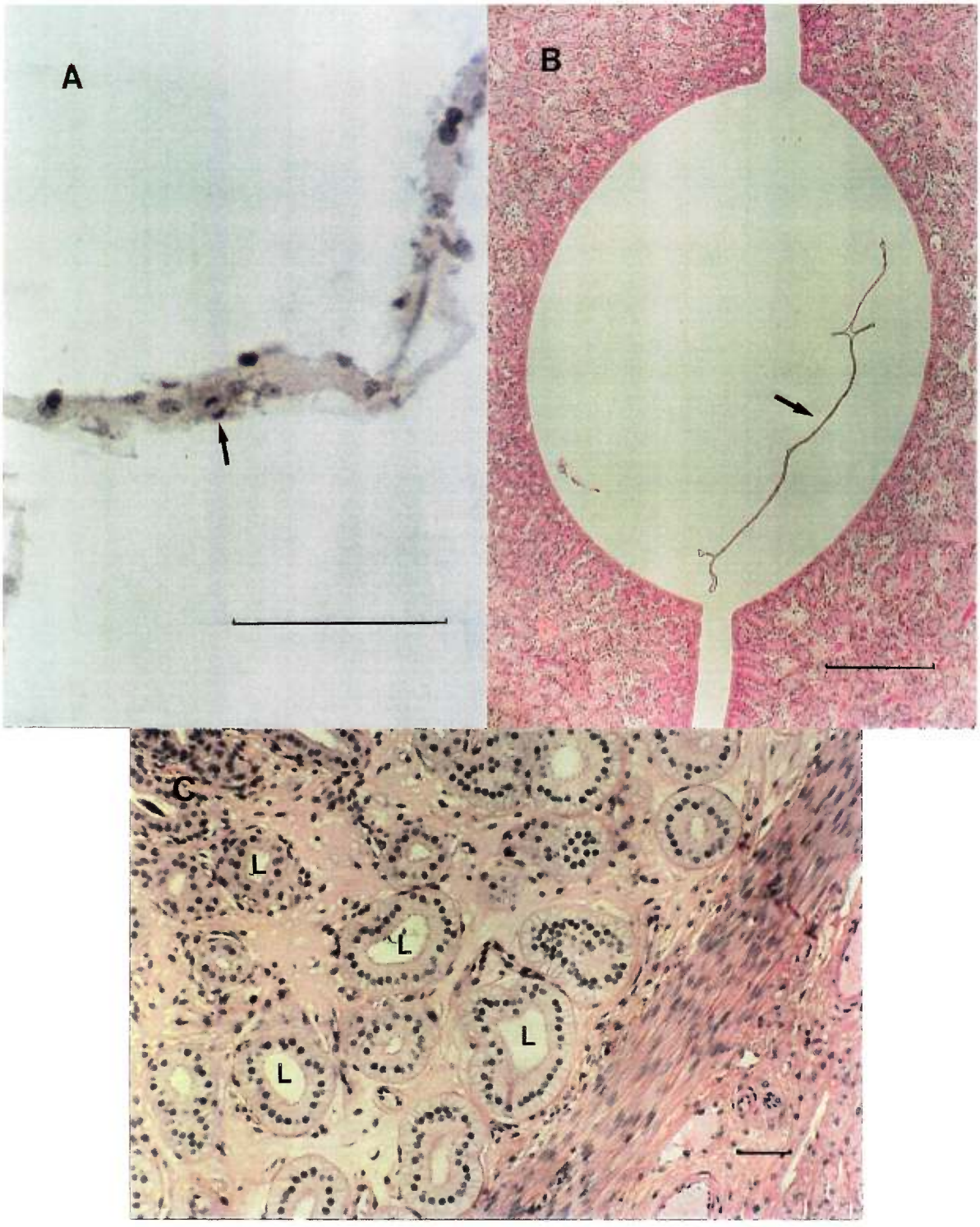
A
B
C
L
L
L
L

Figure 3-3. The mink uterus and embryo at the time of blastocyst expansion and implantation chamber formation. 3A. Close-up of the trophoblastic plaque of an expanded, unimplanted embryo found in an implantation chamber. Note the presence of a mitotic figure (arrow). The horizontal bar represents 10 μm. 3B. Cross section of the uterine implantation chamber containing an embryo (arrow) which has collapsed during histological preparation (defined as Day 0 of postimplantation). The horizontal bar represents 50 μm. 3C. The fundi of uterine glands demonstrating columnar gland cells, central nuclei foamy cytoplasm and a pronounced brush border indicative of secretion. The glandular lumena are designated by L, and the horizontal bar represents 10 μm.

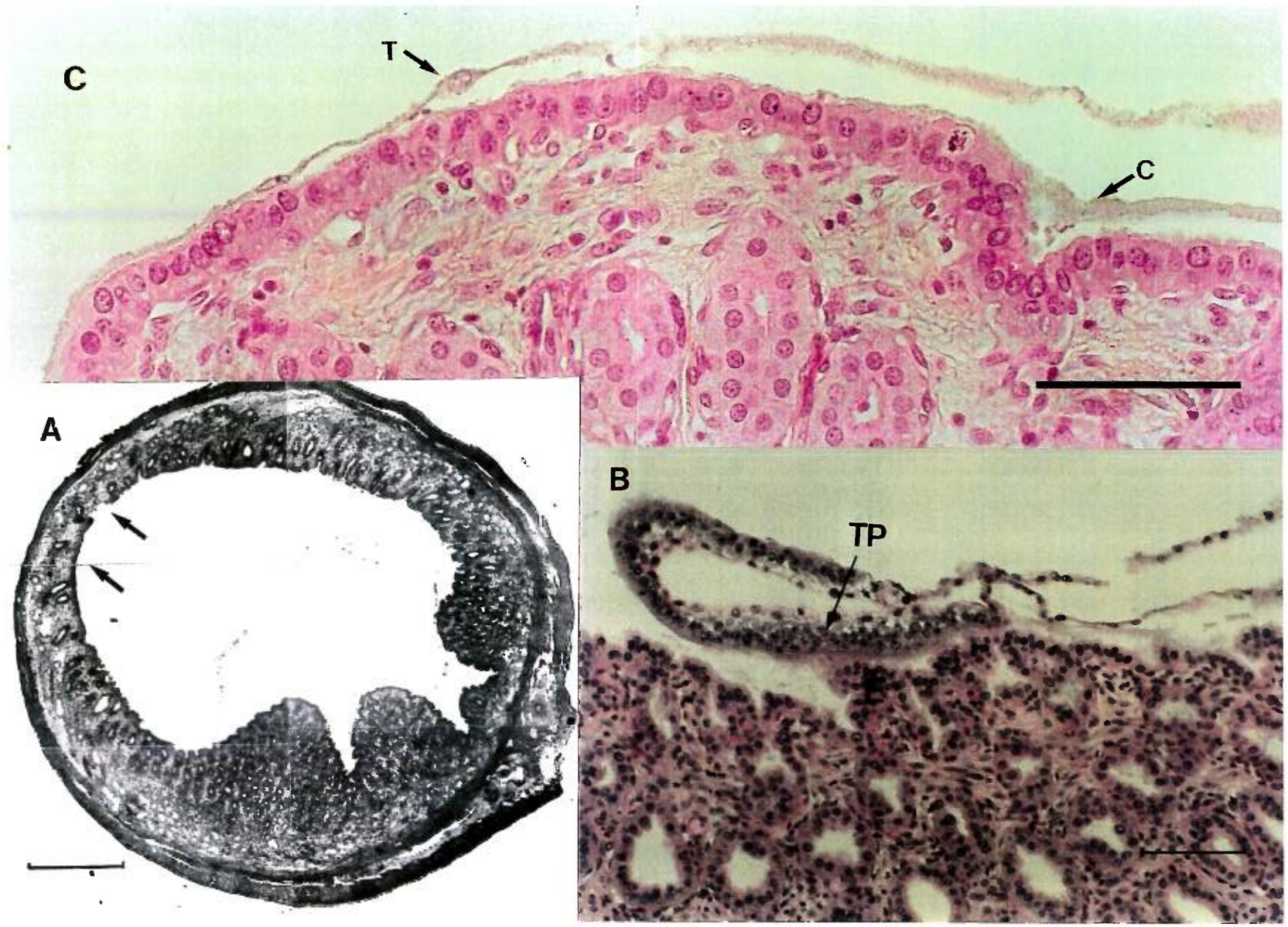
C
T
C
A
B
TP

Figure 3-4 A, B, C. Uterus at the time of adhesion of the trophoblastic plaques to the endometrium. 4A. Cross section of the uterus showing the flattening of the antimesometrial aspect of the endometrium at the site of embryo attachment (arrows) and the apparent straightening of the endometrial glands. 4B. Attachment of a trophoblastic plaque (TP) to the lumenal epithelium (E). 4C (Day 1). Apposition of squamous portions of the trophoblast (T) to the endometrium and a fragment of the embryonic capsule (C). The horizontal bar represents 100 μm 4A and 10 μm in 4B, 4C.

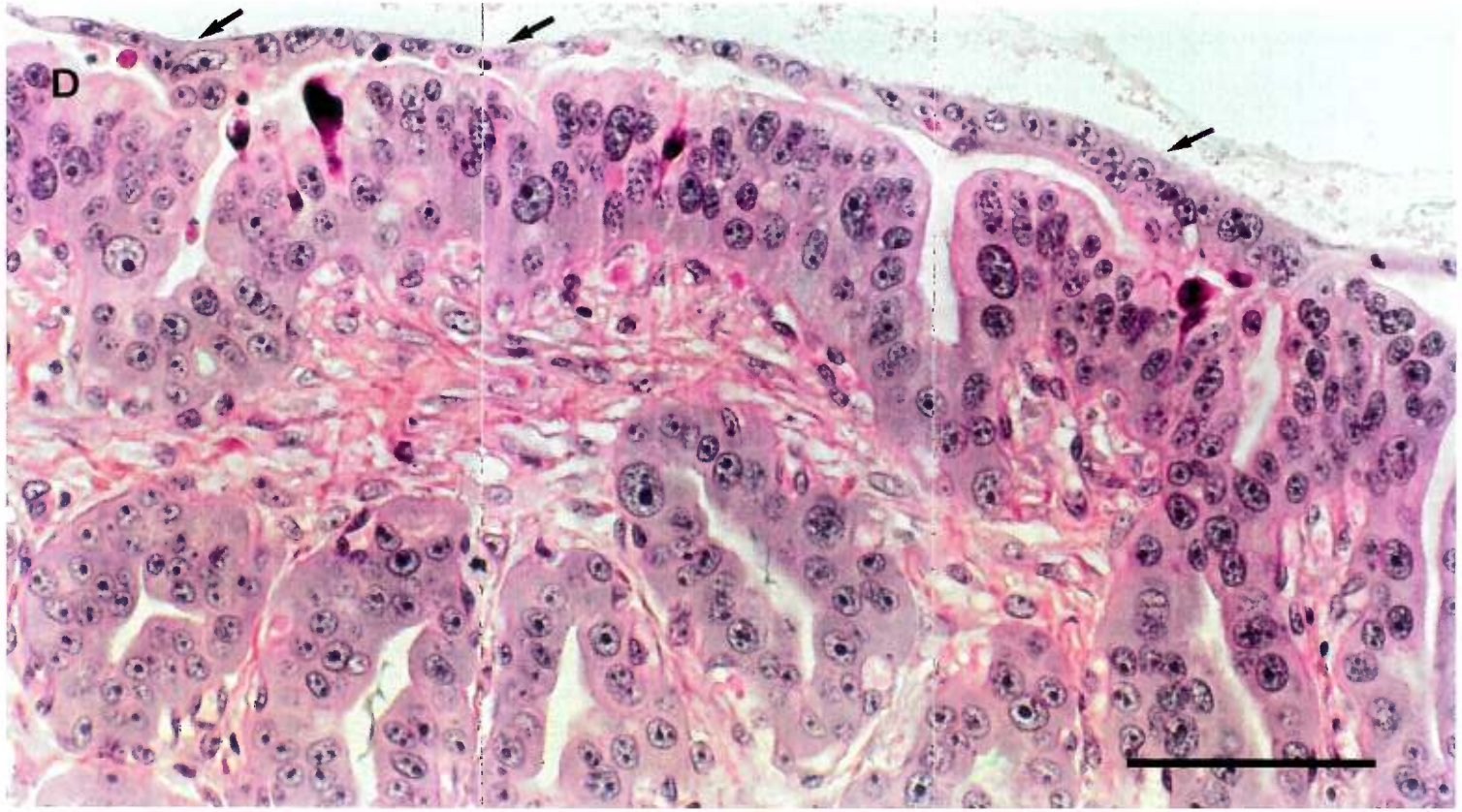
D

Figure 3-4D. In panel 4D, the attachment of trophoblast to the uterine epithelium is evident (arrows). The horizontal bar represents 10 μm in 4D.

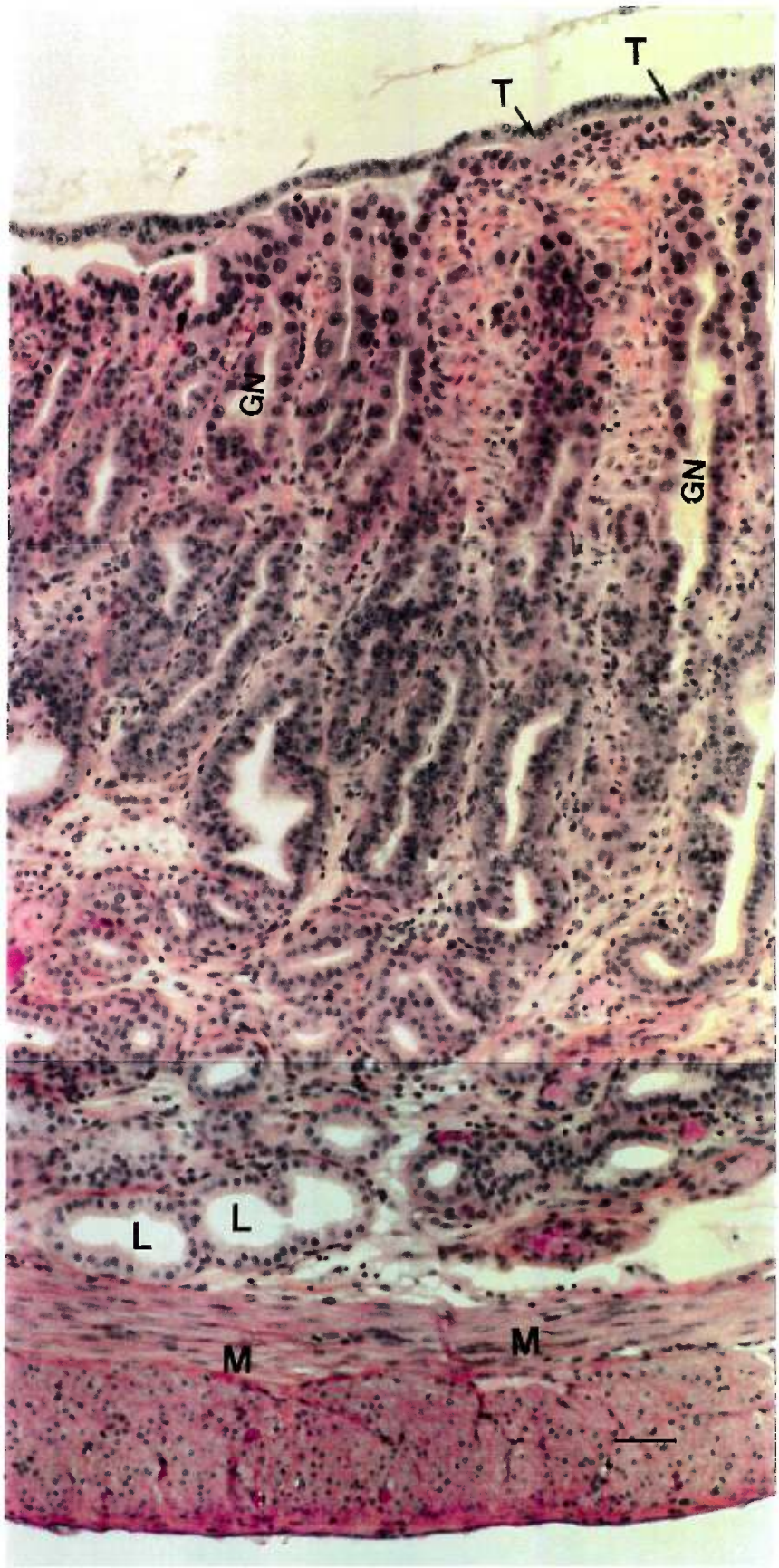
T
T
T
GN
GN
L
L
M
M

Figure 3-5. The uterus during the period of early invasion of the trophoblast (Day 2). This cross section demonstrates the trophoblastic replacement of the uterine epithelium (T), the lengthening and straightening of the uterine gland necks (GN) and the dilated fundi of the uterine glands (L), the letter M is present to indicate the circular layer of the myometrium. The horizontal bar represents 10 μM.

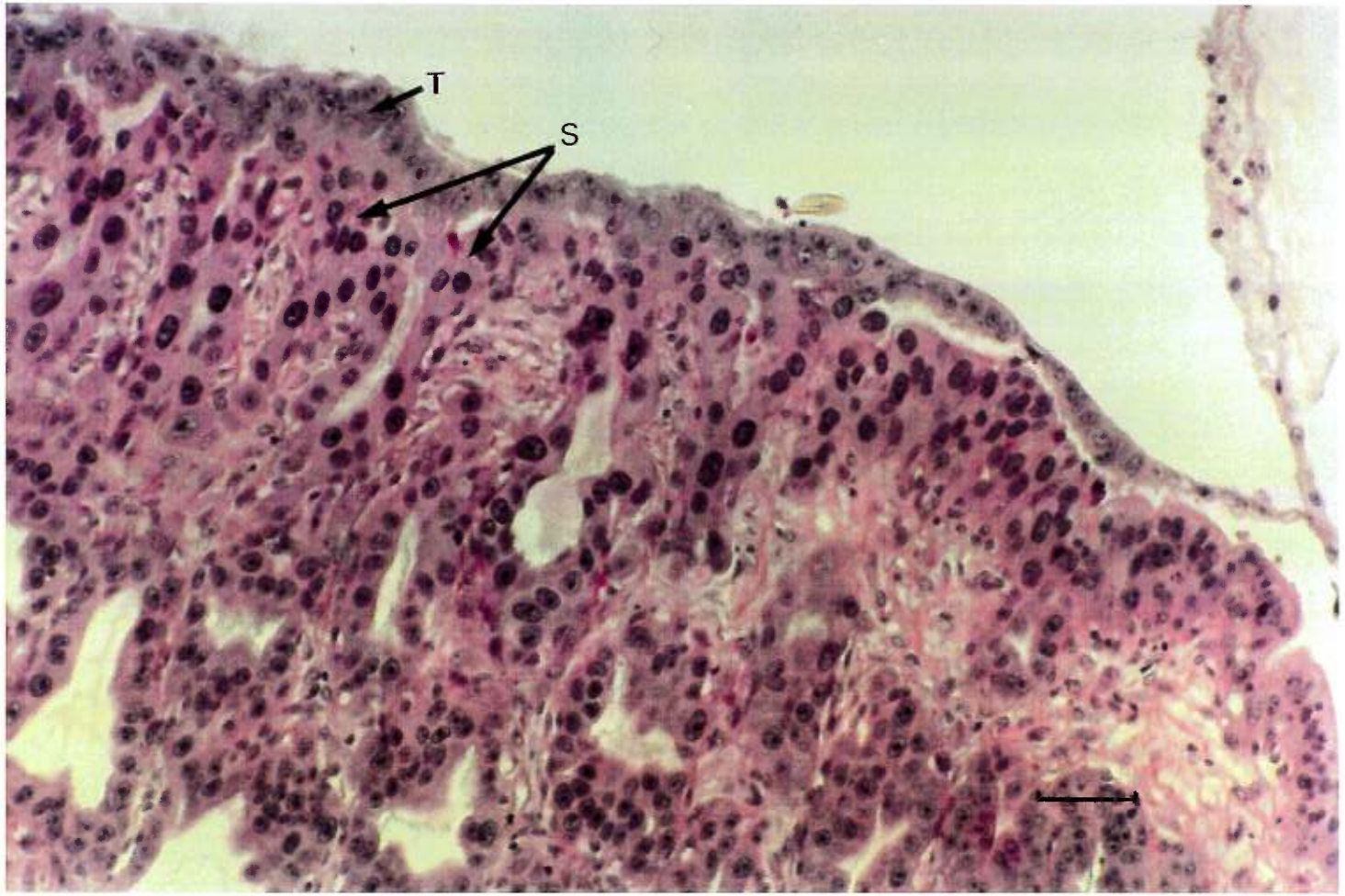
T
S

Figure 3-6. Higher power view (Day 3) of the endometrium close to the lumen of the uterus showing early trophoblastic invasion and replacement of the uterine epithelium (T) and direct abutment of the trophoblast on the endometrial stroma (S). The horizontal bar indicates 10 μm.

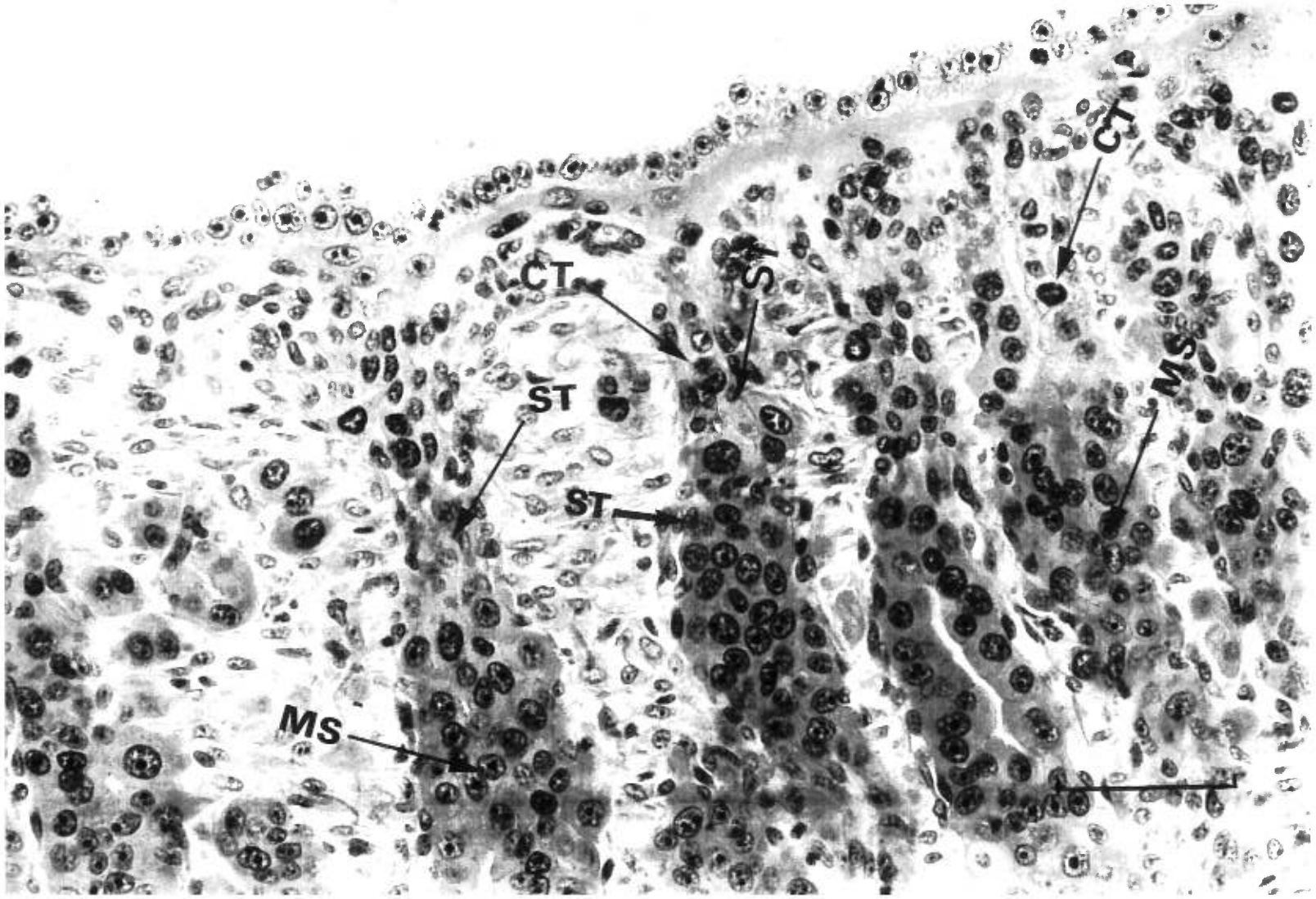
CT
CT
CT
ST
ST
MS
MS

Figure 3-7. Intrusion of the trophoblast into the endometrium (Day 3). Trophoblastic villi comprised of cytotrophoblast (CT) with syncytiotrophoblast at the leading edge (ST) have invaded down the necks of the uterine glands. In advance of the invasion are aggregations of nuclei displaced from the uterine gland necks which are the incipient maternal symplasma (MS). The bar indicates a distance of 10 μm.

