Université de Montréal

REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR DURING THE PERI-IMPLANTATION PERIOD IN THE AMERICAN MINK, *Mustela vison*

par

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Faculté des études supérieures

Cette thèse intitulée :

REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR DURING THE PERI-IMPLANTATION PERIOD IN THE AMERICAN MINK, *Mustela vison*

présentée par :
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Résumé

La formation de nouveaux vaisseaux sanguins ainsi que l'extension du réseau pré - existant sont des processus indispensables au bon déroulement de la gestation. Une augmentation de l'apport sanguin au fœtus durant son développement est en effet essentielle pour répondre rapidement aux besoins croissants en échanges gazeux, en apport nutritionnel et à la nécessité d'éliminer les déchets. Cette amplification de la vascularisation s'effectue par l'intermédiaire de facteurs angiogéniques. Le principal facteur impliqué dans ce processus est le facteur de croissance de l'endothélium vasculaire (VEGF). Grâce à ses récepteurs à activité tyrosine kinase, VEGF induit la proliferation des cellules endothéliales des vaisseaux sanguins et augmente la perméabilité vasculaire. Dans cette étude, nous avons caractérisé l'expression de VEGF et de ses récepteurs au niveau de l'uterus et du placenta pendant la période de péri-implantation embryonnaire. Nous avons utilisé en guise de modèle animal, le vison américain, un carnivore qui présente une diapause embryonnaire obligatoire.

Notre premier objectif a été de caractériser l'expression de VEGF et de ses récepteurs au cours des étapes précoces de la gestation chez le vison. Nous avons cloné la séquence codante de VEGF exprimé chez le vison ainsi que des portions de séquences codante pour ses récepteurs. Toutes les séquences clonées ont témoigné d'une homologie élevée avec les séquences déjà identifiées chez d'autres espèces. L'expression des ARNm correspondant à ces gènes a été évalué dans l'utérus de vison durant la diapause, la

reactivation de l'embryon, l'implantation et la pseudo-gestation. Nous avons démontré que les trois isoformes les plus abondants de VEGF et ses récepteurs étaient sur-régulés au moment l'implantation. Le niveau d'expression de l'ARNm de VEGF était élevé dans l'uterus en pseudo-gestation tandis que celui de ses récepteurs était relativement bas, ce qui suggère que l'embryon est impliqué dans la regulation de l'expression des récepteurs en presence de VEGF. Sous sa forme protéique, VEGF a été localisé dans l'épithélium glandulaire pendant la diapause, et sa progression vers l'épithélium luminal a été observé lors de la réactivation de l'embryon. La présence de VEGF a également été détecté durant l'implantation au niveau de l'épithélium luminale et glandulaire ainsi que du stroma, et de manière plus intense dans les cellules invasives du trophoblaste.

Nous avons ensuite étudié l'expression de la prostaglandine E_2 (PGE_2) dans l'utérus au début de la gestation afin de déterminer son rôle dans la régulation de VEGF. Des embryons de vison mis en culture ont secrété une quantité importante de PGE_2. La présence de PGE synthase, enzyme responsable de la formation de PGE_2, a par ailleurs été détecté dans les cellules du stroma utérin en réponse à l'invasion de l'embryon au début de l'implantation. Deux des récepteurs de PGE_2 , EP2 et EP4, ont de plus été localisé dans l'utérus au même moment. En transfectant des cellules du stroma utérin avec le gène rapporteur de la luciférase placé sous le contrôle du promoteur de VEGF exprimé chez le vison, nous avons démontré que PGE_2 stimulait la transcription de VEGF.

Nous avons finalement déterminé les mécanismes de régulation de la transcription de VEGF par PGE_2. En utilisant le système de transfection précédemment décrit, nous

avons montré que PGE_2 dépendait de l'activation des voies de signalisation par la PKA. Grâce à des expériences de délétions et de mutations dans la séquence promotrice de VEGF, nous avons identifiés la presence de boîtes AP2/SP1 dans la région proximale du promoteur et démontré que ceux-là étaient responsables de la transactivation du promoteur de VEGF par PGE_2. Cela a ensuite été confirmé par des essais d'immnuoprécipitation de la chromatine qui ont permis d'observer que AP2/SP1 se liaient effectivement au promoteur endogène de VEGF après traitement à la PGE_2 chez le vison. En plus d'induire la liaison des facteurs au promoteur, PGE_2 est impliqué dans l'acétylation de l'histone H3 qui est associée à la chromatine active.

Ces études permettent de conclure que VEGF et ses récepteurs sont sur-régulés au début de la gestation, contribuant ainsi à la formation du placenta et à l'augmentation de la vascularisation utérine. VEGF est en partie régulé par la présence de PGE_2 au niveau de l'embryon et de l'utérus, responsable de la transactivation du promoteur par AP2/SP1 ainsi que de l'acétylation de l'histone H3 associée à cette région promotrice.

Mots-clés : VEGF, angiogénèse, période de peri-implantation, uterus, placenta, prostaglandines, vison, régulation transcriptionnelle.

Abstract

The formation of new blood vessels, as well as the extension of a pre-existing vessel network, are processes required for successful maintenance of pregnancy. An increase in blood supply to the growing fetus is necessary to meet the rapidly expanding need for gas exchange, nutrient supply and waste removal. This amplification of vascularity is achieved through the action of angiogenic factors. The principal factor implicated in the process is the vascular endothelial growth factor (VEGF). Through its tyrosine kinase receptors, VEGF induces proliferation of the endothelial cells of the vessels, and increases vascular permeability. In this study, we characterized the expression of VEGF and its receptors in the uterus/placenta during the embryo peri-implantation period, and identified a key mechanism of regulation of this angiogenic factor during this period. As our model, we utilized the American mink, a carnivore presenting obligate embryonic diapause.

Our first objective was to characterize the expression of VEGF and its receptors during the early stages of pregnancy in the mink model. We cloned the coding sequence of mink VEGF, as well as portions of the receptors sequences. All cloned sequences bore high homology with other species sequenced to date. Expression of the mRNA for these genes was evaluated in the mink uterus during different stages: diapause, embryo activation, implantation and pseudopregnancy. We demonstrated that the three most abundant VEGF isoforms and its receptors are up-regulated associated with implantation. VEGF mRNA levels were high in pseudopregnant uteri, whereas both receptors displayed

low levels of mRNA at this stage, indicating that the embryo may play a role in regulating expression of the receptors as opposed to VEGF. VEGF protein was localized in the glandular epithelium during diapause and progressed to the luminal epithelium as embryo activation ensued. Luminal and glandular epithelium, as well as the stroma, were positive for VEGF protein during implantation. The most intense localization was found at the invasive trophoblast cells at this stage.

Following VEGF localization, we investigated prostaglandin E_2 (PGE_2) in the uterus during early pregnancy to determine its role in VEGF regulation. Activated mink embryos in culture produced high levels of PGE_2. PGE synthase, responsible for formation of PGE_2, was present in the uterine stromal cells in response to the presence of the invading embryo during early implantation. Two of the PGE_2 receptors, EP2 and EP4, were found to be present in the uterus at that time. By transfecting mink uterine stromal cells with the luciferase reporter gene driven by the mink VEGF promoter, we demonstrated that PGE_2 stimulates VEGF transcription.

Lastly, we determined the mechanism involved in regulation of VEGF transcription by PGE_2. Using the transfected stromal cell system described above, we showed that PGE_2 action is dependent on activation of the PKA pathway. Through promoter deletion and mutation studies, we identified an AP2/SP1 cluster at the proximal promoter region and demonstrated that it is responsible for PGE_2 induced VEGF promoter transactivation. This was further confirmed by ChIP assay, which demonstrated AP2/SP1 binding to the endogenous mink VEGF promoter following PGE_2 treatment. In addition to inducing

binding of these factors to the promoter, PGE_2 also induced histone H3 acetylation, which is associated with active chromatin.

From these studies we concluded that VEGF and receptors are up-regulated during early pregnancy, contributing to the formation of the placenta and in the increase in uterine vascularity. Expression of VEGF is regulated in part by the presence of embryonic and uterine PGE_2 at this stage, and its action induces promoter transactivation by AP2/SP1 and histone H3 acetylation associated to this promoter region.

Keywords : VEGF, angiogenesis, peri-implantation period, uterus, placenta, prostaglandins, mink, transcriptional regulation

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List of Abbreviations

Ang1	angiopoietin 1
AP-2	activator protein-1
bFGF	basic fibroblast growth factor
bp	basepairs
cAMP	cyclic adenosine monophosphate
ChIP	chromatin immunoprecipitation assay
CL	corpus luteum
COX	cyclo-oxygenase
DAB	3,3' diaminobenzidine
dbcAMP	dibutyrl cAMP
DNA	deoxyribonucleic acid
ECM	extra-cellular matrix
EGF	epidermal growth factor
ER	estradiol receptor
ERα	estradiol receptorα
ERβ	estradiol receptorβ
ex	exon
FGF-4	fibroblast growth factor 4
Flt-1	fms-like tyrosine kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotrophin releasing hormone
hbEGF	heparin-binding epidermal growth factor
HDAC	histone deacetylase
HIF-1	hypoxia-inducible factor-1
IGF-1	insulin-like growth factor-1
IL	interleukin

Kb	kilobases
Kda	kilo Daltons
KDR	kinase domain region
LT	leukotrienes
LH	luteinizing hormone
LIF	leukemia inhibitory factor
PCNA	proliferating cell nuclear antigen
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGEs	prostaglandin E synthase
PGF2α	prostaglandin F2α
PGG_2	endoperoxide-containing prostaglandin H_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
PI	phosphatidylinositol
PKA	protein kinase A
PLA_2	phospholipase A2
PlGF	placental growth factor
PLC	phospholipase C
PPAR	peroxisome proliferator- activated receptor
PTX3	pentaxin family protein
RNA	ribonucleic acid
RTEF-1	related transcriptional enhancer factor-1
RT-PCR	reverse transcription-Polymerase chain reaction
SP1	stimulating protein 1
TBS	tris-buffered saline
TGF-β	transforming growth factor- β
UTR	untranslated region

VEGF	vascular endothelial growth factor
VPF	vascular permeability factor

To my family and friends

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INTRODUCTION

The American mink (*Mustela vison*) is a carnivore that displays obligate embryonic diapause, which consists of an arrest in mitotic activity of the embryo, resulting in delayed implantation. The mink embryos arrest development at the blastocyst stage, six to seven days after mating, upon entrance to the uterus (Hansson, 1947). Increased secretion of prolactin terminates diapause (Papke et al., 1980; Murphy et al., 1981; Martinet et al., 1981). The increase in prolactin levels is dependent upon the reduction in melatonin levels (Murphy et al., 1990) related to the vernal equinox. Prolactin reactivates the corpus luteum, which following ovulation is barely functional. In the mink, reactivation of embryo development appears to be controlled by maternal rather than embryonic factors (Chang et al., 1968). Following embryonic reactivation and implantation, the mink forms a discontinuous zonary placenta of the endotheliochorial type in which the classic decidual response seen in other species is absent (Enders, 1957).

Development and growth of blood vessels is paramount to the success of early pregnancy. Embryo growth and survival are dependent upon establishment of the placenta, which in turn guarantees the influx of nutrients, gas exchange and elimination of waste. Increase in uterine vascularity occurs at early stages of pregnancy, even preceding embryo attachment (Psychoyos, 1986). This increase is dependent upon up-regulation of angiogenic factors responsible for proliferation of vessel endothelial cells as well as increased permeability.

One of the most important regulators of blood vessel formation and development is the angiogenic factor vascular endothelial growth factor (VEGF) and its tyrosine kinase receptors Flt-1 (fms-like tyrosine kinase, also known as VEGFR-1) and KDR (kinase domain region, also known as VEGFR-2) (Jussila and Alitalo, 2002). Through binding to its receptors, VEGF induces both vascular permeability and serves as a potent endothelial cell-specific mitogen (Senger et al., 1983; Ferrara an Henzel, 1989). VEGF is required for the proper development and viability of embryos, based on findings that inactivation of even a single allele is embryonic lethal at days 11-12 of gestation (Carmeliet et al., 1996). Up-regulation of VEGF and receptors in the uterus, during early pregnancy, has been demonstrated in several species to date (Yi et al., 1999; Ghosh et al., 2000; Chakraborty et al., 1995; Halder et al., 2000; Das et al., 1997).

The prostaglandins, lipid compounds derived from the fatty acid arachidonic acid, are known to have multiple effects on reproduction. These effects can be seen in a variety of reproductive processes, including regulation of ovarian changes, participation in some of the events leading to fertilization, and actions on implantation, placental formation, and parturition. An array of prostaglandins can be derived from arachidonic acid. The synthetic cascade begins with the action of phospholipase A_2, which liberates arachidonic acid from the phospholipids of the membrane. This substrate is then acted on by two isoforms of the cyclo-oxygenase enzyme, COX-1 and COX-2, also known as prostaglandin H synthase, giving rise to prostaglandin H_2. The action of prostaglandin synthases on

prostaglandin H_2 bring about formation of specific prostaglandins. Expression of each product will vary according to cell type and biological context, resulting in a panoply of potential effects. The importance of prostaglandin to reproduction is summarized in the work of Lim et al. (1997), who demonstrated that mice null for COX-2, the rate-limiting enzyme on inducible prostaglandin formation, present failures in ovulation, fertilization, implantation and decidualization.

Prostaglandin E_2 is produced from Prostaglandin H_2 following the action of prostaglandin E synthase (PGE synthase). It binds to the EP receptor, which has 4 subtypes: EP1 through EP4, which elicit different intracellular pathways. This specific prostaglandin can partially rescue the ovarian phenotype of anovulation seen in the COX-2 null mice (Davis et al., 1999) and, in fact PGE synthase is highly expressed in pre-ovulatory follicles (Filion et al., 2001). Besides its action in the ovary, PGE_2 appears to modulate early pregnancy events. PGE synthase is co-expressed with COX-2 in the uterus of several species, and it appears to be modulated by the presence of the embryo (Wang et al., 2004; Ni et al., 2002). In the mink, our laboratory has previously demonstrated that COX-2 is expressed in the uterus following implantation and it appears to localize to the stroma surrounding the invading trophoblast cells (Song et al., 1998). In the work presented herein, we demonstrate that PGE synthase appears to follow the same temporal and spatial pattern of expression of COX-2 in the mink pregnant uterus.

Prostaglandin E_2 has been shown to be an important regulator of VEGF. In studies mainly involving cancer cell lines, PGE_2 has been reported to significantly up-regulate VEGF transcription (Eibl et al., 2003; Sales et al., 2004; Casibang et al., 2001). In a considerable number of these investigations, ligand binding to EP2 and EP4, and subsequent increase in intracellular cAMP, seems to be the pathway involved in the VEGF transcriptional induction.

The present work aims: 1) To characterize the expression and localization of the isoforms of the principal angiogenic factor VEGF, as well as its receptors KDR and Flt-1, in the mink uterus during diapause, embryo activation, at early stages of implantation and placental formation, as well as in the pseudopregnant uterus. Its goal is to verify the effect of embryonic and maternal factors on the regulation of VEGF and receptors; 2) To identify prostaglandin E_2 as an important regulator of VEGF and therefore vasculogenesis and angiogenesis at early stages of placenta formation; and 3) To establish the molecular mechanisms involved in PGE_2 induced increase in VEGF transcription in mink uterine cells.

Chapter I

LITERATURE REVIEW

EMBRYONIC DIAPAUSE

Embryonic Diapause and Its Regulation

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REPRODUCTION
REVIEW

Focus on Implantation

Embryonic diapause and its regulation

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Abstract

Embryonic diapause, a condition of temporary suspension of development of the mammalian embryo, occurs due to suppression of cell proliferation at the blastocyst stage. It is an evolutionary strategy to ensure the survival of neonates. Obligate diapause occurs in every gestation of some species, while facultative diapause ensues in others, associated with metabolic stress, usually lactation. The onset, maintenance and escape from diapause are regulated by cascades of environmental, hypophyseal, ovarian and uterine mechanisms that vary among species and between the obligate and facultative condition. In the best-known models, the rodents, the uterine environment maintains the embryo in diapause, while estrogens, in combination with growth factors, reinitiate development. Mitotic arrest in the mammalian embryo occurs at the G0 or G1 phase of the cell cycle, and may be due to expression of a specific cell cycle inhibitor. Regulation of proliferation in non- mammalian models of diapause provide clues to orthologous genes whose expression may regulate the reprise of proliferation in the mammalian context.

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Introduction

Embryonic diapause, also known as discontinuous development or, in mammals, delayed implantation, is among the evolutionary strategies that ensure successful reproduction. It comprises the uncoupling of mating and fertilization from birth and serves to maintain developmental arrest of the embryo, usually to ensure that postnatal development can be completed under more favorable environmental conditions. Its wide distribution among unrelated taxa, from plants to insects to vertebrates, suggests that it has arisen numerous times during evolution. The defining characteristic of diapause in plants and animals is dramatic reduction or cessation of mitosis in the embryo. Cell cycle arrest can occur at the G0/G1 or G2 phase, depending on the species, and is induced by mechanisms that are poorly understood in virtually every species so far investigated. The exit from diapause can be defined as the resumption of mitotic activity. It is regulated by numerous factors, often specific to the species in question.

Recent comprehensive syntheses of literature have appeared on the evolutionary aspects of diapause (Thom *et al.* 2004), and on the maintenance and termination of diapause (Renfree & Shaw 2000). The molecular regulation of implantation from the uterine perspective has recently been discussed in depth (Dey *et al.* 2004). In this presentation we address the characteristics of the embryo in diapause and focus on the mechanisms of regulation of this phenomenon, including the environmental and metabolic stimuli that induce and terminate this condition, the hormonal regulatory pathways, and the phenomenon of cell cycle arrest and reactivation.

The embryo in diapause

In most mammals displaying discontinuity of development, the progression to the blastocyst stage of the embryo and post-implantation development of the embryo and fetus follow a preordained, species-specific program. There is an arrest in development that initiates diapause occuring at the blastocyst stage in most species. Notable exceptions are found in the bat family, where variation in the rate of post-implantation development has been documented (Rasweiler & Badwaik 1997). Among species displaying diapause at the blastocyst stage there is significant variation in morphology of the arrested embryo. In many species that display pre-implantation delay, including the rodents (Zhao & Dean 2002), the roe deer (Aitken 1975) and the nine-banded armadillo (*Dasypus novemcinctus*; A C Enders, personal communication), the embryo hatches

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ABSTRACT

Embryonic diapause, a condition of temporary suspension of development of the mammalian embryo occurs due to suppression of cell proliferation at the blastocyst stage of development. It is an evolutionary strategy to ensure the survival of neonates. Obligate diapause occurs in every gestation of some species, while facultative diapause ensues in others, associated with metabolic stress, usually lactation. The onset, maintenance and escape from diapause are regulated by cascades of environmental, hypophysial, ovarian and uterine mechanisms that vary among species and between the obligate and facultative condition. In the best-known models, the rodents, the uterine environment maintains the embryo in diapause, while estrogens, in combination with growth factors, reinitiate development. Mitotic arrest in the mammalian embryo occurs at the G0 or G1 phase of the cell cycle, and may be due to expression of a specific cell cycle inhibitor. Regulation of proliferation in non-mammalian models of diapause provide clues to orthologous genes whose expression may regulate the reprise of proliferation in the mammalian context.

INTRODUCTION

Embryonic diapause, also known as discontinuous development or, in mammals, delayed implantation, is among the evolutionary strategies that ensure successful reproduction. It comprises the uncoupling of mating and fertilization from birth and serves

to maintain developmental arrest of the embryo, usually to ensure that postnatal development can be completed under more favorable environmental conditions. Its wide distribution among unrelated taxa, from plants to insects to vertebrates, suggests that it has arisen numerous times during evolution. The defining characteristic of diapause in plants and animals is dramatic reduction or cessation of mitosis in the embryo. Cell cycle arrest can occur at the G0/G1 or G2 phase, depending on the species, and is induced by mechanisms that are poorly understood in virtually every species so far investigated. The exit from diapause can be defined as the resumption of mitotic activity. It is regulated by numerous factors, often specific to the species in question.

Recent comprehensive syntheses of literature have appeared on the evolutionary aspects of diapause (Thom *et al.* 2004), and on the maintenance and termination of diapause (Renfree & Shaw 2000). The molecular regulation of implantation from the uterine perspective has recently been discussed in depth (Dey *et al.* 2004). In this presentation we address the characteristics of the embryo in diapause and focus on the mechanisms of regulation of this phenomenon, including the environmental and metabolic stimuli that induce and terminate this condition, the hormonal regulatory pathways, and the phenomenon of cell cycle arrest and reactivation.

THE EMBRYO IN DIAPAUSE

In most mammals displaying discontinuity of development, the progression to the blastocyst stage of the embryo and post-implantation development of the embryo and fetus

follow a preordained, species-specific program. There is an arrest in development that initiates diapause and that occurs at the blastocyst stage in most species. Notable exceptions are found in the bat family, where variation in the rate of post-implantation development has been documented (Rasweiler & Badwaik 1997). Among species displaying diapause at the blastocyst stage there is significant variation in morphology of the arrested embryo. In many species that display preimplantation delay, including the rodents (Zhao & Dean 2002), the roe deer (Aitken 1975) and the nine-banded armadillo (*Dasypus novemcinctus;* A.C. Enders, personal communication) the embryo hatches from its zona pellucida before entering into diapause. The embryo of the roe deer has a modest complement of 30-40 cells (Aitken 1975). The mouse embryo has a similar cell number at hatching, but this number increases to approximately 130 cells within 72 h, and this cell complement is maintained through diapause (Spindler *et al.* 1996). The blastocyst of the armadillo is much larger, consisting of an inner cell mass in excess of 100 cells, and approximately 600 trophoblast cells (Enders 1962). In marsupials, the embryo in diapause comprises 60-100 cells (Smith 1981) surrounded by a glycoprotein investment comprising the zona pellucida of the oocyte, supplemented by two further investments derived from the oviduct (Selwood 2000). The carnivore embryo in diapause consists of 200-400 cells, with a zona that persists until implantation (Desmarais *et al.* 2004). The carnivore zona appears to be supplemented with layers of glycoprotein acquired during the passage of the embryo from the oviduct to the uterus (Enders & Mead 1996). There is evidence from studies of the western spotted skunk *(Spilogale putorius)* and the badger *(Taxidia taxus)* that embryo

diameter and the total number of cells in the blastocyst increase during diapause, although this proliferation is restricted to the trophoblast cells (Mead 1993). In other mustelids, the total cell number does not seem to increase during diapause (Mead 1993). In the mink *(Mustela vison)*, blastocyst diameter increases and cells proliferate only after reactivation (Desmarais *et al.* 2004). In the tammar wallaby *(Macropus eugenii)*, neither the number of cells in the embryo nor its diameter increase during diapause (Renfree 1981). In contrast, a low level of mitosis characterizes preimplantation delay in the roe deer (Lengwinat & Meyer 1996).

TWO VARIATIONS ON THE THEME OF DIAPAUSE

Two functionally distinct categories of mammalian embryonic diapause are recognized (Table 1). Facultative diapause, best known in rodents and marsupials, is the developmental arrest induced by environmental conditions related to the survival of the dam and her ability to nourish developing embryos. Facultative diapause can be produced experimentally in rodents by ovariectomy of the female soon after fertilization, followed by progesterone treatment (Paria *et al.* 2002). In contrast, obligate diapause is present during every gestation of a species, and is believed to be a mechanism for synchrony of parturition with environmental conditions favorable to neonatal survival. While common in mustelid carnivores, it is also found in the roe deer and some bats (Sandell 1990) In some species, there is seasonal diapause superimposed on diapause resulting from metabolic factors or lactation (Renfree & Shaw 2000).

Given the selective advantages of diapause in temperate climates, it is somewhat surprising that species that are closely related do not always express the trait. Examples can be found in the mustelids, where gestation undergoes an ordered progression without evidence of diapause in the European ferret, while in the mink, every pregnancy includes a period of preimplantation delay. Almost every other aspect of reproduction (induced ovulation, postimplantation gestation etc.) is identical between these species. There are examples in which obligate diapause is restricted to subspecies of animals that are geographically isolated, most notably the spotted skunk (Mead 1993). There is similar selectivity in the occurrence of facultative diapause, it is found in rodents of the subfamily Sigmodontinae in North America (e.g. *Peromyscus spp.*), while completely absent in South American species of this subfamily. Lindenfors et al. (2003) argue for a single evolutionary origin of embryonic diapause in carnivores, followed by loss of the trait in some subgroups. It is possible to induce diapause in species where it does not normally occur; for example, blastocysts from the ferret transplanted to the mink uterus, cease development (Chang 1968). A pre-implantation delay can be induced in ferrets by experimental manipulation of either pituitary (Murphy 1979) or ovarian (Foresman & Mead 1978) endocrine function. Evidence based on the appearance of chorionic gonadotropin secretion suggests that diapause or developmental delay, can occur in human embryos (Tarin & Cano 1999). The anecdotal data, then, allow the speculation that many mammalian species might be capable of expressing diapause under appropriate conditions.

REGULATION OF DIAPAUSE BY EXTERNAL FACTORS

Environmental regulation of diapause, including its onset, maintenance and termination is imposed directly on the exposed embryo in invertebrates. In contrast, it is regulated by means of the maternal organism in viviparous vertebrates. Most mammals that have survived in temperate and variable climates have evolved a pattern of seasonal breeding to maximize their reproductive success. The most common environmental cue that synchronizes both estrus and male reproductive competence in mammalian species is photoperiod. Nonetheless, there are numerous examples of species whose reproductive cycle is dictated by the availability of nutrients, often secondary to rainfall, and by environmental temperature.

Photoperiod and temperature

Reduced ambient temperature is one of the principal factors inducing diapause in invertebrates (Kostal *et al.* 2000). Low temperature will induce diapause in some reptiles (Shanbhag *et al.* 2003). In mammals, the role of temperature is best known in the regulation of delayed development in bats (Mead 1993). There is, nonetheless, evidence that elevated temperature induces facultative diapause in rodents (Marois 1982), and that low temperatures can prolong obligate diapause in some carnivores (Canivenc & Bonnin 1979). The physiological mechanisms in mammals are currently unknown.

It was recognized early that photoperiod played an important role in termination of diapause and the consequent induction of implantation (Pearson & Enders 1944). In mustelids, diapause is terminated during lengthening photoperiod, and the lengthening of

days prior to and after the vernal equinox influences the timing of implantation in numerous species, including the spotted skunk (Mead 1971) and the mink (Murphy & James 1974). In the seal family, implantation occurs under a regime of decreasing day length (Atkinson 1997). Day length, or more precisely, a regime of photoperiod in which mink are exposed to light during a critical period from 12-16 h after dawn, provides a facultative signal that induces implantation (Murphy & James 1974). Studies in both mink and skunks indicate that the requirement for long days is not absolute, as implantation occurs in animals maintained in constant dark as well as in blinded animals (Mead 1993). The pineal gland was first implicated in studies in which its denervation by cervical sympathetic ganglionectomy disrupted photoperiodic regulation of the termination of diapause (Murphy & James 1974), later confirmed by pinealectomy and melatonin replacement (Bonnefond *et al.* 1990). While chronic melatonin treatment of mink does not interfere with puberty, ovulation or blastocyst formation in mink, it prevents termination of diapause and implantation (Murphy *et al.* 1990). Implantation can be rescued by exogenous prolactin in this species, suggesting a single mechanism for photoperiod induction of implantation. In some macropod marsupials, seasonal regulation of diapause is superimposed on lactational diapause, and long days associated with the summer solstice are the cue that reinitiates embryo development (Renfree & Shaw 2000). Diapause can be terminated by denervation of the pineal in marsupials, implicating melatonin as the effector (Renfree *et al.* 1981). The environmental cues and their translation into physiological events are less well studied and more difficult to discern in species such as the roe deer,

where diapause is terminated during short days (Sempere *et al.* 1992), or, as in the case of the ursids, when implantation occurs during hibernation (Harlow & Beck 2002).

Metabolic stress and lactation

There is evidence to suggest, at least in the European badger *(Meles meles)*, that reduced nutrition of the dam lengthens diapause (Ferguson *et al.* 1996). In the classic paradigm of facultative diapause in rodents, mating occurs at a postpartum estrus, and implantation is delayed by the presence of suckling young, with larger litters causing a longer delay (Weichert 1940). In marsupials, the presence of suckling young, independent of number, represents the stimulus for entry and for maintenance of diapause, and removal of pouch young results in rapid reactivation of the embryo and consequent implantation (Renfree & Shaw 2000). Social stress, including crowding or introduction of new males, will induce facultative diapause in rodents (Marois 1982).

REGULATION BY ENDOGENOUS FACTORS

Maternal control

Rodent blastocysts survive, but do not implant when transferred to the uterus of ovariectomized, progesterone-treated adult females (Weitlauf & Greenwald 1968) or the oviducts of intact, immature females (Papaioannou & Ebert 1986). Under both conditions, embryos retain their capability to implant and develop normally, indicating that the maternal environment is the crucial factor that maintains diapause. Evidence that the uterus inhibits the renewal of embryonic development in obligate diapause comes from transplant

experiments where blastocysts from the ferret (a non-diapause species) were arrested in development when transferred to the mink uterus, while mink blastocysts reinitiated embryogenesis in the ferret uterus (Chang 1968). Mink embryos in diapause co-cultured with conspecific uterine cell lines displayed the capacity for reprise of embryonic development in vitro, providing further evidence that uterus maintains diapause in this species (Moreau *et al.* 1995).

Control by the pituitary gland

Regulation of embryonic diapause via hypophysial prolactin demonstrates the principle that existing hormones have been co-opted for variable, often diametrically opposed uses, during evolution (Figure 1). Prolactin is the key factor essential for embryo implantation in mustelid carnivores. Its circulating concentrations increase some days prior to implantation in both the mink (Murphy & Rajkumar 1985) and the spotted skunk (Mead 1993). Treatment of mink in diapause with prolactin precociously terminates diapause, while dopamine agonists, at doses that prevent prolactin secretion, prevent implantation (Papke *et al.* 1980). Withdrawal of the dopamine agonist (Papke *et al.* 1980) or administration of dopamine antagonists (Murphy 1983) terminates diapause in mink. Indeed, prolactin alone induced implantation in hypophysectomized mink (Murphy *et al.* 1981), as did administration of prolactin to animals in protracted diapause due to chronic melatonin treatment (Murphy *et al.* 1990). In macropod marsupials, prolactin plays an inhibitory role. In these species, hypophysectomy terminates diapause, and it has been shown that suckling-induced prolactin secretion during lactational delay prevents

implantation (Renfree & Shaw 2000). During the seasonal delay in marsupials, a pulse of prolactin secretion is necessary for inhibition of implantation, and this pulse can be blocked by long photoperiods (Renfree & Shaw 2000).

Rodent facultative diapause is terminated by a short-lived surge of estrogen from the ovary (Dey *et al.* 2004). Hypophyseal secretion of luteinizing hormone (LH) is essential for this release (Macdonald *et al.* 1967). It is of interest that both LH and prolactin will activate the corpus luteum in the long-fingered bat *(Miniopterus schreibersii)*, but only prolactin terminates diapause (Bernard & Bojarski 1994).

Ovarian events

Following ovulation in mustelids displaying an obligate diapause, the ovarian follicle collapses and forms the corpus luteum (Hanssen 1947). The CL body undergoes a remarkable structural reduction in size as diapause ensues, all the time secreting low levels of progesterone (Mead 1993, Murphy *et al.* 1993). In contrast to the pattern of terminal differentiation that characterizes CL development in most species, the mink CL retains its mitotic potential during the period of diapause (Douglas *et al.* 1998). In response to the pituitary prolactin signal that terminates diapause, the CL is rejuvenated, a process characterized by a several fold increase in volume and in progesterone output (Murphy *et al.* 1993). In contrast to models of facultative delay, it has not been possible to terminate diapause in carnivores by steroid administration. Studies in the ferret (Foresman & Mead 1978) and mink (Murphy *et al.* 1983) indicated that a luteal protein in combination with progesterone are required for successful implantation. A credible candidate protein,

glucose-6-phosphate isomerase, also known as autocrine motility factor, has recently been shown to be secreted by the ferret CL during the appropriate pre-implantation window (Schulz & Bahr 2004) and to be required for implantation (Schulz & Bahr 2003). In mink, circulating concentrations of glucose-6-phosphate isomerase are low during diapause and increase with activation of the CL, and are elevated at the time of implantation (R.D. Bennett and B.D. Murphy, unpublished observations), suggesting that it might also play a role in reactivation of the mink embryo in diapause.

In marsupials, as exemplified by the wallaby, the CL develops during the estrous cycle, only to be inactivated by lactational or seasonal prolactin secretion (Renfree & Shaw 2000). Reactivation ensues when this inhibitory influence is removed. In rodents, it is the absence of an ovarian estrogen pulse that maintains diapause, but the ovarian structure (CL or follicle) from which the steroid issues has not been resolved.

The most unusual manifestation of ovarian regulation of diapause is found in the nine-banded armadillo. In this species, there appears to be no regression of the corpus luteum associated with delay, as indicated by plasma progesterone concentrations (Peppler & Stone 1980). Nonetheless, implantation is induced some 14 days after ovariectomy (Mead 1993), suggesting ovarian inhibition of nidation. The basis for this inhibition remains undiscovered.

Uterine factors

As noted above, reciprocal embryo transfers have demonstrated that the maternal uterine environment induces and maintains the embryo in its developmental arrest. An

important question is whether diapause is due to the absence of uterine factor(s) necessary for development beyond the blastocyst, or whether the uterus actively maintains diapause by inhibition of development. Support for the former view can be found in studies that have shown large scale increase in uterine protein synthesis and secretion concurring with the termination of obligate diapause (Mead 1989, Lambert *et al.* 2001). Furthermore, cascades in the synthesis of several classes of proteins, including adhesion factors, cytokines, and growth factors, follow the estrogen pulse that induces mouse implantation (Dey *et al.* 2004). A simplified view of uterine and ovarian regulation of diapause in the embryo is presented in Figure 2.

The requirement for uterine expression of the cytokine, leukemia inhibitory factor (LIF), for implantation has been demonstrated by targeted mutation in mice (Dey *et al.* 2004). In this species LIF injection can replace the nidatory estrogen pulse (Sherwin *et al.* 2004), indicating an important role in termination of diapause. LIF transcripts are detected in the uterus of carnivores during the early stages of embryo reactivation (Song *et al.* 1998, Hirzel *et al.* 1999) rendering it a candidate for an uterine factor that terminates mitotic arrest in the embryo. Evidence is lacking for a direct stimulatory role of LIF on the embryo to reinitiate development in any species, indeed, mouse embryos bearing inactivating mutation of the LIF receptor develop beyond the blastocyst stage and successfully implant (Ware *et al.* 1995).

Epidermal growth factor (EGF) is a potent mitogen, and thus a candidate for a uterine paracrine or autocrine factor regulating embryo mitosis. It can terminate diapause

in ovariectomized rats in the absence of the estrogen pulse (Johnson & Chatterjee 1993), and members of the EGF family of growth factors, including heparin-binding EGF (hbEGF) and amphiregulin are expressed in overlapping patterns by the uterus during rodent implantation (Dey *et al.* 2004). The expression pattern of hbEGF at sites of implantation prior to embryo activation implicates it as a uterine factor effecting termination of diapause in rodents (Das *et al.* 1994). DNA microarray comparison of dormant and activated mouse blastocysts indicates that activation is associated with the expression of the gene encoding hbEGF, as well as the EGF receptor isoforms ErbB1 and ErbB4 (Hamatani *et al.* 2004). Further, hbEGF expression is induced in the uterus by estrogen, (Zhang *et al.* 1998) the proximal signal for the termination of diapause. In addition, EGF receptors are present in dormant carnivore embryos, and their signaling activity is increased associated with escape from diapause (Paria *et al.* 1994). It is therefore reasonable to speculate that estrogen-induced expression of EGF and EGF-like factors from the uterus and embryo, acting on cognate receptors in the blastocyst, reinitiates development.

Microarray analysis comparing the mouse uterus before and after the nidatory estrogen pulse indicates upregulation of other growth factor-related transcripts (Reese *et al.* 2001). A pentraxin family protein (PTX3) is expressed at nearly four-fold greater intensity in the post-delay uterus. This protein is involved in complement binding and in innate immune responses, and is secreted in response to inflammatory cytokines (Fulop *et al.* 2003). PTX3 gene deletion disrupts ovarian function by interfering with cumulus

formation (Fulop *et al.* 2003). While there are no investigations of its role in termination of diapause, its expression pattern, and its known role in glycoprotein synthesis identify it as a potential downstream target of the growth factor and cytokine cascade that terminates embryo arrest.

There is evidence to suggest that the uterus actively inhibits development of the embryo, thereby inducing and maintaining diapause. Flushings from the uteri of ovariectomized, progesterone treated mice (the delayed implantation model) contain protein fractions that inhibit DNA synthesis of embryos in vitro (Weitlauf 1978). Recent studies have revealed that the endogenous cannabinoid, anandamide, at high, but nonetheless physiologically relevant concentrations, inhibits mouse embryo development (Wang *et al.* 2003). Low levels of anandamide, in stark contrast, activate the dormant mouse blastocyst via mitogen activated kinase pathways. Thus, differential expression of cannabinoids may regulate facultative diapause.

Uterine microarray analysis indicates that several interferon-γ induced-genes are downregulated in the activated, relative to the delayed mouse uterus (Reese *et al.* 2001), suggesting that this cytokine might play a role in induction or maintenance of mitotic quiescence of the embryo.

REGULATION OF DIAPAUSE BY CELLULAR FACTORS

Cell cycle arrest

The mammalian embryo develops from the zygote by cell division and

differentiation. The common theme in diapause is the inhibition of the mitotic cell cycle in embryonic cells, such that proliferation ceases or is greatly reduced. Cells enter a quiescent state, and apoptosis is prevented by the maintenance of the basal metabolism, with protein and RNA synthesis, as well as oxygen consumption (Renfree & Shaw 2000). Entry into dormancy occurs first in the trophoblast subpopulation of the mouse blastocyst, followed by a more gradual entry of the inner cell mass cells (Given 1988). In insects, the mitotic arrest most commonly occurs at the G0/G1 stage of the cell cycle, but there are examples of G2-arrest in some species (Tammariello 2000). Quantification of DNA (Sherman & Barlow 1972) suggests that the arrest in mammalian embryos occurs prior to the S phase of the cell cycle. The absence of 5-bromo-2-deoxyuridine uptake by mink embryos in diapause (Desmarais *et al.* 2004) supports the case for G0/G1 arrest in this species.

By definition, the quiescent embryonic cells also retain the ability to resume the cell cycle when diapause terminates (Renfree & Shaw 2000). In the mouse, proliferation is initiated first in the inner cell mass of the blastocyst, almost immediately after the estrogen signal, and follows 6 to 12 h later in the trophoblast (Given & Weitlauf 1981). An intriguing new report suggests that reactivation of development in the trophoblast compartment of the spotted skunk embryo engenders endocycles, resulting in endopolyploidy (Isakova & Mead 2004). The significance of this finding to the termination of diapause awaits further investigation.

Cell cycle arrest has not been extensively studied in the mammalian embryo in diapause. Given the conservation of genes during evolution, investigations of diapause in

invertebrate and submammalian vertebrate models might be expected to provide insight into the maintenance of mammalian diapause. It has been shown that, in the fruit fly *(Drosophila melanogaster)*, the developmental arrest during embryogenesis can be attributed to the *dacapo* gene, homolog of the mammalian p21, an inhibitor of cyclin E/cdk2 complex activation (Lane *et al.* 1996). This is consistent with inhibition in G1, as cyclin E/cdk2 complex formation is necessary for entry into S phase. Other candidate genes for inhibition of the cell cycle in diapause have been derived from cDNA microarray and subtractive hybridization comparisons of embryos in diapause with their activated counterparts. In insects, proliferating cell nuclear antigen, a factor associated with DNA synthesis and regulated by p21 (Fotedar *et al.* 2004), is not expressed during diapause (Denlinger 2002). These findings are consistent with new information from the mouse embryo where dormancy is associated with the increased expression $p21^{cip1/WAF1}$ and concomitant decrease in a number of DNA replication genes (Hamatani *et al.* 2004). These studies also demonstrated that an inhibitor of G0/G1 transition, the B cell translocation gene 1 (Btg1 (Rouault *et al.* 1992)) is upregulated in the embryo during facultative diapause, providing a mechanism for maintenance of cell arrest. Expression of the classic cell cycle inhibitor, p53 and associated genes did not differ between dormant and activated mouse embryos, suggesting that this common effector of cell cycle arrest is not involved in diapause (Hamatani *et al.* 2004).

Regulation of the cell cycle in diapause and reactivation

Given the variation among mammalian groups displaying embryonic diapause, there may be no single mechanism of reactivation of mitosis. In non-mammalian models, several different proximal signals (temperature, photoperiod, nutrient supply) regulate cell cycle arrest and reactivation. In insects, reduction in ambient temperature induces a decline in ecdysteroid concentrations that in turn signals the initiation of diapause (Denlinger 2002). The earliest intracellular response detected to environmental stimuli that terminate diapause, including increasing temperature, is upregulation of synthesis of ecdysone and expression of its nuclear receptor (Denlinger 2002). In the nematode, *Caenorhabditis elegans*, multiple signals inducing the dauer diapause converge on daf-12, a nuclear receptor for a yet unknown sterol ligand (Gerisch & Antebi 2004). In this species, termination of diapause engenders downregulation of daf-9, a P450 hydroxylase that catalyzes formation of the ligand. Thus, a common theme emerges of termination of diapause in invertebrates by cholesterol or its derivatives, acting through classic nuclear receptor pathways.

Vertebrates have evolved to employ specific cholesterol derivatives, the steroids, in the regulation of reproduction. In all known examples of mammalian diapause, with the possible exception of the armadillo, ovarian progesterone is essential for the termination of delay (Mead 1993). Further, a single estrogen injection terminates diapause in rodents (Dey *et al.* 2004). Treatment of carnivores in obligate (Murphy *et al.* 1982) or marsupials in seasonal (Fletcher *et al.* 1988) delay with estrogen does not induce reactivation of the

embryo. Nonetheless, estrogens have pleiotropic mitogenic and mitotic effects on target tissues, mediated through classic nuclear receptors, membrane estrogen receptors and actions of multiple intracellular effectors (Frasor *et al.* 2003) and may, in the appropriate concentration and temporal sequence, reactivate embryos in diapause. Estrogen receptors (ER) are expressed in all cell types of the dormant and activated mouse blastocyst (Hou *et al.* 1996) and both nuclear receptor subtypes, ERα and ERß, have been identified in the cells of the blastocyst in diapause (Paria *et al.* 1998). Treatment of mice in delay of implantation with estradiol-17ß resulted in S-phase activity in the embryos at the earliest time tested, 6 h (Given & Weitlauf 1981) and a detectable increase in the cell number within 12 h (Spindler *et al.* 1996). Paria et al. (Paria *et al.* 1998) report that the principal mammalian estrogens, estradiol-17ß, estrone and estriol do not directly activate the dormant mouse embryo. This was concluded because estradiol-17ß failed to induce the expression of EGF binding to the embryo, the hallmark of embryo activation, and blastocysts treated with estrogen in vitro failed to implant. These findings notwithstanding, it remains likely, based on the temporal sequence of occurrence of the S-phase after estrogen treatment, that estrogens function as mitogens to terminate cell cycle arrest. The effects of estrogens may not be mediated through the classic nuclear receptors. Paria et al. (Paria *et al.* 1998) make a case for embryo activation (EGF-signaling) by uterus-derived catechol-estrogens, signaling via a nongenomic pathway. EGF is a potent mitogen, and there is evidence for nongenomic signaling between estrogen- and EGF-mediated cellular events (Driggers & Segars 2002). A plausible hypothesis is that mitosis is reinstated in the

embryo by EGF as a downstream event induced by primary estrogen or catechol-estrogen signaling.

Clues to the intracellular events in mitotic renewal can be derived from comparison of the transcriptome between dormant and activated mouse embryos (Hamatani *et al.* 2004). There is upregulation of the estrogen-responsive target, *Brca1,* a gene that promotes proliferation in other tissues (Deans *et al.* 2004). Dormant embryos have greater abundance of the histone deacetylase (HDAC)-5 transcript, a gene whose expression is associated with attenuation of proliferation, and is independent of p53 mechanisms (Huang *et al.* 2002). The mitotic stimulus downregulates this chromatin modifier, allowing for histone acetylation and consequent transcription of previously silenced genes.

Comparative models provide some insight into potential regulation of mitotic reinitiation at the end of diapause. The FoxO genes are the mammalian orthologs of the *C. elegans* Daf genes that regulate diapause (Hosaka *et al.* 2004). Among the roles played by FoxO transcription factors in *C. elegans* are the induction of p21 expression and consequent mitotic arrest (Seoane *et al.* 2004). Indeed, overexpression of FoxO transcription factors induces cell cycle arrest at the G1 phase in mammalian cells in vitro (Burgering & Kops 2002). Although null mutation of FoxO1, 2 or 3 does not interfere with early embryo development or implantation in mammals, ovarian function is disrupted in FoxO3a null mice, with a phenotype of proliferation of the granulosa component of an abnormal number of follicles and consequent precocious depletion of the follicle population (Hosaka *et al.* 2004). Thus, FoxO-induced cell cycle inhibition may be an important

mechanism in the maintenance of diapause. Recent studies have further defined a mechanism of escape from FoxO-induced inhibition of proliferation. Phosphorylation of FoxO genes occurs in response to mitogens, including estrogen (Birkenkamp & Coffer 2003). This modification restricts their translocation to the nucleus, thereby abrogating their cytostatic effects (Seoane *et al.* 2004). Further investigation is required to verify this hypothesis.

SUMMARY AND CONCLUSIONS

Embryonic diapause is an intriguing biological mechanism that has been employed by species in numerous taxa to ensure successful reproduction. In mammals, its onset, maintenance and termination are under maternal control, and are influenced by environmental factors and lactation. Reduction or cessation of mitotic activity in the embryo most likely results from the absence of uterine and ovarian mitogens necessary for development of the embryo beyond the blastocyst stage. Members of the EGF family of uterine origin are the best current candidates for induction of mitotic reprise in the embryo. Ovarian estrogen may also act directly to induce embryo mitosis. Cessation of development occurs before the S phase of the cell cycle in mammals, and may be due to expression of cell cycle inhibitors of the p21 family. Little is known about cell cycle regulation upon reactivation of the dormant embryo (Figure 3). Based on non-vertebrate

models, a case is made for transcriptional regulation of cell cycle inhibitors by the forkhead family of transactivators, inactivated by mitogenic stimulation.