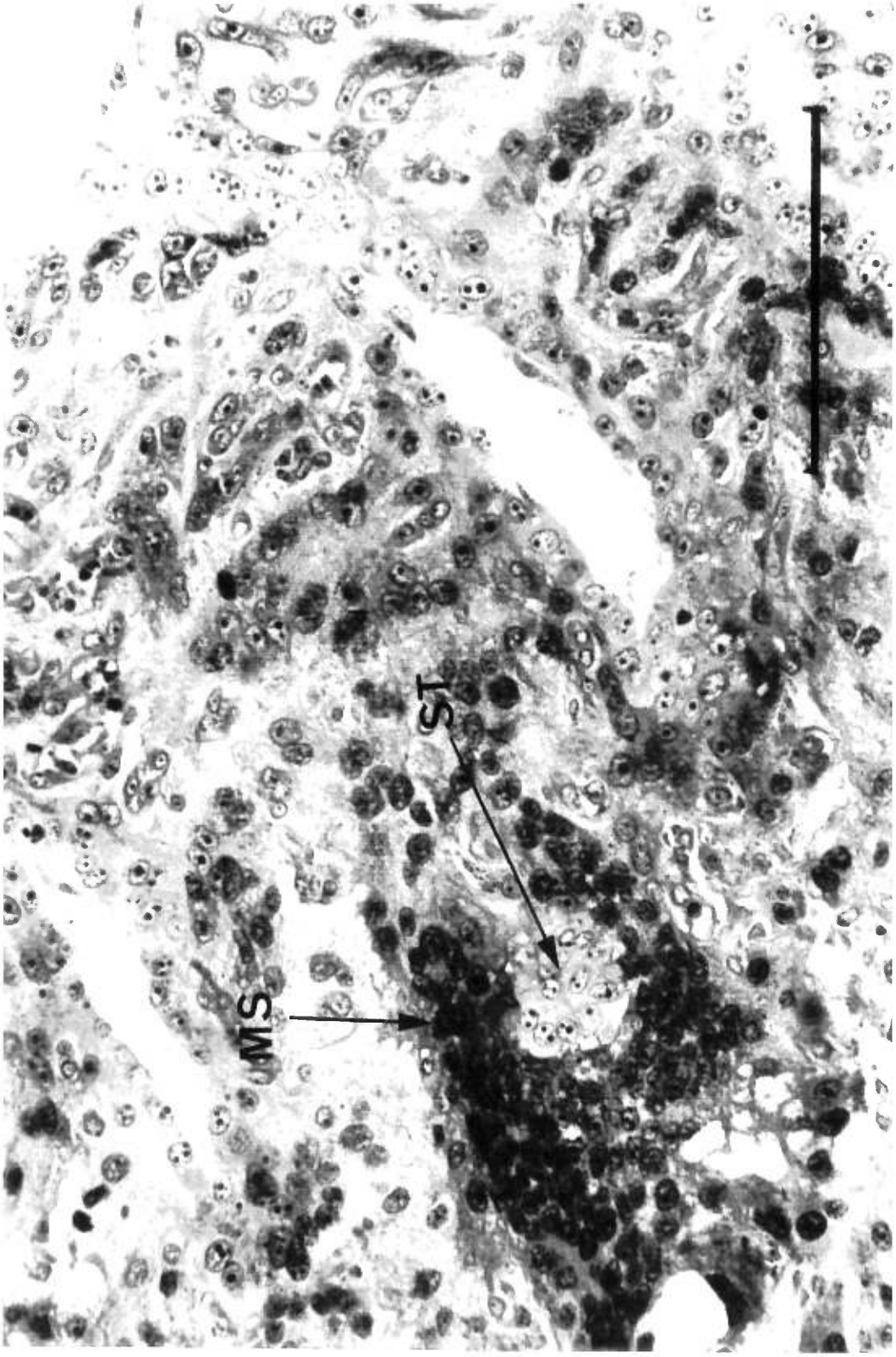
ST
MS

Figure 3-8. Maternal symplasma (MS) surrounding the syncytiotrophoblastic (ST) projection of the trophoblastic villus during the intrusion phase of mink implantation (Day 4). The bar represents 10 μm.

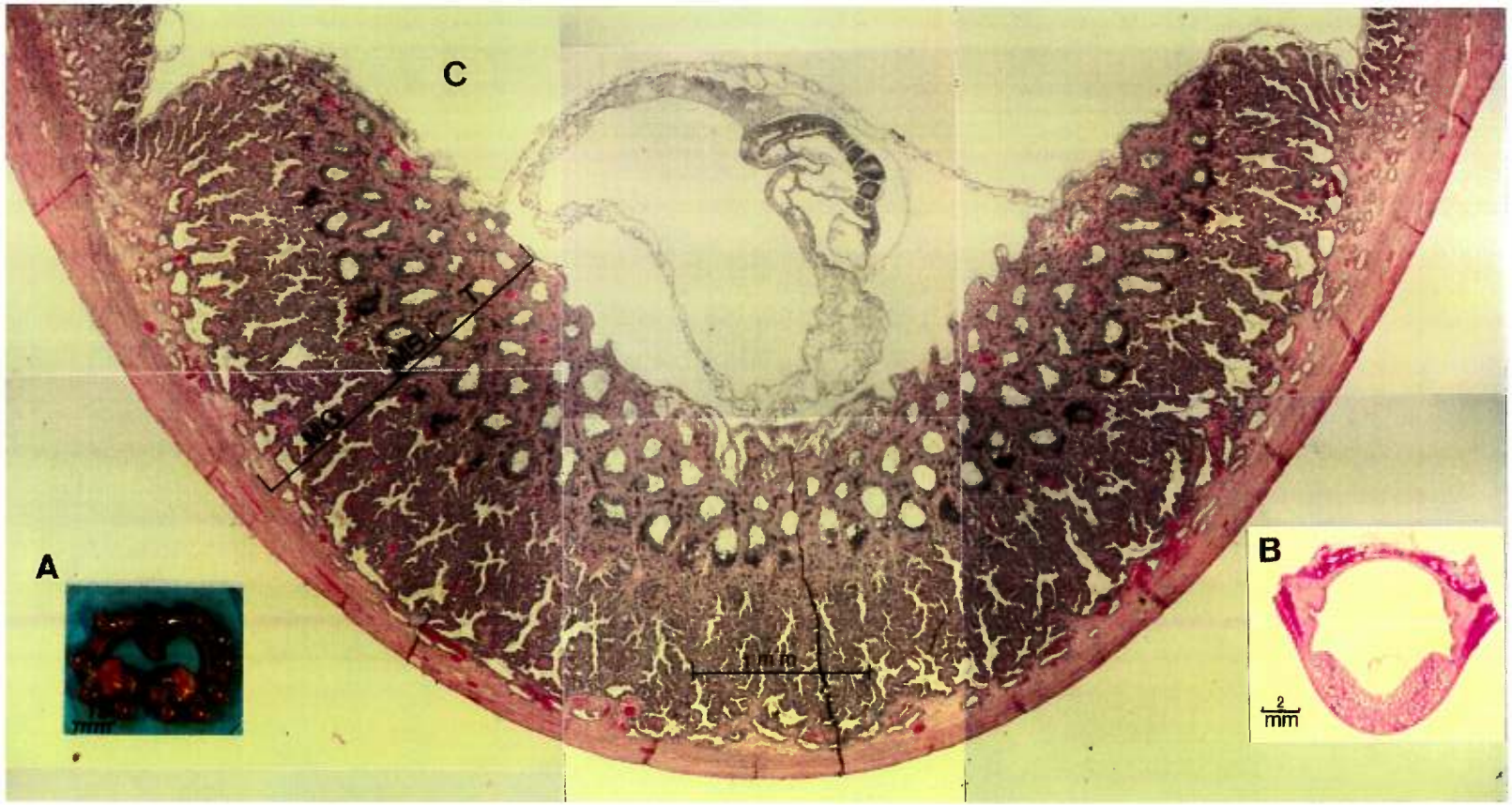
C
A
B
1 mm
2
mm

Figure 3-9. The mink uterus during invasion of the trophoblast and establishment of the placenta (Days 5-6). Panel 9A shows the uterus at excision from the pregnant animal, while 9B provides an overview of a section of one of the uterine swellings demonstrating the zonary nature of the placenta. A composite photo demonstrating the placenta can be found in 9C. Three regions are evident, that of trophoblastic invasion (T), the maternal symplasma (MS) that delineates the limit of invasion, and the maternal uterine glands (MG) overlying the myometrium. Portions of the fetus and the fetal membranes can be seen in the uterine lumen.

A
B
C
T
MS
MG
1mm
1mm

Figure 3-10. The definitive mink placenta (Day 11). A cross section showing the three regions described in the legend to 9C above is present in 10A. The region of invasion is now a functional fetal-maternal labyrinth. 10B, an overview showing the region from which the section was taken and Figure 10C demonstrates the complete uterus from which the swellings were derived.

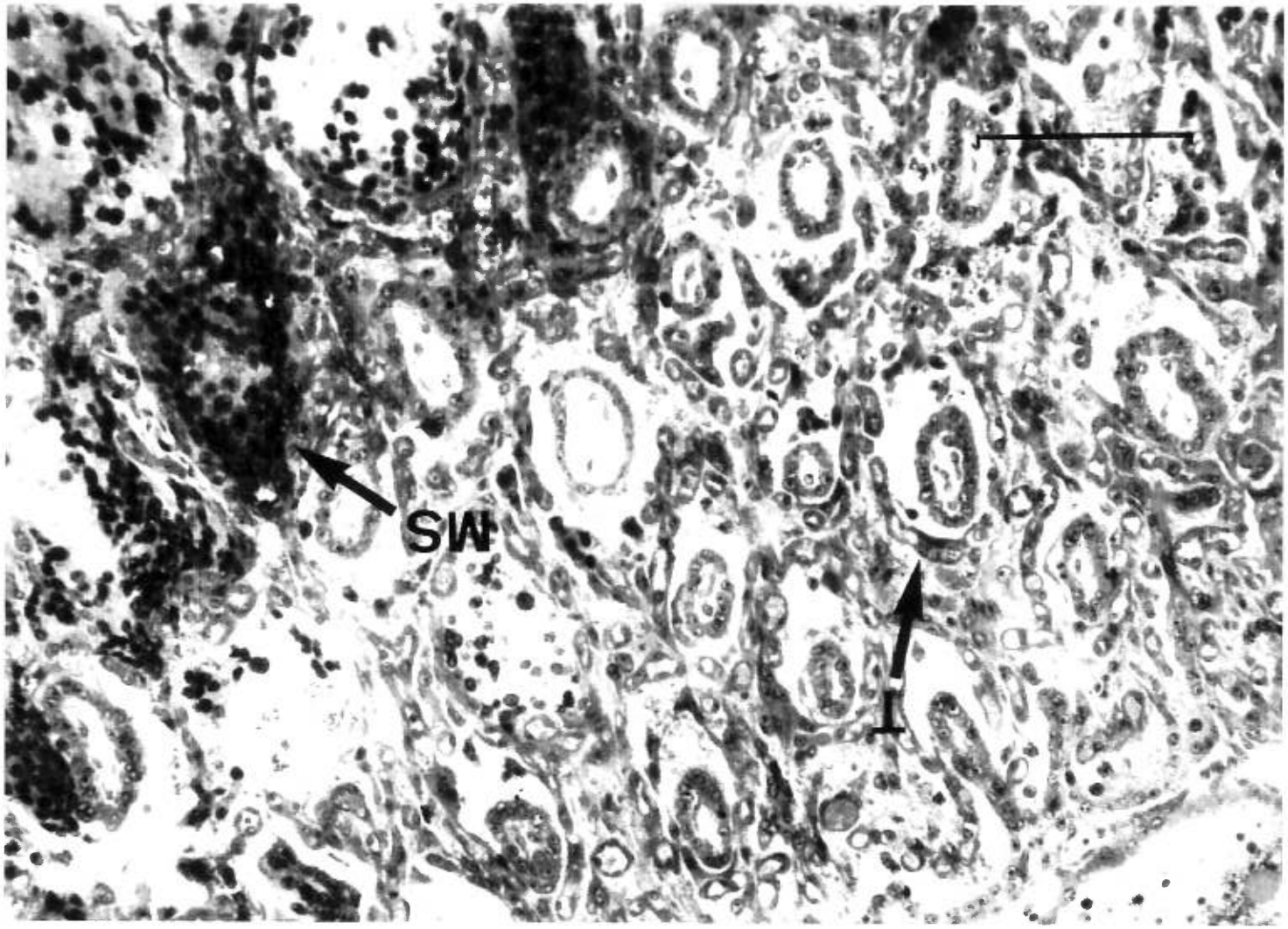
SW

Figure 3-11. Higher power of the fetal-maternal labyrinth (Day 10 postimplantation) showing the trophoblast surrounding the maternal capillaries and sinusoids (T) and maternal symplasma (MS). Bar represents 20 μm.

Cloning of Leukemia Inhibitory Factor (LIF) and Its Expression in the Uterus During Embryonic Diapause and Implantation in the Mink (*Mustela vison*)

JIAN H. SONG, ALAIN HOUDE, AND BRUCE D. MURPHY*

Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Quebec, Canada J2S 7C6

ABSTRACT Leukemia inhibitory factor (LIF) is essential for embryo implantation in mice. Whether LIF plays a role in termination of embryonic diapause and initiation of implantation in carnivores, especially in species with obligate delayed implantation such as the mink, is not known. The objectives of this study were to clone the LIF coding sequence in the mink and determine its mRNA abundance in the uterus through embryonic diapause, implantation, and early postimplantation. We show that the mink LIF cDNA contains 609 nt encoding a deduced protein of 203 amino acids. The homologies are 80.6, 90, 88.2, 87.6, and 86.8% in coding sequence and 79.2, 90.1, 91, 90.1 and 85.4% in amino acid sequence with mouse, human, pig, cow, and sheep respectively. Glycosylation sites and disulfide bonds present in other species are generally conserved in the mink LIF sequence. Quantitation by polymerase chain reaction amplification indicates that LIF mRNA is expressed in mink uterus just prior to implantation and during the first two days after implantation, but not during diapause or later postimplantation pregnancy. The abundance of LIF mRNA was significantly higher in the uterus at the embryo expansion stage ($P < 0.05$) than at days 1–2 ~~of postimplantation~~ after implantation. By immunohistochemical localization it was shown that LIF is expressed in the uterine epithelial glands at time of embryonic expansion and in early postimplantation. The coincidence of LIF expression with implantation in this species suggests that LIF is involved in the implantation process, and may be a maternal signal which terminates obligate embryonic diapause. *Mol. Reprod. Dev. 00:000–000, 1998.* © 1998 Wiley-Liss, Inc.

Key Words: leukemia inhibitory factor; gene expression; uterus; embryonic diapause; implantation; mink

INTRODUCTION

Embryonic diapause, resulting in a period of delay prior to implantation, is found in several species of mustelids (Mead, 1993). In mink, preimplantation delay is considered obligate because it occurs in every gestation (Enders, 1952; Hansson, 1947). Early studies by Hansson (1947) and Enders (1952) chronicled the reproductive cycle in this species. In brief, ovulation is induced by copulation and occurs at 36–48 hr after initiation of mating. Fertilization takes place in the oviduct some 60 hr after copulation, and development of zygotes to a blastocysts occurs over the next 6–8 days, as the embryos travel to the upper reaches of the uterine horns. Development is arrested at the blastocyst stage for a varying period of time, with a minimum of 6 days, and an average of about 20 days. Delays of 50 days or more can be induced experimentally (Murphy and James, 1974). Embryo expansion is the first morphological indication that the developmental progression has been re-initiated (Hansson,1947; Moreau et al., 1995). Postimplantation gestation is predictable, in that parturition occurs at 30–31 days after embryo attachment (Hansson, 1947; Enders, 1952). Reciprocal transfer of embryos between the mink and the ferret, a related species in which there is no delay (Chang, 1968), indicates that termination of embryonic diapause is under maternal control. To date, the nature of the maternal factor that induces or permits the termination of embryonic diapause remains unknown.

Implantation in mustelid carnivores has unique characteristics. One is the maintenance of the zona pellucida-derived embryonic capsule until it is perforated by trophoblastic plaques just prior to the attachment of the embryo (Enders and Schlafke, 1972; Enders and Mead, 1996). The trophoblast then insinuates itself between endometrial cells in an implantation process that has been described as intrusive (Schlafke and Enders, 1975), and the resultant placenta is endothelial-chorial, decidual and zonary (Enders, 1957).

Progress has recently been made in determining the role of cytokines, a group of low molecular weight

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Dr. Houde's current address: CRDA, Agriculture et Agroalimentaire Canada, St-Hyacinthe QC, J2S 8E3.

*Correspondence to: B.D. Murphy, Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, 3200 rue Sicotte, St-Hyacinthe, Québec, Canada. E-mail: murphyb@medvet.umontreal.ca

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4.0 ARTICLE TWO

CLONING OF LEUKEMIA INHIBITORY FACTOR (LIF) AND ITS EXPRESSION IN THE UTERUS DURING EMBRYONIC DIAPAUSE AND IMPLANTATION IN THE MINK *(Mustela vison)*

Jian H. Song, Alain Houde[1], and Bruce D. Murphy[2]

Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal;
3200 rue Sicotte, St-Hyacinthe, Québec, Canada J2S 7C6

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Key words: Leukemia inhibitory factor, gene expression, uterus, embryonic diapause, implantation, mink.

Running Title: Expression of LIF in mink uterus

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[1] Present address: CRDA, Agriculture et Agroalimentaire Canada, St-Hyacinthe QC, J2S 8E3
[2]Correspondence and reprint requests to B.D. Murphy, Fax (514) 778-8103, email murphyb@medvet.umontreal.ca

4.2 ABSTRACT

Leukemia inhibitory factor (LIF) is essential for embryo implantation in mice. Whether LIF plays a role in termination of embryonic diapause and initiation of implantation in carnivores, especially in species with obligate delayed implantation such as the mink, is not known. The objectives of this study were to clone the LIF coding sequence in the mink and determine its mRNA abundance in the uterus through embryonic diapause, implantation and early postimplantation. We show that the mink LIF cDNA contains 609 nt encoding a deduced protein of 203 amino acids. The homologies are 80.6, 90, 88.2, 87.6, and 86.8 % in coding sequence and 79.2, 90.1, 91, 90.1 and 85.4 % in amino acid sequence with mouse, human, pig, cow, and sheep respectively. Glycosylation sites and disulfide bonds present in other species are generally conserved in the mink LIF sequence. Quantitation by polymerase chain reaction amplification indicates that LIF mRNA is expressed in mink uterus just prior to implantation and during the first two days after implantation, but not during diapause or later postimplantation pregnancy. The abundance of LIF mRNA was significantly higher in the uterus at the embryo expansion stage ($P<0.05$) than at days 1-2 after implantation. By immunohistochemical localization it was shown that LIF is expressed in the uterine epithelial glands at time of embryonic expansion and in early postimplantation. The coincidence of LIF expression with implantation in this species suggests that LIF is involved in the implantation process, and may be a maternal signal which terminates obligate embryonic diapause.

4.3 INTRODUCTION

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Progress has recently been made in determining the role of cytokines, a group of low molecular weight regulatory proteins which exhibit autocrine and paracrine actions (Abbas et al., 1994), in the process of embryo implantation. Studies in rodents have suggested that cells at the site of blastocyst invasion produce cytokines and growth factors (Cross et al., 1994). Furthermore, receptors for cytokines and growth factors have been detected on embryonic cells (Haimovici and Anderson, 1993; Jokhi et al., 1994). An interesting candidate for maternal influence on implantation is leukemia inhibitory factor (LIF), a cytokine that regulates proliferation, differentiation and function of many cell types (Smith et al., 1992). Transgenic mice bearing a null mutation in which LIF is not expressed have normal reproductive function including blastocyst development and hatching, but the embryos fail to implant (Stewart et al., 1992). The coincidence of LIF expression and embryo implantation in species including the rabbit (Yang et al., 1994) and mouse (Bhatt et al., 1991; Yang et al., 1994) further suggests its role in the events of embryo attachment and invasion. LIF is also expressed in the elongating porcine conceptus (Yelich et al., 1997) and at the time of attachment of the non-invasive porcine embryo to the maternal endometrium (Anegon et al., 1994). The LIF receptor has been localized both to preimplantation embryos and to the endometrium of rodents (Stewart, 1994a; Tartakovsky and Ben-Yair, 1991; Yamaguchi et al., 1995). Hirzel and Mead (1997) presented preliminary evidence to indicate that LIF is expressed at the time of embryo activation in the spotted skunk, indicating a role for this cytokine in termination of obligate embryonic diapause in a carnivore species.

The objectives of this study were to determine whether LIF was present in the mink, and to establish its cDNA homology with other species. We further sought to profile LIF expression in the mink uterus during diapause, embryo activation and postimplantation gestation.

4.4 MATERIALS AND METHODS

4.4.1 Animals and sample collection

Adult female mink were maintained on a commercial mink ranch (Morrow Fourrures; St-Paul d'Abbotsford, QC). All procedures were conducted in accordance with Canadian Council of Animal Care regulations. Animals received a standard wet ration and water *ad libidum*. Breeding was performed March 5-20 by exposing the females to males every two days until mated. The females were then remated to different males 7-9 days after the first mating. Success was determined by the presence of sperm in vaginal smears.

The length of diapause and, thus the date of implantation, varied among animals. To ensure that samples were representative of delay, peri-implantation and postimplantation gestation, uteri were collected from 10-20 pregnant mink randomly selected from the experimental population every 3 days between March 21 and April 11. Animals were anesthetized using ketamine-promazine (0.3 ml/animal, Rogar/STB, Montreal, PQ) and terminated by injection of euthanol (T-61 Hoechst Canada, Regina, SK). In the absence of visible swelling sites, uteri were flushed with PBS, and the developmental stage of the recovered blastocysts was determined. Those at 0.8 mm diameter or less were considered to be in diapause (Baevsky, 1963; Stoufflet et al., 1989) while those which had expanded (0.9-2.0 mm) were considered peri-implantation embryos (Stoufflet et al., 1989). Uterine swellings indicative of implantation were measured by vernier caliper, and representative enlargements and adjacent tissue were collected and processed for histological evaluation. A visible implantation chamber, in the absence of evidence of trophoblastic invasion, was considered day 0 of implantation. The postimplantation age of embryos found in uterine swellings was estimated by the means we have previously described (Murphy and James, 1974, Song et al.,1995). Samples collected for determination of LIF expression comprised uterine tissue from the

diapause and peri-implantation phases of gestation, uterine swellings representative of postimplantation pregnancy, and segments of the uterus between implantation sites.

For gene expression studies, tissues were immediately placed in 4 M guanidinium isothiocyanate (GITC, Gibco/BRL, Burlington, ON) solution containing 0.12 M 2β -mercaptoethanol (Sigma, St. Louis, MO.), snap frozen in liquid N_2 and stored at -70 C until total RNA extraction by CsCl (Gibco/BRL) gradient ultracentrifugation (Chirgwin, 1979). Briefly, tissue samples were homogenized with a PT 3000 polytron (Brinkmann, Rexdale, ON) in 4 M GITC solution. The homogenate was then layered onto a 5.7 M CsCl gradient and centrifuged at 174,000 x g using a SW-41 rotor (Beckman, Mississauga, ON), for 20 hours at 21 C. The RNA pellet was then dissolved in 360 µl diethyl pyrocarbonate (DEPC, Sigma) treated distilled water and precipitated twice in 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. After the final washing the pellet was dissolved in DEPC-treated water and stored at -70 C until use. The total RNA concentration was determined by spectrophotometric measurement at 260 nm.

4.4.2 Oligonucleotide primers

The primers (Gibco/BRL) for LIF gene cloning were designed from conserved regions of cDNA sequences from LIF coding sequence of mouse and human (Stahl et al., 1990), pig and sheep (Willson et al., 1992) and cow (Kato et al., 1996). The primers for mink ribosomal protein S26 were designed according to the sequence in GeneBank (Accession # X79237). The sense primers were:

mkLIF-B (5′-ACCTCATGAGCCAGATCA), nt 128-145 of the open reading frame;

mkLIF-H (5′-GGTACCCGGCTAAATATAGC), nt (-149)-(-129) from the 5′-untranslated region, containing the BamHI restriction site (GGTACC);

mkLIF 165 (5′-CAATGGCAGTGCCAATGCTC), nt 165-185 of the coding region; and

mkS26-A (5′-AGATGACTAAGAGAGAGAGAG).

The antisense primers were:

mkLIF-2 (5′-TGGTCCCGGGTGATGTT), nt 386-352;

mkLIF-6 (5′-GAATTCTAGAAGGCCTGGGCCA), nt 609-592 with an EcoRI restriction site (GAATTC) added;

mkLIF-461 (5′-GGTATTTGTTACACAGGC), nt 479-461; and

mk26S-1 (5′-CGATACGAACTTCTTAATGG).

4.4.3 Mink LIF cDNA cloning and sequencing

The reverse transcription-polymerase chain reaction (RT-PCR) strategy adopted for cloning the mink LIF cDNA (mkLIF) is outlined in Figure 4-1. The RT reaction was performed for 90 min at 42 C using Superscript-II (Gibco/BRL) in total volume of 20 µl containing 5 µg of total RNA from early post implantation uterus and 20 pmol antisense primer (mkLIF-6) corresponding to the last 16 nt of conserved termination region of the LIF open reading frame. PCR reactions were made to final volume of 100 µl by addition of DEPC-treated H_2O, containing 10 µl 10x PCR buffer (0.5 M Tris pH 9, 15 mM $MgCl_2$, 0.2 M $(NH_4)_2SO_4$), 20 pmol of each sense and antisense primers, 20 nmol of dNTP, 1 µl (5 units) Taq DNA polymerase (Pharmacia, Baie D'Urfé, PQ) and 10 µl RT product. The PCR amplifications were performed by Hybaid Omnigene Thermal Cycler (Intersciences, Markham, ON) for 40 cycles programmed 94 C, 45 sec; 50 C, 45 sec and 72 C, 1 min 30 sec and followed by an extension amplification at 72 C, 10 min at the end of the PCR reaction.

The PCR products were size-fractionated by electrophoresis on 1% agarose gels and the gel was stained with ethidium bromide (Sigma) for visualization under UV illumination. Bands were excised from the gel and purified by using Sephaglas bandpreps (Pharmacia), ligated into pGEM-t vector using T4 DNA ligase according to the method provided by the manufacturer (Promega, Nepean, ON) and ligation products were

transformed into *Escherichia coli* (JM 109) cells by the RbCl method (Sambrook et al., 1989). Positive clones were selected and subsequently sequenced by using T7 polymerase (T7 Sequencing Kit, Pharmacia). To guard against misincorporation of nucleotides by Taq polymerase during PCR amplification, 3 independent clones were sequenced for each gene fragment and the consensus sequence taken.

4.4.4 Mink LIF mRNA RT-PCR quantification

The sense (mkLIF-165) and antisense primers (mkLIF-461) which generate a 315 nt PCR fragment of cDNA were chosen because they bracket an intron, which we amplified and sequenced from mink genomic DNA (data not shown). If genomic DNA were present in the RT samples, the amplified product would comprise 1004 nt and be readily distinguishable from the cDNA product. To control for the efficiency of amplification, an internal control was employed (Siegling et al., 1994). It consisted of a cDNA fragment (600 nt) generated from muskrat genomic DNA using the primers mkLIF-165 and mkLIF-461. The 121 nt fragment of mink S26 was used as a control for RT efficiency and loading. Given the greater abundance of this gene product relative to the LIF gene fragment, it served as an external control, in that it was amplified in separate tubes, as we have previously described (Emond et al., 1998). The RT reaction was carried out as described above, except two antisense primers (mkLIF-6 and mk26S-1) were used. To determine the abundance of LIF mRNA, 10 µl of RT product and 1 pg internal control cDNA were amplified by means of 20 pmol each of mkLIF-165 and mkLIF-461 primers. One µl of RT product was used for S26 in amplification by 20 pmol of mk26S-A and mk26S-1. The PCR amplification program consisted of 94 C (45 sec), 50 C (45 sec) and 72 C (60 sec). PCR was optimized by performing 20-40 cycles for each amplicon. Final amplifications of 30 cycles for mink LIF and 25 cycles for S26 were chosen as they were on the linear portion of the respective curves. Then 30 µl LIF and 10 µl for S26 of PCR products were combined and applied to 2% agarose gels. The gels were stained with ethidium

bromide (Sigma) and the presence of cDNA bands was determined by visualization under UV illumination. The PCR fragments were quantified first by scanning (Collage software, Photodyne, New Berlin, WI) and their intensity was determined by the NIH image program. Three independent RT-PCR assays for each sample were performed. The results are expressed as a dimensionless abundance value, the calculated ratio of the mean of LIF density value divided by the internal control value and then by the S26 (external control) value.

4.4.5 Immunohistochemistry of LIF in the uterus

Attempts to localize LIF in paraffin sections were not successful. Therefore, for immunohistochemical investigation, pieces (5 mm) of uterus were frozen in liquid N_2, sectioned on a cryostat at 8 μm and then fixed in methanol (BDH, Canada) containing 0.3% H_2O_2 (Fisher Scientific) for 15 min. Following 3 X 5 min washes in PBS, the sections were incubated with 5% normal rabbit serum (Vectastain, Vector Laboratories, Burlington, ON) at room temperature for 45 min. The excess serum was blotted off and 200 μl of goat anti-human LIF antiserum (R&D, Minneapolis, MN) was applied to each slide at 1:20 dilution in PBS, followed by incubation at 4 C for 18 hours. After the primary antibody reaction, slides were washed in PBS (2 X 5 min) at room temperature, then incubated with biotinylated second antibody (rabbit anti-goat) for 45 min followed by 2 X 5 min washes in PBS according to the manufacturer's instructions (Vectastain). Subsequently, a complex of avidin-biotin-peroxidase was applied for 45 min followed by 2 X 5 min washes in PBS. All incubations were performed in a humidified chamber to minimize evaporation. A negative control for each slide was subject to the same procedure except that a dilution of normal goat serum replaced the first antibody. The positive reaction was identified by the application of the peroxidase substrate 3, 3'-diaminobenzidine (DAB, Sigma) which produces a red-brown stain at the site of second antibody binding. Sections were

lightly counterstained with hematoxylin #1 (Fisher), dehydrated and mounted.

4.4.6 Statistical analysis

Animals were grouped according to gestational status as determined by the morphological analysis described above. There were 3-6 animals represented at each stage. The mean ± SEM for the non-dimensional values for the abundance of LIF, calculated as described above, was determined for each group. A t-test was used to determine if in the abundance of LIF gene expression in the uterus differed between the two times when the transcript could be detected. $P<0.05$ was the level of statistical significance.

4.5 RESULTS

4.5.1 Mink LIF open reading frame sequence and deduced amino acid sequence

A cDNA consisting of 758 nt was cloned and sequenced. This fragment comprised the open reading frame for mink LIF of 609 nt encoding a deduced protein of 203 amino acids and 149 nt of the 5′ untranslated region (Figure 4-2). Figure 4-3 presents the homology comparison of mink LIF with known LIF sequences in other species. The deduced amino acid sequence of mink LIF contains four potential sites for N-linked glycosylation (asparagines at 34, 56, 95, 118) and three potential disulfide bonds (cysteines at 34 and 156, 40 and 153, and 95 and 118).

4.5.2 Expression of LIF transcripts in the mink uterus throughout gestation

Expression of LIF in the mink uterus was a transitory event, with maximal amplification present in the uterus at the peri-implantation phase of gestation (Figure 4-4A and B). At this time embryos were expanded, but not attached. Transcripts were amplified, at a lower level ($P<0.05$) in uterine swellings, comprised of the embryo-uterine complex at day 2 of postimplantation gestation (Figure 4-4A and B). LIF transcripts were not detectable in the uterus by PCR analysis during preimplantation

delay, and were likewise not detectable at 5 or more days after implantation (Figure 4-4A and B).

4.5.3 Immunohistochemistry of LIF in the mink uterus

The immunohistochemical observations concurred with the PCR findings. LIF was present in greatest abundance in the fundus of uterine glands taken from uteri with an implantation chamber just prior to embryo attachment (Figure 4-5A). It was also present in uterine glands in specimens from early postimplantation, where trophoblastic intrusion had eliminated the luminal epithelium (data not shown). LIF distribution was most concentrated in the basal regions of gland cells, but was also found at lower density the periluminal portions of the cells. No LIF protein could be detected in the uterus during the delay phase of gestation, nor was it evident in the uterine stroma, invading trophoblast, or in the incipient decidual cells at any time during gestation. The negative control samples, i.e. those with no first antibody, showed no deposition of the DAB precipitate, indicating specificity of the analysis (Figure 4-5B).

4.6 DISCUSSION

This is the first known report of LIF gene sequence in a carnivore species and it indicates that the gene is conserved relative to other mammals. The homologies are, respectively, 80.6, 90, 86.8, 88.2, 87.6 and 80.5 % in nucleotide and 79.2, 90.1, 86.7, 91, 90.1 and 85.4 % in amino acids with the complete sequences in the mouse and human (Stahl et al., 1990), sheep and pig (Willson et al., 1992) and cow (Kato et al., 1996), and with the partial muskrat sequence (GenBank Accession # AF034742). Of six potential glycosylation sites present in rat (Yamimori et al., 1989) and human (Stahl et al., 1990) LIF, only four appear conserved in the deduced amino acid sequence of mink LIF. Sistine residues for the formation of three disulfide bonds present in other species, are conserved in the mink LIF sequence.

LIF transcripts were identified through a narrow window of gestation in the mink. Our PCR analysis indicated that LIF was expressed after diapause has been terminated at the time when expanded, reactivated embryos are present (Stoufflet et al., 1989). LIF is also expressed during the first few days after trophoblast intrusion into the endometrium. As PCR amplification is theoretically capable of recognition of a single transcript, the absence of LIF amplicons in the uterus prior to implantation and after day 2 postimplantation suggests that this gene is not expressed at these times. It should be noted that PCR was performed on uteri, without embryos, during the delay phase of gestation. It remains possible that there is embryonic expression of the gene, as has been demonstrated in the mouse (Lavranos et al., 1995), human (Cullinan et al., 1996) and sheep (Vogiagis et al., 1997a; 1997b).

The transience of mink LIF expression is in contrast with findings in other species. While maximal LIF expression coincided with early embryo implantation in mice, both transcripts and protein could be found during the estrous cycle and earlier in gestation (Bhatt et al., 1991; Stewart, 1994a; Yang et al., 1995). In rabbits, LIF protein, as detected by immunolocalization, is most abundant at day 5 of gestation, two days prior to implantation, but it can also be found both earlier and later in pregnancy (Yang et al., 1994).

In the present study, LIF protein localized to the fundus and neck of epithelial glands in the uterus at the time of embryo implantation. It was not found in uterine stroma nor in the decidual cells of early pregnancy. In the mouse, the highest concentration of LIF transcripts are found in uterine glands (Bhatt et al., 1991), but it was also present in endometrial epithelium and stroma by immunolocalization using species-specific LIF antiserum (Yang et al., 1995). In the rabbit (Yang et al., 1994) and human (Kojima et al., 1995), LIF protein was also more widely distributed than in the mink, being present in uterine epithelium, glands and stroma. We were unable to detect LIF in mink by Northern analysis, nor to determine

its presence by immunolocalization in paraffin sections. It therefore appears to be in low abundance, and it is possible that the restricted distribution we report reflects the sensitivity of our immunohistochemistry procedure and the heterologous antibody employed in the frozen sections.

The transgenic null mutation which ablates LIF expression in the mouse prevents embryo implantation, nonetheless embryos in null mice develop to the blastocyst stage and hatch in the expected fashion (Stewart et al., 1992). Maternal LIF expression is essential, as embryos from the LIF knockout mice can implant and develop normally in the uterus of a normal mice. Further, the pseudopregnant mouse uterus expresses LIF in the absence of the embryo (Bhatt et al., 1991), indicating that the embryo is not the source of peri-implantation LIF. Lactation in mice will induce a facultative delay in implantation, which can be mimicked by ovariectomy on day 3 of gestation and progesterone treatment (Psychoyos, 1976). Termination of embryonic diapause in mice in lactation-induced delay by weaning of suckling pups, or termination of experimental delay by estrogen injection, precipitates LIF expression within 24 h (Stewart, 1994b). LIF upregulates proteinase activity in mouse blastocysts *in vitro* (Harvey et al., 1995), and this may be a mechanism that establishes the invasive capacity of the trophoblast. Together these observations argue for LIF as the proximal signal which induces embryo implantation in the mouse. Alternatively, LIF may activate expression of uterine factor(s) which terminate diapause. Recent studies have identified the receptor gp49B1 as a LIF-stimulated protein which is expressed specifically in the endometrium at the time of implantation in the mouse (Matsumoto et al., 1997).

In mink, obligate embryonic diapause and implantation are under maternal control (Chang, 1968). Pituitary prolactin activates the quiescent corpus luteum prior to embryo expansion and implantation (Murphy et al., 1981). The uterine cue which terminates diapause and initiates

embryo expansion is not known. Coincidence of LIF expression with the reactivation of embryo development in mink suggests that this cytokine may be the elusive uterine signal to the embryo in obligate delayed implantation. This supposition is supported by observations in vitro, where BRL cells, known to secrete LIF (Smith and Hooper, 1987), and recombinant human LIF itself increase the frequency of mink blastocyst survival and expansion in vitro (Moreau et al., 1995; Moreau, Song and Murphy, unpublished observations).

The ovarian event that terminates diapause in mink is the activation of the quiescent corpus luteum of delay and the production of progesterone and other factors is essential to implantation (Murphy et al., 1983). This coincides with embryo expansion and attachment (Stoufflet et al., 1989). The correlation of LIF expression with the reinitiation of embryo development in the present study suggests for a role of steroid hormones in regulation of LIF. In other species, the importance of estrogens and progesterone in uterine LIF expression varies, depending on the model studied (Yang et al., 1996). Administration of estrogen, but not progesterone alone to pseudopregnant mice results in uterine LIF expression (Bhatt et al., 1991; Yang et al., 1996), while in the rabbit, progesterone, but not estrogen alone stimulates the appearance of LIF in the endometrium (Yang et al., 1996). In humans, in vivo inhibition of progestin action by the antiprogestin, mifepristone, reduces endometrial LIF expression at the time of expected implantation (Gemzell-Danielsson et al., 1997). In vitro estrogen, but not progesterone, induces LIF production by human decidual cells (Sawai et al., 1997). Nevertheless, progestins activate the LIF promoter in transcriptional studies (Bamberger et al., 1997). Vogiagis et al. (1997b) reported that treatment of ovariectomized ewes with estrogen or a combination of estrogen and progesterone reduces, rather than increases, LIF expression in the endometrium. The role of steroid hormones in control of LIF expression

in the uterus is complicated, may vary between species, and clearly merits further investigation.

In summary, the cDNA for mink LIF has been cloned and sequenced, and RT-PCR analysis indicates that it is present in the mink uterus during the period when the embryo escapes diapause and during early postimplantation gestation. Immunohistochemistry revealed LIF in uterine glands just prior to and shortly following embryo implantation. The close correlation between the events of implantation and the expression of LIF suggests that it may be involved in the implantation process in the mink.

4.7 ACKNOWLEDGMENTS

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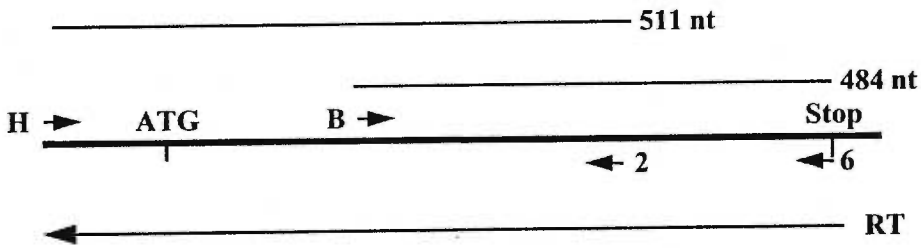
511 nt
484 nt
H
ATG
B
Stop
2
6
RT

Figure 4-1. Cloning strategy for mink leukemia inhibitory factor (LIF). First strand cDNA was generated by reverse transcription (RT) of total uterine mRNA from the pregnant mink uterus. PCR amplification was achieved by sense primers mink LIF-H (H) and mink LIF-B (B) and antisense primers mink LIF-6 and mink LIF-2. Double lines represent the mink LIF cDNA, overlapping clones are represented above, and the dark line indicates the direction of reverse transcription.

```
-149  ggatcccggctaaatatagctgatttccctgtcttacaacacaggctccagtatataaatca
 - 87  ggcaaattccccgttgagcatgaacctctgaaaactgccggcatctaaggtctcctccaa
 - 27  ggaaggccctctggagtccagcccataATGAAGGTCTTGGCGGCAGGAGTTGTGCCCCTG
   1                                M  K  V  L  A  A  G  V  V  P  L

  34  CTGCTGGTTCTGCACTGGAAACACGGAGCAGGGACCCCCCTTCCCATCACCCCAGTCAAC
  12   L  L  V  L  H  W  K  H  G  A  G  T  P  L  P  I  T  P  V  N

  94  GCCACCTGTGCCACCCGCCACCCATGTCACAGCAACCTCATGAACCAGATCAGGAACCAA
  32   A  T  C  A  T  R  H  P  C  H  S  N  L  M  N  Q  I  R  N  Q

 154  CTGGCGCACGTCAATGGCAGTGCCAATGCTCTCTTTATTCTCTATTACACGGCCCAGGGG
  52   L  A  H  V  N  G  S  A  N  A  L  F  I  L  Y  Y  T  A  Q  G

 214  GAGCCGTTCCCCAACAACCTGGACAAGTTGTGTGGCCCCAATGTGACGGACTTCCCGCCA
  72   E  P  F  P  N  N  L  D  K  L  C  G  P  N  V  T  D  F  P  P

 274  TTCCACCGCAACGGCACGGAGAAGACACGGCTCGTGGAGCTCTACCGTATCATCGCATAC
  92   F  H  R  N  G  T  E  K  T  R  L  V  E  L  Y  R  I  I  A  Y

 334  CTTGGCGCCTCCCTGGGCAACATCACCCGGGATCAGAAGGTCCTCAATCCCAATGCCCTC
 112   L  G  A  S  L  G  N  I  T  R  D  Q  K  V  L  N  P  N  A  L

 394  AGCCTCCACAGCAAGCTGAAGGCCACGGCGGACATCCTGCGGGGCCTCCTCAGCAATGTG
 132   S  L  H  S  K  L  K  A  T  A  D  I  L  R  G  L  L  S  N  V

 454  CTCTGCCGCCTGTGTAACAAATACCATGTGGCCCACGTGGACGTGGCCTATGGCCCTGAC
 152   L  C  R  L  C  N  K  Y  H  V  A  H  V  D  V  A  Y  G  P  D

 514  ACCTCGGGCAAGGACGTCTTTCAGAAGAAGAAGCTGGGCTGTCAGCTCCTGGGGAAGTAT
 172   T  S  G  K  D  V  F  Q  K  K  K  L  G  C  Q  L  L  G  K  Y

 574  AAGCAGGTCATTGCCGTGGTGGCCCAGGCCTTCTAG  609
 192   K  Q  V  I  A  V  V  A  Q  A  F  *   203
```

Figure 4-2. Nucleotide and deduced amino acid sequences derived from the cloned mink LIF cDNA. The translation initiation (ATG) and termination sites (TAG) are marked in boldface type. The partial 5′ untranslated sequence is indicated in lower case.

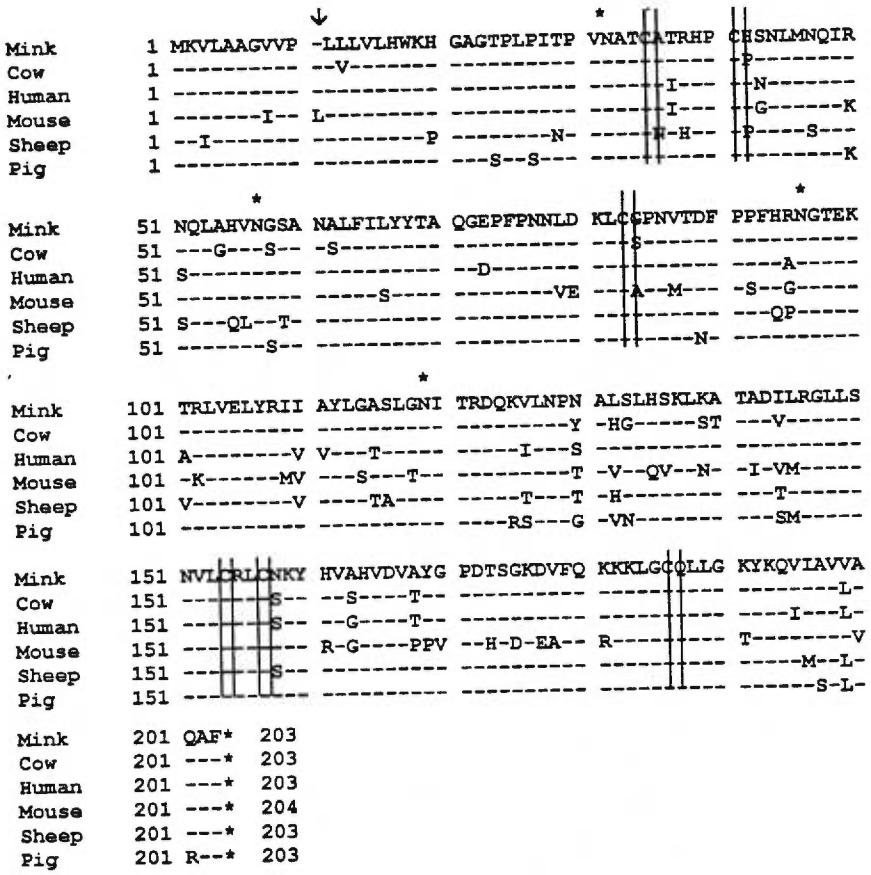
Mink 1 MKVLAAGVVP -LLLVLHWKH GAGTPLPITP VNATCATRHP CHSNLMNQIR
Cow 1 ---------- --V------- ---------- ---------- -P--------
Human 1 ---------- ---------- ---------- ------I--- --N-------
Mouse 1 -------I-- L--------- ---------- ------I--- --G------K
Sheep 1 --I------- ---------P -------N- -----N-H-- -P----S---
Pig 1 ---------- ---------- ---S--S--- ---------- --------K
Mink 51 NQLAHVNGSA NALFILYYTA QGEPFPNNLD KLCGPNVTDF PPFHRNGTEK
Cow 51 ---G---S-- -S-------- ---------- ---S------ ----------
Human 51 S--------- ---------- --D------- ---------- ----A-----
Mouse 51 ---------- -----S---- --------VE ---A--M--- -S--G-----
Sheep 51 S---QL--T- ---------- ---------- ---------- ---QP-----
Pig 51 -------S-- ---------- ---------- ---------N- ----------
Mink 101 TRLVELYRII AYLGASLGNI TRDQKVLNPN ALSLHSKLKA TADILRGLLS
Cow 101 ---------- ---------- ---------Y -HG-----ST ---V------
Human 101 A--------V V---T----- -----I---S ---------- ----------
Mouse 101 -K------MV ---S---T-- ---------T -V--QV--N- -I-VM-----
Sheep 101 V--------V ----TA---- -----T---T -H-------- ---T------
Pig 101 ---------- ---------- ----RS---G -VN------- ---SM-----
Mink 151 NVLCRLCNKY HVAHVDVAYG PDTSGKDVFQ KKKLGCQLLG KYKQVIAVVA
Cow 151 ------S-- --S----T-- ---------- ---------- --------L-
Human 151 ------S-- --G----T-- ---------- ---------- ----I---L-
Mouse 151 ------R-- R-G----PPV --H-D-EA-- R--------- T--------V
Sheep 151 ------S-- ---------- ---------- ---------- -----M--L-
Pig 151 ---------- ---------- ---------- ---------- ------S-L-
Mink 201 QAF* 203
Cow 201 ---* 203
Human 201 ---* 203
Mouse 201 ---* 204
Sheep 201 ---* 203
Pig 201 R--* 203

Figure 4-3. Comparison of deduced amino acid sequences between the mink and known sequences from other species. The mouse sequence contains a leucine residue at position 11 (arrow) which is not found in other species. The conserved sequences are indicated by a dash and the amino acid abbreviations are present where the sequence is not conserved. Asterisks represent potential N-linked glycosylation sites, and conserved cysteines believed to be associated with disulfide bonds are marked with vertical bars.