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REFERENCES

Aitken RJ 1975 Ultrastructure of the blastocyst and endometrium of the roe deer (Capreolus capreolus) during delayed implantation. *J Anat* 119:369-384

Atkinson S 1997 Reproductive biology of seals. *Rev Reprod* 2:175-194

Bernard RT & Bojarski C 1994 Effects of prolactin and hCG treatment on luteal activity and the conceptus during delayed implantation in Schreibers' long-fingered bat (Miniopterus schreibersii). *J Reprod Fertil* 100:359-365

Birkenkamp KU & Coffer PJ 2003 Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors. *Biochem Soc Trans* 31:292-297

Bonnefond C, Martinet L & Monnerie R 1990 Effects of timed melatonin infusions and lesions of the superchiasmatic nuclei on prolactin and progesterone secretion in pregnant and pseudopregnant mink. *J. Neuroendocrinology* 2:583-591

Burgering BM & Kops GJ 2002 Cell cycle and death control: long live Forkheads. *Trends Biochem Sci* 27:352-360

Canivenc R & Bonnin M 1979 Delayed implantation is under environmental control in the badger (Meles meles L.). *Nature* 278:849-850

Chang MC 1968 Reciprocal insemination and egg transfer between ferrets and mink. *J. Exp. Zool.* 168:49-60

Das SK, Wang XN, Paria BC, Damm D, Abraham JA, Klagsbrun M, Andrews GK & Dey SK 1994 Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development* 120:1071-1083

Deans AJ, Simpson KJ, Trivett MK, Brown MA & McArthur GA 2004 Brca1 inactivation induces p27(Kip1)-dependent cell cycle arrest and delayed development in the mouse mammary gland. *Oncogene* 23:613-645

Denlinger DL 2002 Regulation of diapause. *Annu Rev Entomol* 47:93-122

Desmarais JA, Bordignon V, Lopes FL, Smith LC & Murphy BD 2004 The escape of the mink embryo from obligate diapause. *Biol Reprod* 70:662-670

Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T & Wang H 2004 Molecular cues to implantation. *Endocr Rev* 25:341-373

Douglas DA, Song JH, Moreau GM & Murphy BD 1998 Differentiation of the corpus luteum of the mink (Mustela vison): mitogenic and steroidogenic potential of luteal cells from embryonic diapause and postimplantation gestation. *Biol Reprod* 58:1163-1169

Driggers PH & Segars JH 2002 Estrogen action and cytoplasmic signaling pathways. Part II: the role of growth factors and phosphorylation in estrogen signaling. *Trends Endocrinol Metab* 13:422-427

Enders AC 1962 The structure of the armadillo blastocyst. *J Anat* 96:39-48

Enders AC & Mead RA 1996 Progression of trophoblast into the endometrium during implantation in the western spotted skunk. *Anat Rec* 244:297-315

Ferguson SH, Virgl JA & Larivière S 1996 Evolution of delayed implantation and associated grade shifts in life history traits of of North American carnivores. *Ecoscience* 3:7-17

Fletcher TP, Jetton AE & Renfree MB 1988 Influence of progesterone and oestradiol-17 beta on blastocysts of the tammar wallaby (Macropus eugenii) during seasonal diapause. *J Reprod Fertil* 83:193-200

Foresman KR & Mead RA 1978 Luteal control of nidation in the Ferret (Mustela putorius). *Biol Reprod* 18:490-496

Fotedar R, Bendjennat M & Fotedar A 2004 Functional Analysis of CDK Inhibitor p21WAF1. *Methods Mol Biol* 281:55-72

Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR & Katzenellenbogen BS 2003 Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* 144:4562-4574

Fulop C, Szanto S, Mukhopadhyay D, Bardos T, Kamath RV, Rugg MS, Day AJ, Salustri A, Hascall VC, Glant TT & Mikecz K 2003 Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development* 130:2253-2261

Gerisch B & Antebi A 2004 Hormonal signals produced by DAF-9/cytochrome P450 regulate C. elegans dauer diapause in response to environmental cues. *Development* 131:1765-1776

Given RL & Weitlauf HM 1981 Resumption of DNA synthesis during activation of delayed implanting mouse blastocysts. *J Exp Zool* 218:253-259

Given RL 1988 DNA synthesis in the mouse blastocyst during the beginning of delayed implantation. *J Exp Zool* 248:365-370

Hamatani T, Daikoku T, Wang H, Matsumoto H, Carter MG, Ko MS & Dey SK 2004 Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. *Proc Natl Acad Sci U S A* 101:10326-10331

Hanssen A 1947 The physiology of reproduction in the mink (*Mustela vison* Schreb.) with special reference to delayed implantation. *Acta Zool.* 28:1-136

Harlow HJ & Beck TDI 2002 Body mass and lipid changes by hibernating reproductive and nonreproductive black bears *(Ursus americanus)*. *J Mammol* 83:1020-1025

Hirzel DJ, Wang J, Das SK, Dey SK & Mead RA 1999 Changes in uterine expression of leukemia inhibitory factor during pregnancy in the Western spotted skunk. *Biol Reprod* 60:484-492

Hosaka T, Biggs WH, 3rd, Tieu D, Boyer AD, Varki NM, Cavenee WK & Arden KC 2004 Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc Natl Acad Sci U S A* 101:2975-2980

Hou Q, Paria BC, Mui C, Dey SK & Gorski J 1996 Immunolocalization of estrogen receptor protein in the mouse blastocyst during normal and delayed implantation. *Proc Natl Acad Sci U S A* 93:2376-2381

Huang Y, Tan M, Gosink M, Wang KK & Sun Y 2002 Histone deacetylase 5 is not a p53 target gene, but its overexpression inhibits tumor cell growth and induces apoptosis. *Cancer Res* 62:2913-2922

Isakova GK & Mead RA 2004 Occurrence of amitotic division of trophoblast cell nuclei in blastocysts of the western spotted skunk (Spilogale putorius latifrons). *Hereditas* 140:177-184

Johnson DC & Chatterjee S 1993 Embryo implantation in the rat uterus induced by epidermal growth factor. *J Reprod Fertil* 99:557-559

Kostal V, Shimada K & Hayakawa Y 2000 Induction and development of winter larval diapause in a drosophilid fly, Chymomyza costata. *J Insect Physiol* 46:417-428

Lambert RT, Ashworth CJ, Beattie L, Gebbie FE, Hutchinson JS, Kyle DJ & Racey PA 2001 Temporal changes in reproductive hormones and conceptus-endometrial interactions during embryonic diapause and reactivation of the blastocyst in European roe deer (Capreolus capreolus). *Reproduction* 121:863-871

Lane ME, Sauer K, Wallace K, Jan YN, Lehner CF & Vaessin H 1996 Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during Drosophila development. *Cell* 87:1225-1235

Lengwinat T & Meyer HH 1996 Investigations of BrdU incorporation in roe deer blastocysts in vitro. *Anim Reprod Sci* 45:103-107

Lindenfors P, Dalen L & Angerbjorn A 2003 The monophyletic origin of delayed implantation in carnivores and its implications. *Evolution Int J Org Evolution* 57:1952-1956

Macdonald GJ, Armstrong DT & Greep RO 1967 Initiation of blastocyst implantation by luteinizing hormone. *Endocrinology* 80:172-176

Marois G 1982 [Inhibition of nidation in mice by modification of the environment and pheromones. Re-establishment by prolactin and thioproperazine]. *Ann Endocrinol (Paris)* 43:41-52

Mead RA 1971 Effects of light and blinding upon delayed implantation in the spotted skunk. *Biol Reprod* 5:214-220

Mead RA 1993 Embryonic diapause in vertebrates. *J Exp Zool* 266:629-641

Moreau GM, Arslan A, Douglas DA, Song J, Smith LC & Murphy BD 1995 Development of immortalized endometrial epithelial and stromal cell lines from the mink (Mustela vison) uterus and their effects on the survival in vitro of mink blastocysts in obligate diapause. *Biol Reprod* 53:511-518

Murphy BD & James DA 1974 The effects of light and sympathetic innervation to the head on nidation in mink. *J Exp Zool* 187:267-276

Murphy BD 1979 The role of prolactin in implantation and luteal maintenance in the ferret. *Biol Reprod* 21:517-521

Murphy BD, Concannon PW, Travis HF & Hansel W 1981 Prolactin: the hypophyseal factor that terminates embryonic diapause in mink. *Biol Reprod* 25:487-491

Murphy BD, Concannon PW & Travis HF 1982 Effects of medroxyprogesterone acetate on gestation in mink. *J Reprod Fertil* 66:491-497

Murphy BD 1983 Precocious induction of luteal activation and termination of delayed implantation in mink with the dopamine antagonist pimozide. *Biol Reprod* 29:658-662

Murphy BD, Mead RA & McKibbin PE 1983 Luteal contribution to the termination of preimplantation delay in mink. *Biol Reprod* 28:497-503

Murphy BD & Rajkumar K 1985 Prolactin as a luteotrophin. *Can J Physiol Pharmacol* 63:257-264

Murphy BD, DiGregorio GB, Douglas DA & Gonzalez-Reyna A 1990 Interactions between melatonin and prolactin during gestation in mink (Mustela vison). *J Reprod Fertil* 89:423-429

Murphy BD, Rajkumar K, Gonzalez Reyna A & Silversides DW 1993 Control of luteal function in the mink (Mustela vison). *J Reprod Fertil Suppl* 47:181-188

Papaioannou VE & Ebert KM 1986 Development of fertilized embryos transferred to oviducts of immature mice. *J Reprod Fertil* 76:603-608

Papke RL, Concannon PW, Travis HF & Hansel W 1980 Control of luteal function and implantation in the mink by prolactin. *J Anim Sci* 50:1102-1107

Paria BC, Das SK, Mead RA & Dey SK 1994 Expression of epidermal growth factor receptor in the preimplantation uterus and blastocyst of the western spotted skunk. *Biol Reprod* 51:205-213

Paria BC, Lim H, Wang XN, Liehr J, Das SK & Dey SK 1998 Coordination of differential effects of primary estrogen and catecholestrogen on two distinct targets mediates embryo implantation in the mouse. *Endocrinology* 139:5235-5246

Paria BC, Reese J, Das SK & Dey SK 2002 Deciphering the cross-talk of implantation: advances and challenges. *Science* 296:2185-2188

Pearson OP & Enders RK 1944 Duration of pregnancy in certain mustelids. *J. Exp. Zool.* 95:21-35

Peppler RD & Stone SC 1980 Plasma progesterone level during delayed implantation, gestation and postpartum period in the armadillo. *Lab Anim Sci* 30:188-191

Rasweiler JJt & Badwaik NK 1997 Delayed development in the short-tailed fruit bat, Carollia perspicillata. *J Reprod Fertil* 109:7-20

Reese J, Das SK, Paria BC, Lim H, Song H, Matsumoto H, Knudtson KL, DuBois RN & Dey SK 2001 Global gene expression analysis to identify molecular markers of uterine receptivity and embryo implantation. *J Biol Chem* 276:44137-44145

Renfree MB 1981 Embryonic diapause in marsupials. *J Reprod Fertil Suppl* 29:67-78

Renfree MB, Lincoln DW, Almeida OF & Short RV 1981 Abolition of seasonal embryonic diapause in a wallaby by pineal denervation. *Nature* 293:138-139

Renfree MB & Shaw G 2000 Diapause. *Annu Rev Physiol* 62:353-375

Rouault JP, Rimokh R, Tessa C, Paranhos G, Ffrench M, Duret L, Garoccio M, Germain D, Samarut J & Magaud JP 1992 BTG1, a member of a new family of antiproliferative genes. *Embo J* 11:1663-1670

Sandell M 1990 The evolution of seasonal delayed implantation. *Q Rev Biol* 65:23-42

Schulz LC & Bahr JM 2003 Glucose-6-phosphate isomerase is necessary for embryo implantation in the domestic ferret. *Proc Natl Acad Sci U S A* 100:8561-8566

Schulz LC & Bahr JM 2004 Potential endocrine function of the glycolytic enzyme glucose-6-phosphate isomerase during implantation. *Gen Comp Endocrinol* 137:283-287

Selwood L 2000 Marsupial egg and embryo coats. *Cells Tissues Organs* 166:208-219

Sempere AJ, Mauget R & Bubenik GA 1992 Influence of photoperiod on the seasonal pattern of secretion of luteinizing hormone and testosterone and on the antler cycle in roe deer (Capreolus capreolus). *J Reprod Fertil* 95:693-700

Seoane J, Le HV, Shen L, Anderson SA & Massague J 2004 Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* 117:211-223

Shanbhag BA, Saidapur SK & Radder RS 2003 Lowering body temperature induces embryonic diapause during prolonged egg retention in the lizard, Calotes versicolor. *Naturwissenschaften* 90:33-35

Sherman MI & Barlow PW 1972 Deoxyribonucleic acid content in delayed mouse blastocysts. *J Reprod Fertil* 29:123-126

Sherwin J, Freeman T, Stephens R, Kimber S, Smith A, Chambers I, Smith S & Sharkey A 2004 Identification of genes regulated by Leukaemia Inhibitory Factor in the mouse uterus at the time of implantation. *Mol Endocrinol* 18:2185-2195

Smith MJ 1981 Morphological observations on the diapausing blastocyst of some macropodid marsupials. *J Reprod Fertil* 61:483-486

Song JH, Houde A & Murphy BD 1998 Cloning of leukemia inhibitory factor (LIF) and its expression in the uterus during embryonic diapause and implantation in the mink (Mustela vison). *Mol Reprod Dev* 51:13-21

Spindler RE, Renfree MB & Gardner DK 1996 Carbohydrate uptake by quiescent and reactivated mouse blastocysts. *J Exp Zool* 276:132-137

Tarin JJ & Cano A 1999 Do human concepti have the potential to enter into diapause? *Hum Reprod* 14:2434-2436

Thom MD, Johnson DD & MacDonald DW 2004 The evolution and maintenance of delayed implantation in the mustelidae (mammalia: carnivora). *Evolution Int J Org Evolution* 58:175-183

Wang H, Matsumoto H, Guo Y, Paria BC, Roberts RL & Dey SK 2003 Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. *Proc Natl Acad Sci U S A* 100:14914-14919

Ware CB, Horowitz MC, Renshaw BR, Hunt JS, Liggitt D, Koblar SA, Gliniak BC, McKenna HJ, Papayannopoulou T, Thoma B & et al. 1995 Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development* 121:1283-1299

Weichert CK 1940 The experimental shortening of delayed pregnancy in the albino rat. *Anat Rec* 77:31-48

Weitlauf HM & Greenwald GS 1968 Survival of blastocysts in the uteri of ovariectomized mice. *J Reprod Fertil* 17:515-520

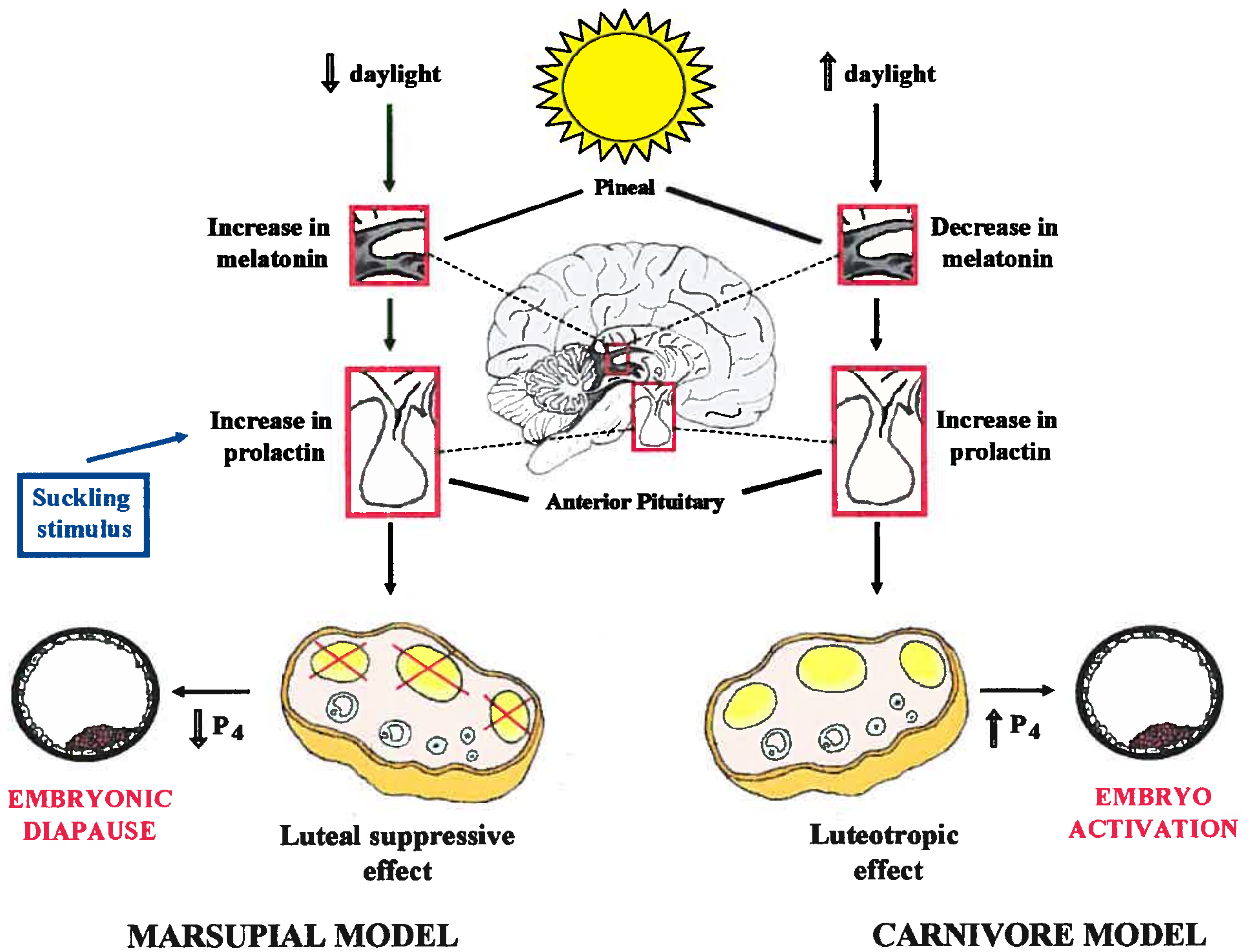
Weitlauf HM 1978 Factors in mouse uterine fluid that inhibit the incorporation of [3H]uridine by blastocysts in vitro. *J Reprod Fertil* 52:321-325

Zhang Z, Laping J, Glasser S, Day P & Mulholland J 1998 Mediators of estradiol-stimulated mitosis in the rat uterine luminal epithelium. *Endocrinology* 139:961-966

Zhao M & Dean J 2002 The zona pellucida in folliculogenesis, fertilization and early development. *Rev Endocr Metab Disord* 3:19-26

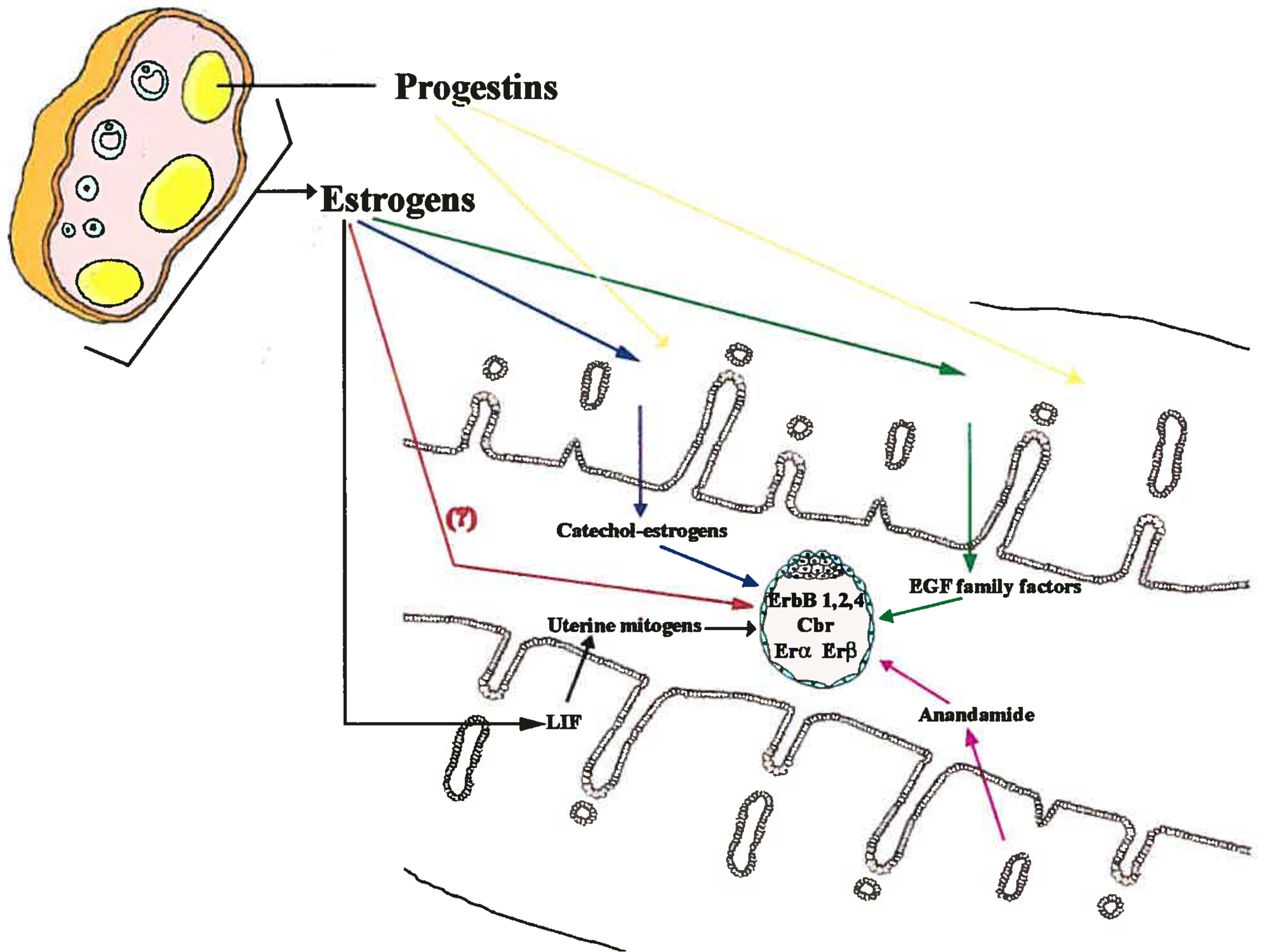
Characteristic	Facultative diapause	Obligate diapause
Distribution	Rodents, marsupials	Mustelid, ursid and phocid carnivores, roe deer, some bats and armadillos
Developmental status of blastocyst in diapause	Hatched in rodents, Encapsulated in marsupials	Encapsulated in carnivores, hatched in roe deer and armadillo
Mitotic activity in the embryo in diapause	None	Minor proliferation in some species restricted to trophoblast
Stimulus for entry into diapause	Lactation and metabolic stress	Developmental stage in all gestations
Exogenous stimulus for exit from diapause	Weaning (photoperiod in some marsupials)	Photoperiod
Endogenous stimulus for exit from diapause	Ovarian estrogen (rodents) Prolactin withdrawal (marsupials)	Prolactin secretion, unknown ovarian factors.

Table 1. Characteristics of facultative vs. obligate diapause of mammalian embryos.



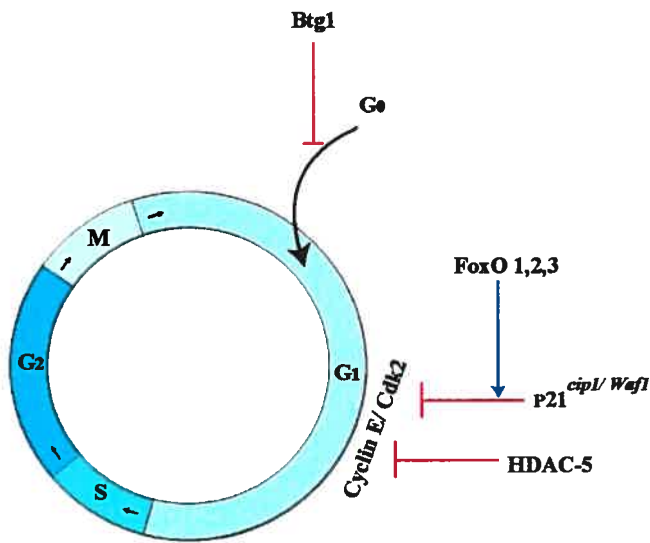
daylight
daylight
Pineal
Increase in melatonin
Decrease in melatonin
Increase in prolactin
Increase in prolactin
Suckling stimulus
Anterior Pituitary
P_4
P_4
EMBRYONIC DIAPAUSE
Luteal suppressive effect
Luteotropic effect
EMBRYO ACTIVATION
MARSUPIAL MODEL
CARNIVORE MODEL

Figure 1. Strategies for photoperiodic modulation of diapause employ melatonin and prolactin for contrasting purposes. In the marsupial model, both suckling stimulus, and increased melatonin secretion associated with nocturnal periods in excess of the summer solstice upregulate prolactin which then inhibits luteal activation, thereby initiating and maintaining diapause. In the carnivore model, photoperiod associated with the vernal equinox decreases melatonin secretion, releasing prolactin from inhibition. Prolactin activates the corpus luteum, provoking release of progesterone and other factor(s) that terminate diapause.