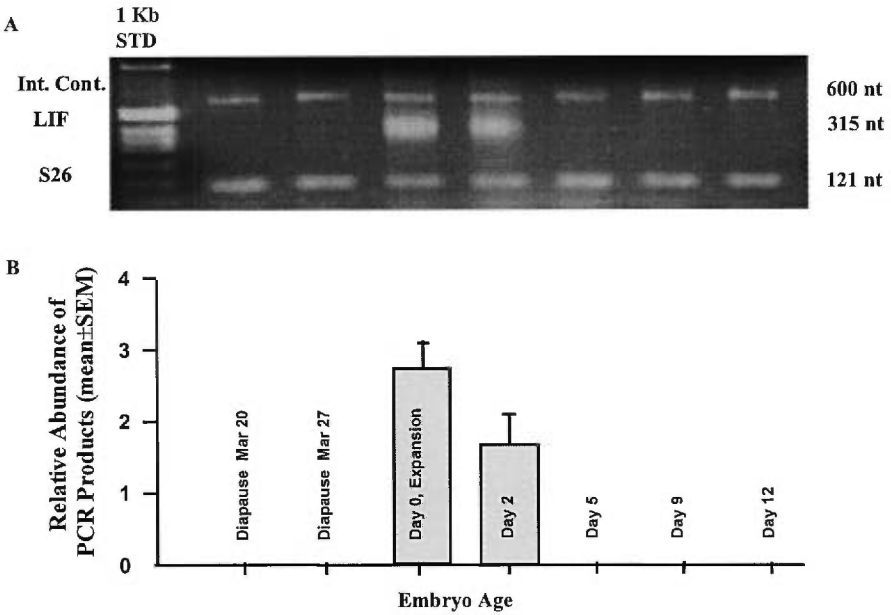
A
1 Kb
STD
Int. Cont.
LIF
S26
600 nt
315 nt
121 nt
B
Relative Abundance of
PCR Products (mean±SEM)
4
3
2
1
0
Diapause Mar 20
Diapause Mar 27
Day 0, Expansion
Day 2
Day 5
Day 9
Day 12
Embryo Age

Figure 4-4. Quantitative RT-PCR analysis of LIF expression in the mink uterus through diapause and early postimplantation. **Panel A** is a representative agarose gel loaded with 30 µl of PCR products, including LIF, with an expected size of 315 nt and the internal control (1 pg) of muskrat genomic DNA expected to yield a product of 600 nt, amplified by the same primers in the same reaction. The external control (S26, 121 nt) was reverse transcribed in the same reaction and amplified in a separate reaction. An aliquot of 10µl of the S26 product was added to the other amplicons prior to electrophoresis. **Panel B** depicts the mean ± (SD) abundance of the LIF amplicon (315 nt) from 3-10 animals from various stages of early gestation. The asterisk indicates that the mean at Day 0 was greater than that at Day 2 of implantation ($p<0.05$).

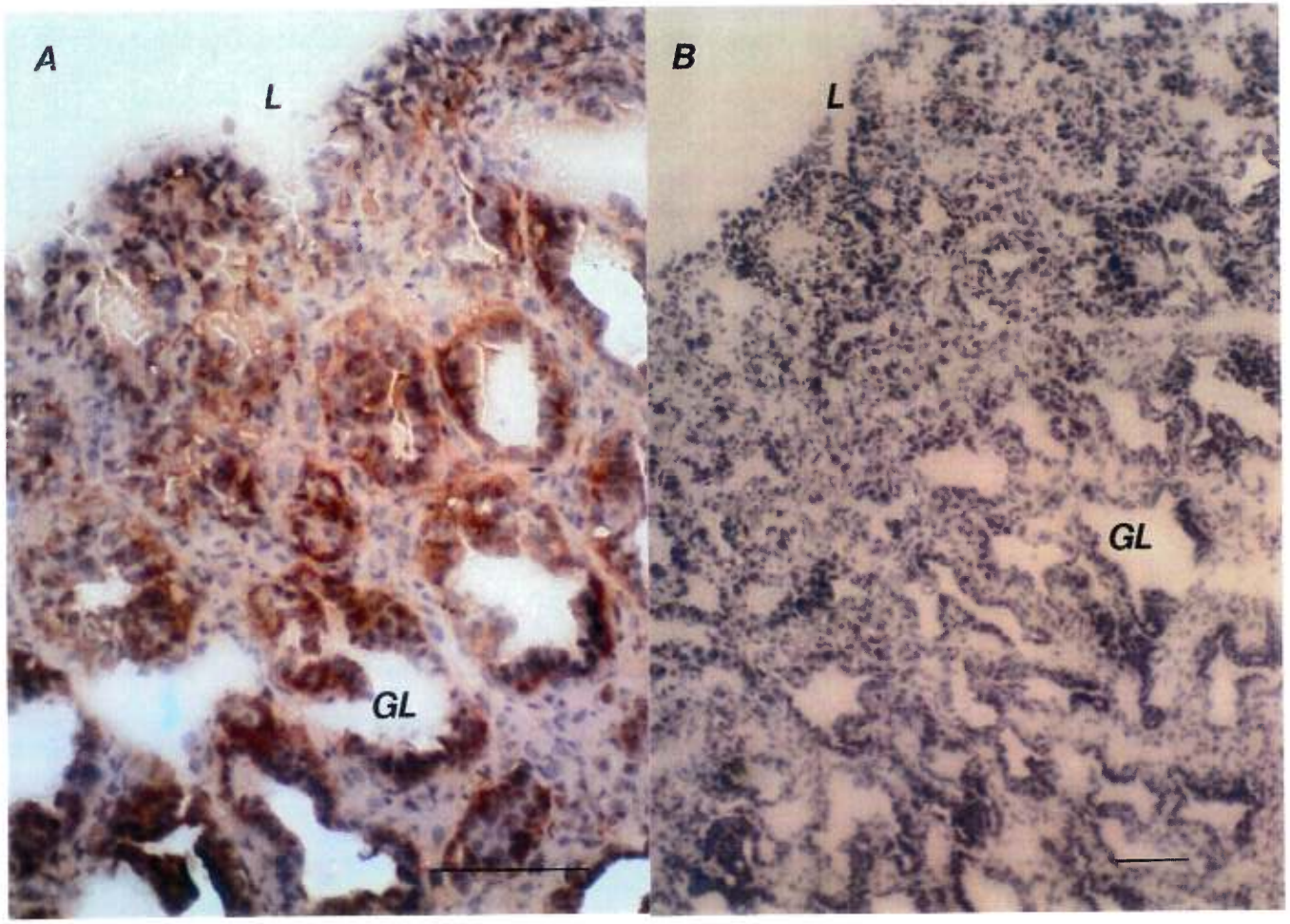
A
L
GL
B
L
GL

Figure 4-5. Immunohistochemical localization of LIF in endometrial glands from a the region of an implantation chamber of the mink uterus on Day 0 (day of embryo expansion). In **Panel A**, the red brown precipitate marks sites of antibody binding. **Panel B** is a control section which was subject to all of the procedures, but dilute normal goat serum was employed in place of the anti-LIF antibodies. The arrow designates the lumenal epithelium; the asterisk indicates an example of the fundi of uterine glands. The bars represent 10 μm.

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Cloning, Developmental Expression, and Immunohistochemistry of Cyclooxygenase 2 in the Endometrium during Embryo Implantation and Gestation in the Mink (*Mustela vison*)*

JIAN H. SONG, JEAN SIROIS, ALAIN HOUDE†, AND BRUCE D. MURPHY

Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Quebec, Canada J2S 7C6

ABSTRACT

Cyclooxygenase (COX) is the first rate-limiting enzyme in the biosynthesis of PGs. There are two isoforms, COX-1, a constitutive enzyme and COX-2, the induced form, products of two different genes. In this study, we report COX-2 complementary DNA cloning, uterine expression, and immunohistochemical localization in the mink uterus during postimplantation gestation. The open reading frame of mink COX-2 contains 1812 nucleotides encoding 604 amino acids. The homologies are 86%, 83%, 83%, 83%, and 85% in nucleotides and 86%, 87%, 87%, 85%, 86%, and 88% in amino acids with human, mouse, rat, guinea pig, sheep, and rabbit, respectively. All domains associated with biological activity are conserved in the mink. Northern analysis revealed a transcript of 4.2 kb for COX-2 in mink uterus and adrenal. Semiquantitative RT-PCR showed that COX-2 messenger RNA is not present during diapause. The abundance of COX-2 messenger RNA reached its maxima ($P < 0.05$) on days 3–5 of postimplantation, gradually decreased through day 9, and was not present thereafter. By immunohistochemistry, COX-2 was present in uterine epithelium, stroma, and necks of endometrial glands at sites of implantation. COX-2 expression appears to be induced in the endometrium by the embryo and may play a role in implantation and placentation in the mink. (*Endocrinology* 139: 0000–0000, 1998)

OBLIGATE embryonic diapause is a condition in which, during every pregnancy, there is a period of arrest in mitotic activity in the embryo. In a number of mustelid carnivores, including the mink, an obligate delay of implantation of variable duration has been identified (1). In the mink, the length of diapause can be as brief as 6 days (2) or can be extended under experimental conditions to more than 55 days (3). Embryonic diapause in mink is believed to be under maternal control, based on reciprocal transfer of embryos between the mink and the ferret, a related species that does not normally display a delay of implantation (4). Further evidence for maternal control can be found in the capability of embryos in diapause to reinitiate development *in vitro* when provided with the appropriate culture conditions (5, 6).

Mustelid embryos do not hatch from the zona pellucida-derived glycoprotein coat before implantation, as occurs in other species. Rather, syncytial trophoblastic knobs pierce the zona at several sites on the antimesometrial side of the uterus and adhere focally to the endometrial endothelium (7, 8). The trophoblast intrudes between endometrial epithelial cells, the endometrial epithelium is then eliminated over broad areas, and the trophoblast invades down the necks of the epithelial glands (9). The endometrial glands respond by undergoing hyperplasia and elongation (8). The mature placenta is zonary and epitheliochorial (10).

The role of PG synthesis in embryo implantation was first demonstrated by Lau *et al.* (11), who blocked implantation with the cyclooxygenase (COX) inhibitor, indomethacin, and overcame the blockade by administration of PGs. Subsequent investigations confirmed a role for PG synthesis in embryo implantation in rodents (12). There is clear evidence that PGs, primarily PGE_2, are necessary for increased vascular permeability at the site of implantation (13) and for increased local blood flow (14). PGs are implicated in decidualization of rodent (15) and human (16) stromal cells. Transcripts coding for PG receptors have been found in mouse luminal endometrial epithelium coincident with the time of expected implantation (17). PGs are believed to be involved in adhesion of the ovine trophoblast to the endometrium (18) and in regulation of local immune responses (19). PGs also initiate endometrial plasminogen activator expression in the rat and, therefore, may be involved in the tissue remodeling associated with trophoblast invasion (20, 21). PG regulation of expression of maternal tissue inhibitors of metalloproteinase enzymes has been suggested as a mechanism of limitation of the extent of ovine trophoblast invasion (18, 22, 23).

There is little information on the role of PGs in carnivore implantation. Treatment of ferrets with indomethacin reduced the number and size of implantation sites, but did not alter uterine vascular permeability at the time of implantation (24).

PG synthesis from arachidonic acid is catalyzed by two isoforms of the COX enzyme that are derived from two different genes (reviewed in Ref. 25). The isoform known as COX-1 is widely distributed and appears to be constitutively

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Address all correspondence and requests for reprints to: Dr. Bruce D. Murphy, Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, 3200 rue Sicotte, St-Hyacinthe, Quebec, Canada J2S 7C6. E-mail: murphyb@medvet.umontreal.ca.
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† Present address: Centre de Recherche en Agroalimentaire, Agriculture Canada, St-Hyacinthe, Quebec, Canada J2S 8E3.

5.0 ARTICLE THREE

CLONING, DEVELOPMENTAL EXPRESSION AND IMMUNOHISTOCHEMISTRY OF CYCLOOXYGENASE 2 (COX-2) IN THE ENDOMETRIUM DURING EMBRYO IMPLANTATION AND GESTATION IN THE MINK (*Mustela vison*)

Jian H. Song, Jean Sirois, Alain Houde[1] and Bruce D. Murphy

Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe Québec, Canada J2S 7C6

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Address all correspondence and requests for reprints to: Bruce D. Murphy, CRRA, Faculté de médecine vétérinaire, 3200 rue Sicotte, St-Hyacinthe, Québec, Canada J2S 7C6. E-mail: murphyb@medvet.umontreal.ca

[1]Present address: Centre de recherche en agroalimentaire, Agriculture Canada, St-Hyacinthe, Québec, Canada J2S 8E3.

Key words: COX-2, embryo implantation, endometrium, prostaglandins, mink, delayed implantation

Running Title: COX-2 expression in mink uterus

5.2 ABSTRACT

Cyclooxygenase (COX) is the first rate-limiting enzyme in the biosynthesis of prostaglandins . There are two isoforms, COX-1, a constitutive enzyme and COX-2, the induced form, products of two different genes. In this study, we report COX-2 cDNA cloning, uterine expression and immunohistochemical localization in the mink uterus during postimplantation gestation. The open reading frame of mink COX-2 contains 1812 nucleotides (nt) encoding 604 amino acids. The homologies are 86, 83, 83., 83, and 85% in nt; and 86, 87, 87, 85, 86 and 88% in amino acids with human, mouse, rat, guinea pig, sheep and rabbit, respectively. All domains associated with biological activity are conserved in the mink. Northern analysis revealed a transcript of 4.2 kb for COX-2 in mink uterus and adrenal. Semi-quantitative RT-PCR showed that COX-2 mRNA is not present during diapause. The abundance of COX-2 mRNA reached its maxima ($P<0.05$) at days 3-5 of postimplantation, gradually decreased through day 9, and was not present thereafter. By immunohistochemistry, COX-2 was present in uterine epithelium, stroma and necks of endometrial glands at sites of implantation. COX-2 expression appears to be induced in the endometrium by the embryo and may play a role in implantation and placentation in mink.

5.3 INTRODUCTION

Obligate embryonic diapause is a condition in which there is a period of arrest in mitotic activity in the embryo. In a number of mustelid carnivores, including the mink, an obligate delay of implantation of variable duration has been identified (1). In the mink, the length of diapause can be as brief as 6 days (2), or can be extended under experimental conditions to more than 55 days (3). Embryonic diapause in mink is believed to be under maternal control, based on reciprocal transfer of embryos between the mink and ferret, a related species which does not normally display a delay of implantation (4). Further evidence for maternal control can be found in the capability of embryos in diapause to reinitiate development in vitro when provided with the appropriate culture conditions (5, 6).

Mustelid embryos do not hatch from the zona pellucida derived glycoprotein coat prior to implantation, as occurs in other species. Rather, syncytial trophoblastic knobs pierce the zona at several sites on the antimesometrial side of the uterus and adhere focally to the endometrial epithelium (7, 8). The trophoblast intrudes between endometrial epithelial cells, the endometrial epithelium is then eliminated over broad areas, and the trophoblast invades down the necks of the epithelial glands (9). The endometrial glands respond by undergoing hyperplasia and elongation (8). The mature placenta is zonary and epitheliochorial (10).

The role of prostaglandin synthesis in embryo implantation was first demonstrated by Lau et al. (11) who blocked implantation with the cyclooxygenase (COX) inhibitor, indomethacin, and overcame the blockade by administration of prostaglandins. Subsequent investigations confirmed a role for prostaglandin synthesis in embryo implantation in rodents (12). There is clear evidence that prostaglandins, primarily prostaglandin E_2, are necessary for increased vascular permeability at the site of implantation (13) and for increased local blood flow (14). Prostaglandins are implicated in decidualization of rodent (15) and

human (16) stromal cells. Transcripts coding for prostaglandin receptors have been found in mouse luminal endometrial epithelium coincident with the time of expected implantation (17). Prostaglandins are believed to be involved in adhesion of the ovine trophoblast to the endometrium (18) and in regulation of local immune responses (19). Prostaglandins also initiate endometrial plasminogen activator expression in the rat, and therefore may be involved in the tissue remodeling associated with trophoblast invasion (20, 21). Prostaglandin regulation of expression of maternal tissue inhibitors of metalloproteinase enzymes has been suggested as a mechanism of limitation of the extent of ovine trophoblast invasion (18, 22, 23).

There is little information on the role of prostaglandins in carnivore implantation. Treatment of ferrets with indomethacin reduced the number and size of implantation sites, but did not alter uterine vascular permeability at the time of implantation (24).

Prostaglandin synthesis from arachidonic acid is catalyzed by two isoforms of the COX enzyme, which are derived from two different genes (reviewed in (25)). The isoform known as COX-1 is widely distributed, and appears to be constitutively expressed, while COX-2 is regulated by a number of intra- and extracellular stimuli (25). Jacobs et al. (26) showed that COX-2 localizes to uterine stroma in regions of mouse blastocyst attachment. A more complete investigation by Chakraborty et al. (27) showed that COX-1 is present prior to implantation in mouse in uterine luminal epithelium and subepithelial stromal cells. COX-2, however, is locally expressed in the uterus in the region surrounding the implanting blastocyst at the time of embryo attachment (day 4), and persists until early on day 5 (27). Coincidence of implantation and COX-2 expression in uterine and/or trophoblastic tissue has been reported in the ovine (18, 22), bovine (28), and human uterus (29). Transgenic mice bearing a mutation that eliminates expression of the COX-1 gene were capable of reproduction (30). In contrast, knockout of COX-2 had profound negative consequences

on reproduction, including interference with the ovulatory process and the failure of embryo implantation (30). Further, specific COX-2 inhibitors severely compromise embryo implantation in the mouse (30).

The role of prostaglandins in carnivore embryo implantation has not been clearly established, nor has it been shown whether mustelid carnivores such as the mink possess the COX-2 isoform. Thus, the objectives of this study were (1) to determine whether COX-2 is present in the mink by cDNA cloning and (2) to investigate COX-2 gene expression and the patterns of occurrence of COX-1 and COX-2 proteins in the mink uterus during early gestation.

5.4 MATERIALS AND METHODS

5.4.1 Animals and sample collection

Primiparous Standard Dark variety female mink were maintained on a commercial farm (Morrow Fourrures, St Paul d'Abbotsford, QC), under approved husbandry conditions. Breeding was performed by exposing the females to males every two days until mated. Females were then remated to different males 7-9 days after the first mating. All matings were confirmed by observation of sperm in vaginal smears. All animal treatment protocols were approved by the Faculté de médecine vétérinaire, Comité de déontologie, in accordance with the regulations of the Canadian Council of Animal Care.

To bracket the period of implantation, uteri were collected every 3 days from 10-20 pregnant mink randomly selected from the experimental population, beginning at least 6 days after the final mating and continuing through the periods of embryonic diapause and implantation and into late gestation. Animals were terminated by injection of euthanol (T-61, Hoechst, Regina SK) and uteri were removed by midventral laparatomy. The embryos were judged to be in diapause when they could be readily flushed from the uterus, and had a diameter not greater than 1.0 mm (31) and when there was no implantation chamber in the uterus. The expansion or peri-implantation phase of gestation was identified by the

presence of embryos that were expanded to 1.5-2.0 mm, but that were not attached to the endometrium. At this time, the implantation chamber was visible on inspection of the outer surface of the uterus. Implantation was characterized by visible enlargements of the uterine horns and by attachment and invasion of the endometrium by the trophoblast. Postimplantation age was determined by swelling size and by embryonic characteristics, as previously described (3, 32). Uteri from diapause, implantation chambers, swelling sites and portions of uterus from regions between swelling sites in the uterine horn were collected for RNA and histological analysis. For comparative purposes, adrenal glands were also processed for histological scrutiny.

Samples for total RNA extraction were immediately placed in 4 M guanidinium isothiocyanate (GITC, Gibco/BRL, Burlington, ON) solution containing 0.12 M β-mercaptethanol (Sigma Chemical Co., St Louis, MO), snap frozen in liquid nitrogen and stored at -70 C until total RNA was extracted. For immunohistochemical investigation, pieces of uterus were placed in Bouin's fixative overnight and then transferred into 70 % ethanol and stored at 4 C until embedded in paraffin. Longitudinal and cross sections of the uterine horns were prepared by standard histological procedures.

5.4.2 RNA purification

Tissue total RNA was isolated by CsCl (Gibco/BRL) gradient ultracentrifugation (33). Briefly, tissue samples were homogenized with a PT 3000 polytron (Brinkmann, Rexdale, ON) in 4 M GITC solution. The homogenate was then layered onto a 5.7 M CsCl gradient and centrifuged at 174,000 X g using a SW-41 rotor (Beckman, Mississauga, ON), for 20 hours at 21 C. The RNA pellet was then dissolved in 360 µl diethyl pyrocarbonate (DEPC, Sigma) treated distilled water and precipitated twice in 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. After the final washing, the pellet was dissolved in DEPC-treated

water and stored at -70 C until use. The total RNA concentration was determined by spectrophotometry at 260 nm.

5.4.3 Oligonucleotide primers

Primers used in this study were prepared by Gibco/BRL. The sequences of the primers were designed from conserved regions of COX-2 from mouse (34), rat (35), human (36) and sheep (37) sequences. The primers for mink ribosomal S26 protein were designed from the sequence in GeneBank (accession # X79237).

Sense primers were

COX-2-A, 5'-ACAGATCTCGAGCGAGGACC, nt 592-611;

COX-2-B, 5′-CAGTCAAAGACACTCAGGTGG, nt 752-772;

COX-2-C, 5′-CAGCAAATCCTTGCTGTTCC, nt 50-69;

COX-2-D, 5′-CGCCGCTGCGATGCTC, nt (-10)-(+6);

MkS26-A, 5′-AGATGACTAAGAGAGAGGAG.

Antisense primers were

COX-2-1, 5′-CTACAGCTCCGTTGAACGTTCCTTTAGTAGGACTG, nt 1815-1781;

COX-2-2, 5′-CCGCAGCCATTTCCTTCTCTCCTG, nt 1426-1403;

COX-2-3, 5′-ATCGATTACCTGGTATTTC, (mink sequence, nt 732-714);

mkS26-1, 5′-CGATACGAACTTCTTAATGG.

5.4.4 Strategy for mink COX-2 cDNA cloning

Mink COX-2 cDNA was cloned by the reverse transcription-polymerase chain reaction (RT-PCR) method, as outlined in Figure 5-1. The RT reactions were performed by using Superscript-II kit (Gibco) according to manufacturer's instructions. A final volume of 20 µl containing 5 µg total RNA from the mink uterine implantation sites, 20 nmol of dNTP (dATP, dCTP, dGTP and dTTP), 40 units Superscript II M-MLV reverse transcriptase (Gibco), 10 mM DTT, single-strength first strand synthesis buffer and 20 pmol of downstream primers COX-2-1, or COX-2-2. PCR amplifications were performed in a Hybaid Omnigene

Thermal Cycler (Intersciences, Markham, ON) for 40 cycles consisting of 94 C for 45 sec, 52 C for 45 sec, and 72 C for 1 or 2 min, and 72 C for 10 min extension at the last cycle. PCR reactions were made in a total volume of 100 µl containing 10 µl of 10x PCR buffer (0.5 M Tris pH 9, 15 mM $MgCl_2$, 0.2 M $(NH_4)_2SO_4$), 20 pmol of each sense and antisense primers, 20 nmol dNTP, 5 units Taq DNA polymerase (Pharmacia, Baie D'Urfé, QC), 5 µl of RT products .

PCR products were size-fractionated by electrophoresis on 1% agarose gels and fragment size were compared to 1 kb DNA ladder (Gibco). Amplicons of expected sizes were excised from the gel and purified with Sephaglas Bandpreps kit (Pharmacia). The purified cDNA was ligated into pGEM-t vector using T4 DNA ligase according to manufacturer's instructions (Promega, Nepean, ON), and ligation products were transformed into *Escherichia coli* JM 109. Plasmid inserts from positive clones were sequenced with the T7 Sequencing Kit (Pharmacia). To guard against misincorporation of nucleotides by Taq polymerase during PCR amplification, three independent clones were sequenced for each gene fragment and the consensus sequence taken.

5.4.5 Northern analysis

In addition to mink uterine samples, ovaries, adrenals, pieces of the cerebral cortex, large intestine, kidney and skeletal muscle were collected for Northern analysis. Samples of 20 or 40 µg of total uterine RNA were denatured and separated by formaldehyde-agarose gel electrophoresis, and transferred to a nylon membrane. RNA was cross-linked to the membrane by UV irradiation. The blot was prehybridized, hybridized and washed as described previously (38). A 1063 nt probe (fragment mkCOX-2 B-1) was generated from mink uterine total RNA by RT-PCR with upstream primer COX-2-A and downstream primer COX-2-1. This probe (50 ng) was labeled by Random-Primed DNA Labeling Kit (Boehringer Mannhem, Laval, Canada) and hybridized at 60 C overnight. The blots were rinsed with 2X SSC containing 0.1% of SDS 1X and following by two

washes at 65 C for 15 min each. The blots were then exposed to Kodak X-5 film at -80 C for two weeks. To monitor the total RNA loading, the blot was stripped and rehybridized with human 28S probe (39). Transcript size was determined by comparison with a RNA ladder (Gibco) which was subjected to concurrent electrophoresis.

5.4.6 Semi-quantitative RT-PCR

COX-2 transcript abundance in the uterus was determined by semi-quantitative PCR. The mink ribosomal protein S26 gene PCR fragment (121 nt) was used as a control for RNA loading and for the efficiency of reverse transcription (RT). The S26 fragment was subcloned into pGEM-t vector and its sequence confirmed. The quantification procedure was standardized as described (40, 41) with a modification. Briefly, both downstream primers, COX-2-1 and S26-1, were used in RT with 5 µg of total RNA from each sample. RT reactions were performed as described above and products were diluted to a final volume of 200 µl with DEPC-treated water and stored at -20 C. A negative control was carried out by omission of reverse transcriptase in the RT reaction and followed in the subsequent PCR assay. All PCR reactions were performed in final volume of 100 µl containing single strength PCR buffer, 1.5 mM $MgCl_2$, 20 nmol dNTPs, 20 nmol of each primer and 5 U Taq polymerase (Pharmacia) and RT products, 20 µl for COX-2 and 2 µl for S26.

A pool of total RNA from early postimplantation uterine samples was used to optimize the conditions of PCR quantification for COX-2 and S26. Primers COX-2-B and COX-2-2 were employed because they bracket several introns of genomic version of COX-2 in other species. They generate a 675 nt fragment of cDNA, thus contamination of RT products with genomic DNA would be readily evident by the size of the amplified products. RT products equivalent to 0.25, 0.5, 0.75, 1, 1.25, 2.5, and 5 µg of total RNA were amplified using COX-2 primers for 30 cycles in an amplification program consisting of 94 C for 45 sec, 52 C for 45 sec and 72 C for 1 min followed by an extension amplification step at 72 C for 10 min at

the end of the PCR reaction. The amount of 0.5 μg of total RNA was on the linear portion of the amplification curve. Similarly, primers S26-A and S26-1 amplified a 121 nt fragment of S26, and an amount of 50 ng RNA was on the linear portion of the curve. To determine optimal amplification conditions, RT products were subject to 15-40 PCR cycles. The 30 cycle point for COX-2 and the 25 cycle point for S26 were on the linear portion amplification curves just below the asymptote. These cycle numbers were therefore employed in the subsequent semi-quantitative PCR assay, which were carried out in separate reactions as we have previously described (19). For each COX-2 assay sample, 20 μl of COX-2 and 10 μl of S26 PCR products were combined and subjected to electrophoresis on a 2% agarose gel containing ethidium bromide (Sigma). The density of amplified fragments was analyzed by Collage computer software (Photodyne, New Berlin, WI). Three independent RT-PCR assays for each sample were performed and the results were expressed as a density ratio of COX-2 to the external control, the PCR amplified S26 DNA fragment.

5.4.7 Immunohistochemistry of COX-2

A Vectastain ABC Kit (Vector Laboratories, Burlington, ON) was used for the immunohistochemistry staining according to the manufacturer's protocol. Deparaffinized and hydrated sections were immersed in methanol (BDH, Ville St-Laurrent, Canada) containing 0.3% hydrogen peroxide (Fisher Scientific) for 15 min. All incubations were performed in a humidified chamber to minimize evaporation. Following 3 X 5 min washes in PBS, the sections were incubated with 5% normal goat serum at room temperature for 45 min. The excess serum was carefully blotted off and 200 μl of COX-2 (rabbit against human COX-2, PG26, Oxford Biomedical Research Inc., MI) antibodies were applied on each slide at 1:50 dilution in PBS and incubated at 4 C for 18 hours. Following the primary antibody reaction, slides were washed in PBS (2 X 5 min) at room temperature, then the slides incubated with biotinylated

second antibody for 45 min. Following 2 X 5 min washes in PBS, a complex of avidin-biotin-peroxidase was applied for 45 min. The positive reaction was then identified by the application of the peroxidase substrate, 3, 3'-diaminobenzidine (Sigma). For comparative purposes, COX-1 staining was performed on separate sections according to the same procedure, employing antibodies raised in rabbits against ovine COX-1 at 1:50 dilution (42, 43). Sections were then washed in distilled water and lightly counterstained with hematoxylin #1 (Fisher Scientific). Control sections were subject to the same procedure except that dilute rabbit serum replaced the first antibody.

5.4.8 Statistical analysis

The mean density ratio of the PCR fragments COX-2 to S26 from three separate amplifications was taken as an assay value for each sample. Means were then calculated for six stages of gestation, diapause, the period of embryo expansion, days 2-4 , 6-7, 8-9, and 12-15 after implantation. There were a minimum of three and a maximum of 12 different animals represented at each 4 stage of gestation. Where detected (days 2-4, 6-7 and 8-9) COX-2 abundance was compared by one way analysis of variance, followed by individual comparisons by means of Dunnett's test. The value $P<0.05$ was considered significant.

5.5 RESULTS

5.5.1 Cloning and sequence analysis of mink COX-2 cDNA

We cloned the open reading frame of 1812 nt of mink COX-2 cDNA by the RT-PCR method (Figure 5-2, GeneBank, accession number AF047841). The coding region of mink COX-2 cDNA shows high homology with its human counterpart at the nucleotide and amino acid level (86 and 86%, respectively) (36). Homologies to other species are: guinea pig 83 and 85% (44), rat 83 and 87% (35), rabbit 84 and 88% (45), mouse 83 and 87% (34) and sheep 85 and 86% (37) respectively. The ATG translation start site and the surrounding nucleotides are almost entirely conserved relative to other species. The transmembrane domain and

other important functional sites such as those for heme coordination, aspirin acetylation, putative glycosylation and active site tyrosine are conserved (Figure 5-2).

5.5.2 Characteristics of COX-2 mRNA in the mink

Northern analysis employing 20 μg total RNA demonstrated the COX-2 probe hybridized with a single transcript of approximately 4.2 Kb in the adrenal. There was a suggestion of hybridization in implantation sites, which was confirmed when larger amounts of RNA were employed (below). There was weak hybridization with skeletal muscle, but no signal was detected in brain, intestine, kidney, or liver (Figure 5-3A). When 40 μg of uterine RNA was loaded on the gels, the COX-2 probe hybridized with message from embryo-uterine complexes at implantation sites at early stages of postimplantation (Days 2-8, Figure 5-3B) but not in the uterus of diapause nor in the areas of the postimplantation uterus between swellings (data not shown).

5.5.3 Abundance of COX-2 transcripts in the uterus

COX-2 transcripts quantified by RT-PCR were detected only in uterine swellings during early postimplantation gestation. No COX-2 mRNA was detected in uterine samples from the diapause phase of pregnancy, from the peri-implantation period when the blastocysts were expanded but not attached, nor from the interswelling regions between implanted embryos. Transcripts were more abundant in early implantation (days 2-4) than at days 6-7 postimplantation ($P<0.05$, Figure 5-4). No expression could be detected in uterine swellings or interswelling regions of the uterus taken from days 12-15 postimplantation.

5.5.4 Immunolocalization of COX proteins

In the uterus taken during diapause, staining for COX-1 protein was found in low intensity in endometrial epithelium, and in the bases of the uterine glands adjacent to the myometrium. No COX-2 staining was detected at this time. At the peri-implantation stage, when the blastocyst was expanded and the uterine diameter at the site of imminent

implantation was approximately 3.5 mm, staining for COX-1 was restricted to the deep regions of the uterine glands with minor concentrations present in the subepithelial stroma, and as before, no COX-2 protein localization was detectable. At the time of adhesion and early invasion, or day 2 of the implantation process, there was strong signal for COX-2 in the luminal epithelium (Figure 5-5A), the subepithelial stroma and in necks of the uterine glands at the anti-mesometrial pole of the uterus. This phase of implantation was characterized by a swelling size with an external diameter approximately 4.0 mm, by attachment of the trophoblast plaques to the endometrium and by the presence of minor focal invasion. At this time, COX-2 staining was strongest in the antimesometrial regions and associated with the region of attachment of the trophoblast. At the adhesion phase, low levels of COX-1 could be detected in gland bases in samples taken from regions of the uterus where no implantation was present, but no COX-2 signal could be found at these sites (data not shown). When the swelling size attained 5.5-6.0 mm, approximately day 4 postimplantation, the glands were elongated, their cells hypertrophied and the trophoblast had eliminated the endometrial epithelium at sites of attachment. There was COX-2 immunoreactivity in the maternal stroma, but none in the trophoblast (Figure 5-5B). At this time there was a strong COX-2 signal in the necks of the hypertrophied glands, most pronounced in the basal portions of the cells (Figure 5-5B, C and D). No binding of the COX-1 antibody was detected. When the uterine swelling diameter reached 8-9 mm, 7-8 days after implantation, the trophoblast had begun to invade down the uterine glands. COX-2 staining was strong in basal region of the cells, down the length of the extended uterine gland necks (Figure 5-5E), and had spread to mesometrial side of the uterus, where invasion does not occur (data not shown). COX-1 was not widely detected in the uterus at any time after implantation. The fundal regions of maternal gland cells in areas between uterine swellings showed some COX-1 activity (Figure 5-5F), while COX-2 did not localize to these sites

(Figure 5-5G). By day 12 postimplantation, when the swelling size attained 10 or more mm, the maternal-fetal labyrinth portion of the placenta was formed. At this time, no COX-1 nor COX-2 could be localized in the endometrium and there was no detectable binding of the COX-2 antibody to either the maternal or fetal component of the placenta.

Immunohistochemistry demonstrated COX-2 also localized to the adrenal cortex in mink, in keeping with the distribution of its mRNA by the Northern analysis (data not shown)

5.6 DISCUSSION

The mink sequence proved to have substantial homology with other known species, in the range of 83-88 %. All of the active sites were conserved, including the heme coordination sites, the aspirin acetylation site, the putative transmembrane domain and glycosylation sites (44, 46). The mink sequence contained a potential signal peptide of 24 amino acids which preceded the amino terminal, as first shown in the sheep sequence (46). There were three areas of variability with sequences in other species, one in the signal peptide region, a second at nt 1141-1320, and the carboxyl terminal region, nt 1747-1775. Interspecies variation in signal peptide and carboxyl terminus regions has previously been noted in comparisons between the sheep and human sequences (46).

Prostaglandin synthesis is essential to successful embryo implantation in rodents (30). Its involvement in carnivores is indicated by the ability of indomethacin treatment to delay implantation in the ferret (24). This is the first indication of a role for COX in implantation in a species displaying obligate embryonic diapause. RT-PCR analysis demonstrates that COX-2 expression is a transient event which occurs at the time of trophoblast attachment and invasion in the mink, over the first 8-9 days after initiation of implantation. Histochemical findings concur. The first detectable localization was in uterine stroma and glands at sites where the trophoblast had adhered, but had not yet penetrated the endometrial epithelium. The temporal expression of COX-2, i.e. its

relation to the early stages of the implantation process, concurs with findings in the mouse, where in situ hybridization and immunohistochemical localization of COX-2 demonstrated its presence at the time of embryo attachment (27). Spatial localization was similar, COX-2 was found associated with the presence of the embryo in both species and it appears first in the luminal endometrial epithelium and subepithelial stroma. The pronounced localization in the necks of uterine glands in the mink at the time of early attachment does not occur in the mouse, where fewer gland structures are present. Nonetheless, COX-2 is found in endometrial glands from the human uterus, as well as in the decidual tissues (29, 47). The COX-2 signal is likewise present later in gestation in the decidual cells at the mesometrial pole of the uterus of the mouse (27). COX-2 was not found in the mink embryo, nor was it found prior to implantation. In contrast, COX-2 is present in both the endometrium (22) and the trophoblast (18) prior to attachment of the ovine embryo.

The essential role of prostaglandins in decidualization in rodents was demonstrated by Yee and Kennedy (15). Recent evidence indicates that COX-2 is implicated in decidualization in mice, as this process is defective in transgenic mice bearing the null mutation for this gene (30). The cellular analog of the rodent decidual cell (48) appears not to exist in the carnivores (10, 49), suggesting an alternate role for prostaglandins the establishment of the mink conceptus and consequent placental development.

In the uteri of ovariectomized, steroid treated mice, COX-2 was not inducible by ovarian steroids alone, rather it required the presence of the blastocyst for its expression (27). In the mink uterus, COX-2 transcripts and protein were detected only at sites of embryo attachment and invasion. In addition, it was associated with the ablation of the endometrial epithelium and with early establishment of the placenta. Together these findings suggest that there may be a trophoblastic signal

which induces COX-2 expression in mink. We have previously shown that mink embryos can escape diapause in vitro in co-culture with mink uterine cell lines (5). Recent investigations indicate that the presence of the mink embryo elevates prostaglandin E_2 accumulation in uterine stromal cell cultures (Moreau GM, Song JH, Smith LC, Murphy BD, unpublished). Factors which induce COX-2 and prostaglandin expression in other tissues include interleukin-1 (50) and epidermal growth factor (51). Little information is available with respect to carnivore implantation. Nonetheless, there is clear evidence for EGF receptors in the endometrium of the spotted skunk at the time of implantation (52). Further, a cytokine, leukemia inhibitory factor is expressed in the mink uterus at the time of blastocyst expansion and during the first two days of postimplantation (53). To date there is no evidence to indicate that either of these factors is of trophoblastic origin.

COX-1 in the present study was distributed throughout the uterus, and present in uterine glands and endometrial epithelium prior to implantation. Its presence in these two sites is consistent with its occurrence in the preimplantation mouse uterus (27). COX-1 was detectable in the basal region of uterine glands in areas between uterine swellings, but not in at the site of implantation, and could not be found later in gestation. COX-1 is not essential for implantation in the mouse (30), and its distribution in the mink indicates that it may not be involved in the implantation process in this species.

In summary, we have determined the cDNA sequence of the COX-2 enzyme in a domestic carnivore in which the reproductive cycle is characterized by obligate delayed implantation. This cDNA has high homology with other known species, and the known biologically active domains are conserved. The enzyme is locally expressed at sites of embryo invasion, particularly in the necks of the uterine glands during early implantation. Its coincidence with embryo implantation,

decidualization and placenta formation suggests that locally produced prostaglandins are proximate inducers of these processes.

5.7 ACKNOWLEDGMENTS

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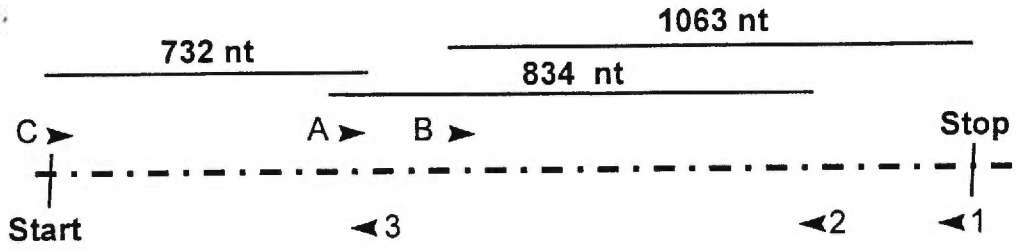
1063 nt
732 nt
834 nt
C
A
B
Stop
Start
3
2
1

Figure 5-1. Cloning strategy for the mink COX-2 cDNA. Cloning was via first strand cDNA generation by reverse transcription (RT) from uterine total RNA and followed by PCR amplification using primers described in Materials and Methods. The sense primers are indicated by capital letters and the right arrow heads, while the antisense primers are indicated by numbers and left arrowheads. The broken line represents the coding region of the COX-2 cDNA structure and overlapping cloned sequences are represented above the composite diagram of the mink COX-2 cDNA.

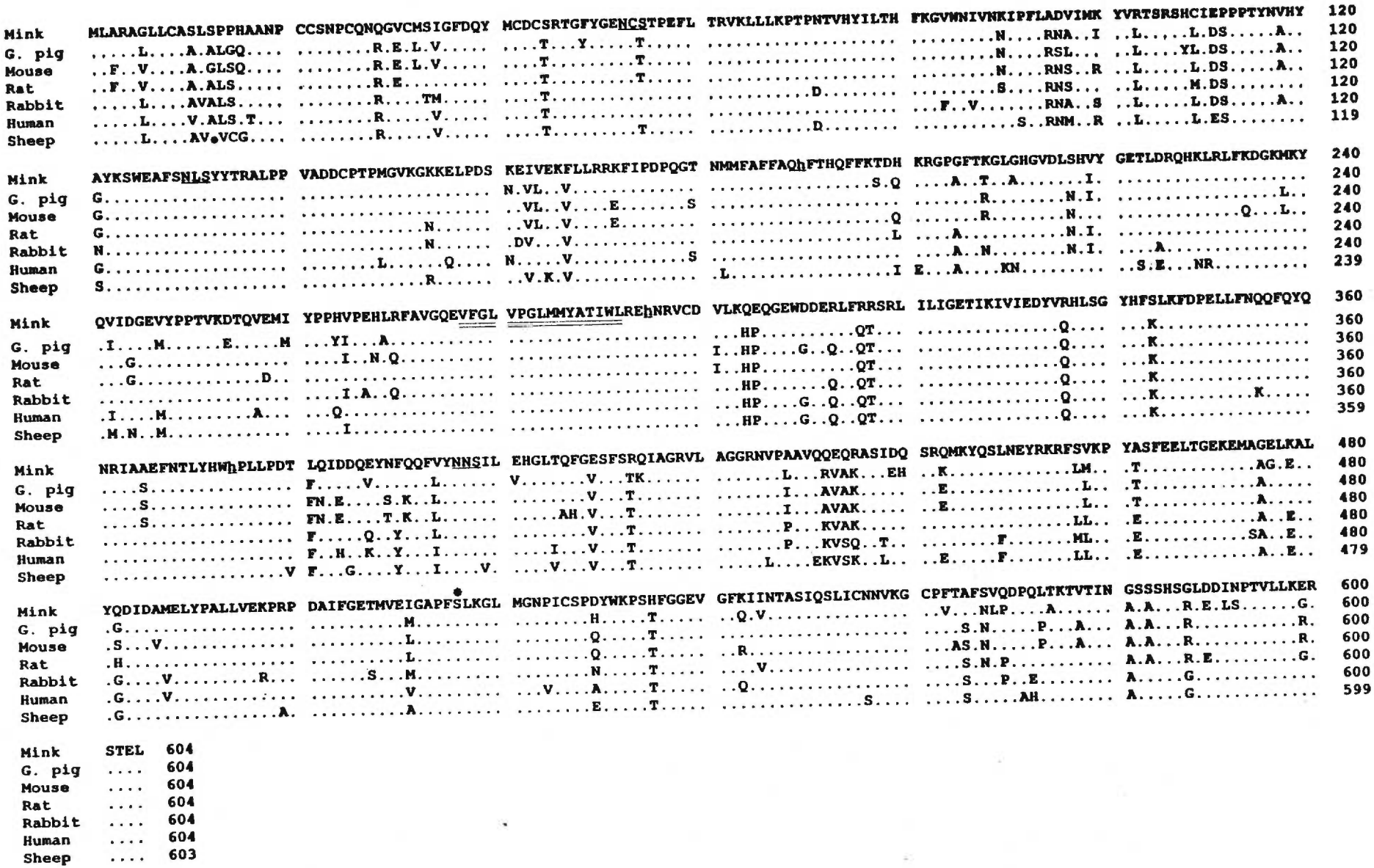

Figure 5-2. Deduced amino acid sequence of mink COX-2 compared with other known sequences. A period identifies identical amino acids, while mismatches are shown with the differing one letter code. A shift in the sheep sequence to maximize alignment is indicated by (•). The potential sites of N-glycosylation are underlined and the putative membrane spanning domain is marked by double underlines. The heme coordination sites are indicated by an underlined lower case letter and the aspirin acetylation site by an asterisk. G. pig designates the guinea pig sequence.