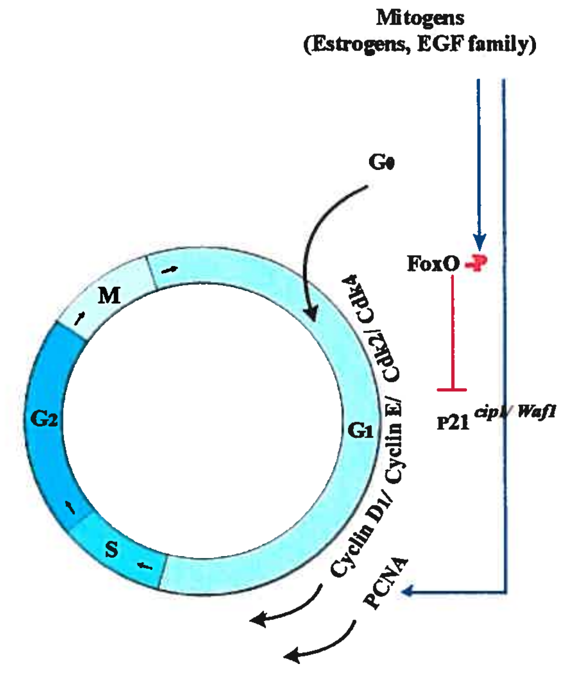


Progestins
Estrogens
(?)
Catechol-estrogens
ErbB 1,2,4
Cbr
Erα Erβ
EGF family factors
Uterine mitogens
LIF
Anandamide

Figure 2. Summary of uterine influences that could be acting on the dormant embryo in the rodent model to terminate the mitotic arrest of diapause. Against a background of luteal progesterone (P4), an ovarian pulse of estradiol 17ß (E2) is the proximal stimulus for developmental recrudescence of the embryo. It may have direct mitogenic effects on the dormant embryo via its nuclear receptors, ERα and ERß. It can be replaced by leukemia inhibitory factor (LIF) acting on the uterus to induce secretion of factor(s) mitogenic to the dormant embryo. E2 provokes expression of members of the epidermal growth factor (EGF) family, including EGF, heparin-binding EGF, and amphiregulin, acting through the EGF receptors Erb1 and Erb4 to reactivate mitotic activity of the embryo in diapause. Catechol estrogens derived from uterine conversion of E2, act via an unknown receptor to activate the embryo via the mitogen-activate protein kinase pathway. Finally, anandamide, an endogenous cannabinoid from the endometrium acts in low concentrations on its cognate receptors (Cbr), to stimulate the reprise of embryo development.



Entry and maintenance
of mitotic arrest
Btg1
G0
M
G2
G1
S
FoxO 1,2,3
P21 cip1/ Waf1
Cyclin E/ Cdk2
HDAC-5



Mitotic reactivation
Mitogens
(Estrogens, EGF family)
G0
FoxO -P
M
G2
G1
S
P21 cip1/ Waf1
Cyclin D1/ Cyclin E/ Cdk2/ Cdk4
PCNA

Figure 3. Some of the potential cell cycle regulatory mechanisms controlling the entry of the mammalian embryo into diapause by mitotic arrest and consequent developmental recrudescence by reinitiation of mitotic activity. Entry and maintenance of diapause result from expression of cell cycle inhibitors of the p21 family that interfere with cyclin E/cdk2 complex formation necessary for progression through G1. Transcription factors of the FoxO family upregulate p21 expression, to initiate and maintain this effect. In addition, histone deacetylase 5 (HDAC-5) is upregulated in the dormant embryo, which can prevent chromatin modification necessary for transcription of cell cycle genes. Further, dormant embryos express Btg1, a factor that prevents entry from G0 to G1, providing a further mechanism for cell cycle arrest. Embryo reactivation occurs due to mitogens from the ovary (estrogens) and from the uterus (EGF family and other factors). These have pleiotropic effects on the mitotic cycle, first by upregulation of proliferating cell nuclear antigen (PCNA), second by phosphorylating FoxO transcription factors, thereby preventing their translocation to the nucleus and consequent upregulation of p21, and third by direct stimulation of several components of G1 regulation, including cyclins D and E and cdk2 and cdk4.

PLACENTATION IN THE MINK

Classification of mammalian placental morphology has traditionally been based on the number of tissue layers present between maternal and fetal blood systems. The most often used system is the Grosser's histological classification method (Steven 1975). The most invasive placenta is found in humans and rodents, where the embryonic tissue is in direct contact with maternal blood, therefore with no layer present between maternal blood and chorionic tissue. This placental type is called hemochorial. The carnivore species also present a high degree of erosion of maternal tissues at the site of embryo attachment, but in these species however, the maternal vessels are surrounded by trophoblast cells but vessel integrity is maintained (Steven 1975), resulting in the endothelial-chorial designation. The American mink is a carnivore and like its counterparts it displays an endotheliochorial placenta of the discontinuous zonary type (Enders 1957).

THE PROCESS OF VESSEL FORMATION

The vascular system is amongst one of the first developed systems in the early embryo and placenta, in order to accommodate the necessity for nutrients, gas exchange and elimination of waste products.

In the early stages of tissue development, i.e. placental formation, formation of vessels occurs through a process termed vasculogenesis. This is understood to be differentiation of endothelial cells from primitive angioblasts, giving rise to a primitive tubular network in a previously avascular tissue (Risau, 1995; 1997; Yancopoulos et al., 2000). Following initial formation of this tubular structure, other processes take place in order to transform this primitive system into a well organized, and highly functional network responsible for the supply of blood to the tissues. This process of transforming the primary vascular system into a mature network is known as angiogenesis. Angiogenic remodelling can take place by two distinct processes, sprouting and non-sprouting. True sprouting angiogenesis involves extra-cellular matrix degradation allowing for migration and proliferation of the pre-formed endothelial cells (Risau, 1997). Most of the currently identified angiogenic factors orchestrate this form of angiogenesis either by directing the migration by serving as chemoatractants, and/or by inducing the proliferation of these cells (Risau, 1997). Non-sprouting angiogenesis occurs based on enlargement of the lumen of pre-existing vessels by proliferation of endothelial cells, which will in turn form bridges inside the lumen thereby initiating the splitting of the primary vessel (Risau, 1997).

Angiogenic factors

Several factors are currently known to play a role in formation and modification of the vascular system. VEGF and its tyrosine kinase receptors, as well as the basic fibroblast growth factor (bFGF) are involved in regulating angioblast differentiation (reviewed by

Carmeliet, 2000). Angiopoietin-1 (Ang1) and ephrin-B2 are required during remodelling and maturation of the vasculature (Yancopoulus et al., 2000). Ang1, acting through its receptor Tie2, is believed to optimize the integration of endothelial cells with their supporting cells, allowing for an interaction between them, which in turn guarantees successful signalling of further angiogenic factors (Suri et al., 1996). Ang1 has also been demonstrated to be responsible for maintaining a quiescent stage to the mature vasculature, and induction of its antagonist Ang2 will perturb this quiescence and re-initiate vascular remodelling (Yancopoulus et al., 2000). Of utmost importance and at the forefront of vessel formation is VEGF, which is required for both initiation of vascular formation (vasculogenesis) to sustain development in forming tissues, and for re-arrangement and maturation of pre-existing or newly formed primary vasculature.

VEGF, first named vascular permeability factor (VPF), was identified from a partial purification from a guinea-pig hepatocarcinoma cell line as a protein that promoted increased vascular permeability with a high potency (Senger et al., 1983). In 1989, Ferrara and Henzel isolated a diffusible endothelial cell-specific mitogen from the medium of bovine pituitary folliculostellate cells, and they determined it was a potent mitogen specific to endothelial cells and named it VEGF, due to its restricted target, the endothelial cells (Ferrara an Henzel, 1989). Since the original isolation, the VEGF family has been shown to include several forms derived from different genes, and to date VEGF-A, -B, -C, -D and -E, have been identified.

VEGF-A is a homodimeric glycoprotein of 40–45 kDa, and the isoforms coded by the human VEGF-A gene are formed due to alternative splicing of eight exons. Four major isoforms are present in the majority of the tissues, varying according to the final number of aminoacids in the mature form: VEGF121, VEGF165, VEGF189, VEGF206 (Tischer et al., 1991; Houck et al., 1991). Other less common isoforms have been identified in a more tissue specific manner (Neufeld et al., 1999). Differential heparin-binding properties are related to the bioavailability of the isoforms (Houck et al., 1992). The shorter VEGF form, VEGF121, does not bind heparin, making this isoform freely diffusible, whereas the two longer forms, 189 and 206 are almost found exclusively bound to the extra-cellular matrix (ECM). The most predominant form is VEGF165, and it can be found either in the diffusible form or bound to the ECM (Park et al., 1993). The diffusible form 121 has reduced mitogenic activity given that mitogenicity is associated with the heparin binding properties (Keyt et al., 1996).

VEGF exerts its action through binding to tyrosine kinase receptors, Flt-1 (fms-like tyrosine kinase, also known as VEGFR-1) and KDR (kinase domain region, also known as VEGFR-2) (Jussila e Alitalo, 2002). These tyrosine kinase receptors are comprised of seven extracellular immunoglobulin (Ig)-like domains, a membrane-spanning region and an intracellular tyrosine kinase domain (Shibuya et al., 1990; reviewed by Robinson and Stringer, 2001). A soluble form of Flt-1 has been identified. This form lacks the last Ig-like domain, nonetheless it binds VEGF with very high affinity. This truncated form binds

to VEGF and sequesters it, causing inhibition of VEGF activity (Kendall and Thomas, 1993). This soluble form was found at increasing levels in the mouse placenta starting at day 13 of gestation (He et al., 1999) and its presence may indicate a role in regulating VEGF activity at later stages of pregnancy.

VEGF165 can also bind to a receptor called neuropilin-1, which has been shown to be a neuronal receptor involved in axon growth (Takagi et al., 1995). Neuropilin-1 is a co-receptor to VEGF165, enhancing its binding to KDR (Soker et al., 1998).

VEGF-B binds Flt-1 and appears to be involved in degradation of the ECM, in cell adhesion and in cell migration (Olofsson et al., 1996). VEGF-C and VEGF-D both bind KDR and Flt-4. The latter is a further tyrosine kinase receptor on precursor cells that originate the endothelial cells, as well as on endothelial cells of the lymphatic system (Kukk et al., 1996). Both forms seem to be involved primarily with regulation of lymphatic system development (Kukk et al., 1996). VEGF-E is coded by the parapoxvirus Orf virus (Orf) and binds selectively to the KDR receptor and has its effects on angiogenesis through this receptor (Meyer et al., 1999). Another member of the VEGF family that presents angiogenic effects is the placental growth factor (PlGF); this protein has no mitogenic or permeability activities, nonetheless it can dimerize with VEGF and it is known to potentiate the actions of low concentrations of VEGF (Meyer et al., 1999). For the purposes of the present work, we focus on the VEGF-A gene and its isoforms.

VEGF and receptors are required during early pregnancy for embryo and placental development

The importance of VEGF for the proper development and viability of embryos was elegantly demonstrated by Carmeliet et al. (1996), who showed that inactivation of even a single allele to be embryonic lethal at days 11-12 of gestation. A hypermorphic model of VEGF-A is also embryo lethal (Miquerol et al., 2000), which indicates the need for precisely controlled levels of VEGF during embryonic development.

Importance of the Flt-1 receptor during vasculogenesis has been demonstrated through the use of null mice, which die at embryonic day 8.5 (Fong et al., 1995). Despite formation of endothelial cells in both embryonic and maternal compartments of the placenta in these animals, organized vessels fail to form. However, null mutation of the tyrosine kinase domain of Flt-1 does not eliminate embryonic vascular development in mice (Hiratsuka et al., 1998), which in turn indicates that actual Flt-1 signalling is not required for embryo development. It appears that the role for Flt-1 during vascular development in embryos lies on sequestering VEGF-A and preventing its binding to KDR. Studies inactivating Flt-1 in a trophoblast specific manner demonstrated that this receptor is not necessary for the initial establishment of the feto-maternal interface (Hirashima et al., 2003). Mice null for KDR receptor die at 9.5 days of gestation due to problems in the development of endothelial precursors (Shalaby et al., 1995).

Using a mice model containing the reporter LacZ at the VEGF gene, Miquerol et al. (1999) demonstrated that VEGF is expressed as early as day 4 in blastocysts, and expression was also observed soon after in the primary giant cells of the placenta, those invading the decidual layer of the uterus. This mechanism coordinates the beginning of vasculogenesis of the placenta. By day 7 post-conception, extensive expression of the reporter LacZ was observed in the extra-embryonic membranes, whereas expression in the embryo proper is noticeable only a day later.

Expression of angiogenic factors during early pregnancy

Several studies to date have demonstrated the expression of VEGF-A and its most common receptors, Flt-1 and KDR, during stages of the reproductive cycles in several species. In humans, intense staining for VEGF was observed in both stromal and glandular epithelial uterine cells during the mid-secretory phase. Accompanying decidualization, expression of both VEGF and KDR is increased. Even though expression of Flt-1 is also high during early stages of pregnancy, it does not seem to vary with the decidualization process (Sugino et al., 2002). In the golden hamster, expression of VEGF and both of its receptors was observed in the uterus at early stages of pregnancy (Yi et al., 1999). Ghosh et al. (2000) demonstrated that VEGF increased in levels in the decidua as implantation progressed, and that cytotrophoblast cells also express VEGF. In mice, luminal epithelial and stromal cells accumulated VEGF mRNA (Chakraborty et al., 1995). On day 5 of gestation, following initial attachment and invasion, the luminal epithelial and stromal cells

immediately surrounding the blastocysts exhibited accumulation of VEGF mRNA. On the embryonic side, mainly the trophoblast giant cells accumulated VEGF mRNA on day 8. In situ hybridization studies showed accumulation of Flk-1 mRNA in a subset of cells in the stromal bed on day 4. On days 5-8, cells in both the mesometrial and antimesometrial decidual beds exhibited accumulation of Flk-1 and Flt-1 mRNAs (Chakraborty et al., 1995). Also in the mouse, Halder et al., (2000) have shown that VEGF164 is the predominant form of VEGF during early gestation, and that VEGF expression is temporally coordinated with expression of the receptors KDR and neuropilin-1 in the endothelial cells. The pregnant rabbit uterus also displays high levels of VEGF at the peri-implantation stages and a pronounced localization was observed in the invading trophoblast cells (Das et al., 1997).

Regulation of VEGF

The vast majority of studies presented to date on regulation of the angiogenic factor VEGF have dealt with tumor vascularity, given that tumor growth is dependent upon transactivation of VEGF transcription. One of the most potent and known regulators of VEGF is hypoxia (reviewed by Josko and Matzurek, 2004). Its up-regulation is exerted through increased transcription, which is mediated by binding of the hypoxia-inducible transcription factor (HIF-1) to the HRE region of the VEGF promoter, as well as increased stability of the mRNA (Liu et al., 2002). HIF-1α is induced by hypoxia and it heterodimerizes with the constitutively expressed HIF-1β form (Jiang et al., 1997).

Hypoxia can also induce other transcriptional mediators, such as related transcriptional enhancer factor-1 (RTEF-1), which binds to a SP1 site in the proximal promoter region of the human VEGF gene (Shie et al., 2004). Hypoxia plays a central role in the vasculogenesis and angiogenesis that must take place during early gestation, in order to meet the increasing demands for gas exchange and nutrient supply of the growing tissue.

Non-hypoxic regulation of VEGF is controlled by several classes of substances, including cytokines, other growth factors, products of oncogenes, hormones such as estrogens and progestins (reviewed by Loureiro e D'Amore, 2005). Of the growth factor class, one very important regulator of angiogenesis that acts both directly by inducing endothelial cell proliferation, and indirectly by up-regulating VEGF, is fibroblast growth factor-4 (FGF-4) (Deroanne et al., 1997). Transforming growth factor-β (TGF-β) has been shown to stimulate VEGF in a variety of cancerous cells by several different mechanisms, varying according to the cancer type. Some of these mechanisms involve activation of the SMAD signalling (Sugano et al. 2003), or transactivation of SP1 (Benckert et al., 2003). Members of the epidermal growth factor family (EGF) and their receptors (EGFR and ErbB) have been linked with increased expression of VEGF, and different mechanisms appear to be involved (Goldman et al., 1993; Yen et al., 2002; Petit et al., 1997). Another example of growth factors regulation of VEGF is the insulin-like growth factor-1 (IGF-1), which dictates VEGF expression in endometrial adenocarcinoma cells at the post-transcriptional level by enhancing the stabilization of the 2 major VEGF isoforms mRNAs

(VEGF121 and VEGF165), and also by directly inducing VEGF expression (Bermont et al., 2001).

Cytokines are also known to regulate VEGF, such as some forms of interleukin (IL-1β and IL-6; Kawaguchi et al., 2004 and Huang et al., 2004, respectively) and tumor necrosis factor-α, which has been reported to both stimulate and inhibit VEGF according to cell system investigated (Ryuto et al., 1996; Patterson et al., 1996).

Steroids have been extensively reported to be regulators of VEGF and angiogenesis. Several studies dealt with the control of angiogenesis by the changing levels of steroids during the reproductive cycle and pregnancy in the ovaries and uterus (Cullinnan-Bove and Koos, 1993; Shifren et al., 1996; Ma et al., 2001). Estradiol-17β increases all isoforms of VEGF in human endometrium, whereas progesterone selectively stimulates VEGF189 (Ancelin et al., 2002). In breast cancer cells, and endometrial adenocarcinoma cells, both nuclear receptors ERα and ERβ are involved in up-regulation of VEGF in response to estradiol (Buteau-Lozano et al., 2002; Mueller et al., 2000).

Prostaglandin-dependent regulation of VEGF has also been demonstrated in other systems and will be discussed in later sections of this literature review.

Given the variety of elements controlling VEGF, it is of interest to evaluate the effects and mechanisms involved on VEGF regulation by the hormones, cytokines and other regulators previously demonstrated to be present during early pregnancy in the mink. This comprises the main purpose of the present study.

PROSTAGLANDINS AND THEIR RECEPTORS

Prostaglandins are bioactive lipid compounds derived from the essential fatty acid arachidonic acid that were first identified in the 30s by von Euler (1939) from semen and the prostate, and later had their chemical structures elucidated by Bergström and Samuelsson (1962) and van Dorp (1964).

Arachidonic acid can be metabolized into three different categories of eicosanoids: 5-HpETE, that will be further processed into leukotrienes (LT A4, B4, C4, D4 and E4) through the action of the enzyme 5-lipoxygenase; it can also be processed by 11-, 12- or 15-lipoxygenase into 11-, 12- or 15-HETE or HpETE; and finally prostaglandins and thromboxanes following the action of COX-1 and COX-2.

Arachidonic acid is most commonly found in an esterified form in phospholipids of the membrane (Irvine, 1982). In order for the arachidonic acid to be available for formation of prostanoids, it must be cleaved from the membrane. The main lipase

responsible for this cleavage is phospholipase A_2 (PLA_2), on its two forms, cytosolic PLA_2 and the non-pancreatic type II secretory PLA_2 (Dennis, 1994). Following cleavage, the free arachidonate migrates to the luminal surface of the endoplasmic reticulum (ER) or to the nuclear membrane where it enters the prostanoid formation cascade (Morita et al., 1995). The first stages of prostaglandin formation involve the cyclo-oxygenases (COX-1 and -2) present at the ER or nuclear membranes, which will first diooxygenate the original arachidonic acid in order to form the endoperoxide-containing prostaglandin G_2 (PGG_2) that will then have a hydroperoxyl reduced to form a hydroxyl group to give rise to prostaglandin H_2 (PGH_2), also by COX-1 and COX-2. PGH_2 can then be converted by the specific synthases (prostaglandin and thromboxane synthases) to its biologically active products.

Leukotrienes, also derived by PLA_2-cleaved arachidonic acid, are formed following the action of 5-lipoxygenase present at the nuclear membrane, and are synthesized mainly in inflammatory cells, such as mast cells, leukocytes and macrophages (Peters-Golden and Brock, 2001). These substances contain an oxane ring instead of the cyclopentane ring, as found in the prostaglandins.

Several prostaglandins are formed from the PGH_2 precursor, PGI_2, PGD_2, PGE_2, $PGF_{2}\alpha$ and they have a variety of biological functions, as well as a diverse spatial

localization, according to the functions they exert. Each of these prostaglandins is formed by the action of a synthase carrying the name of the final prostaglandin formed from PGH_2.

The prostanoids (prostaglandin and thromboxanes) are secreted substances that act as autocrine and/or paracrine modulators of biological responses. Prostaglandins G, H, I and thromboxane are very unstable and have a half-life of 30s to a few minutes. The remaining prostaglandins, although stable, are metabolized and inactivated in a single passage through the lungs, which makes local production of prostanoids a requirement for their action (reviewed by Narumiya et al., 1999).

The prostanoid receptors have been identified as part of the G protein-coupled rhodopsin type receptors, containing seven transmembrane domains. There is a different receptor for each prostanoid (Narumiya et al., 1999). Although each prostaglandin binds to its receptor with high affinity, a certain degree of cross-reactivity is found amongst the ligands. Eight different receptors, coded by separate genes, have been identified to date: DP – PGD; IP – PGI; FP – PGF; TP – thromboxane; EP – PGE. Four subtypes are known for the EP class of receptors, termed EP1 through EP4. The intracellular pathway elicited by these receptors is also variable. The FP receptor activates the Phospholipase C (PLC) pathway via the G protein Gq (Ito et al., 1994). The IP receptor was reported to stimulate adenylate cyclase following ligand binding, however activation of phosphatidylinositol (PI), as well as elevation of Ca2+ levels, were also observed (Namba et al., 1994; Vassaux

et al., 1992). Ligand binding to the prostanoid receptor DP induces increases in cAMP given that this receptor is coupled to the Gs protein (Boie et al., 1995). The isoforms α and β of the thromboxane receptor TP both induce a PI dependent response through Gq (Hirata et al., 1996), but they differ on their cAMP related response, where the α isoform inhibits and the β activates cAMP. The PGE receptors of the EP type present a variety of intracellular pathways. EP1 activation elevates Ca^{2+} concentration (Watabe et al., 1993) and activation of the PLC pathway (Kimura et al., 2001). EP2 and EP4 are coupled to Gs and are therefore responsible for increase in cAMP. EP3 is a complex series of receptor variants (3A, B, C and D) with signalling systems varying from Gi (inhibitory) to Gs (stimulatory) in relation to cAMP responses, as well as Gq, related to PI responses. The major pathway however, is inhibition of cAMP through Gi activation (Namba et al., 1993).

A variety of actions throughout the body have been described for the prostanoids. Their actions elicit a diversity of responses: contraction and relaxation of smooth muscles, modulation of neurotransmitter release, fever response, sleep induction, secretion of gastrointestinal enzymes, as well as regulation of tract motility, transport of water and ions to the kidney, apoptosis, cell differentiation, immune responses, platelet aggregation and vascularity (Clyman et al., 1978; Andersen et al., 1980; Coleman and Sheldrick 1989; Hayaishi, 1991; Phipps et al., 1991; Akarsu and Ayhan, 1993; Dorn et al., 1992; Dinchuck et al., 1995; Matlhagela et al., 2005; and reviewed by Narumiya et al., 1999). They also

play major roles in regulation of reproductive functions as discussed in the following section of this review.

Prostaglandins and their effects on reproduction

Prostaglandins have been shown to exert multiple effects on reproduction, varying from effects on ovarian changes, to involvement in fertilization events, to implantation and regulation of parturition. A study utilizing a null mice model demonstrated the important effects of prostaglandins on reproduction. In this study, Lim et al. (1997) revealed the multiple reproductive failures observed in COX-2 deficient mice. Given that COX-2 is the rate-limiting enzyme on inducible prostaglandin formation, this study increased the interest in uncovering the specific prostaglandin involved in each aspect of the reproductive processes.

Mice null for COX-2 present normal follicular development but compromised ovulation even following exogenous gonadotropin treatment, indicating that the defect in ovulation is not due to gonadotropin levels (Lim et al.,1997). The authors suggested that it is likely due to defective oocyte maturation given that the cumulus cells surrounding ovulated oocytes in wild-type mice present a peri-nuclear accumulation of COX-2 protein. Ovulation was rescued in part due to PGE_2 administration to the COX null mice (Davis et al., 1999), suggesting that COX-derived PGE_2 participates in the ovulatory process. In fact, the requirement for COX-2 during the ovulatory process was shown earlier in several

species like the rabbit (Grinwich et al., 1972), the rat (Orczyc et al., 1972); the pig (Ainsworth et al., 1979), the ewe (Murdoch et al., 1983) and the monkey (Wallach et al., 1975). In the mice null model, fertilization was also reduced in oocytes collected from the null mice, likely due to lack of maturation (Lim et al., 1997). Through the use of exogenous injections of steroids and transplantation of wild-type blastocysts, the authors demonstrated that implantation is also defective in the COX-2 knockout females, and this was not due to improper steroid priming of the uterus. The process of decidualization was also found to be impaired in the COX-2 deficient mice.

Mice null for the prostaglandin E_2 receptor subtype EP2 have impaired fertility (Kennedy et al., 1999; Tilley et al., 1999) and it is likely due to reduced ovulation and impairment in fertilization. It was demonstrated that expansion of the cumulus cells surrounding the oocytes, a process required for oocyte maturation, was reduced, as well as unovulated oocytes were present in the null ovaries (Hizaki et al.,1999).

Lim et al. (1999) has also demonstrated that impaired implantation in the COX-2 knockout mice can be rescued through the administration of a PGI_2 analog and also by analogs of PPARδ, keeping in mind that PGI_2 is a ligand for PPARδ, suggesting involvement of this nuclear receptor and PGI_2 in the implantation process, according to the authors.

Perhaps the best known reproductive functions of prostaglandins is the effect of $PGF_{2\alpha}$ in ovarian cycles. This prostaglandin is well known for its major role in inducing luteolysis in Artiodactyls, such as the cow and the ewe (Nancarrow et al., 1973; Peterson et al., 1975; 1976). In ruminants, prostaglandin $F_{2\alpha}$ is released from the uterus in pulses during the process of luteolysis, reaching the ovary via a counter-current transfer from the uterine vein to the ovarian artery (Ginther, 1974). This prostaglandin is then responsible, following binding to its receptors in the ovary, for the regression of the progesterone producing corpus luteum, in order for a new cycle to be initiated by a new ovulation.

Prostaglandin E_2 and reproduction

Prostaglandin E_2 originates from PGH_2 through the action of two enzymes, PGE synthase of the cytosolic and microsomal isoforms, products of separate genes. The microsomal version is a membrane-bound protein, part of the membrane-associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) superfamily (Jakobsson et al., 1999), that has high specificity for the substrate PGH_2. The cytosolic form was characterized, almost at the same time, as an also highly specific enzyme that converts PGH_2 into PGE_2 in the presence of glutathione as a co-factor (Tanioka et al., 2000). This enzyme acts preferentially on the conversion of COX-1 induced PGH_2, rather than on the COX-2 derived PGH_2. The microsomal form is associated to the inducible form of COX, and is therefore related to delayed generation of PGE_2, given that it depends on induction of COX-2 for production of its substrate PGH_2 (Murakami et al., 2000).

As mentioned above, PGE_2 is capable of rescuing ovulation in mice lacking COX-2, which present defective ovulation as a phenotype (Davis et al., 1999). Likewise, mice null for the EP2 receptor of PGE_2 have reduced ovulation. More recently, microsomal PGE synthase was co-localized with COX-2 in the granulosa cells of pre-ovulatory bovine follicles, and expression was related to gonadotropin treatment (Filion et al., 2001). In the present work, we focus on microsomal PGE synthase produced PGE_2, given that this form is related to the induced COX-2 and is more likely to be regulated during the reproductive processes.

In the uterus of hamsters, PGE_2 was identified as the major prostaglandin in implantation sites, and mPGE synthase is co-expressed with COX-2 at this site (Wang et al., 2004). In mice, mPGE synthase mRNA and protein were localized in the subluminal stroma surrounding the implanting blastocyst. The authors suggested that the embryo regulates PGE synthase expression, given that a similar pattern of expression was absent in pseudopregnant females, as well as in the interimplantation sites in the uterus (Ni et al., 2002). In bovine endometrial cells, the expression of PGE synthase is also correlated with that of COX-2 (Parent et al., 2002).

Expression of EP4 mRNA by in situ hybridization was detected in the epithelial and stromal layers of the mouse uterus during days 3-5 of early pregnancy. Furthermore, the same level of expression was found in pseudopregnant females, indicating that maternal

factors, rather than the embryo, regulate expression of this receptor (Yang et al., 1997). The mRNA for the EP2 receptor was highly expressed in the luminal epithelial cells primarily on days 4 and 5 of pregnancy, suggesting a possible role for these factors in the implantation process (Lim and Dey, 1997). Elevated expression of the receptor EP2 mRNA as well as its protein was detected in the luminal epithelium at implantation sites of female rats on day 6 of pregnancy (Shi et al., 2005).

Prostaglandin E_2 and regulation of VEGF

Although some other prostaglandins have been reported to participate in the regulation of VEGF, for the purpose of the present work, we have focused on the effects of PGE_2 in the transcription of VEGF, as this appears to be a very important regulatory process in the uterus.

Given the importance of angiogenesis to cancer biology, it is not surprising that the vast majority of work published in the regulation of VEGF by PGE_2 deals with the use of a variety of cancer cells as models. In human pancreatic cancer cells, PGE_2 stimulated the mRNA for VEGF and this effect was blocked by an EP2 receptor antagonist (Eibl et al., 2003). EP2 acts by increasing cAMP levels, and in their study the blocking of VEGF induction by the EP2 antagonist was corroborated by the findings that intracellular levels of cAMP increased in response to PGE_2 treatment. In lung cancer cells (squamous cell carcinoma), Casibang and colleagues (2001) reported that PGE_2 induces a rapid increase in

cAMP and that this increase resulted in elevated mRNA and secreted protein levels of VEGF. Further, the protein kinase A inhibitor H89 was successful in blocking the PGE_2 induced VEGF response. Increased VEGF expression was observed in mammary tumor cells null for the EP2 receptor following adenoviral delivery of the gene coding for this receptor. Further, the authors reported that the induction was indeed dependent on the cAMP/PKA pathway (Chang et al., 2005). Fukuda et al. (2003) placed HIF-1 as the transcription factor involved in induction of VEGF by PGE_2 in HCT116 human colon carcinoma cells. PGE_2 induced release of VEGF in human uterine cancer cells (Yshikawa cells) is dependent on intracellular cAMP mediated transactivation of the EGFR and ERK1/2 pathways, and this pathway is induced by ligand binding to EP2 receptor (Sales et al., 2004). Homozygous deletion of the EP2 receptor caused decreases in number and size of intestinal polyps in Apc (Delta 716) mice (mouse model for human familial adenomatous polyposis). The authors conclude that EP2 is the major receptor mediating the PGE_2 induced increase in cellular cAMP and subsequently VEGF (Sonoshita et al., 2001).

In a study on carpal tunnel syndrome, a disease that causes extensive extra-cellular matrix remodelling, PGE_2 and VEGF are co-expressed in the lesion areas, indicating a possible association of these two factors in this disease (Hirata et al., 2004). In rat gastric microvascular endothelial cells, PGE_2 stimulates VEGF mRNA, as well as protein, through transactivation of JNK1 by ERK2 (Pai et al., 2001). In a cell model using human airway

smooth muscle cells, Bradburry and colleagues (2005) have recently shown that PGE_2 stimulates VEGF transcription following binding to EP2/EP4 and cAMP activation. The binding site for SP1 appears to be involved in this cAMP dependent pathway. In human synovial fibroblasts, binding of PGE_2 to EP2 and EP4, and increase in cAMP are also involved in regulation of VEGF (Inoue et al., 2002).

As reviewed above, PGE_2 stimulation of VEGF acts through a variety of second messenger systems. It appears that the majority of the work published thus far, indicates that the receptors EP2 and EP4, and therefore the PKA dependent pathway, are the mechanisms of choice in the regulation of this specific angiogenic factor by PGE_2. In the work presented herein, we have succeeded in demonstrating the regulation of VEGF by PGE_2 and we have characterized the factors involved in this regulation in a mink uterine cell system.

Chapter II

Expression of Vascular Endothelial Growth Factor (VEGF) Isoforms and Receptors Flt-1 and KDR During the Peri-Implantation Period in the Mink, *Mustela vison.*

Short Title : Angiogenesis in carnivore implantation

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Keywords : Implantation, angiogenesis, VEGF, VEGF receptors, diapause

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ABSTRACT

Expression of vascular endothelial growth factor (VEGF) isoforms and its receptors, Flt-1 and KDR, was investigated during the period of peri-implantation in mink, a species that displays obligate embryonic diapause. Uterine samples were collected during diapause, embryo activation, and implantation from pseudopregnant and anestrous animals and analyzed by semiquantitative reverse transcription polymerase chain reaction and immunohistochemistry. The abundance of mRNA of VEGF isoforms 120, 164, and 188 was highest during late embryo activation and at implantation. VEGF protein was localized to the glandular epithelium at all stages of peri-implantation, whereas the luminal epithelium lacked VEGF reactivity during diapause. Endometrial stroma and luminal and glandular epithelia were positive for VEGF in implanted uteri. The invasive trophoblast cells of the implanting embryo were intensively stained. High levels of VEGF mRNA in pseudopregnant uteri indicates that VEGF upregulation leading to implantation is dependent upon maternal rather than embryonic factors. The abundance of the two receptors, KDR and Flt-1, increased in the uterus during implantation. Low levels of the receptors in pseudopregnant uteri compared with those containing activated or implanted embryos indicates that the embryo regulates receptor expression. These results demonstrate VEGF and VEGF receptor expression during early gestation in mink and suggest that maternal and embryonic input regulates different aspects of the angiogenic process.

placenta, pregnancy, implantation, seasonal reproduction, uterus

INTRODUCTION

The American mink, *Mustela vison*, is among a number of carnivores that display obligate embryonic diapause [1], characterized by an arrest in mitotic activity of the embryo and leading to a delay in implantation. Mink are seasonal breeders, and their mating season is during spring in the Northern Hemisphere. Ovulation is induced by mating, and the arrest in embryo development occurs at the blastocyst stage 6 days after mating, concurrent with embryo entrance into the uterus [2]. The length of embryonic diapause is associated with photoperiod and averages 18–25 days [3]. The termination of diapause is associated with increased levels of prolactin [4–6] under the regulatory influence of a reduction in melatonin [7] associated with the vernal equinox. The corpus luteum, which functions at a diminished level following ovulation, is reactivated by prolactin [4, 5]. The proximal regulation of delayed implantation appears to be dependent on uterine factors. Using reciprocal transfers, Chang [8] demonstrated that mink embryos in diapause reinitiated development in the ferret uterus, whereas ferret embryos, which do not display diapause, underwent developmental arrest when transferred into mink uterus. The process of implantation in mustelids begins with focal adhesion of the trophoblast to the endometrial endothelium, followed by rapid invasion at the sites of attachment [9]. Consequent differentiation of trophoblast cells in the mink leads to formation of a discontinuous zonary placenta of the endotheliochorial type in which the classic decidual response seen in other species is absent [10]. Postimplantation pregnancy is a consistent 30–31 days in this species [2].

The peri-implantation period in mammals is characterized by morphological and functional changes in the uterine cells accompanied by vascular remodelling. Angiogenesis is a key event for the proliferative processes in the uterus and is required for both placental and embryonic development [11–14]. Vascular endothelial growth factor (VEGF) is the principal factor responsible for regulation of vascular changes [15]. VEGF is a homodimeric glycoprotein of 40–45 kDa and is best known for its potent endothelial cell-specific mitogenic activity, but it also plays a role in increasing vascular permeability [16–19]. Several isoforms have been identified to date, and these differ in the number of amino acids in the final protein. The VEGF gene has eight exons, seven introns, and a coding region of around 14 kilobases [20]. The isoforms are the result of alternative exon splicing from a single gene [20]. The isoforms share the same function, and the main difference among them lies in their ability to bind to heparin [15, 21]. Such differential heparin-binding properties are related to the bioavailability of the several isoforms [22]. The major isoforms identified in humans are comprised of 121, 165, and 189 amino acids [23]. One of these, VEGF 121, is a soluble protein in its free form and has no heparin-binding properties. VEGF 165 is secreted and bound to the cell surface and extracellular matrix (ECM), and VEGF 189 is almost completely bound to the ECM [23]. VEGF protein appears to become available to endothelial cells in at least two ways: as freely diffusible proteins (VEGF 121 and 165 in humans) or after protease activation and cleavage of the longer isoforms bound to the ECM [15]. Among the factors that upregulate VEGF are the ovarian steroid hormones (see [24] for review) and prostaglandins (see [25] for review). VEGF is expressed in the endometrium [26] and is an important fac-

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ABSTRACT

Expression of vascular endothelial growth factor (VEGF) isoforms and its receptors, Flt-1 and KDR, was investigated during the period of peri-implantation in mink, a species that displays obligate embryonic diapause. Uterine samples were collected during diapause, embryo activation, implantation, from pseudopregnant and anestrous animals and analyzed by semi-quantitative RT-PCR and immunohistochemistry. The abundance of mRNA of VEGF isoforms 120, 164 and 188 was highest during late embryo activation and at implantation. VEGF protein localized to the glandular epithelium at all stages of peri-implantation, whereas the luminal epithelium lacked VEGF reactivity during diapause. Endometrial stroma, and luminal and glandular epithelia were positive for VEGF in implanted uteri. The invasive trophoblast cells of the implanting embryo were intensively stained. High levels of VEGF mRNA in pseudopregnant uteri indicates that VEGF upregulation leading to implantation is dependent upon maternal rather than embryonic factors. The abundance of the two receptors, KDR and Flt-1, increased in the uterus during implantation. Low levels of the receptors in pseudopregnant uteri compared to those containing activated or implanted embryos indicates that the embryo regulates receptor expression. These results demonstrate VEGF and VEGF receptor expression during early gestation in mink and suggest that maternal and embryonic inputs regulate different aspects of the angiogenic process.

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of the longer isoforms bound to the ECM [15]. Among the factors that upregulate VEGF are the ovarian steroid hormones ([24] for review) and prostaglandins ([25] for review). VEGF is expressed in the endometrium [26], and is an important factor in regulation of the events of early implantation and establishment of the placenta [27].

VEGF effects on angiogenesis are dependent upon its binding to tyrosine kinase receptors, Flt-1 (fms-like tyrosine kinase, also known as VEGFR-1) and KDR (kinase domain region, also known as VEGFR2) ([28] for review). These receptors play an important role in transduction of the VEGF signal during implantation [27].

Gestation in mink displays unique characteristics including obligate diapause, implantation through the zona pellucida and formation of an endotheliochorial placenta. Further, mitogenic activity of the endothelial cells of the maternal placental microvascular is accelerated during the early part of gestation.[29]. In addition, mink placenta differs from other carnivores in retention the fetal chorionic villi, allowing the maternal blood vessels to maintain their architecture [30]. Given the peculiarities in this species, it was of considerable interest to explore the expression of the angiogenic factors through the implantation process, including VEGF isoforms, and the VEGF receptors KDR and Flt-1. A further goal was to evaluate the involvement of the embryo in the angiogenic process.

MATERIALS AND METHODS

Animas and Sample Collection

All animal treatment protocols were approved by the Comité de déontologie, Faculté de médecine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care.

Mink of the Dark and Pastel varieties were purchased and maintained on a commercial farm (A. Richard, St. Damase, Canada). Females were mated to fertile males twice, 7 days apart throughout the first two weeks of March, according to standard husbandry procedures. Prolactin injection has been previously shown to induce embryo activation [4] and implantation [5,6]. A standard protocol, consisting of injections of 1 mg/kg of prolactin (Sigma, St. Louis, MO) given i.m. daily beginning approximately one week following last mating, was employed. Injections were carried on for twelve days. Implantation, as indicated by the presence of uterine swelling(s), occurred on the thirteenth day after initiation of prolactin injections.

Uterine tissues were collected from three animals selected randomly every second day starting on the day of the first prolactin injection (d 0) to 19 days thereafter. Uteri were flushed for embryo recovery (non-implanted females) and frozen in liquid nitrogen immediately following flushing or, in the case of implanted uteri, implantation chambers were frozen individually. Samples were kept at - 70° C until analyzed.

The assignment of samples into categories was based upon gross and microscopic inspection of the uterus and embryos. Diapause samples were collected prior to prolactin

injection, the uterus had no implantation chambers and only embryos in diapause (approximately 200 μm) were found during flushing. The activation period was divided into early and late activation, according to the number of prolactin injections received as well as the size of flushed embryos [31], i.e. late activated embryos were near 2 mm in diameter. Implantation was confirmed by microscopic evidence of embryo attachment and trophoblast invasion. Pseudopregnant animals were not mated, rather received two injections of GnRH (10 μg/kg; Factrel, Ayerst, Canada) 7 days apart during the mating period to induce ovulation, and samples were obtained 30 days later, allowing for their natural increases in prolactin and progesterone levels to take place. To establish whether there is basal expression of VEGF, uteri from anestrous females were obtained prior to beginning of the mink breeding season, when ovarian steroids are not present in significant amounts and therefore, are not expected regulate VEGF.

RNA Extraction, Purification and Reverse transcriptase (RT) Reaction

Tissues were homogenized in buffer RLT (Qiagen, Mississauga, Canada) with 0.12M β-mercaptoethanol (Sigma) and RNA was purified using a RNeasy Protect Mini kit (Qiagen) as recommended by the manufacturer. Total RNA was measured by spectrophotometry at 260 nm and 1.5 μg/sample of total RNA was used for the RT reaction using the Omniscript RT kit (Qiagen) according to the instructions from the manufacturer.

Mink Specific cDNA Cloning

VEGF primers were designed based on homologous sequences between human (GenBank Accession No. AF022375), mouse (GenBank Accession No. NM_009505) and bovine (GenBank Accession No. M32976) (Table 1). Homologous sequences of human (GenBank Accession No. for KDR: AF035121, for Flt-1: AF063657), rat (GenBank Accession No. for KDR: U93306, for Flt-1: D28498) and mouse (GenBank Accession No. for KDR: X70842, for Flt-1: L07297) were also used for designing primers for the VEGF receptors (Table 1). PCR products of the expected size, obtained from the primers mentioned above were excised and purified using a Gel Extraction kit (Qiagen). Purified cDNA was then ligated into a pGEM-T Easy Vector System I (Promega Corp., Nepean, Canada) according to the instructions of the manufacturer, and further transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated by the used of a QIAprep Spin Miniprep kit (Qiagen) and sequenced using a ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA), and at least 3 independent samples were sequenced for verification of the transcripts. The primers for KDR and Flt-1 based on the homologous sequences mentioned above were further used for our studies.

Mink specific primers (GenBank Accession No. AY158156), with exception to primer ex6 which was based on the homologous sequences (above), were chosen for all 3 VEGF isoforms, and are presented in Fig. 1. Briefly the forward primer (ex3) utilized for

all 3 VEGF isoforms was the same, located at the third exon, whether the reverse primers varied to allow for amplification of the individual isoforms (Table 1).

Mink specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used (GenBank Accession No. AF076283) as a control (Table 1).

Semi-quantitative RT-PCR

Uterine tissues from three animals in each of the following reproductive states: anestrous, diapause, early embryo activation, late embryo activation, implanted (implantation sites and inter-implantation sites), and pseudopregnant were studied. All analyses are presented as the mean ± SEM of the 3 individual samples per group.

Relative abundance of the VEGF isoforms 120, 164 and 188, as well as the receptors KDR and Flt-1, was determined by semi-quantitative PCR using GAPDH as a control for RNA quantity and RT efficiency. For each individual product analyzed, the number of cycles was chosen by subjecting the RT products to PCR reactions of 17 – 35 cycles. The quantification procedure was performed by choosing the number of cycles retaining amplification in the exponential phase. The number of cycles chosen for GAPDH, VEGF 120 and 164 were 23, 32 and 28, respectively. For VEGF 188 and the receptors KDR and Flt-1, 31 cycles were employed.

The semi-quantitative reactions were carried at the chosen number of cycles in a final volume of 50 μl and using Taq DNA polymerase (Amersham Biosciences Corp., Baie

d'Urfe, Canada). Amplifications were carried out with annealing conditions of 58° C/40 s for VEGF 120 and 59° C/40 s for the remaining products. PCR products were separated in a 1.8% agarose gel and stained in ethidium bromide. Densities of the amplified fragments were analysed using the Collage software (Photodyne, New Berlin, WI). Results were expressed as a density ratio of the target gene to the control (GAPDH).

Immunohistochemical Analysis of VEGF

Tissues fixed in Zamboni's solution were used to demonstrate expression of VEGF through the peri-implantation period. A Vectastain ABC kit (Vector Laboratories, Burlington, Canada) was used according to the manufacturer's protocol. Deparaffinized and hydrated sections were immersed in methanol containing 0.75% hydrogen peroxide for 20 min for quenching of any endogenous peroxidase activity. Sections were then washed in TBS and retrieval of antigens was performed by microwave treatment twice for 5 minutes in TBS. Sections were then incubated with normal goat serum at room temperature for 30 min. A rabbit polyclonal antibody, (VEGF: A-20, Santa Cruz Biotechnology, CA) raised against residues 1-20 at the amino-terminus of human VEGF, thus recognizing all isoforms used during this study, was added at a concentration of 0.6 μg/ml diluted in normal serum and tissues were incubated overnight at 4° C. The same antibody has been previously used to immunolocalize VEGF in mink [32]. After washes in PBS, incubation with biotinylated second antibody took place for 45 min. Following PBS

washes, a complex of avidin-biotin-peroxidase was applied for 45 min. Positive reactions were identified by the use of the peroxide substrate 3,3' diaminobenzidine (Sigma) at a concentration of 0.6 mg/ml.

Statistical Analysis

The ratio of target gene/GAPDH was used as a value for each sample and data analyzed using the least square analysis of variance by the General Linear Model procedures of SAS. When significant differences in treatments were found, comparisons of means were further performed by the methods of Orthogonal Contrasts, and Duncan's Multiple Range Test.

RESULTS

Semi-Quantification of VEGF Isoforms 120, 164 and 188

All three of VEGF isoforms were present in anestrous, diapause, activated (early and late), implanted (implantation site and inter-implantation site) and pseudopregnant uteri of mink. The relative abundance for all three isoforms of VEGF increased through implantation (Fig. 2). Expression of VEGF 120 was significantly different among groups

(P<0.01) (Fig. 2). Individual mean comparisons revealed that anestrous uteri had the lowest levels of VEGF 120 mRNA, significantly lower relative to implanted and pseudopregnant samples (P<0.05). Diapause and early activated uteri displayed significantly lower VEGF 120 levels relative to implanted uteri (P<0.05) (Fig. 2).

Expression of VEGF 164 mRNA also differed significantly among groups (P<0.01). Anestrous uteri had the lowest levels of VEGF 164 mRNA, which were significantly lower than early and late activated, implanted and pseudopregnant uteri (P<0.05). Samples from diapause and early activation had lower VEGF 164 levels than late activated, implanted and pseudopregnant uteri (P<0.05), with diapause VEGF 164 levels being similar to anestrous levels (Fig. 2).

Expression of VEGF 188 differed significantly among groups (P<0.01). Anestrous uteri also had the lowest levels of VEGF 188 mRNA relative to late activated, implanted and pseudopregnant uteri levels (P<0.05). Late activated and pseudopregnant samples had the highest mRNA levels and differed from anestrous, diapause and early activated samples (P<0.05); while implanted samples had levels in between early activated and the highest groups, late activated and pseudopregnant (Fig. 2).

Further analyses were performed by comparing the groups by Orthogonal Contrasts, in which the different stages were grouped and contrasted to allow comparisons of biological interest. The contrasts chosen were as follows: anestrous vs. pregnant (including all the other groups); diapause vs. activated (early and late); early activated vs. late activated; late activated and implanted vs. pseudopregnant; implanted vs. non-implanted

(diapause, early and late activated); implantation site vs. inter-implantation site. Results for VEGF isoforms are depicted in Table 2.

VEGF Localization During the Peri-implantation Period

Mink uterine tissues from the peri-implantation period were stained for VEGF protein. In uteri samples taken from diapause, or obligate delay, presence of VEGF staining was observed in the glandular epithelium, very little or no staining present on the luminal epithelium, whereas the subepithelial stroma presented no staining for VEGF (Fig. 3A,B). Following activation of the embryo, VEGF staining was observed in the luminal and glandular epithelium, while the stromal bed consistently lacked expression of VEGF (Fig. 3E,F). In implanted uteri, VEGF was strongly expressed in the luminal and glandular epithelium as well as in the subepithelial stroma (Fig. 3G,H) . At the site of embryo attachment and implantation the first layer of trophoblast cells, leading the invasion into the uterus, displayed a strong VEGF signal (Fig 3G,H). Samples taken from pseudopregnant animals presented a similar pattern of VEGF localization as activated samples, with positive staining in the luminal and glandular epithelium and lack of signal in the stromal bed (Fig. 3I,J). In uterine samples collected during anestrous, no significant localization of VEGF protein could be observed (Fig. 3L,M).

Semi-Quantification of VEGF Receptors KDR and Flt-1

We also investigated expression of the VEGF receptors KDR and Flt-1 through the process of implantation. Expression of KDR mRNA was different among groups ($P<0.05$). Group comparisons revealed that anestrous and pseudopregnant uteri had lower levels of this receptor than implanted uteri ($P<0.05$) (Fig. 4).

Expression of Flt-1 mRNA was different among groups ($P<0.01$). Group comparisons revealed that anestrous and pseudopregnant uteri had lower levels in comparison to activated and implanted uteri ($P<0.05$); diapause levels were found to be significantly lower than implantation site samples ($P<0.05$) but did not differ from any other group (Fig. 4). Orthogonal Contrast analysis was performed for the receptors using the same comparisons as for the VEGF isoforms. Results are shown in Table 2.