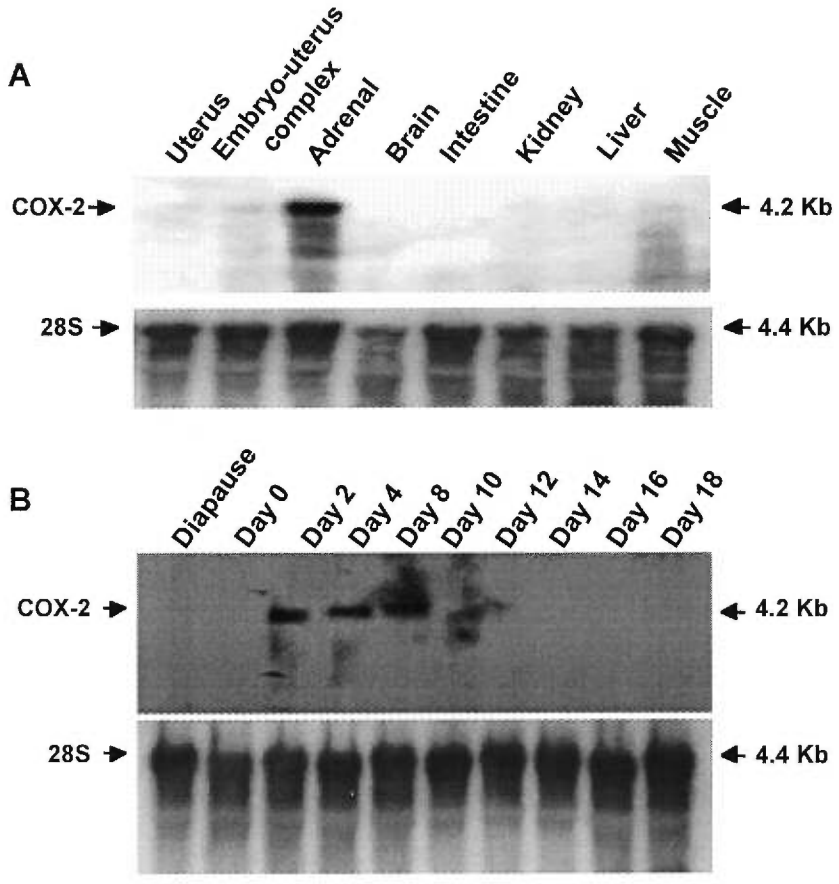
A
Uterus
Embryo-uterus complex
Adrenal
Brain
Intestine
Kidney
Liver
Muscle
COX-2
4.2 Kb
28S
4.4 Kb
B
Diapause
Day 0
Day 2
Day 4
Day 8
Day 10
Day 12
Day 14
Day 16
Day 18
COX-2
4.2 Kb
28S
4.4 Kb

Figure 5-3. (A) Detection of COX-2 mRNA in mink tissues by Northern blot employing 20 μg total RNA. (B) Northern blot of COX-2 in the uterus through diapause and late postimplantation. An aliquot of 40 μg of total RNA was loaded for each sample. Following hybridization with the homologous COX-2 probe, blots were hybridized to a human 28S probe. Diapause indicates the uterus of delay prior to implantation, expansion (day 0) refers to the time when the embryo is reactivated and expanded, but has not yet attached to the endometrium. Days 2, 4 and 8, 10-14 and 15-18 represent samples of embryo-uterine complexes. The postimplantation age was determined by swelling size and morphological characteristics of the embryo.

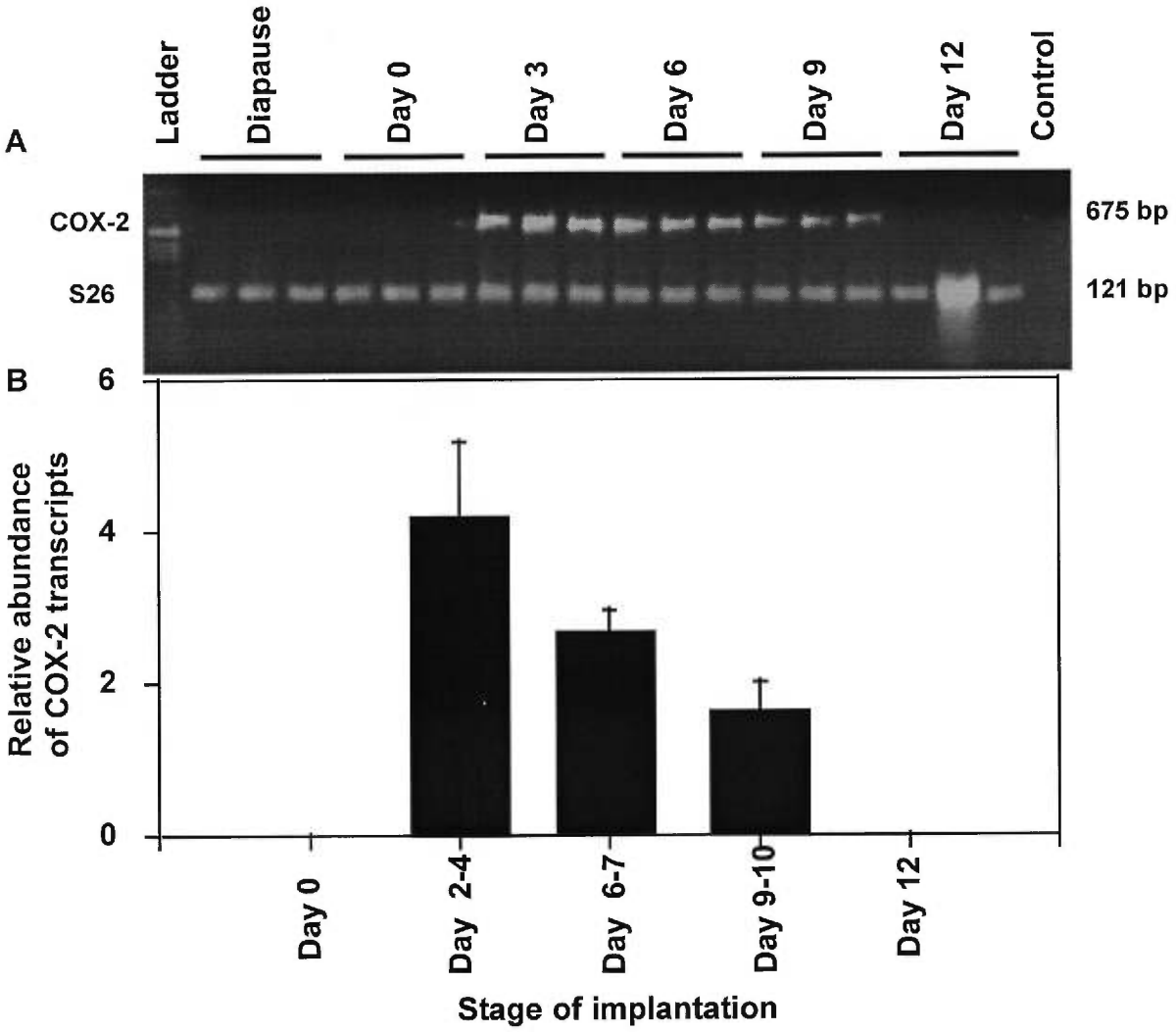
A
Ladder
Diapause
Day 0
Day 3
Day 6
Day 9
Day 12
Control
COX-2
S26
675 bp
121 bp
B
Relative abundance of COX-2 transcripts
6
4
2
0
Day 0
Day 2-4
Day 6-7
Day 9-10
Day 12
Stage of implantation

Figure 5-4. Illustration of semi-quantitative RT-PCR for COX-2 transcripts in the mink uterus during diapause and postimplantation gestation. (A). A representative agarose gel demonstrating migration of triplicate RT-PCR products (upper band 20 µl of COX-2, lower band, 10 µl of S26). Samples were subject to the same reverse transcription, amplified in separate tubes and then combined for electrophoretic analysis. The first lane on the left is the 1 kb DNA ladder. (B) Mean ± SEM of the dimensionless ratio of COX-2 to S26 amplicons from embryo-uterus complexes of various postimplantation ages in the mink. Day 0 represents the day of embryo expansion prior to attachment. All means were different from each other at $P<0.05$.

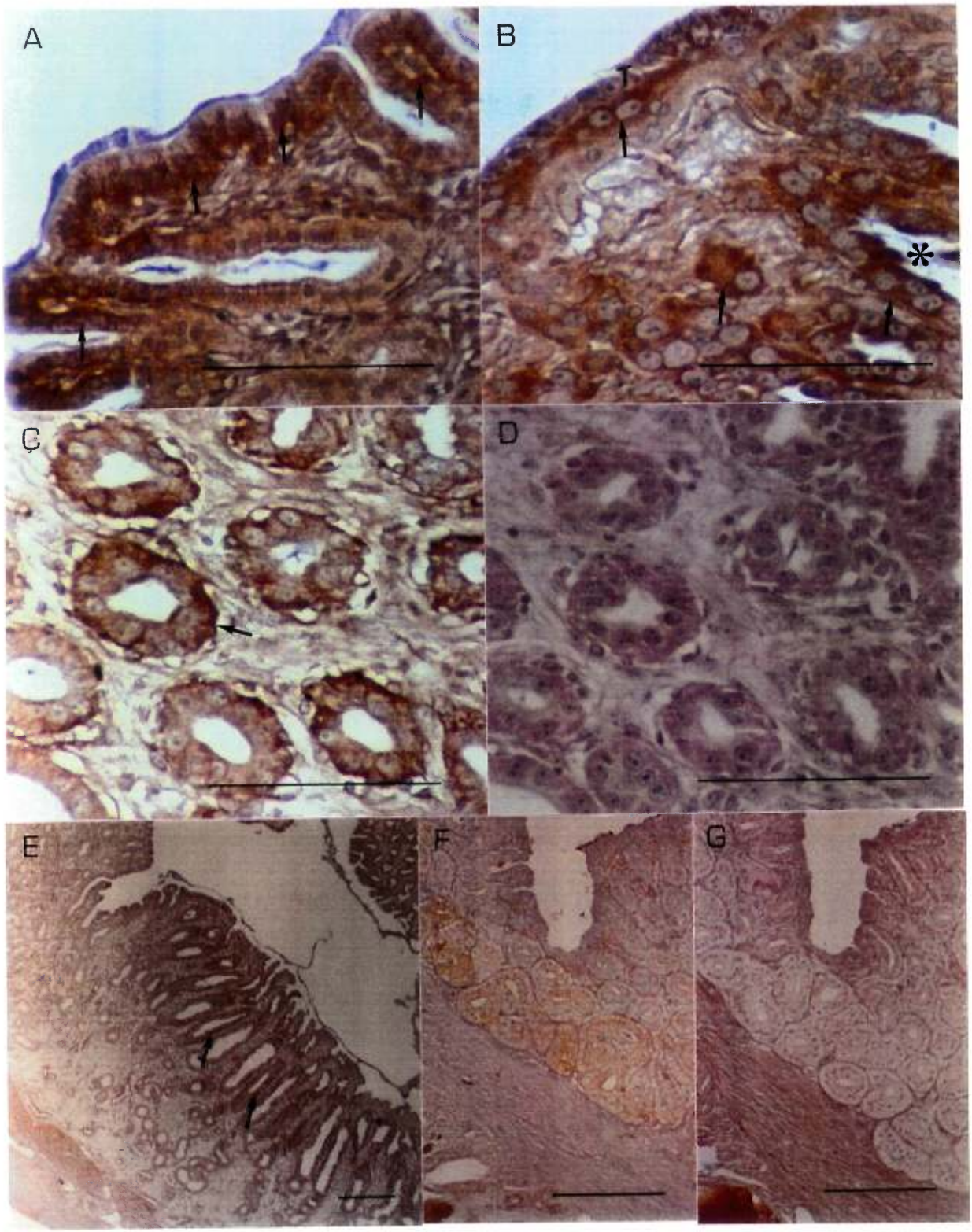
A
B
C
D
E
F
G

Figure 5-5. Immunohistochemical localization of COX isoforms in the mink uterus. (A) Expression of COX-2 in the uterine epithelium at the site of embryo attachment on day 2 postimplantation. The trophoblast overlying the epithelium is blue-purple, examples of epithelial expression of COX-2 are indicated by arrows. (B) At day 4 postimplantation, the trophoblast (T) has eliminated the epithelium at the sites of invasion and COX-2 localized to uterine stroma (arrows) and to uterine gland cells (asterisks). (C) COX-2 localization in the basal regions of uterine gland necks on Day 4 postimplantation. (D) Negative control for localization in Figure 5-5C, tissue sections were subject to the same treatment except dilute rabbit serum replaced the anti-COX-2 antibody. (E) Lower power micrograph of the mink uterus showing the extent of COX-2 expression in the necks of elongated uterine glands (arrows) at day 4 postimplantation. (F) COX-1 localization in the fundi of uterine glands in the inter-swelling area at day 4 postimplantation. (G) COX-2 antiserum treated section adjacent to that in F (above) demonstrating the absence of localization of this isoform of the enzyme. The bar on each photo represents 10 μm.

6.0 GENERAL DISCUSSION

This study provides new information on the uterine morphological changes during embryo implantation, and on the formation of the symplasma and the maternal-fetal labyrinth in the mink. The initiation of attachment in mink is the result of embryo expansion to form an implantation chamber rather than by closure of the uterine lumen to grasp the embryo, as seen in the mouse (Abrahamsohn and Zorn, 1993). Closure of uterine lumen in rodents is triggered by P_4 (Finn, 1977). Similarly, the increased P_4 level in mink during preimplantation phase coincides with blastocyst expansion (Møller, 1973; Murphy and Moger, 1977; Stoufflet et al., 1989) and results in an intimate relationship between embryo and endometrial surface. The role of P_4 in expansion is not known, as expansion occurs in vitro (Moreau et al., 1995) and may be caused simply by fluid uptake (Daniel, 1967).

The typical decidual cell reaction seen in rodents has not been observed in the mink. Instead, the maternal symplasma derived from epithelial cells may function as does the mouse decidua to regulate trophoblast invasion. Decidual cells in rodents synthesize PGs throughout pregnancy (Anteby et al., 1975; Parr et al., 1988) and PGs from the decidual cells may participate in an autocrine or paracrine manner in maintenance of increased vascular permeability and differentiation of decidual tissue (Kennedy, 1985) and placentation. Whether the mink symplasmal mass plays the same role in producing PGs is unknown. In the present study, the precursors of the symplasma, the uterine gland neck cells strongly expressed COX-2 during placenta formation, suggesting that the symplasma may be an important source of uterine PGs.

There have been detailed investigations of the uterine morphological changes associated with implantation and placentation in dog (Anderson, 1969), ferret (Enders and Schlafke, 1972; Gulamhusein and Beck, 1973; Lawn and Chiquoine 1965), cat (Leiser, 1982; Leiser and Koob, 1993) and western spotted skunk (Mead, 1981; Sinha and Mead, 1976). In

the mink, the placental type and uterine morphology during delayed implantation have only been superficially described (Bychkova, 1971; Enders, 1957; Enders and Enders, 1963). In this study, mink were sampled to provide a sequence of the morphological changes in the uterus during diapause, preimplantation, implantation and postimplantation gestation.

This study is the first known report on LIF and COX-2 gene cDNA sequences and their expression throughout gestation in a carnivore species. LIF and COX-2 are expressed in the mink uterus during implantation and early postimplantation gestation, respectively. LIF has been shown to have an essential role in implantation in mouse (Stewart et al., 1992). The expression of LIF coincides with implantation in several species (Anegon et al., 1994; Bhatt et al., 1991; Charnock-John et al., 1994; Stewart, 1994b; Yang et al., 1994). The present study shows that LIF expression coincides with termination of embryonic diapause and initiation of implantation, and that it ceases shortly after implantation occurs. This suggests that the role of LIF in implantation is stimulatory rather than inhibitory to the preimplantation embryo in this species as suggested by Murphy (1992). In the present study, immunohistochemistry seems to indicate that the uterine endometrium may be the only source of LIF protein. It remains possible that a homologous antibody may have revealed other sites, where LIF was synthesized or stored. Nonetheless, similar expression patterns have been observed in the mouse (Bhatt et al., 1991), rabbit (Yang et al., 1994) and several other species including the human (Charnock-John et al., 1994). In contrast to these observations, LIF expression was detected in trophoblast cells by immunohistochemistry in ewe at Day 17 of pregnancy (Vogiagis et al., 1997). We found no LIF in mink embryos or trophoblast, although RT-PCR analysis of embryos alone was not undertaken.

In this study, the expression in vivo of COX-2, determined by RT-PCR assay and Northern analysis, was elevated at trophoblast invasion and declined through Day 9 postimplantation. Immunohistochemical

studies revealed that COX-2 is expressed in the endometrial glands at implantation sites, but not in the inter-swelling areas of the uterus nor in trophoblast cells. Preliminary observations by Western blot revealed the presence of COX-2 protein in mink uterine cell lines incubated with embryos (Figure 9-6). Other preliminary evidence indicates that PGE_2 accumulates in culture medium when mink endometrial cell lines are cultured in the presence of blastocysts (Moreau, Song and Murphy, unpublished observation). Together these fragmentary results suggest that COX-2 expression and PG synthesis may be induced by the presence of embryo. This concurs with the evidence on localization of COX-2 in vivo in this study, where its expression was restricted to implantation sites. Evidence for embryo-induced expression of COX-2 and embryonic induction of local PG secretion in endometrium have been shown in mouse (Chakraborty et al., 1996; Kennedy, 1994). These PGs may be locally involved in vascular permeability associated with trophoblast invasion in mink uterus. As COX-1 is expressed at all stages of gestation in mink uterus, its contribution to PG synthesis is not known.

The failure of implantation in LIF deficient mice is due to failure of decidualization (Stewart, 1994b), and LIF and COX-2 may be involved in decidualization in rodents (Stewart, 1994b; Chakraborty et al., 1996). The role of these elements in mink, an adecidual species, is less clear. The temporal expression pattern of LIF suggests that LIF primes the uterine endometrium and initiates the implantation process. It is followed soon after by COX-2 expression, particularly in the uterine stroma and uterine glands. In mink there is coincidence of symplasma formation with COX-2 expression and these events take place in the same cells, the epithelium of the necks of the uterine glands. It is possible to speculate that PGs play a role in development of the maternal symplasma or in promoting trophoblast invasion.

LIF transcripts are present in low abundance in the mink uterus, not detectable by Northern blot, even where 40 μg total RNA was loaded.

The Southern blot (Figure 9-1) determined that the mink LIF cDNA C-6 fragment is a valid probe for blot analysis. Nonetheless, the present study indicates that RT-PCR is a reliable method to study LIF expression in mink during delayed implantation and early postimplantation.

We have proposed that LIF terminates embryonic diapause and initiates implantation in the mink. However, infusion of human recombinant LIF (hrLIF) into the uterine lumen of the western spotted skunk during delayed implantation phase did not induce termination of delay and initiation of implantation (Mead RA, 1998 personal communication). Further, addition of hrLIF to embryo-cell line co-cultures did not increase the frequency of mink embryo hatching in vitro (Moreau, Song and Murphy, unpublished observations). This suggests that the LIF involvement in the implantation process is complicated in mustelid carnivores and that it may be one of several factors of ovarian and uterine origin necessary for the endometrial function (Foresman and Mead, 1978; Mead, 1981; Murphy et al., 1983; Huang et al., 1993). A recent study has revealed that the LIF is involved in activating the hypothalamus-pituitary-adrenal axis during stress and inflammation (Chesnokova et al., 1998). There is a possiblility that LIF may also be a factor in reactivation of the hypothalamus-pituitary-CL axis in the mink. More comprehensive investigation of LIF in implantation is merited.

LIF and PGs are involved in the regulation of ECM-degrading enzymes in vitro in endometrial cells which are subject to decidual transformation (Harvey et al., 1995; Zhang et al., 1996). ECM-degrading enzymes, including MMPs and their tissue inhibitors, TIMPs, are under precise control in the process of trophoblast invasion and decidualization in mouse and human uterus (Lala and Graham, 1990; McMaster et al., 1994; Alexander et al., 1996). Preliminary immunohistochemical localization revealed that a gelatinase, MMP-9 is present in trophoblast cells at the limit of invasion in the mink (Song and Murphy, 1998,

unpublished observations). The nature of LIF and PG involvement in regulation of MMPs and TIMPs is not understood in any species.

Delayed implantation has provided an useful model in studies of reproduction in mammals because it permits separation of the endometrial and embryonic changes preceeding implantation from those of implantation. The advantage in mice and rats is that it can be experimentally manipulated to more precisely control the developmental events in the embryo and uterus. Ovarian steroid hormones, P_4 and estrogens are the key elements which are shown to induce uterine secretion of elements such as LIF and growth factors (Johnson and Chatterjee, 1993a; 1993b; Paria et al., 1993b; Psychoyos, 1973a; Stewart et al., 1992; Taga, 1992). In contrast, the regulation of delayed implantation in mustelids has not yielded to the same approaches. Administration of steroid hormones has not successfully been employed to induce implantation in either intact or ovariectomized mink (Hansson, 1947; Enders, 1952; Cochrane and Shackelford, 1962; Murphy and James, 1974; Rose et al., 1996). No success was recorded in other mustelids, such as the ferret (Wu and Chang, 1973; Foresmen and Mead, 1978), the badger (*Meles meles*, Canivenc and Laffargue, 1958; Canivenc et al., 1967), the weasel (*Mustela nivalis*, Wright, 1963; Shelden, 1973) or the western spotted skunk (Mead, 1981). Mead and Eroschenko (1995) reported that the failure of attempts to hasten implantation by administration of steroid hormones, P_4 and E_2 is not due to deficiencies in the number or functional status of the receptors for these steroids in the western spotted skunk. Other luteal factors, presumably proteins, are necessary for implantation (Foresman and Mead, 1978; Murphy et al., 1983; Huang et al., 1993). To date, the nature of the elements in not known.

Overall, there remain several problems limiting our understanding in control of delayed and initiation of implantation and invasion in the mink. Further attention could be given to a number of aspects. First of all, the LIFR and gp130 protein have been localized on the blastocyst in

mouse and rabbit (Yang et al., 1995a; 1995b), which provides a mechanism for interaction between embryo and uterus at implantation. Determination of the expression of LIFR and gp130 protein in the mink preimplantation embryo by RT-PCR or in situ hybridization would provide new information to further understand the role of LIF in termination of diapause and initiation of implantation in this species. Although Mead failed to induce implantation reaction in the western spotted skunk in vivo by administration of hrLIF (Mead RA, 1998 personal communication), we observed an increase of embryo survival when mink embryos co-cultured with cell lines that express LIF (Moreau et al., 1996). These contradictions suggest that the involvement of LIF in termination of diapause is not simple, and dose and time of administration may be important. LIF may also involve in the regulation of Prl secretion or in expression of Prl receptors since disruption of LIF gene results in an inhibition of hypothalamus-pituitary function in the mouse (Chesnokova et al., 1998). If it were possible to disrupt the LIF gene in mink, a difinitive proof of its role in implantation might emerge, as it has seen in the mouse (Stewart et al., 1992; Chesnokova et al., 1998).

Secondly, cytokines other than LIF could also involved in the process of implantation in other species and they may be also responsible for the stimulation of the reinitiation of development of the blastocyst in diapause. Colony-stimulating factor 1 (CSF-1) is a steroid-regulated protein, produced by the uterus in mouse (Arceci et al., 1989). CSF-1 is present on the mouse uterine epithelium prior to implantation, and stimulates trophoblast invasion of the maternal epithelium (Pollard et al., 1987). The receptor for CSF-1 has been localized on trophoblast cells (Pollard et al., 1987). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by the uterine glands and is known to induce the growth of mouse trophoblast and is also involved in the process of implantation in the mouse (Kanzaki et al., 1991; Crainie et al., 1990). The other cytokines, such as interleukins (IL-1 and IL-6), IFN-τ, tumor necrosis

factor-α (TNF-α), transforming growth factor-β (TGF-β) have been implicated in implantation process in humans and rodents (see reviews by Robertson et al., 1994; Chard, 1995; Simon et al., 1995; Sharkey, 1998). However, none of these have yet been investigated in mustelid carnivores.