DISCUSSION

This investigation provides the first information about the expression of angiogenic factors in uterine and embryonic tissues during the peri-implantation period in a species that displays obligate embryonic diapause and the distinct carnivore pattern of implantation. The defining characteristics of obligate diapause are the developmental arrest of the embryo and the lack of an active corpus luteum, with attendant low level of progesterone secretion [33]. In other species it has been shown that estrogen and progesterone upregulate VEGF expression [24]. The differential effects of the two steroids were revealed by a recent study

in which estrogen was shown to inhibit angiogenesis while increasing vascular permeability in the mouse uterus [34]. Progesterone, on the other hand, stimulated angiogenesis, VEGF and Flk-1, but had no effect on vascular permeability [34]. Based on these findings, we postulated that reactivation the CL and its attendent increase in progesterone synthesis would be reflected in the expression of VEGF and its receptors. This proved to be the case, as VEGF expression in the mink uterus is low during diapause and is upregulated during embryonic activation and implantation. Concurrence with ovarian changes provides strong evidence for ovarian steroid hormone control.

A second candidate for the regulation of angiogenic factors during implantation is prolactin, as this hormone is an important effector of the termination of diapause [5]. Indeed, prolactin receptors are present in the mink uterus during early gestation, and their abundance is influenced by ovarian steroids [35]. No evidence for or against direct regulation of VEGF expression in the mink uterus by prolactin is yet available.

A third possibility is that the activated or implanting embryo plays a role in regulating VEGF either locally or globally in the uterus. This has been previously suggested for the hamster [36] and is supported in the present study by the distinct differences that were noted in VEGF expression between mink uteri containing blastocysts in diapause and those with activated embryos. We showed that VEGF staining was limited to the glandular epithelium during diapause, whereas activated uteri presented VEGF localization in the glandular epithelium as well in the luminal epithelium. Nonetheless, similar increases in the quantity and extent of VEGF expression, both in terms of mRNA

abundance and protein localized to glandular and endometrial epithelium during the implantation process, were also observed in uteri of pseudopregnant animals. The pattern in the uterus of the pseudopregnant animals resembles that seen during late embryo activation and early implantation. Further, mRNA results revealed that the increase in VEGF did not differ in samples taken from the implantation site relative to those from inter-implantation areas, indicating that the changes are not specific local effects of embryo invasion. Thus, it can be concluded that VEGF expression is independent of embryonic influence in the mink. This distinguishes this species from other mammals studied to date.

An interesting finding of the present study is the very strong VEGF localization present in invasive hypertrophied trophoblast cells. In contrast, the outermost layer of trophoblast cells, which are not in direct contact with uterine tissue, had no positive staining for VEGF. A similar VEGF localization within the invasive trophoblast cells has been observed in murine implantation [37]. At later times in gestation, VEGF positive staining is present in the cytotrophoblast and syncytiotrophoblast layers of the mink placenta [32]. This indicates evolution of VEGF expression in the two trophoblast cell types as gestation progresses.

Matsumoto et al. [27] have suggested that, while preimplantation expression of VEGF is steroid regulated, the onset of implantation and decidualization shift this regulation to COX-2-derived prostaglandins. Uterine expression of COX-2 is associated with implantation in mice, and COX-2 deficient mice display defective implantation and decidualization [38,39]. Further studies by Lim et al. [40] revealed that COX-2-derived

PGI_2 is involved in implantation and decidualization, and its action is mediated by the peroxisome proliferator-activated receptor delta (PPARδ). We have previously shown in the mink that COX-2 expression is a transient event that occurs at the time of trophoblast attachment and invasion [41] and this concurs with elevated expression of VEGF in the present study. Further, endometrial expression of PPARδ has been observed in this species following implantation [42]. This reinforces the view that prostaglandins contribute to upregulation of VEGF during this time in early gestation. PGE_2 has been shown to effectively induce VEGF expression in other tissues [43], and its expression was shown in the mouse uteri during implantation [40]. Preliminary data indicates that the mink activated embryo produces PGE_2 [42]. Other candidates include PGJ, which has been shown to stimulate VEGF expression in human macrophages, activating gene expression through a PPARγ mediated processes [43]. PPARγ, as well as its heterodimerization partner RXRα, are strongly expressed in human trophoblasts; RXR is also present in decidual cells [44]. Preliminary information indicates that PPARγ is expressed in the trophoblast at the time of implantation [42], placing PPARγ as a proximal candidate for VEGF regulation following implantation. The occurrence of the ligands for PPARγ at implantation remains to be investigated.

VEGF acts through the tyrosine kinase receptors KDR and Flt-1 [45]. In this study, expression of KDR in the uteri of pseudopregnant mink was low in comparison to samples from implanted animals. In addition, uterine samples derived from implantation sites displayed greater expression of Flt-1 relative to either uteri from diapause or from

pseudopregnancy. Studies in the mouse show very low accumulation of KDR mRNA in the uterus on the first two days of pregnancy; however, on days 3 and 4, these genes were distinctly expressed in the stromal bed [37]. On days 5-8 of mouse gestation, the decidual beds accumulated KDR as well as Flt-1 mRNAs [37]. In the rabbit, mRNA for both receptors were present in the uterus at several stages, with high levels at estrus and just prior to implantation [46]. In the hamster, expression of the receptors were also correlated with the progression of embryo implantation [36]. In the current studies we shown that elevation of VEGF receptor expression likewise appears to be associated with implantation in mink. Unlike the VEGF isoforms, both receptors studied were found to be at lowest levels in pseudopregnant uteri. This indicates that the implanting embryo plays a role in regulating expression of VEGF receptors. The factors produced by embryos, or by the uterus induced by the presence of the embryo during implantation remain obscure, but the eicosanoids and their receptors described above are excellent candidates.

In conclusion, we have used an unique animal model that presents an obligate developmental arrest in the embryo, progesterone-dependent embryo activation, a lack of decidual response and an endotheliochorial placenta, to study the factors involved in angiogenesis during the peri-implantation period. We have shown that three VEGF isoforms are upregulated during the peri-implantation period, as are the VEGF receptors, KDR and Flt-1. Upregulation of VEGF during the implantation process is dependent on maternal factors, presumably gonadal steroids, whereas the presence of the embryo appears to regulate the VEGF receptors.

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REFERENCES

1. Mead RA. Embryonic diapause in vertebrates. J Exp Zool 1993; 266: 629-641.
2. Hansson A. The physiology of reproduction in mink (*Mustela vison*) with special reference to delayed implantation. Acta Zool 1947; 28: 1-136.
3. Murphy BD, James DA. The effects of light and sympathetic innervation to the head on nidation in mink. J Exp Zool 1974; 187: 267-276.
4. Papke RL, Concannon PW, Travis HF, Hansel W. Control of luteal function and implantation in the mink by prolactin. J Anim Sci 1980; 50: 1102-1107.
5. Murphy BD, Concannon PW, Travis HF, Hansel W. Prolactin: the hypophyseal factor that terminates embryonic diapause in mink. Biol Reprod 1981; 25: 487-491.
6. Martinet L, Allain D, Meunier M. Regulation in pregnant mink (Mustela vison) of plasma progesterone and prolactin concentrations and regulations of onset of spring moult by daylight ratio and melatonin injection. Can J Zool 1983; 61: 1959-1963.
7. Murphy BD, DiGregorio GB, Douglas DA, Gonzalez-Reyna A. Interactions between melatonin and prolactin during gestation in mink (Mustela vison). J Reprod Fertil 1990; 89: 423-429.
8. Chang MC. Reciprocal transplantation of eggs between rabbit and ferret. J Exp Zool 1966; 161: 297-305.

9. Enders AC, Mead RA. Progression of trophoblast into the endometrium during implantation in the western spotted skunk. Anat Rec 1996; 244: 297-315.

10. Enders AC. Histological observations on the chorio-allantoic placenta of the mink. Anat Rec 1957; 127: 231-245.

11. Breier G, Albrecht U, Sterrer S, Risau W. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 1992; 114: 521-532.

12. Klauber N, Rohan RM, Flynn E, D'Amato RJ. Critical components of the female reproductive pathway are suppressed by the angiogenesis inhibitor AGM-1470. Nat Med 1997; 3: 443-446.

13. Risau W. Mechanisms of angiogenesis. Nature 1997; 386: 671-674.

14. Smith SK. Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update 1998; 4: 509-519.

15. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997; 18: 4-25.

16. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989; 161: 851-858.

17. Gospodarowicz D, Abraham JA, Schilling J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci U S A 1989; 86: 7311-7315.

18. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989; 246: 1309-1312.

19. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989; 246: 1306-1309.

20. Tischer E, Gospodarowicz D, Mitchell R, Silva M, Schilling J, Lau K, Crisp T, Fiddes JC, Abraham JA. Vascular endothelial growth factor: a new member of the platelet- derived growth factor gene family. Biochem Biophys Res Commun 1989; 165: 1198-1206.

21. Gitay-Goren H, Soker S, Vlodavsky I, Neufeld G. The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. J Biol Chem 1992; 267: 6093-6098.

22. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 1992; 267: 26031-26037.

23. Park JE, Keller GA, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 1993; 4: 1317-1326.

24. Hyder SM. The role of steroid hormones on the regulation of vascular endothelial growth factor. Am J Pathol 2002; 161: 345-346.

25. Gately S. The contributions of cyclooxygenase-2 to tumor angiogenesis. Cancer Metastasis Rev 2000; 19: 19-27.

26. Smith SK. Regulation of angiogenesis in the endometrium. Trends Endocrinol Metab 2001; 12: 147-151.

27. Matsumoto H, Ma WG, Daikoku T, Zhao X, Paria BC, Das SK, Trzaskos JM, Dey SK. Cyclooxygenase-2 differentially directs uterine angiogenesis during implantation in mice. J Biol Chem 2002; 277: 29260-29267.

28. Jussila L, Alitalo K. Vascular growth factors and lymphangiogenesis. Physiol Rev 2002; 82: 673-700.

29. Winther H, Leiser R, Pfarrer C, Dantzer V. Localization of micro- and intermediate filaments in non-pregnant uterus and placenta of the mink suggests involvement of maternal endothelial cells and periendothelial cells in blood flow regulation. Anat Embryol (Berl) 1999; 200: 253-263.

30. Pfarrer C, Winther H, Leiser R, Dantzer V. The development of the endotheliochorial mink placenta: light microscopy and scanning electron microscopical morphometry of maternal vascular casts. Anat Embryol (Berl) 1999; 199: 63-74.

31. Stoufflet I, Mondain-Monval M, Simon P, Martinet L. Patterns of plasma progesterone, androgen and oestrogen concentrations and in-vitro ovarian steroidogenesis during embryonic diapause and implantation in the mink (Mustela vison). J Reprod Fertil 1989; 87: 209-221.

32. Winther H, Dantzer V. Co-localization of vascular endothelial growth factor and its two receptors flt-1 and kdr in the mink placenta. Placenta 2001; 22: 457-465.

33. Douglas DA, Song JH, Moreau GM, Murphy BD. Differentiation of the corpus luteum of the mink (Mustela vison): mitogenic and steroidogenic potential of luteal cells from embryonic diapause and postimplantation gestation. Biol Reprod 1998; 58: 1163-1169.

34. Ma W, Tan J, Matsumoto H, Robert B, Abrahamson DR, Das SK, Dey SK. Adult tissue angiogenesis: evidence for negative regulation by estrogen in the uterus. Mol Endocrinol 2001; 15: 1983-1992.

35. Rose J, Slayden O, Stormshak F. Melatonin-induced downregulation of uterine prolactin receptors in mink (Mustela vison). Gen Comp Endocrinol 1996; 103: 101-106.

36. Yi XJ, Jiang HY, Lee KK, O WS, Tang PL, Chow PH. Expression of vascular endothelial growth factor (VEGF) and its receptors during embryonic implantation in the golden hamster (Mesocricetus auratus). Cell Tissue Res 1999; 296: 339-349.

37. Chakraborty I, Das SK, Dey SK. Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation. J Endocrinol 1995; 147: 339-352.

38. Chakraborty I, Das SK, Wang J, Dey SK. Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus

and their differential regulation by the blastocyst and ovarian steroids. J Mol Endocrinol 1996; 16: 107-122.

39. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 1997; 91: 197-208.

40. Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM, Dey SK. Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. Genes Dev 1999; 13: 1561-1574.

41. Song JH, Sirois J, Houde A, Murphy BD. Cloning, developmental expression, and immunohistochemistry of cyclooxygenase 2 in the endometrium during embryo implantation and gestation in the mink (Mustela vison). Endocrinology 1998; 139: 3629-3636.

42. Desmarais J, Bennett RD, Gevry N, Murphy BD. The role of peroxisome proliferator-activated receptor delta in the process of implantation in the mink *Mustela vison*. Biol Reprod 2002; 66 (Suppl 1): Abstr 63.

43. Bamba H, Ota S, Kato A, Kawamoto C, Fujiwara K. Prostaglandins up-regulate vascular endothelial growth factor production through distinct pathways in differentiated U937 cells. Biochem Biophys Res Commun 2000; 273: 485-491.

44. Tarrade A, Schoonjans K, Guibourdenche J, Bidart JM, Vidaud M, Auwerx J, Rochette-Egly C, Evain-Brion D. PPAR gamma/RXR alpha heterodimers are

involved in human CG beta synthesis and human trophoblast differentiation. Endocrinology 2001; 142: 4504-4514.

45. Matsumoto T, Claesson-Welsh L. VEGF receptor signal transduction. Sci STKE 2001; 2001: RE21.

46. Das SK, Chakraborty I, Wang J, Dey SK, Hoffman LH. Expression of vascular endothelial growth factor (VEGF) and VEGF- receptor messenger ribonucleic acids in the peri-implantation rabbit uterus. Biol Reprod 1997; 56: 1390-1399.

Gene	Primer	Sequence 5' - 3'
VEGF	forward	CCTCCGAAACCATGAACTTTCTG
	reverse	GAGTTAAACGAACGTACTTGCAGA
KDR	forward	AAGTGGCTAAGGGCATGGAG
	reverse	CTGCCTACCTCACCTGTTTCC
Flt-1	forward	GAAGGAGAGGACCTGAAACTG
	reverse	GCACGCTGTTTATTGAAAGAGTCAC
VEGF120, 164, 188	forward	CCGTCCCATTGAGACCCTG
VEGF 120	reverse	GACAAGAAAAATGTGACAAGCCG
VEGF 164	reverse	GCAAGAAAATCCCTGTGGGC
VEGf 188	reverse	GAGGAAAGGGAAAGGGGCA
GAPDH	forward	GTCCATGCCATCACTGCCAC
	reverse	CAAGAAGGTGGTGAAGCAGG

Table 1. Sequences of oligonucleotides used for mink specific cDNA cloning or RT-PCR of VEGF isoforms and receptors KDR and flt-1.

Contrasts	VEGF 120	VEGF 164	VEGF 188	KDR	Flt-1
anestrous vs. pregnant	**	**	**		*
diapause vs. activated		*			*
early vs. late activated		**	**		
late activated, implanted vs. pseudopregnant				**	**
implanted vs. non-implanted	*	**		*	
implantation site vs. inter-implantation site	*				

*P < 0.05; **P < 0.01

Table 2. Orthogonal contrast analysis of the abundance of VEGF isoforms and receptors during mink early gestation.

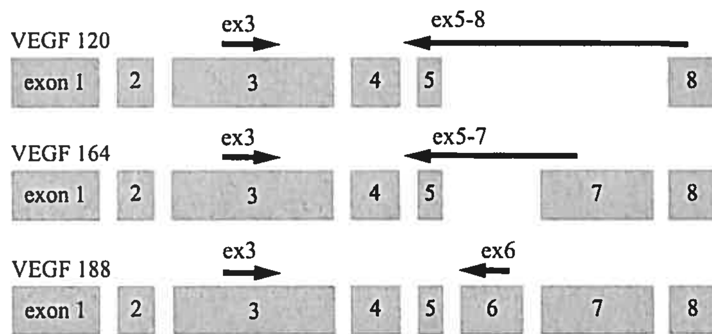
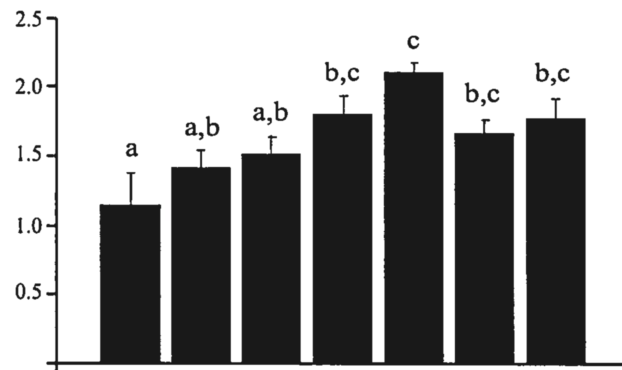
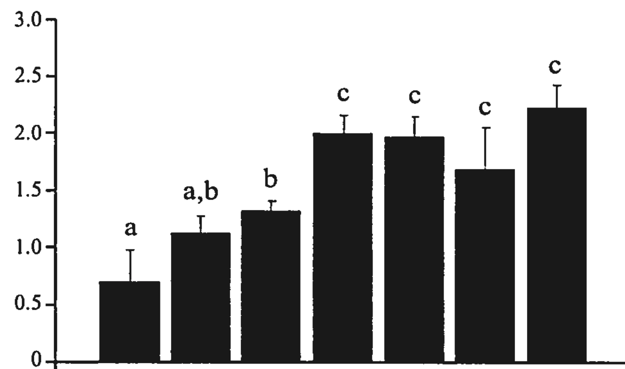

VEGF 120
ex3
ex5-8
exon 1
2
3
4
5
8
VEGF 164
ex3
ex5-7
exon 1
2
3
4
5
7
8
VEGF 188
ex3
ex6
exon 1
2
3
4
5
6
7
8

Fig. 1. Structure of the VEGF gene and selection of primers for specific semi-quantitative RT-PCR for VEGF isoforms 120, 164 and 188 in mink uterine and embryo-uterine tissue samples.

A
VEGF
120
280nt
GAPDH
262nt


2.5
2.0
1.5
1.0
0.5
a
a,b
a,b
b,c
c
b,c
b,c

B
VEGF
164
278nt
GAPDH
262nt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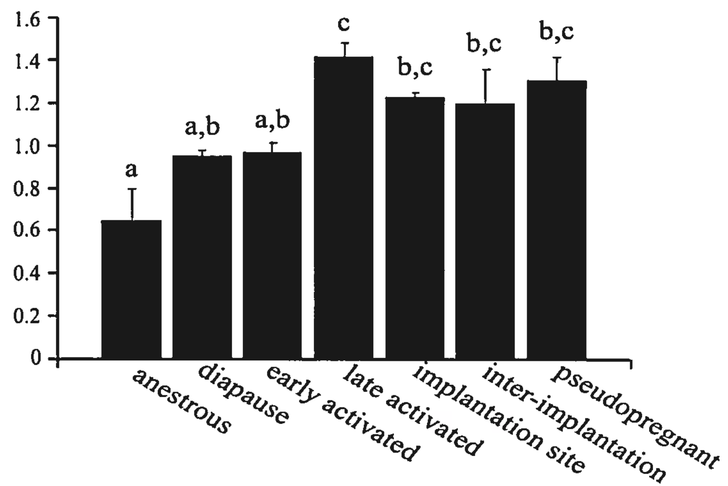
2.5
2.0
1.5
1.0
0.5
0
a
a,b
b
c
c
c
c

C
VEGF
188
296nt
GAPDH
262nt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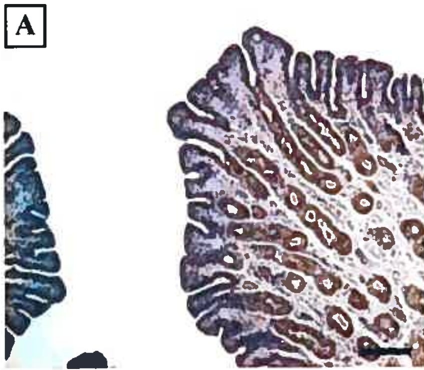
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
a
a,b
a,b
c
b,c
b,c
b,c
anestrous
diapause
early activated
late activated
implantation site
inter-implantation
pseudopregnant

Fig. 2. Semi-quantitative RT-PCR for VEGF isoforms 120, 164 and 188 in the anestrous, diapause, activated (early and late), implanted (implantation site and inter-implantation region) and pseudopregnant uteri in mink. Graphs represent the ratio of VEGF 120/GAPDH (A); VEGF 164/GAPDH (B); and of VEGF 188/GAPDH (C). The quantification represents mean ± SEM of three individual samples. Different superscripts represent differences in means at $P<0.05$.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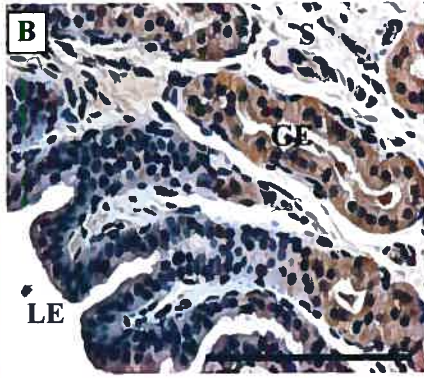
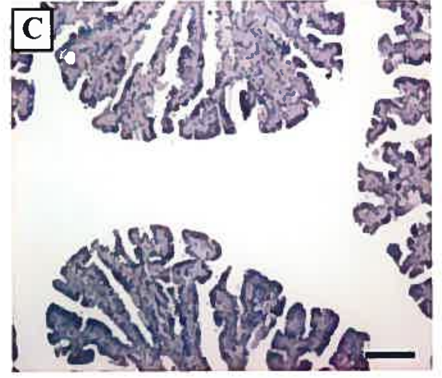

B
S
GE
LE

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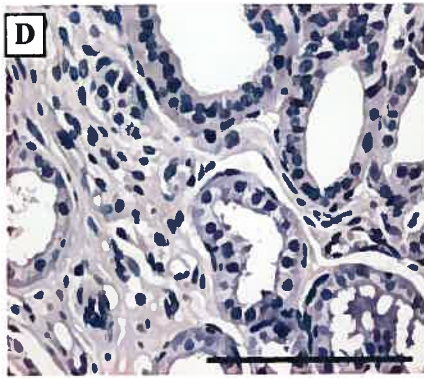
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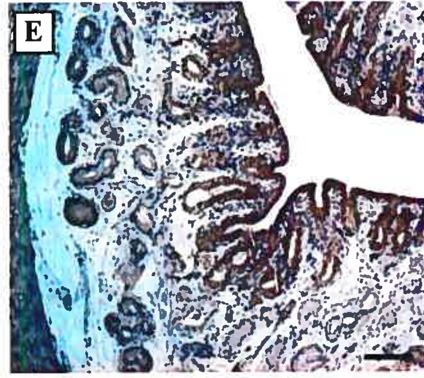
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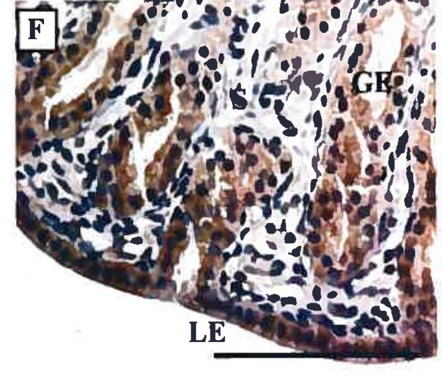
F
GE
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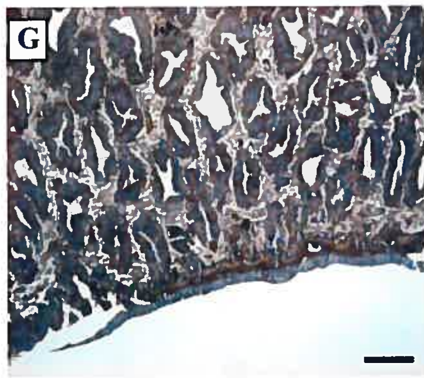
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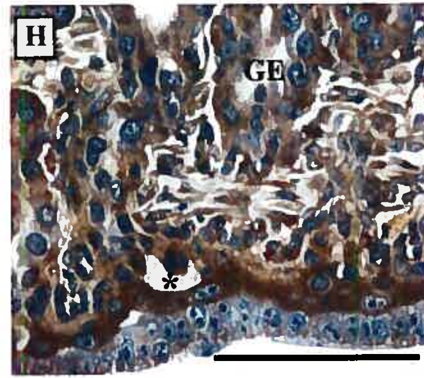
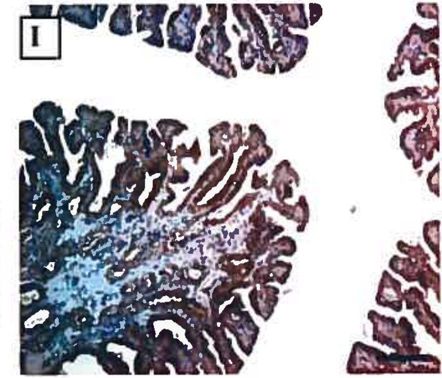