Thirdly, it has been shown that COX-2 expression is associated with trophoblast invasion and placenta formation in the present study. COX-2 was localized to the implantation sites in this study,as also found in mouse (Chakraborty et al. 1996). This local COX-2 expression may result in a local synthesis of PG in the mink uterus and this local PG production may be essential to the completion of the process of implantation. To date, there is no evidence on the role of PGs in implantation in this species.

Fourthly, it is well known that Prl reactivates the CL thus increasing production of P_4 and other ovarian factors resulting advanced implantation in mink. It is also known that Prl terminates mink embryo diapause and stimulates growth of embryonically derived stem-like cell in vitro (Polejaeva et al., 1997). Prl binding sites and receptor mRNA transcripts have been found on mink uterus, and they are regulated during gestation by steroids and melatonin (Rose et al. 1983; Douglas et al. 1998; Rose et al., 1996). These observations suggest that Prl may have a direct action on the uterus and/or on the embryos. A precise function of Prl in the process of implantation needs to be investigated. It is possible Prl induces an uterine receptivity for embryo implanating and terminates the diapause of the embryos in vivo.

Next, there are morphological changes in the endometrium associated with embryo reactivation and trophoblast invasion in mink, as seen in other mustelid species with obligate delayed implantation (Given and Enders 1989). The mechanisms of trophoblast penetration and invasion are not completely known in any species. Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) have been

implicated in the tissue invasion process in humans and laboratory rodents (Lala and Graham 1990; Strickland and Richards 1992). Our preliminary results have shown MMP-9 and TIMP-3 localized in the interface of the trophoblast and endometrial epithelium (Song and Murphy, 1998, unpublished observations). This suggests that the process of invasion may be under the control of MMPs and TIMPs in the mink. Investigation of the regulation of matrix-degrading enzymes, may provide valuable insight onto the process of trophoblast invasion.

And last, symplasmae appear in the mink during trophoblast invasion and placenta formation instead of decidual cells as seen in human and rodent. The symplasma is also seen in other mustelid such as the ferret (Lawn, 1965; Enders, 1972), but its role is unknown in these species, including the mink. The symplasma may have a similar role in carnivores as decidual cells do in the human and rodent, i.e. production of hormones and proteins (Parr and Parr, 1989; Weitlauf, 1994). Whether symplasmal cells produce PGs merits further investigation.

The mink is a seasonal breeder and they breed but once per year. The variation in delayed implantation limits the studies of the interaction between embryo and uterus during implantation in this species. In vitro model would provide tools to resolve this problem. Co-culture of cell lines derived from mink trophoblast cells (Smith and Murphy, unpublished data) with immortalized endometrial cell lines from mink uterus (Moreau et al., 1995) could be employed. To address the hypothesis that embryonic cells induce COX-2 expression and PG production by co-culture of trophoblast cell lines and endometrial cell lines. There also appear possiblilities to test the effects of hormones, cytokines, and growth factors on the differentiation of embryonic and endometrial cell lines in this in vitro model.

As previous reported, we have successfully cultured embryo collected from mink uteri during diapause and reactivated stages in various complex culture media containing amino acids and serum

(Moreau et al., 1995; 1996). Further this culture system allows some embryos to escape diapause and resume development in vitro. One problem has been seen during the past few years, the failure of survival of a proportion of the cultured mink blastocysts in some experiments. This may be the result of frequent changes of medium. The blastocysts can survive for 4 weeks or even longer in culture without changes of medium, and some of the embryos can hatch in this condition (Song, Smith, Murphy unpublished observations 1994-1998). During diapause, the mink embryos survive in a relatively unchanged environment in vivo (Daniel 1967). Uterine secretion is low and the uterine fluid is expected to be in lower abundance than at the time of activation and preimplantation (Aplin 1989). Blastocysts and endometrial epithelium may produce unknown factors which are necessary for embryo survival. Frequent changes of medium dilute the concentration of these factors and result in failure of support for the embryos.

Implantation is complex events, essential to mammalian reproduction, including in the mink. Biochemical, cytological, morphological, endocrinological, immunological, and physiological changes are involved in this process. Similarities in the process argue for similarity in mechanisms in different species. The mustelid carnivores are unique models for the study of nidation. By employment of molecular tools, a number of advances have been made in understanding implantation in the mink. A number of challenges remain.

7.0 GENERAL CONCLUSIONS

7.1 Trophoblast invasion and placentation

This study has described morphological changes associated with trophoblast invasion and placentation in the mink. These observations made it possible to precisely study the time of gene expression of uterine factors associated with implantation in this species. The sequence of trophoblast invasion and embryonic characteristics are summarized in Table 7-1.

Table 7-1. Sequences of trophoblast invasion and embryonic characteristics of mink

Stages of implantation	Diameters of the uterus and swelling (mm)	Characteristics of the embryo	Characteristics of the uterus
Delayed implantation	Uterus 1-1.5	Bl: mitosis ceases Ø: 200-800 µm	Numerous crypts Ep: tall columnar
Preimplantation reactivated phase	Uterus 1.5-2	Bl: expanded, Ø: 800-2000 µm	ES: mucoid apical fringe appears
Day 0 of implantation	Uterus 2-3	Bl: expended Ø: 2000 µm	Crypts are eliminated
Day 1 of postimplantation	Swelling 4-5	TP differentiates and attaches to Ep	Ip chambers appear
Day 2	Swelling 4-5	Em capsule is broken	
Day 3-4	Swelling 4-5	Tr intrudes down EG, abuts on St. Em NT is in evidence	Ep is replaced by trophoblast
Day 5-6	Swelling 4-5	C-shaped fetus	MS appears
Day 8-9	Swelling 4-5	Crl is 1-2 mm	Pl forms, but not yet matured and 3 zones can be seen.
Day 10-12	Swelling 4-5	Crl reaches 2-4 m m	La & Mature Pl formed

Notes: Bl: blastocysts; Ø: blastocyst diameter; TP: trophoblast plaque; Ep: epithelium; ES: epithelial surface; St: stromal cells; Em: embryo; Ca: capsule; Ip: implantation; Tr: trophoblasts; EG: epithelial glands; MS: maternal symplasma; La: labyrinth; Crl: crown-rump length; Pl: placenta; NT: neural tube;

7.2 LIF and COX-2 gene cloning

This study is the first known report on the cDNA sequences for LIF and COX-2 in a mustelid carnivore. The mink LIF and COX-2 coding sequences consist 609 and 1812 nucleotides, with proteins of 202 and 604 amino acids, respectively. The homology comparison with other known cDNA sequences for LIF and COX-2 and their deduced sequences of amino acids in mammal species are listed in table 7-2.

Table 7-2. Homology comparison of mink LIF and COX-2 cDNA and their deduced amino acid (aa) among species known. (%)

Mink	Mouse	Rat	Rabbit	Human	Sheep	Guinea pig	Pig	Cow
LIF cDNA	80.6	NI	NI	90	86.8	NI	88.2	87.6
LIF aa	79.2	NI	NI	90.1	85.4	NI	91	90.1
COX-2 cDNA	83	83	84	86	85	83	NI	NI
COX-2 aa	87	87	88	86	86	85	NI	NI

NI, no information.

7.3 Uterine LIF and COX-2 expression

High levels of LIF mRNA and protein are detected in the mink uterine epithelial glands at the embryo expansion stage and in early postimplantation. The abundance of COX-2 mRNA reaches its maxima at days 3-5 of postimplantation, gradually decreases through day 9, but is not present thereafter. COX-2 protein is localized in uterine epithelium, stroma and necks of endometrial glands at sites of implantation. COX-2 mRNA and protein were not detected in the uterus during diapause or between implantation sites. The transcript for COX-2 is of 4.2 kb in this species.

In summary, the patterns of the transcript appearance and relative levels of abundance of LIF and COX-2 in uterus/embryo complex throughout diapause, implantation and early stage of gestation by RT-PCR assay are listed in table 7-3.

Table 7-3. Patterns of LIF, COX-1 and COX-2 expression in uterus during early stages of gestation.

Factors	Dia-pause	Day -1,-2	Day 0*	Day 2	Day 5	Day 7	Day 10
LIF	--	--	+++	++	--	--	--
COX-2	--	--	--	++++	+++	++	+
COX-1	++	++	+	+	+	+	+

Notes: -- indicates absent while + indicates detective levels. *Visible expansion of blastocyst in implantation chamber, but no attachment was considered as Day 0 of implantation.

The coincidence of LIF expression with implantation in mink suggests that LIF is involved in the implantation process, and may be a maternal signal which terminates obligate embryonic diapause. COX-2 expression appears to be induced in the endometrium by the embryo and may play a role in implantation and placentation in mink.

Together the data in this study suggest that expression of LIF and COX-2 are consequently associated with implantation process in the mink. LIF may induce the the termination of diapause and initiation of implantation. Subsequently, embryonic factors induce COX-2 expression which may increase a local PG production and the changes of vascular permeability in the endometrium. Whether LIF directly induces COX-2 in the uterus at the time of implantation is not clear. It is also worth while to further investigate the roles of LIF and PGs in implantation in this species to derive a better understanding of the mechanisms in initiation of implantation in those carnivores with an obligate delay of implantation. The relatively short in obligate delayed implantation and availibity of mink render it a good model for these studies.

8.0 GENERAL REFERENCES

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9.0 APPENDIX

9.1 Southern analysis of leukemia inhibitory factor gene

Genomic DNA was purified from mink liver as described by (Sambrook et al., 1989). Ten µg of genomic DNA in each sample was digested at 37 C, overnight by different restriction enzymes (Pharmacia). The products were separated by agarose gel electrophoresis and the gel was stained with ethidium bromide for UV light visualization and DNA ladder (1 kb) was employed to measure the DNA fragment size. The DNA fragments were then capillary-transferred onto a nylon membrane from the gel and cross-linked by UV irradiation. The blot was prehybridized, hybridized and washed as described previously Northern analysis. The mink LIF cDNA fragment C-6 (411 nt) was used as probe. This probe (50 ng) was labeled by Random-Primed DNA Labeling Kit (Boehringer Mannhem) and hybridized at 60 C overnight. The blots were rinsed with 2X SSC containing 0.1% of SDS 1X and followed by two washes at 65 C for 15 min each. The blots were then exposed to Kodak X-5 film at -80 C for 10 days.

Southern analysis of LIF gene

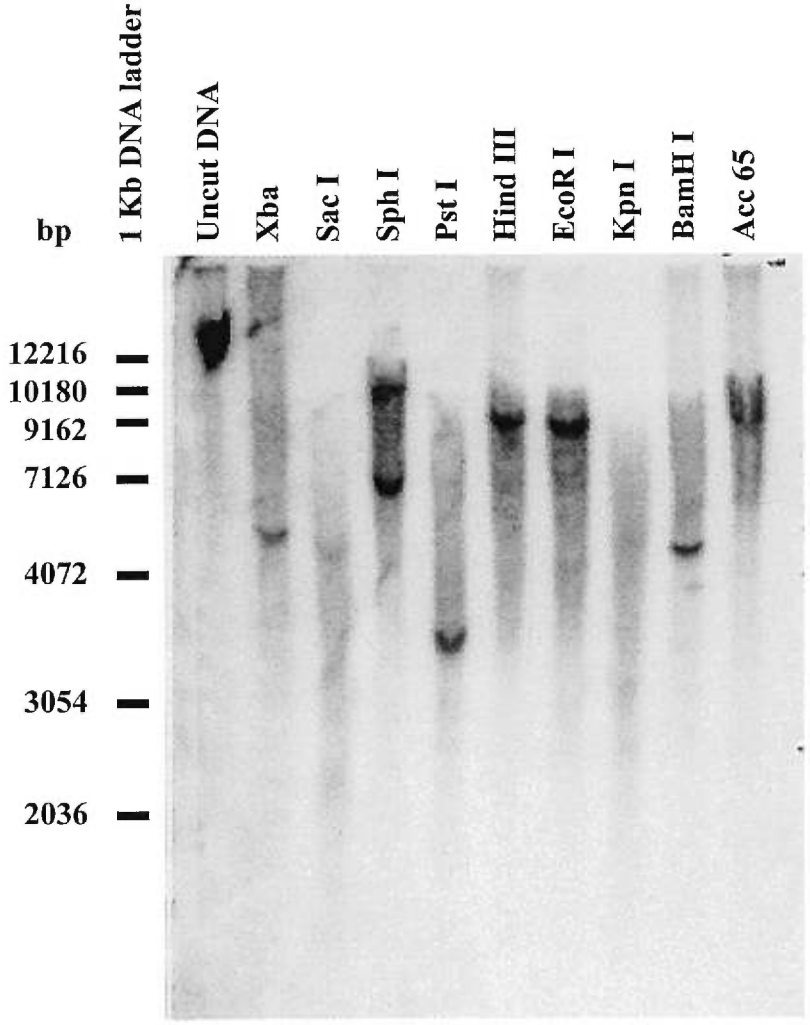
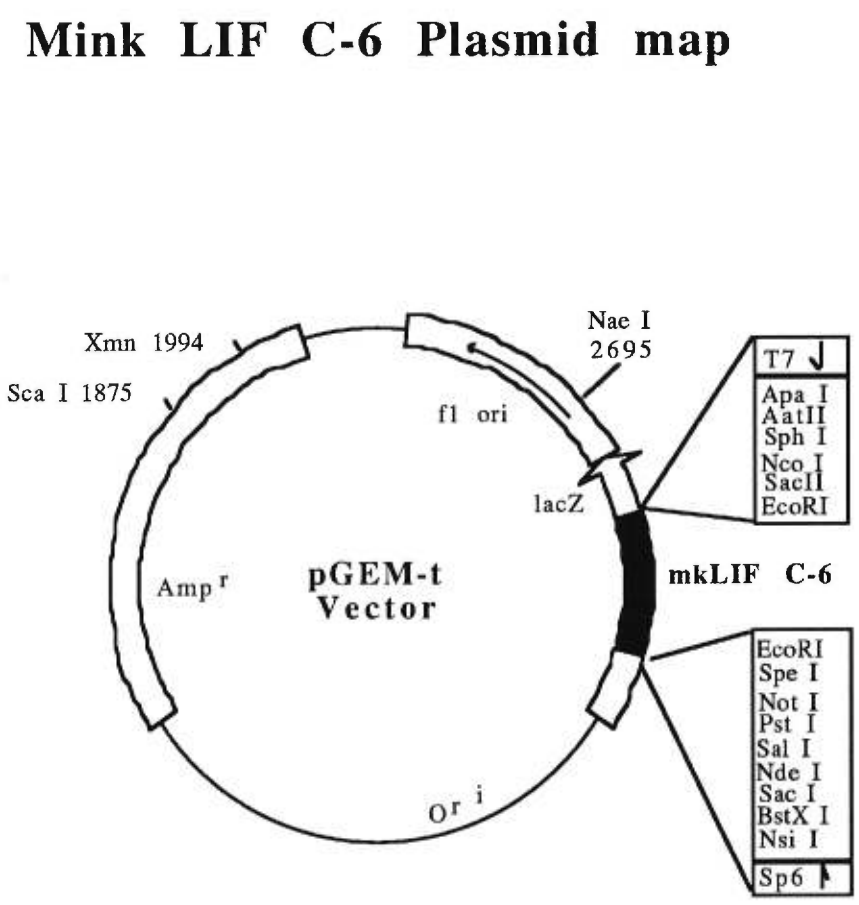


Figure 9-1 Autoradiograph of Southern blot for LIF gene. Restriction enzymes used are indicated on top of the graph. The mink LIF cDNA C-6 probe was labeled with ^{32}P-α-dCTP. Uncut DNA bound to the probe at high (over 12 kb) fragment size. Binding fragments corresponding to restriction enzymes and approximate sizes: Xba (5.5 kb), Sac I (5 kb), Sph I (6.5 and 10 kb), Pst I (3.3 kb), Hind III (9 kb), EcoR I (8.9 kb), Kpn I (unknown), BamH I (4.2 kb), and Acc65 (10 kb) are shown in the radiograph, respectively. A DNA ladder (1 kb) was used to measure the DNA fragment size.

9.2 Plasmid map of mink leukemia inhibitory factor cDNA fragment C-6

A



B

TTACACGGCCCAGGGGGAGCCGTTCCCCAACAACCTGGACAAGTTGTGTGGCCC

CAATGTGACGGACTTCCCGCCATTCCACCGCAACGGCACGGAGAAGACACGGCT

CGTGGAGCTCTACCGTATCATCGCATACCTTGGCGCCTCCCTGGGCAACATCAC

CCGGGATCAGAAGGTCCTCAATCCCAATGCCCTCAGCCTCCACAGCAAGCTGAA

GGCCACGGCGGACATCCTGCGGGGCCTCCTCAGCAATGTGCTCTGCCGCCTGTG

TAACAAATACCATGTGGCCCACGTGGACGTGGCCTATGGCCCTGACACCTCGGG

CAAGGACGTCTTTCAGAAGAAGAAGCTGGGCTGTCAGCTCCTGGGGAAGTATAA

GCAGGTCATTGCCGTGGTGGCCCAGGCCTTCTAG

Figure 9-2. Plasmid map (A) and sequence (B) of mink LIF cDNA fragment C-6. Characteristics of the plasmid are shown in the map. A fragment (mk LIF C-6) of 411 nucleotides was generated by RT-PCR from mink uterus taken at the preimplantation phase. PCR conditions used are described in Material and Methods in Article 2. The C-6 fragment was ligated into pGEM-t vector by T4 ligase at room temperature for 2 hrs and subsequently sequenced as described in Materials and Methods in Article 2. Primers are underlined.

9.3 Sequence of intron 2 for mink leukemia inhibitory factor gene

```
  1 GTAAGTCCCCTATTCCAGGTCCTGAGGTGGGGGGAAGGTGTTGCTCCACA
 50 GAGTTTGGAGCTGGCAGGAGAGTATGGGGAAAGGGCTTGGTTCAACAACA
101 ATCCCCTACCCCCCACAGCTTCAGGGGCGTGGGGGTGCCCCTCCCCCAGC
151 CCAGGCTCAGACTGGAAGCCCCTTTTCCAAGGTGCCCAGTTGGGTCTGGG
201 CAGAGCTGAGGGCAGAAGCGGGAAGCTGGGCACTGCTGGTCTAGGCTTTC
251 CTCCACCACGTGGACAGGAGGCCAATGAGGGACAGAGAGGAAGTGGGGTG
301 ACGGGGAGATGCTGCTGTTGGGAGAGAAAGTGGGCTGTGGGTGTCTGGGG
351 TGCAAAGCCAGAGCAGGAAGAAGGCGCGTACGGTGGGTGAAAGGGCAAGT
401 GTGTGTGGCACTCTCACAGGAGGTGAGACGGGGTGTTTTCCTTTCCGTCC
451 CCATACGATCTTGAGTTAAGGGACGAGAGTCCCTTGATGCTTCACCACGT
501 GCATTCACACCATGATTACTCCCGGCTCTCACGGTGCTCCTGAGATGCCT
551 AGAGGGGCGAGTAGCTGGGTCTCTATGACAGAGGCAGGAAACGGACCCAA
601 GAGCTTGCCCAGAGGCTTGGCGGTAGGGCTGGAACCCTCAACCCTGACCA
651 CCCCCAACGTCACCTCCCCTCTCCCTCTCTGCTCCTCAG      689
```

Figure 9-3. Intron 2 sequence of mink LIF gene. The intron 2 fragment of mink LIF gene was generated by PCR from mink genomic DNA using mkLIF-B and mkLIF-2 primers. The PCR conditions used and sequencing are described in Materials and Methods in Article 2. The sequence of intron 2 contains 689 nucleotides. The coding sequence regions are not shown. This intron fragment was used as control of genomic DNA contamination in mRNA transcript RT-PCR assay.

9.4 Sequence of mink ribosomal protein S26

AGATGACTAA GAAGAGGAGG AATAATGGTC GTGCCAAAAA GGGCCGCGGC CACGTGCAGC
CTATTCGCTG CACCAACTTG GCCCGTTGTG TGCCCAAGGA CAAGGCCATT AAGAAGTTCG
T

Figure 9-4. Sequence of mink ribosomal protein S26. Fragment (121 nt) was generated by RT-PCR. The RT-PCR conditions used and primers were described in the Materials and Methods in Article chapters 2 and 3. PCR products were size-fractionated on 2% agarose gel. The expected fragment was inserted into pGEM-t vector by T4-ligase and subsequently sequenced by the method as describe for LIF and COX-2 gene cloning.

9.5 Sequence of exon 3 for muskrat leukemia inhibitory factor

LOCUS AF034742 411 bp DNA ROD 18-DEC-1997

DEFINITION *Ondatra zibethicus* leukemia inhibitory factor (LIF) gene, exon 3, partial cds.

ACCESSION AF034742 in GenBank

SOURCE muskrat

ORGANISM *Ondatra zibethicus; Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Arvicolinae; Ondatra.*

REFERENCE 1 (bases 1 to 411)

AUTHORS Song JH, Houde A and Murphy BD

TITLE Muskrat (*Ondatra zibethicus*) sequence of leukemia inhibitory factor (LIF), partial cds

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 411)

AUTHORS Song JH, Houde A and Murphy BD

TITLE Direct Submission

JOURNAL Submitted (14-NOV-1997) Agriculture and Agri-Food Canada, Food Research and Development Centre, 3600 Casavant Blvd. west, St-Hyacinthe, Quebec J2S 8E3, Canada

FEATURES Location/Qualifiers

source 1..411
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/db_xref="taxon:10060"
/tissue_type="liver"

gene <1..411
/gene="LIF"

exon <1..411
/gene="LIF"
/number=3

CDS <1..411
/gene="LIF"
/codon_start=1
/product="leukemia inhibitory factor"
/db_xref="PID:g2695675"

(A)

/translation="YTAQGEPFPNNLDKLCGPNMTDFPPFHANGTE
KTKLVELYRMVAYLSVFLGNITREQRILNPNALSLHSKLNATI
DIMRGLLSNVLCRLCNKYHVGHVDVTYAPDTSSKDVFQKKK
LGCQLLGTYKQVISVVAQAF"

(B)

BASE COUNT 101 a 114 c 108 g 88 t

ORIGIN

```
  1 TACACAGCTC AAGGGGAGCC ATTTCCCAAC AACTTGGACA AGCTGTGTGG GCCCAACATG
 61 ACAGATTTCC CGCCTTTCCA TGCCAATGGG ACTGAGAAGA CCAAGTTGGT GGAGCTGTAT
121 CGGATGGTCG CGTATCTGAG TGTCTTCCTG GGCAATATCA CCCGGGAGCA GAGGATCCTG
181 AACCCCAATG CCCTGAGCCT CCACAGCAAG CTCAATGCTA CTATAGACAT CATGCGTGGG
241 CTCCTCAGCA ATGTGCTTTG CCGGCTGTGC AACAAGTACC ACGTGGGCCA TGTGGATGTG
301 ACCTATGCCC CCGACACCTC CAGCAAAGAT GTTTTCCAAA AGAAAAAGTT GGGCTGCCAG
361 CTCCTGGGGA CATACAAGCA AGTCATTAGC GTGGTGGCCC AGGCCTTCTA G //
```

Figure 9-5. Sequence of nucleotide (B) and deduced aa (A) of leukemia inhibitory factor gene from exon 3 for the muskrat. Complementary DNA was generated by PCR from muskrat liver genomic DNA. Primers (mkLIF-C and mkLIF-6) were designed for mink LIF gene cloning and the PCR conditions were used as previously described (see Materials and Methods, Article chapter 2). Muskrat LIF cDNA fragment C-6 contains 411 nt and deduced 136 aa.