H
GE
*

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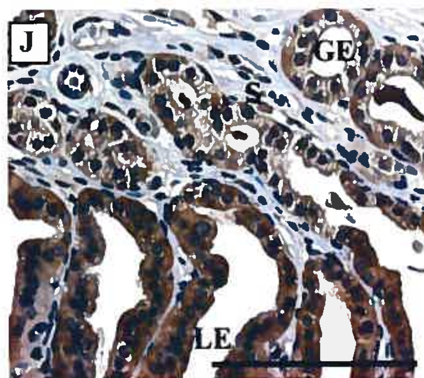
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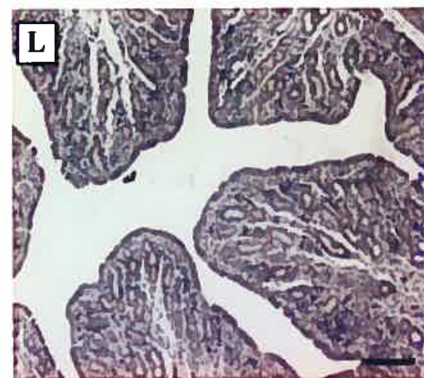
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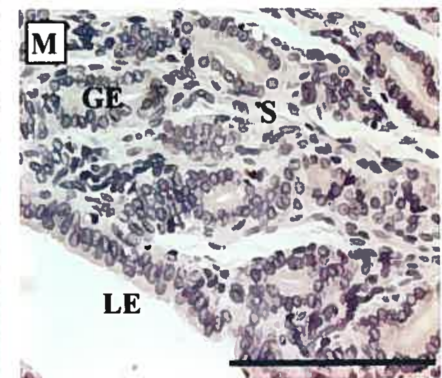
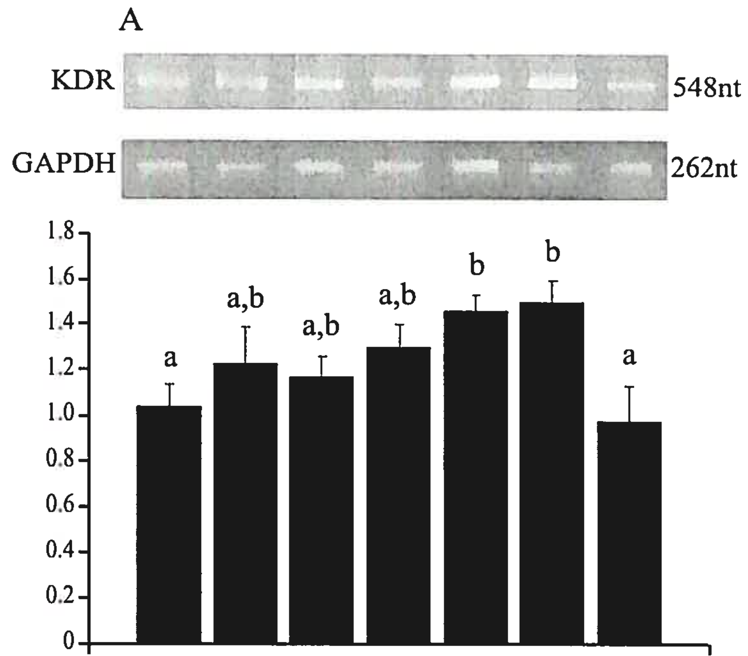

M
GE
S
LE

Fig. 3. Immunohistochemical characterization of VEGF in the mink uterus. A and B, VEGF mainly localized to the glandular epithelium (GE) in the diapause uterus. C and D, Negative control for localization. E and F, VEGF expressed in both the GE and luminal epithelium (LE) in the activated uterus. G and H, VEGF localization in the subepithelial stroma (S), GE, LE and intensively in the invasive trophoblast cells (*) in the implanted uterus. I and J, VEGF in both the GE and LE in the pseudopregnant uterus. L and M, lack of VEGF localization in anestrous uterus. The bar in each photo represents 500 μm.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GAPDH
262nt
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1.6
1.4
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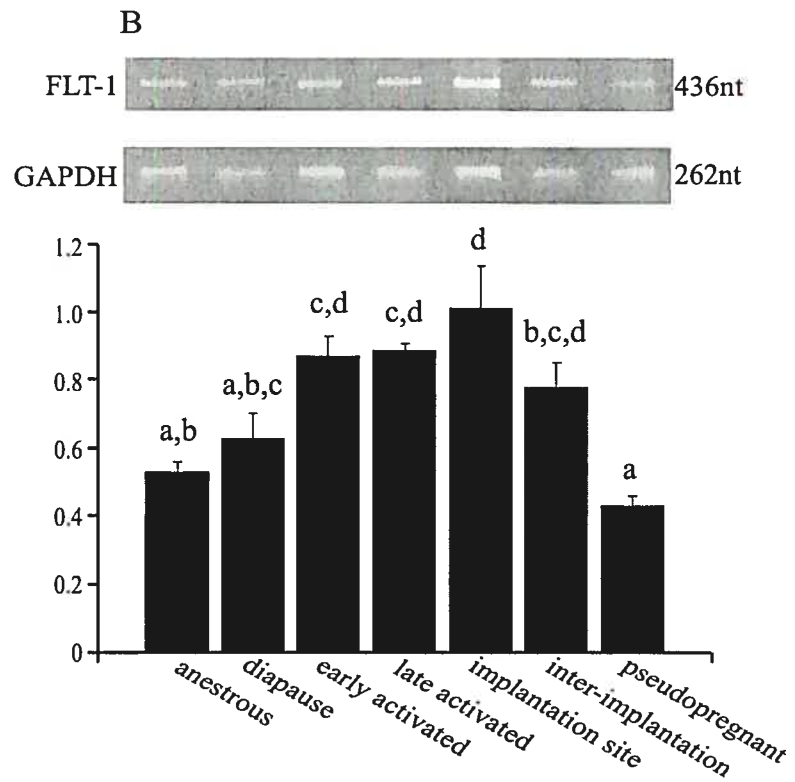
0
a
a,b
a,b
a,b
b
b
a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c,d
c,d
d
b,c,d
a
anestrous
diapause
early activated
late activated
implantation site
inter-implantation
pseudopregnant

Fig. 4. Semi-quantitative RT-PCR for VEGF receptors KDR and Flt-1 in the anestrous, diapause, activated (early and late), implanted (implantation site and inter-implantation region) and pseudopregnant uteri in mink. Graphs represent the ratio of KDR/GAPDH (A); and Flt-1/GAPDH (B). The quantification represents mean ± SEM of three individual samples. Different superscripts represent differences in means at $P<0.05$.

Chapter III

Transcriptional regulation of uterine vascular endothelial growth factor during early gestation in a carnivore model, *Mustela vison**

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Running Title: PGE_2 regulation of the VEGF gene

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ABSTRACT

Vascular endothelial growth factor (VEGF) is an essential angiogenic signalling element. Acting through its two tyrosine kinase receptors, it induces both proliferation of vessel endothelial cells and vascular permeability. Given the importance of vasculogenesis and angiogenesis to early pregnancy, it is of interest to understand the mechanisms regulating vascular development at this stage. We previously demonstrated that VEGF and its receptors are up-regulated during embryo implantation in the mink, a species displaying obligate embryonic diapause. In the present investigation, we examined the role of prostaglandin E_2 (PGE_2) as a regulator of VEGF during early pregnancy and established the mechanisms of this regulation. We demonstrate that activated mink embryos secrete PGE_2 and that expression of the PGE synthase protein in the uterus is dependent upon direct contact with the invading trophoblast cells during implantation. Using mink uterine stromal cells transfected with the mink VEGF promoter driving the luciferase reporter gene, we show that PGE_2 induces promoter transactivation and that this response can be eliminated by blockade of PKA. There was no PGE_2 induced response in transfected cells pre-treated with antagonists to the PGE_2 receptors EP2 and EP4. Deletional studies of the promoter revealed that a region of 99 basepairs (bp) upstream of the transcription start site is required for PGE_2 induced transactivation. Mutation of the AP2/SP1 cluster, found within the 99 bp, completely eliminated the PGE_2 response. Furthermore, chromatin immunoprecipitation assays confirmed binding of these two transcription factors to the

endogenous mink VEGF promoter in the uterine cells. PGE_2 stimulation increased the acetylation of histone H3 associated with promoter region containing the AP2/SP1 cluster. Taken together, these results demonstrate that in this unique model, PGE_2 plays an important role in regulating uterine and thus placental vascular development, acting through its receptors EP2 and EP4, provoking PKA activation of AP2 and SP1, as well as acetylation of histone H3, to transactivate the VEGF promoter.

INTRODUCTION

Prostaglandin E2 (PGE_2) is a prostanoid synthesized through the cyclo-oxygenase pathway characterized by the initial step of formation of PGH2 from arachidonic acid catalyzed by the cyclo-oxygenases 1 and 2 (COX-1 and -2). Formation of PGE_2 follows formation of PGH_2 from arachidonic acid and is dependent on the presence of prostaglandin E synthase (PGE synthase). Two isoforms of the PGE synthase have been identified, one is a cytosolic form (cPGE synthase), that acts mostly on COX-1 derived PGH_2. The second is a microsomal form (mPGE synthase), preferentially coupled with the inducible COX-2 induction of PGE_2 generation (Murakami et al, 2000). PGE_2 exerts its effects following binding to specific receptors containing seven transmembrane domains (see Narumiya et al., 1999 for review). Four receptor subtypes have been identified to date: EP1, 2, 3 and 4, each activating different intracellular pathways. Knockout models for each

subtype have been investigated, and mice deficient for EP2 presented impaired ovulation and fertilization (Tilley et al.,1999).

The role of prostaglandins in reproductive processes have been extensively investigated. COX-2 deficient mice have impaired ovulation, fertilization, implantation, and decidualization (Lim et al.,1997). PGE_2 is luteoprotective (Arosh et al., 2004); it also plays a role in regulation of immune responses at the site of embryo attachment (Lala et al., 1989), in ovulation (Matsumoto et al., 2001), and in the decidualization process in rats (Johnston and Kennedy, 1984; Kennedy and Doktorcik, 1988). Recently, Wang et al. (2004) identified PGE_2 as the major PG at implantation sites in hamsters, and expression of mPGE synthase was correlated with expression of COX-2. In mice, both mPGE synthase mRNA and protein were localized in the subluminal stroma surrounding the implanting blastocyst (Ni et al.,2002). The authors suggested embryonic regulation of PGE synthase expression since a similar pattern of expression was absent in pseudopregnant females as well as at the inter-implantation sites in the uterus.

Early pregnancy in mammals is associated with morphological and functional changes in uterine cells, accompanied by vascular remodelling. These changes are required for both placental and embryonic development (Breier et al., 1992; Klauber et al., 1997; Risau, 1997; Smith, 1998). Vascular endothelial growth factor (VEGF) is the major regulator of angiogenesis (Ferrara and Davis-Smyth, 1997) and is an important factor in

regulation of the events of early implantation and establishment of the placenta (Matsumoto et al., 2002). VEGF is a homodimeric glycoprotein of 40–45 kDa and while best known for its potent endothelial cell-specific mitogenic activity, it also plays a role in increasing vascular permeability (Ferrara and Hanzel, 1989; Gospodarowicz et al., 1989; Keck et al., 1989; Leung et al., 1989). Prostaglandins are among the factors reported to regulate VEGF (see Gately, 2000 for review). VEGF effects on angiogenesis are dependent upon binding to tyrosine kinase receptors, Flt-1 (fms-like tyrosine kinase, also known as VEGFR-1) and KDR (kinase domain region, also known as VEGFR-2) (see Jussila and Alitalo, 2002 for review). We have previously demonstrated that VEGF and its receptors are up-regulated during peri-implantation stages of gestation in the mink uterus (Lopes et al., 2003).

Earlier, Kennedy and colleagues (1979) showed that PGE_2 is a regulator of increased vascular permeability at implantation sites in the rat. Since then, PGE_2 has been shown to up-regulate VEGF in a number of tissues, including umbilical cord blood-derived mast cells (Abdel-Majid and Marshall, 2004), colon cancer cells (Fukuda et al., 2003), endothelial cells (Pai et al., 2001), human pancreatic cancer cells (Eibl et al., 2003), gastric cancer cells (Ding et al., 2005), and mouse mammary tumor cells (Chang et al., 2005).

In the present investigation, we aimed to verify the expression of PGE synthase in the uterus during early pregnancy and to investigate the role of PGE_2, as well as its mechanism of regulation of the angiogenic factor VEGF.

MATERIALS AND METHODS

Animals and Sample Collection - All treatment protocols involving the use of animals were approved by the Comité de déontologie, Faculté de médecine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. Mink of the Dark and Pastel varieties were purchased and maintained on a commercial farm (A. Richard, St. Damase, PQ, Canada). Females were mated twice, 7 days apart, throughout the first 2 weeks of March, according to standard husbandry procedures. It has been previously demonstrated that prolactin injections induce embryo activation and implantation in the mink (Papke et al., 1980; Murphy et al., 1981; Martinet et al., 1981) and a standard protocol consisting of daily i.m. injections of 1 mg/kg prolactin (Sigma, St. Louis, MO) was employed beginning 1 week following last mating and continuing for 12 days. Implantation takes place approximately on the 13th day after initiation of prolactin injections, verified by the presence of uterine swelling(s). Pseudopregnancy was induced by two injections of GnRH (10 µg/kg Factrel; Ayerst, Guelph, ON, Canada) 7 days apart given to non-mated females to induce ovulation. Uterine tissues were collected at the early stages of implantation from randomly selected females at days 13 and 15 following the first prolactin injections, as well as from pseudopregnant animals. Pseudopregnant samples were obtained 30 days later, allowing natural increases in prolactin and progesterone levels to take place. Samples were frozen immediately in liquid nitrogen and stored at -70°C until analyzed.

Cell Culture - A mink uterine stromal immortalized cell line previously described by our laboratory (Moreau et al., 1995) was used for the in vitro experiments described herein. Cells were cultured in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), containing 1% of Penicillin/Streptomicin (Invitrogen) and 0.5% of Fungizone (Invitrogen). A naturally immortalized cell line prepared in our lab (Douglas and Murphy, unpublished) from an ovarian tumor of the mink was also used for transfection experiments. This cell line was maintained in OPTI-MEM supplemented with 5% FBS and the antibiotics as above. A human breast cancer cell line, MCF-7, was kindly made available by Dr. Wilson Miller and was also used during our experiments. The MCF-7 cells were maintained in the same medium as the mink ovarian cells described above.

Embryo collection and RIA for PGE_2 - Embryos were collected by repeated flushing of the uterine horns of females in diapause and 9 days following initial embryo activation (Desmarais et al., 2004) with TC-199 medium (Invitrogen, Burlington, ON, Canada) containing 10% fetal bovine serum (Invitrogen). Embryos (in groups of 5) were incubated in 500 µl INRA Menezo B2 medium (Pharmascience, Paris France) supplemented with 5% FBS (Invitrogen) for 48 h, in the presence or absence of mink uterine cells. For radioimmunoassay evaluation, 100 µl of embryo or cell culture medium were employed. The RIA for PGE_2 was performed according to Xiao et al. (1999). Briefly, antiserum from Assay Design (Ann Arbor, MI) was used with reactivity with PGE, $PGF_{1\alpha}$, $PGF_{2\alpha}$ and keto-$PGF_{1\alpha}$ of 70%, 1.4%, 0.7% and 0.6%, respectively. Assay sensitivity was 4 pg/100 µl, and

the intra-assay coefficient of variation, calculated between duplicates ranged from 0.04% to 6.1%.

Extraction of RNA, Purification and Reverse Transcription - Uterine tissues were homogenized in buffer RLT (Qiagen, Mississauga, ON, Canada) with 0.12 M ß-mercaptoethanol (Sigma). Purification of RNA was performed using an RNeasy Protect Mini kit (Qiagen), following the recommendations of the manufacturer. Total RNA was measured by spectrophotometry at 260 nm, and 1 µg/sample of total RNA was used for reverse transcription (RT) with the Omniscript RT kit (Qiagen) according to the manufacturer's instructions.

PCR for PGE synthase and Receptors in the Mink Uterine Samples - Homologous sequences of rat (accession numbers: PGEs: NM_021583; EP-1: NM_013100; EP-2 : NM_031088; EP-4 : D28860), mouse (PGEs: NM_022415; EP-1: NM_013641; EP-2 :NM_008964 ; EP-3 : NM_011196), and human (EP-1: NM_000955; EP-2 : NM_000956; EP-3 : NM_000957; EP-4 : NM_000958) were used to design primers for PGE synthase, and for PGE receptors (Table 1). PCR products of the expected size were excised and purified using a gel extraction kit (Qiagen). Purified cDNA was ligated into a pDrive vector (Qiagen) following manufacturer's instructions, and further transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated with a QIAprep Spin Miniprep kit (Qiagen) and sequenced by automated DNA sequencing for verification

(Service d'Analyze et de Synthèse d'Acides Nucléiques de Université Laval, Québec, Canada). PCR reactions were carried out in a final volume of 50 µl using Taq DNA polymerase (Amersham Biosciences Corp., Baie d'Urfe, PQ, Canada). PCR products were separated in a 1.5% agarose gel and visualized with ethidium bromide.

Cloning and Sequencing of the Mink VEGF 5'UTR and Promoter Regions - The 5'-flanking region of the mink VEGF gene was cloned by PCR using the Universal Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA) from a library constructed from mink genomic DNA. The Expand High Fidelity kit served for amplification. The PCR products were cloned into a pGEM-T vector (Qiagen) for sequencing, which was performed by automated DNA sequencing (Service d'Analyze et de Synthèse d'Acides Nucléiques de Université Laval, Québec, Canada). Sequence analysis was undertaken using MatInspector (Abteilung Genetek, Braunschweig, Germany) and TF Search (Yukata Akiyama: TF Search - Searching TF Binding Sites). The transcription start site of the VEGF gene was predicted using the program for promoter prediction of the Berkeley *Drosophila* Genome Project (UC Berkeley, Berkeley, CA). To confirm prediction, we employed the 5'/3' RACE kit (Roche) to identify the site of transcription initiation from 2.9 Kb of the mink 5'flanking region. Primers used are described in Table 2.

Immunohistochemical Analysis of microsomal PGE synthase - Tissues fixed in 4% paraformaldehyde solution were used to demonstrate expression of PGE synthase during

the early implantation stages. Sections were re-hydrated and permeabilized with 0.2% Triton in PBS. Blocking was perfomed for 1 h using 5% BSA in PBS, and sections were incubated overnight at 4°C with rabbit anti-human PGE synthase (Cayman, Ann Arbor, MI) diluted 1:150 in 5% BSA/PBS. A conjugated Cy3 anti-rabbit second antibody (Jackson ImmunoResearch, West Grove, PA) was employed for 1 h to localize the PGE synthase positive staining. Tissues were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim, Indianapolis, IN). Normal rabbit serum was used as negative control.

Plasmid constructions - The 1.7kb sequence of the mink VEGF gene was cloned into a pGL2 basic vector (Promega Corp., Nepean, Ontario, Canada). All deletions were derived from the original construct by PCR using KPNI and XHOI insertions for directional cloning. Mutation of the predicted AP2 and SP1 sites was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). All plasmids used for transient transfection were prepared using the Maxi Prep kit (Qiagen, Mississauga, Ontario, Canada) and sequenced prior to transfection.

Transfections, Luciferase Reporter Assays and Treatments – Just prior to transfection, culture medium was changed to OPTI-MEM lacking FBS and antibiotics in all cell lines used. Transfection and treatments were carried out in 24-well plates in this medium. For transient transfection, Lipofectamine 2000 (Invitrogen) was used according to the

manufacturer's protocol. Cells were transfected with 400 ng/well of the pGL2 basic vector, containing the mink VEGF promoter constructs, for 5 h prior to addition of treatments. Medium was changed following the 5 h of transfection, and treatments were added 1 h following transfection. Cells were cotransfected with the simian virus 40 (SV40) Renilla luciferase control vector pRL.SV40 (Promega) to normalize the results for transfection efficiency. Control transfections received equal amounts of the pGL2 basic vector (Promega) devoid of promoter constructs. Treatments comprised addition of varying doses of PGE_2 (Sigma) from 10 to 100 μM for 6 to 24 h. To test the role of the protein kinase A (PKA) pathway, some cultures were treated with 100 μM and 1 mM dibutyryl cAMP (Bu_2cAMP; Sigma) and chlorophenylthio cAMP (pCPT cAMP; Sigma) for 12 h, or the PKA inhibitor H89 (Sigma) at a dose of 10 μM beginning 1 h prior to PGE2 treatment. To establish which of the PGE2 receptors were involved, antagonists for PGE_2 receptors EP1 (SC19220; 10 μM; Sigma), EP2 (AH6809; 20 μM; Sigma) and EP4 (AH2384B; 30 μM; Sigma) were added to transfected cells 1 h prior to treatment with PGE_2. Luciferase activity was evaluated using the Dual Luciferase Assay System (Promega) and chemiluminescence was measured with a Berthold 9501 luminometer.

Chromatin Immunoprecipitation (ChIP) Assay - ChIP assays were performed as described by Kuo and Allis (1999) with some modifications. Mink uterine stromal cells were plated in a 10 cm plate and treated after confluence with 75 μM PGE_2 for 6 h. Prior to treatment, cells were serum-starved for 20 h. Following treatment, DNA and protein were cross-

linked by addition of formaldehyde to the medium at a final concentration of 1% for 10 min at 37 C. Cells were then washed in PBS, resuspended in 200 μl of ChIP lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and protease inhibitors] and sonicated with a Branson Sonifier 450 (Danbury, CT) at power setting 2 with 10-sec pulses at duty cycle 90. ChIP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris, pH 8.1; 16.7 mM NaCl; and protease inhibitors) was used to dilute the chromatin solution 10-fold. Total DNA used for controlling the amount of DNA/sample was purified from one tenth of the lysate. Each sample was precleared by incubating with 80 μl salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology, Inc., Lake Placid, NY) for 30 min at 4 C. Anti-acetyl histone H-3 (5μg; Upstate Biotechnology, Inc.), anti-AP2 (5 μg; Santa Cruz Biotehnology, Santa Cruz, CA), anti-SP1 (5 μg; Santa Cruz), and rabbit IgG (as negative controls) were added and immunoprecipitated at 4 C overnight. The immunoprecipitate was collected using salmon sperm DNA/protein A-agarose and washed once with buffers in the following order: low-salt wash buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tri-HCl, pH 8.1; 150 mM NaCl); high-salt wash buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; 500 mM NaCl); LiCl wash buffer (0.25 M LiCl; 1% Nonidet P-40; 1% sodium deoxycholate; 1 mM EDTA; 10 mM Tris-HCl, pH 8.1); TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The DNA-protein or histone cross-links were reversed by incubation at 65 C for 4 h followed by proteinase K treatment. DNA was then recovered and purified with the Qiaquik PCR purification column (Qiagen). PCR was carried with an annealing

temperature of 61 C. The primers used for the PCR are depicted in Table 2. Primers derived from the open reading frame of the gene were employed as controls. PCR products were separated on a 1.5% agarose gel and ethidium bromide was used for visualization.

Statistical Analysis - The relative luciferase activity throughout this study was analyzed using the least square ANOVA and the general linear model procedures of SAS (Cary, NC). When significant differences in treatments were found, comparisons of means were further performed by the methods of orthogonal contrasts and the Duncan multiple range test. A probability level of $p<0.05$ was considered significant.

RESULTS

Embryonic production of PGE_2 - Embryos collected during the diapause and activation stages were incubated in the presence or absence of mink uterine cells to determine whether there was production of PGE_2. Embryos in diapause failed to produce and/or secrete identifiable levels of PGE_2. The embryos collected following activation, and that were therefore in active growth (Desmarais et al., 2004), produced copious quantities of PGE_2 (Fig. 1A), either alone or in co-culture with uterine cells. Uterine cells alone failed to produce this prostaglandin.