9.6 Western blot of COX-2 in immortalized mink endometrial cell lines

Uterine cell extracts and immunoblot analysis were performed as described by (Sirois, 1994; Liu et al., 1997). Briefly, epithelial and stromal cells from immortalized cell lines (Moreau et al., 1995) were collected separately with PBS and precipitated in Eppendorf tube. Cell pellets were homogenized on ice in 700 µl TED homogenization buffer (50 mM Tris,10 mM EDTA, 1 mM DEDTC, pH8.0) supplemented with 2 mM octyl glucoside, and centrifuged at 30,000 X g for 1 hr at 4 C. The crude pellets containing membranes, nuclei and mitochondria were sonicated (5 sec/cycle; 4 cycles) in 250 µl TED sonication buffer (20 mM Tris, 50 mM EDTA, 0.1 mM DEDTC, pH 8.0) containing 32 mM octyl glucoside. The sonicates were centrifuged at 13,000 X g for 25 min at 4 C. The supernatants (solublized cell extracts) were stored at -70 C until immunoblot analysis. The protein concentration was determined by the method described by (Lowry et al., 1951).

Proteins (50 µg) from cell extracts were resolved by one-dimensional SDS-PAGE, and electrophoretically transferred onto nitrocellulose filters. Filters were incubated over night at 4 C with rabbit against human COX-2 antibody (1:100, PG26, Oxford Biomedical Research Inc., MI) and ^{125}I-labeled protein A (ICN Pharmaceuticals, Montreal, Quebec, Canada) was used to visualize immunopositive proteins as described (Sirois, 1994). The filter was washed with 0.1% tween 20 in TED buffer, 3X at room temperature and then the blot was exposed to X-ray film at -70 C for 24 hrs.

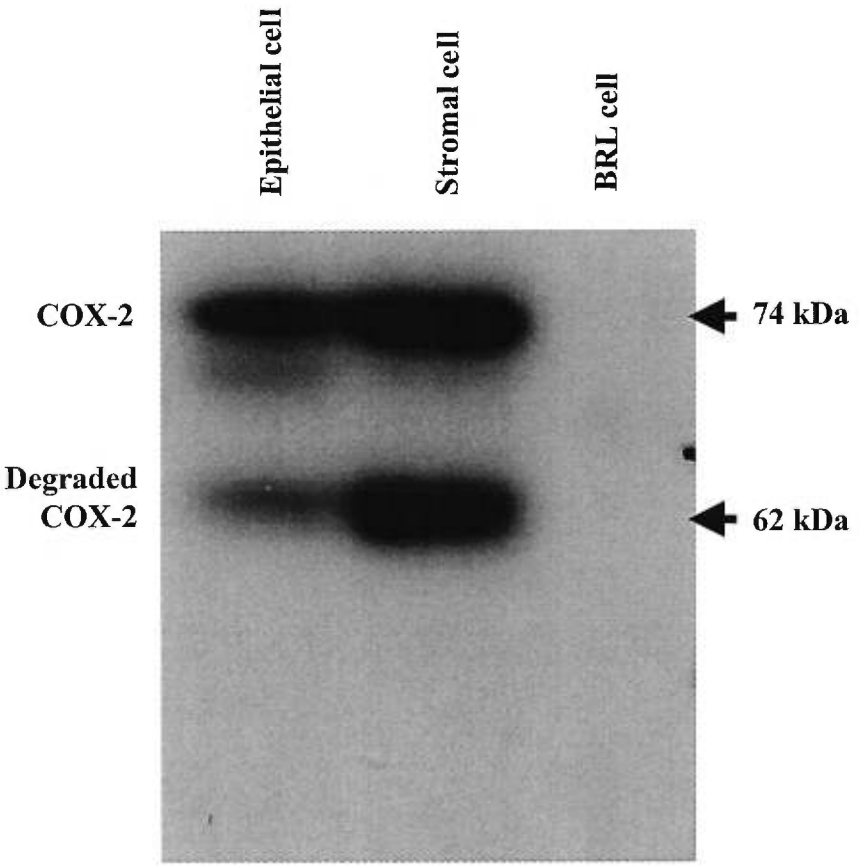
Epithelial cell
Stromal cell
BRL cell
COX-2
Degraded
COX-2
74 kDa
62 kDa

Figure 9-6 Western blot of COX-2. Mink immortalized uterine cell lines (epithelial and stromal cells) were cultured at presence of embryos. An aliquot of 50 μg protein was loaded for each sample. Buffalo Rat Liver (BRL) cell line was used to compare the expression of COX-2 in different cell lines. The COX-2 protein is identified 74 kDa in the mink endometrial cell lines. The expression of COX-2 is stronger in stroma than in epithelium, but no expression in the BRL cells. The lower band (62 kDa) shows a putative proteolytic fragment of COX-2.

9.7 Mink cyclooxygenase-2 cDNA and its deduced amino acid sequences

LOCUS	1825 bp mRNA MAM 12-FEB-1998
DEFINITION	Mink COX-2, complete cds.
ACCESSION	AF047841 in GenBank
SOURCE	American mink.
ORGANISM	*Mustela vison; Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Carnivora; Fissipedia; Mustelidae; Mustela.*
REFERENCE	1 (bases 1 to 1825)
AUTHORS	Song JH, Sirois J, Houde A and Murphy BD
TITLE	Cloning, developmental expression and immunohistochemistry of cyclooxygenase 2 (COX-2) in the endometrium during embryo implantation and gestation in the mink (*Mustela vison*)
JOURNAL	Endocrinology 1998 (in press)
REFERENCE	2 (bases 1 to 1825)
AUTHORS	Song JH, Sirois J, Houde A and Murphy BD
TITLE	Cyclooxygenase 2 (COX-2) cDNA cloning in the mink (*Mustela vison*)
JOURNAL	Centre de recherche en reproduction animale, Faculte de medecine veterinaire, Universite de Montreal, St-Hyacinthe Quebec, Canada J2S 7C6
REFERENCE	3 (bases 1 to 1825)
AUTHORS	Song JH, Sirois J, Houde A and Murphy BD
TITLE	Direct Submission
JOURNAL	Submitted (12-FEB-1998) Centre de recherche en reproduction animale, Faculte de medecine veterinaire, Universite de Montreal, 3200, rue Sicotte, St-Hyacinthe, QC J2S 7C6, Canada

FEATURES Location/Qualifiers

source 1..1825

/organism="*Mustela vison*"

/strain="American mink"

/db_xref="taxon:9667"

/note="Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Carnivora; Fissipeda; Mustelidae;

gene 1..1825

/note="Prostaglandin synthase type 2"

/gene="COX-2"

CDS 11..1825

/gene="COX-2"

/note="Prostaglandin synthase 2"

/codon_start=1

/product="COX-2"

(A)

/translation="MLARAGLLCASLSPPHAANPCCSNPCQNQGVCMSIGFDQYMCDC
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KEIVEKFLLRRKFIPDPQGTNMMFAFFAQHFTHQFFKTDHKRGPGFTKGLGHGVDLSH
VYGETLDRQHKLRLFKDGKMKYQVIDGEVYPPTVKDTQVEMIYPPHVPEHLRFAVGQE
VFGLVPGLMMYATIWLREHNRVCDVLKQEQGEWDDERLFRRSRLILIGETIKIVIEDY
VRHLSGYHFSLKFDPELLFNQQFQYQNRIAAEFNTLYHWHPLLPDTLQIDDQEYNFQQ
FVYNNSILLEHGLTQFGESFSRQIAGRVAGGRNVPAAVQQEQRASIDQSRQMKYQSLN
EYRKRFSVKPYASFEELTGEKEMAGELKALYQDIDAMELYPALLVEKPRPDAIFGETM
VEIGAPFSLKGLMGNPICSPDYWKPSHFGGEVGFKIINTASIQSLICNNVKGCPFTAF
SVQDPQLTKTVTINGSSSHSGLDDINPTVLLKERSTEL"

(B)

BASE COUNT 508 a 439 c 423 g 455 t

ORIGIN

```
   1 CGCCGCTGCG ATGCTCGCCC GAGCCGGTCT GCTGTGCGCT AGCTTAAGTC CACCCCATGC
  61 AGCAAATCCT TGCTGTTCCA ACCCATGTCA AAACCAAGGT GTATGTATGA GCATAGGATT
 121 TGACCAGTAT ATGTGTGACT GCTCTCGAAC AGGATTCTAT GGTGAAAACT GTTCAACACC
```

```
 181 TGAATTTCTG ACAAGAGTAA AATTGCTCCT GAAACCCACT CCGAATACAG TGCACTACAT
 241 ACTTACCCAC TTCAAGGGAG TCTGGAACAT TGTCAATAAA ATTCCCTTCC TGGCAGATGT
 301 AATTATGAAG TACGTGAGGA CGTCCAGATC ACATTGCATT GAGCCTCCAC CAACTTACAA
 361 TGTGCACTAT GCATATAAAA GCTGGGAAGC CTTTTCTAAT CTCTCCTACT ACACCAGAGC
 421 TCTTCCCCCT GTGGCGGATG ACTGTCCAAC ACCCATGGGT GTGAAAGGCA AGAAAGAGCT
 481 TCCTGATTCT AAAGAGATTG TGGAAAAATT TCTTCTAAGA AGAAAGTTCA TTCCTGATCC
 541 CCAGGGCACA AATATGATGT TTGCATTCTT TGCCCAGCAC TTCACCCATC AATTTTTCAA
 601 GACAGATCAT AAGCGAGGGC CAGGTTTCAC CAAAGGACTG GGCCATGGGG TGGACTTAAG
 661 CCATGTTTAT GGGGAAACTT TGGATAGACA ACATAAACTG CGCCTCTTCA AGGATGGAAA
 721 AATGAAATAC CAGGTAATCG ATGGAGAGGT GTATCCTCCC ACAGTCAAAG ATACTCAGGT
 781 GGAGATGATC TACCCACCCC ATGTTCCTGA ACACCTGCGG TTTGCTGTGG GCCAGGAAGT
 841 CTTTGGGCTG GTGCCTGGTC TGATGATGTA TGCCACGATC TGGCTGCGGG AACACAACAG
 901 AGTGTGTGAT GTGCTGAAAC AGGAGCAGGG AGAGTGGGAT GATGAGCGTC TGTTCAGACG
 961 AAGCCGGCTC ATATTGATAG GAGAAACCAT TAAGATTGTG ATTGAAGACT ATGTCAGACA
1021 TTTGAGTGGC TATCACTTCA GCTTGAAGTT TGACCCAGAG CTGCTCTTCA ACCAACAATT
1081 CCAGTACCAA AACCGGATTG CTGCCGAGTT TAACACGCTC TATCACTGGC ATCCCCTTCT
1141 GCCTGACACC TTGCAAATTG ATGACCAGGA GTACAATTTC CAACAGTTTG TCTACAACAA
1201 CTCTATACTG CTGGAACATG GGCTTACCCA GTTTGGGGAA TCCTTCAGCA GGCAAATTGC
1261 TGGCAGGGTT GCTGGTGGTA GGAATGTTCC AGCTGCAGTA CAACAGGAGC AAAGGGCCTC
1321 AATTGACCAG AGCAGACAGA TGAAGTACCA GTCTCTGAAT GAGTACCGCA AACGCTTTTC
1381 CGTGAAGCCC TACGCGTCTT TTGAGGAACT TACAGGAGAG AAGGAAATGG CTGGAGAGTT
1441 GAAAGCCCTG TACCAAGATA TTGATGCCAT GGAGCTGTAT CCAGCCCTCC TGGTAGAAAA
1501 GCCTCGTCCA GATGCCATCT TTGGTGAGAC CATGGTAGAA ATTGGAGCAC CATTCTCCTT
1561 GAAAGGACTT ATGGGTAATC CCATCTGTTC ACCTGACTAC TGGAAGCCTA GTCATTTCGG
1621 TGGAGAAGTA GGCTTTAAAA TCATCAACAC TGCCTCCATT CAGTCTCTCA TCTGCAATAA
1681 CGTGAAGGGC TGTCCTTTTA CTGCATTCAG TGTTCAAGAC CCCCAGCTCA CCAAAACAGT
1741 CACTATTAAT GGAAGCTCTT CACACTCCGG ACTAGATGAC ATCAATCCCA CAGTCCTACT
1801 AAAGGAACGT TCAACGGAGC TGTAG//
```

Figure 9-7 Sequences of COX-2 cDNA (B) and its deduced aa (A).

9.8 Optimization of semi-quantitative RT-PCR for COX-2 transcripts

A pool of total RNA from early postimplantation uterine samples was used to optimize PCR quantification for COX-2. Each RT reaction was performed in total volume of 20 μl containing 5 μg of total RNA and both downstream primers for mink S26 ribosomal protein and COX-2. A series of aliquots of RT products (equivalent to 0.25, 0.5, 0.75, 1, 1.25, 2.5, and 5 μg of total RNA, respectively) were used in further PCR amplification to determine the amount of RT to be used in PCR assay for COX-2 expression in the uterus (Fig 9-8 A). The point of 0.5 μg of total RNA was used in subsequent PCR quantification. The 30 cycle point was employed in the PCR assay (Fig 9-8 B). The abundance of S26 ribosomal protein transcript was used as a total RNA loading control in this study. An aliquot equivalent to 50 ng of total RNA in RT products was used in PCR amplification for each sample at 25 cycles (Fig 9-8 C). The density ratio of COX-2 to S26 PCR amplified DNA was considered as a PCR assay value. Thirty and 10 μl of PCR product for COX-2 and S26, respectively, were size-fractionated by 2% of agarose gel containing 1 μg ethidium bromide and the density for each fragment was determined by computer software (Photodyne, College Inc.). After logarithmic transformation, a linear relationship of the amplified DNA density against to total RNA amount and PCR cycles was obtained (Fig. 9-8 A, B, C). In summary, the 500 ng of total RNA and 30 PCR cycles were determined for COX-2 quantification. For S26, 50 ng of total RNA and 25 cycles was employed as the optimal condition in further RT-PCR assays.

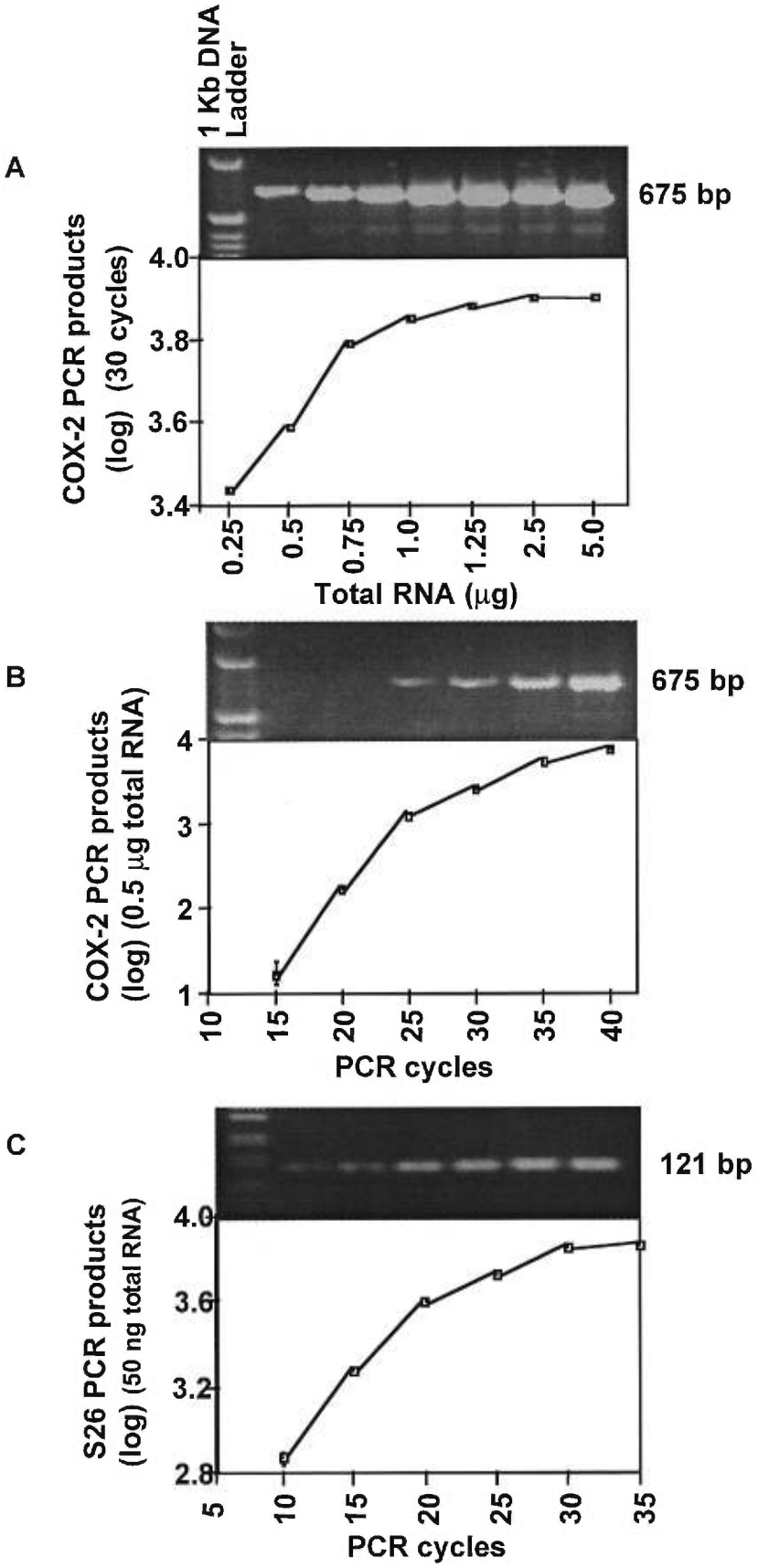
A
1 Kb DNA Ladder
675 bp
COX-2 PCR products (log) (30 cycles)
4.0
3.8
3.6
3.4
0.25
0.5
0.75
1.0
1.25
2.5
5.0
Total RNA (μg)
B
675 bp
COX-2 PCR products (log) (0.5 μg total RNA)
4
3
2
1
10
15
20
25
30
35
40
PCR cycles
C
121 bp
S26 PCR products (log) (50 ng total RNA)
4.0
3.6
3.2
2.8
5
10
15
20
25
30
35
PCR cycles

Figure 9-8. Optimization of semi-quantitative reverse transcription poly-chain reaction (SQ-RT-PCR) assay. Panel A indicates the relationship between PCR cycle (30) and total RNA used in RT reaction. Panel B represents the relationship of 500 ng total RNA used in RT reaction and PCR cycles. Panel C demonstrates the relationship between 50 ng of total RNA used in RT reaction and PCR cycles for mink ribosomal protein S26 PCR amplification.

9.9 Technique de: Hématoxyline-Eosine-Safran

1 Hématoxyiine de Harris

Se commande déja prepare

Se conserve 3 mois

Colorer 5 minutes pour 6 semaines puis 10 minutes les 6 autres semaines

2. Alcool chlorhydrique 1%

Alcool éthylique 100%	1 ml
HCl	10 ml

3. Carbonate de lithium

Carbonate de lithium	15 g
Eau distillée	1000 ml

4 Eosine Phloxine (Working)

1 % Eosine (solution mere)	100 ml
1% Phloxine B (solution mere)	10 ml
95% Alcool éthylique	780 ml

Mélanger, filtrer et ajouter:

Acide acétique glacial	4 ml

1% Eosine (solution mère)

Eosine Y (soluble dans l'eau)	10 g
Eau distilée	1000ml

1% Phioxine B (solution mère)

Phioxine B	1 g
Eau distillée	100ml

L'Eosine-Phioxine se change à toutes les semaines

5. Safran

Safran	5 g
Ethanol 100%	500 ml

Porter à ébuilition dans un erlenmeyer. Arrêter après les premiers gros bouillons.

Décanter la première bouillotte en gardant le safran.

Ajouter un autre 500ml d éthanol et faire bouillir, 1 minute.

Garder la deuxième bouillotte. Répéter avec un autre 500 ml d'alcool éthylique.

Décanter et filtrer. Se conserve environ 2 mois.

Procedure:

1	1 bain de toluoi (Toluène)	5 minutes
2.	1 bain d'alcool absolue	2 minutes
3.	1 bain d'alcool 95%	2 minutes
4.	1 bain d'alcool 80%	2 minutes
5.	Laver à l'eau courante (7-8 fois)	2 minutes
6.	Colorer à l'hématoxyiine	5 ou 10 minutes
7.	Laver à l'eau courante	
8.	Différentier dans l'alcool chiorydrique jusqu'à env. (6 fois) teinte brun rosé pâle ou orange pâle	4-5 secondes
9.	Laver à l'eau courance (7-8 fois)	
10.	Passer au carbonate de lithium	20 secondes
11,	Laver à l'eau courante	
12.	Alcool 70%	10-30 minutes
13.	Colorer dans l'éosine - phloxine (Working)	4 minutes
14.	3 bains d'alcool 95%	2 rinqages, 1 bain de 2 minutes
15.	1 bain d'alcool 1 00%	2 minutes
1 6,	Colorer au safran	~3 minutes
17.	Tremper dans l'alcool 100%	
18.	2 bains de toluoi	
19.	Montage	

Résultats: Noyau bleu; Cytoplasme rouge; Collagène jaune

9.10 Protocole d'immunohistochimie

1 Déparaffinizer les lames en histologic:

Toluène, 5 minutes (1X)

Alcool 100%, 3 minutes (2X)

Alcool 95%, 3 minutes (2X)

Alcool 80%, 3 minutes (1 X)

H20, 5 minutes (1 X)

2. "Quencher" la peroxidase endogène:

Incuber les lames dans 0.3% H_2O_2 dans le methanol (mettre 1 ml de H_2O_2 dans 99 mls de methanol) pendant 30 minutes à la temperature de la piece.

3. Rinser les lames pendant 5 minutes dans le PBS (3X).

4. Incuber chaque lame avec le serum normal provenant du kit Vectastain:

Tablé 9.10-1. Diluer le sérum dans du PBS selon le tableau suivant:

Volume de PBS nécéssaire (ml)	Sérum de blocquage (µl)	Deuxiéme Ac (µl)	Agents A, B (µl)
1	13.5	4.5	9
3	40.5	13.5	27
6	81	27	54
9	121.5	40.5	81
....	....	....	....

Mettre assez de sérum dilué par lame pour couvrir la coupe de lame. Incubation de la coupe pendant 20 minutes à la temperature de la pièce.

NE PAS RINSER LES LAMES APRES L'INCUBATION AVEC LE SERUM.

5. Enlever doucement le sérum en "blottant" chaque lame sur du papier absorbant.

6. Mettre 1'anticorps primaire ou l'anticorps contrôle dilués dans le PBS (couvrir entièrement la section de tissu). lncuber les lames toute la nuit à 4 C (réfrigérateur) dans un plat couvert avec des papiers humides dans le fond.
7. Rinser les lames pendant 5 minutes dans le PBS (2X).
8. Mettre l'anticorps secondaire dilué dans le PBS (selon le tableau au #4). lncuber pendant 45 minutes à la temperature de la pièce. A ce moment, préparer aussi le réactif ABC pour que les complexes aient le temps de se former.
9. Rinser les lames pendant 5 minutes dans le PBS (2X).
10. Mettre le complexe ABC et incuber pendant 45 minutes à la temperature de la pièce.
11. Rinser les lames pendant 5 minutes dans le PBS (2X).
12. Préparer le substrat (DAB).

    ATTENTION: Le DAB est cancérigène. Toujous porter des gants a cette etape.

    Additionner 0. 1 5 gr de Tris buffer à 20 ml d'H_20 et ajouter la tablette de DAB, bien mélanger au vortex pour dissoudre la tablette de DAB complétement.

    Ajouter 300 µl d'H_20_2 3% (le H_20_2 est 30% dans la bouteille, donc faire une dilution 1:10). Filtrer le tout DAB travers du papier Whatman.

    Couvrir le tissu entièrement avec le DAB et incuber pendant 15 minutes à la temperature de la pièce,
13. Rinser les lames dans l'H_20 pendant 5 minutes (1 X).
14. lncuber les lames dans le colorant hématoxyline pendant 3 minutes.
15. Rinser les lames dans l'H_20 pendant 5 minutes (1X).
16. Couvrir chaque lame d'une lamelle.