Expression of PGE synthase and EP receptor mRNA in the uterus - The uterine tissues collected from implantation and inter-implantation sites, as well as uteri collected from pseudopregnant females expressed mRNA for PGE synthase. This enzyme was highly up-regulated (approximately 7 fold) in the tissues collected from the implantation sites, comprised of both embryonic and uterine components, at 3-4 days following implantation (Fig. 1B). EP1 receptor expression was negligible, whereas the receptors of the EP2 and EP4 subtypes were expressed at all stages and locations collected, but no apparent regulation was evident among the samples (Fig. 1B). The EP3 receptor mRNA was not detected at any of the sites or stages investigated (data not shown).

Immuhistochemical localization of PGE synthase in the mink pregnant uterus - Given the increased expression of the PGE synthase observed at implantation sites (Fig. 1B), we were interested in establishing whether this increased expression was of uterine or embryonic origin. We found the PGE synthase protein to be present in the myometrium of all stages evaluated. In the endometrium, PGE synthase was localized in the stromal layer immediately surrounding the implanting embryo (Fig. 2A,B,C), whereas no significant localization was observed in the antimesometrial uterine tissue opposite to the invading trophoblasts (Fig. 2D). Histological sections from inter-implantation sites further confirmed that pattern, in that endometrial cells in these regions, lacking direct contact with embryonic tissue, did not express this protein (Fig. 2E,F).

Cloning and sequencing of the 5'-flanking region of the mink VEGF gene – A 2.9 kb sequence upstream of the ATG triplet was identified by means of the Genome Walker kit (Clontech). The transcription initiation site at 1058 bp upstream if the ATG triplet was predicted by means of the promoter prediction program of the Berkeley Drosophila Genome Project (UC Berkeley, Berkeley, CA), and confirmed by 5'RACE using the RNA of three different mink samples, two of uterine and one of ovarian origin. The VEGF proximal promoter region in the mink bears a high homology to the human (81%) and mouse sequences (72%) (Accession Numbers: AF095785 and U41383, respectively). Matinspector analysis of the mink promoter sequence identified several potential response element sequences previously identified in the human and mouse VEGF promoters, AP1, AP2 and SP1, amongst others. Concurring with the human and mouse counterparts, there was no consensus TATA box motif present, while an important GC rich region was found in the proximal promoter region, about 70 bp upstream of the transcription start site.

PGE_2 induces VEGF transcription in different cell types – We employed the reporter gene luciferase driven by the mink VEGF proximal promoter in mink uterine stromal cells. We observed that PGE_2 was capable of inducing a 3-fold induction in transcription of the reporter gene, in response to the doses of 75 and 100 μM ($p<0.05$), and a 2-fold induction to the dose of 50 μM, whereas 10 μM resulted in a modest and non-significant 50% elevation (Fig. 3A). Similar levels of induction were observed at the three different times tested, 6, 12 and 24 h of PGE_2 treatment (Fig. 3B). Furthermore, we tested transfection of

other cell types and consequent response to prostaglandin treatment in regards to induction of VEGF transcription. The results indicate mink promoter activation in mink ovarian tumor cells (Fig. 3C), as well as in the human breast cancer cells MCF-7 (Fig. 3D).

PGE_2 induction of VEGF transcription is PKA dependent – Mink stromal cells transfected with a 1.5 kb mink VEGF promoter driving the luciferase gene were treated with the cAMP agonists dibutyryl and chlorophenylthio cAMP (100 μM, 1 mM) eliciting inductions of the VEGF promoter comparable to those observed following treatment with PGE_2 (Fig. 4A). To further investigate the involvement of the PKA pathway in PGE_2 induced VEGF transcription, transfected cells were pre-treated with the PKA antagonist H89. The responses to PGE_2 were completely abolished by pre-treatment with H89 (Fig. 4B). H89 blockade of PKA likewise abrogated the PGE_2 response in mink ovarian tumor cells (not shown).

EP2 and EP4 antagonists block the PGE_2 induced up-regulation of VEGF transcription – After verifying that PGE_2 stimulates VEGF through a PKA dependent pathway (Fig. 4), we sought to confirm that the response observed in VEGF transcription was dependent upon ligand binding to the receptors previously known to elicit an increase in cAMP. Antagonists to the receptors EP1, EP2 and EP4 were added to the transfected stromal cells 1 h prior to treatment with PGE_2. Even though ligand binding to EP1 does not elicit PKA dependent responses, the antagonist for EP2 also blocks EP1. We therefore tested an EP1

specific antagonist to control for the blocking of this receptor. The EP1 specific antagonist had no significant effect on the transcription of the reporter gene driven by the mink VEGF promoter (Fig. 5). The antagonist impairing both EP2 and EP1 receptors significantly blocked the transactivation of the VEGF promoter (Fig. 5, $p<0.05$). A significant, if less pronounced attenuation was observed following treatment with the EP4 antagonist (Fig. 5, $p<0.05$). Complete abrogation of promoter activation was observed when the cells were treated with antagonists for EP1+EP2 and EP4 (Fig. 5).

AP2 and SP1 are involved in mediating PGE_2 induced responses in the mink VEGF promoter – We carried out promoter deletion analysis in order to identify the region(s) of the promoter transactivated following PGE_2 treatment. The two longest constructs used, containing 1289 and 708 bp respectively, resulted in comparable levels of induction following PGE_2 treatment. The third construct, spanning only 99 bp upstream of the transcription start site resulted in slightly lower induction by PGE_2, although not significantly different from the two longer versions. Loss of response to PGE_2 was observed when only 51 bp remained upstream of the transcriptional site, and a further deletion of 34 bp completely eliminated basal promoter activity (Fig. 6A, $p<0.05$). Within the 51 bp remaining upstream of the transcription, an AP2 and a SP1 binding site, were predicted by Matinspector. To verify the importance of these two sites in promoter induction, we effected a mutation to render them unable to bind these transcription factors. Given their proximity, and sharing of nucleotides, the mutation interfered with both sites.

Subsequent transfection trials demonstrated that mutation of these sites completely eliminated the PGE_2 induced promoter transactivation (Fig. 6B, $p<0.05$).

AP2 and SP1 interact with the VEGF promoter in response to PGE_2 – Following the mutation of the AP2/SP1 cluster and loss of PGE_2 induced promoter response, we sought to verify if treatment with PGE_2 could induce the binding of these two transcription factors to the endogenous mink VEGF promoter in the uterine stromal cell line by ChIP assay. Confluent cells were serum-starved for 20 h prior to addition of PGE_2. Following 6 h of PGE_2 treatment, cells were cross-linked and immunoprecipitated with the AP2 and SP1 antibodies. Cross-linking was reversed and the immunoprecipitated DNA was then amplified by PCR using primers spanning the proximal promoter region (including the transcription start site). Confirming the deletional studies, binding of both transcription factors to the VEGF promoter region in uterine cells was induced by treatment with PGE_2 (Fig. 7A). A four fold induction of AP2 binding to the VEGF promoter region was observed following PGE_2 treatment, whereas PGE_2 doubled the binding of SP1 to the VEGF promoter.

PGE_2 induces histone modifications – To investigate whether PGE_2 also plays a role in covalent modification of histones, we immunoprecipitated PGE_2 treated cells with an antibody recognizing histone H3 acetylated on lysine 14. Mink stromal cells were serum-starved for 20 h and then were treated with 75 µM PGE_2 for 6 h. Cells were then cross-

linked and immunoprecipitated with the acetylated H3 antibody. Cross-linking was reversed and the immunoprecipitated DNA was then amplified by PCR using primers spanning the proximal promoter region (including the transcription start site). Treatment with PGE_2 was effective in inducing acetylation of histone H3 by two fold in comparison to non-treated control (Fig. 7B), and the site amplified by PCR corresponds to the promoter region containing the binding sites for AP2 and SP1, shown here to be involved in PGE_2 transactivation of the mink VEGF promoter (Fig. 6A, B and Fig. 7A).

DISCUSSION

Extensive angiogenesis is paramount for successful maintenance of gestation (Psychoyos, 1986) to provide the increase in blood supply to the implanting and rapidly developing embryo. VEGF has been demonstrated to be one of the major angiogenic factors inducing proliferation and migration of endothelial cells, as well as permeability in the vessels in a wide array of normal and pathogenic mammalian tissues (Ferrara and Davis-Smyth, 1997).

We previously demonstrated that, in the mink uterus, VEGF mRNA and protein are up-regulated around the time of implantation (Lopes et al., 2003), indicating an important role for this growth factor in this species. The early investigations of Kennedy (1977) demonstrated the importance of prostaglandins in the process of embryo implantation in

rodents. Confirmation came in the form of complete abrogation of implantation in mice bearing null mutation of the COX-2 gene (Lim et al.,1997). We established the occurrence of COX-2 expression by both the trophoblast and uterine stroma at the site of implantation in the unique carnivore model we employ in the current study, the mink (Song et al., 1998). Investigations by Matsumoto and colleagues (2002) on the COX-2 deficient mouse have indicated a functional link between prostaglandin synthesis and VEGF expression in mouse implantation.

In the present study we provide the first evidence to place PGE_2 as an important regulator of VEGF expression during the peri-implantation period in mammals. Firstly, we show that the mink embryo that has escaped from diapause is an important source of PGE_2. Further we have verified that presence of the enzyme responsible for synthesis of PGE_2 from PGH_2, PGE synhase, is regulated in the uterus during early stages of pregnancy, with its up-regulation dependent on the presence of the invading embryo. We have unequivocally placed the expression of PGE synthase in the expression in the stroma surrounding the invading trophoblastic layer, and have demonstrated its absence from the inter-implantation endometrium. This concurs with a similar phenomenon in mice where PGE synthase mRNA and protein occur in the subliminal stroma surrounding the implanting blastocyst (Ni et al., 2002). In the current investigation, the PGE synthase expression we found appears to coincide with expression of COX-2, which is likewise localized at the sites of trophoblast invasion, particularly in the necks of the uterine glands

during early implantation (Song et al., 1998). This co-localization places the presence of the substrate for PGE_2 in a timely manner to allow for PGE_2 synthesis by the PGE synthase.

In the present study, we were interested in investigating possible regulators of VEGF and the mechanisms involved in this regulation. PGE_2 was our choice given its effects on VEGF expression in other tissues, the secretion of PGE_2 by the embryo and the presence of PGE synthase at the site of implantation. By employing a homologous in vitro system using immortalized mink uterine stromal cells with the mink VEGF promoter driving the reporter gene luciferase, we show that PGE_2 induces expression of VEGF as demonstrated. PGE_2 can act through four different G-coupled receptors, EP1 through EP4, each employing its own second messenger system (see Narumiya et al., 1999 for review). EP1 acts through activation of the phospholipase C system and by inducing a rise in intracellular calcium levels (Kimura et al., 2001), while EP2 and EP4 both induce responses through activation of the PKA pathway and both have been involved in PGE_2 induced regulation of VEGF (Chang et al., 2005; Sales et al., 2004; Bradbury et al., 2005). Multiple EP3 isoforms have been identified to date, and they can act through different signalling pathways associated with Gi, Gs and Gq activation (see Narumiya et al., 1999 for review).

We identified the presence of EP2 and EP4 mRNA in the mink uterine tissues during early stages of implantation and placentation, as well as in the pseudopregnant uterus, but no variation in expression, and thus no pregnancy-specific regulation of these

receptors was present. This suggests that regulation of the ligand PGE_2, rather than up-regulation of the receptors, is responsible for the effects seen in VEGF expression. The fact that these two receptors bring about cAMP dependent responses, strongly argues that the increase in VEGF transcription is cAMP dependent.

Intracellular pathways involving cAMP most usually involve phosphorylation of CREB (reviewed by Sassone-Corsi, 1998), but we were unable to find, through the use of the Matinspector software, predicted CRE response elements within the proximal promoter region of the mink VEGF gene. Although responses involving transcription factors to which no putative binding sequences were predicted are quite possible, we were able to place the AP2 and SP1 cis-acting elements downstream of the PGE_2 induction of the VEGF promoter. Mutation of these overlapping regions for AP-2 and SP-1 was sufficient to induce loss of transcription induction by PGE_2, without the loss of basic promoter activity. The use of ChIP has enabled us to verify that PGE_2 does in fact increase binding of these two transcriptional factors to the proximal promoter region of VEGF. This concurs with the studies of human smooth muscles cells of the airway, which have placed SP1 downstream of cAMP in the induction of VEGF by PGE_2 (Bradbury et al., 2005). This AP-2/SP-1 cluster has previously been shown to be involved in transcriptional regulation of VEGF (Milanini et al., 1998; Brenneisen et al., 2003). The partnership between AP-2/SP-1 has also been involved in transcriptional regulation of another gene, CYP17, following increased levels of cAMP (Zhang and Veldhuis, 2004).

The region we found to control the transactivation of the promoter lies in a GC rich region of the mink VEGF promoter, from -71 to -53. This again concurs with previous findings in which the same GC rich region appears to regulate VEGF promoter activation by TGFα through AP2 in humans (Gille et al., 1997), by UVB through AP2/SP1 in humans (Brenneisen et al., 2003), by p42/p44 MAP kinase also through AP2/SP1 binding in hamsters (Milanini et al., 1998), and by PDGF through SP1 in humans (Finkenzeller et al., 1997). Taken together, these previous results in human and the hamster, and our findings with the mink VEGF promoter, argue for the conclusion that this promoter region, along with the transcription factors AP2 and SP1, play a central role in the regulation of VEGF transcription.

In addition to recruitment of cis-acting elements, chromatin modifications are also required in order to allow transactivation of promoter regions. Amongst these modification, histone acetylation has been associated with active chromatin and consequent access of transcription factors to promoter regions (reviewed by Mizzen and Allis, 1998). In the mink model, PGE_2 provoked acetylation of H3 localized to the proximal promoter region, spanning the area containing the response elements for AP-2 and SP-1. This demonstrates that PGE_2 induces activation of the transcription factors in conjunction with induction of chromatin restructuring to bring about promoter binding and transactivation.

In summary, the current study provides evidence that PGE_2 of embryonic and endometrial provenance regulates the expression of VEGF at implantation sites in the uterus of the mink. This indicates that, in this unique model of decidua-free, endothelial-chorial placentation, there is dependence on the presence of local prostaglandin synthesis for establishment of the vascularity required for maintenance of early gestation.

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REFERENCES

Abdel-Majid, R. M., and Marshall, J. S. (2004). Prostaglandin E2 induces degranulation-independent production of vascular endothelial growth factor by human mast cells. *J Immunol* **172,** 1227-36.

Arosh, J. A., Banu, S. K., Chapdelaine, P., Madore, E., Sirois, J., and Fortier, M. A. (2004). Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. *Endocrinology* **145,** 2551-60.

Bradbury, D., Clarke, D., Seedhouse, C., Corbett, L., Stocks, J., and Knox, A. (2005). Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1 transcription factor binding sites. *J Biol Chem* **280,** 29993-30000.

Breier, G., Albrecht, U., Sterrer, S., and Risau, W. (1992). Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* **114,** 521-32.

Brenneisen, P., Blaudschun, R., Gille, J., Schneider, L., Hinrichs, R., Wlaschek, M., Eming, S., and Scharffetter-Kochanek, K. (2003). Essential role of an activator protein-2 (AP-2)/specificity protein 1 (Sp1) cluster in the UVB-mediated induction of the

human vascular endothelial growth factor in HaCaT keratinocytes. *Biochem J* **369,** 341-9.

Chang, S. H., Liu, C. H., Wu, M. T., and Hla, T. (2005). Regulation of vascular endothelial cell growth factor expression in mouse mammary tumor cells by the EP2 subtype of the prostaglandin E2 receptor. *Prostaglandins Other Lipid Mediat* **76,** 48-58.

Desmarais, J. A., Bordignon, V., Lopes, F. L., Smith, L. C., and Murphy, B. D. (2004). The escape of the mink embryo from obligate diapause. *Biol Reprod* **70,** 662-70.

Ding, Y. B., Shi, R. H., Tong, J. D., Li, X. Y., Zhang, G. X., Xiao, W. M., Yang, J. G., Bao, Y., Wu, J., Yan, Z. G., and Wang, X. H. (2005). PGE2 up-regulates vascular endothelial growth factor expression in MKN28 gastric cancer cells via epidermal growth factor receptor signaling system. *Exp Oncol* **27,** 108-13.

Eibl, G., Bruemmer, D., Okada, Y., Duffy, J. P., Law, R. E., Reber, H. A., and Hines, O. J. (2003). PGE(2) is generated by specific COX-2 activity and increases VEGF production in COX-2-expressing human pancreatic cancer cells. *Biochem Biophys Res Commun* **306,** 887-97.

Ferrara, N., and Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. *Endocr Rev* **18,** 4-25.

Ferrara, N., and Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* **161,** 851-8.

Finkenzeller, G., Sparacio, A., Technau, A., Marme, D., and Siemeister, G. (1997). Sp1 recognition sites in the proximal promoter of the human vascular endothelial growth factor gene are essential for platelet-derived growth factor-induced gene expression. *Oncogene* **15,** 669-76.

Fukuda, R., Kelly, B., and Semenza, G. L. (2003). Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1. *Cancer Res* **63,** 2330-4.

Gately, S. (2000). The contributions of cyclooxygenase-2 to tumor angiogenesis. *Cancer Metastasis Rev* **19,** 19-27.

Gille, J., Swerlick, R. A., and Caughman, S. W. (1997). Transforming growth factor-alpha-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. *Embo J* **16,** 750-9.

Gospodarowicz, D., Abraham, J. A., and Schilling, J. (1989). Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc Natl Acad Sci U S A* **86,** 7311-5.

Johnston, M. E., and Kennedy, T. G. (1984). Estrogen and uterine sensitization for the decidual cell reaction in the rat: role of prostaglandin E2 and adenosine 3':5'-cyclic monophosphate. *Biol Reprod* **31,** 959-66.

Jussila, L., and Alitalo, K. (2002). Vascular growth factors and lymphangiogenesis. *Physiol Rev* **82,** 673-700.

Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. (1989). Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* **246,** 1309-12.

Kennedy, T. G. (1977). Evidence for a role for prosaglandins in the initiation of blastocyst implantation in the rat. *Biol Reprod* **16,** 286-91.

Kennedy, T. G. (1979). Prostaglandins and increased endometrial vascular permeabiltiy resulting from the application of artificial stimulus to the uterus of the rat sensitized for the decidual cell reaction. *Biol Reprod* **20,** 560-6.

Kennedy, T. G., and Doktorcik, P. E. (1988). Effects of analogues of prostaglandin E2 and F2 alpha on the decidual cell reaction in the rat. *Prostaglandins* **35,** 207-19.

Kimura, M., Osumi, S., and Ogihara, M. (2001). Prostaglandin E(2) (EP(1)) receptor agonist-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes: the involvement of TGF-alpha. *Endocrinology* **142,** 4428-40.

Klauber, N., Rohan, R. M., Flynn, E., and D'Amato, R. J. (1997). Critical components of the female reproductive pathway are suppressed by the angiogenesis inhibitor AGM-1470. *Nat Med* **3,** 443-6.

Kuo, M. H., and Allis, C. D. (1999). In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. *Methods* **19,** 425-33.

Lala, P. K. (1989). Similarities between immunoregulation in pregnancy and in malignancy: the role of prostaglandin E2. *Am J Reprod Immunol* **20,** 147-52.

Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246,** 1306-9.

Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997). Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91,** 197-208.

Lopes, F. L., Desmarais, J., Gevry, N. Y., Ledoux, S., and Murphy, B. D. (2003). Expression of vascular endothelial growth factor isoforms and receptors Flt-1 and KDR during the peri-implantation period in the mink, Mustela vison. *Biol Reprod* **68,** 1926-33.

Martinet, L., Allais, C., and Allain, D. (1981). The role of prolactin and LH in luteal function and blastocyst growth in mink (Mustela vison). *J Reprod Fertil Suppl* **29,** 119-30.

Matsumoto, H., Ma, W., Smalley, W., Trzaskos, J., Breyer, R. M., and Dey, S. K. (2001). Diversification of cyclooxygenase-2-derived prostaglandins in ovulation and implantation. *Biol Reprod* **64,** 1557-65.

Matsumoto, H., Ma, W. G., Daikoku, T., Zhao, X., Paria, B. C., Das, S. K., Trzaskos, J. M., and Dey, S. K. (2002). Cyclooxygenase-2 differentially directs uterine angiogenesis during implantation in mice. *J Biol Chem* **277,** 29260-7.

Milanini, J., Vinals, F., Pouyssegur, J., and Pages, G. (1998). p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. *J Biol Chem* **273,** 18165-72.

Mizzen, C. A., and Allis, C. D. (1998). Linking histone acetylation to transcriptional regulation. *Cell Mol Life Sci* **54,** 6-20.

Moreau, G. M., Arslan, A., Douglas, D. A., Song, J., Smith, L. C., and Murphy, B. D. (1995). Development of immortalized endometrial epithelial and stromal cell lines from the mink (Mustela vison) uterus and their effects on the survival in vitro of mink blastocysts in obligate diapause. *Biol Reprod* **53,** 511-8.

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh, S., and Kudo, I. (2000). Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem* **275,** 32783-92.

Murphy, B. D., Concannon, P. W., Travis, H. F., and Hansel, W. (1981). Prolactin: the hypophyseal factor that terminates embryonic diapause in mink. *Biol Reprod* **25,** 487-91.

Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999). Prostanoid receptors: structures, properties, and functions. *Physiol Rev* **79,** 1193-226.

Ni, H., Sun, T., Ding, N. Z., Ma, X. H., and Yang, Z. M. (2002). Differential expression of microsomal prostaglandin e synthase at implantation sites and in decidual cells of mouse uterus. *Biol Reprod* **67,** 351-8.

Pai, R., Szabo, I. L., Soreghan, B. A., Atay, S., Kawanaka, H., and Tarnawski, A. S. (2001). PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. *Biochem Biophys Res Commun* **286,** 923-8.

Papke, R. L., Concannon, P. W., Travis, H. F., and Hansel, W. (1980). Control of luteal function and implantation in the mink by prolactin. *J Anim Sci* **50,** 1102-7.

Psychoyos, A. (1986). Uterine receptivity for nidation. *Ann N Y Acad Sci* **476,** 36-42.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386,** 671-4.

Sales, K. J., Maudsley, S., and Jabbour, H. N. (2004). Elevated prostaglandin EP2 receptor in endometrial adenocarcinoma cells promotes vascular endothelial growth factor expression via cyclic 3',5'-adenosine monophosphate-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase 1/2 signaling pathways. *Mol Endocrinol* **18,** 1533-45.

Sassone-Corsi, P. (1998). Coupling gene expression to cAMP signalling: role of CREB and CREM. *Int J Biochem Cell Biol* **30,** 27-38.

Smith, S. K. (1998). Angiogenesis, vascular endothelial growth factor and the endometrium. *Hum Reprod Update* **4,** 509-19.

Song, J. H., Sirois, J., Houde, A., and Murphy, B. D. (1998). Cloning, developmental expression, and immunohistochemistry of cyclooxygenase 2 in the endometrium during embryo implantation and gestation in the mink (Mustela vison). *Endocrinology* **139,** 3629-36.

Tilley, S. L., Audoly, L. P., Hicks, E. H., Kim, H. S., Flannery, P. J., Coffman, T. M., and Koller, B. H. (1999). Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E2 receptor. *J Clin Invest* **103,** 1539-45.

Wang, X., Su, Y., Deb, K., Raposo, M., Morrow, J. D., Reese, J., and Paria, B. C. (2004). Prostaglandin E2 is a product of induced prostaglandin-endoperoxide synthase 2

and microsomal-type prostaglandin E synthase at the implantation site of the hamster. *J Biol Chem* **279,** 30579-87.

Xiao, C. W., Murphy, B. D., Sirois, J., and Goff, A. K. (1999). Down-regulation of oxytocin-induced cyclooxygenase-2 and prostaglandin F synthase expression by interferon-tau in bovine endometrial cells. *Biol Reprod* **60,** 656-63.

Zhang, G., and Veldhuis, J. D. (2004). Requirement for proximal putative Sp1 and AP-2 cis-deoxyribonucleic acid elements in mediating basal and luteinizing hormone- and insulin-dependent in vitro transcriptional activation of the CYP17 gene in porcine theca cells. *Endocrinology* **145,** 2760-6.

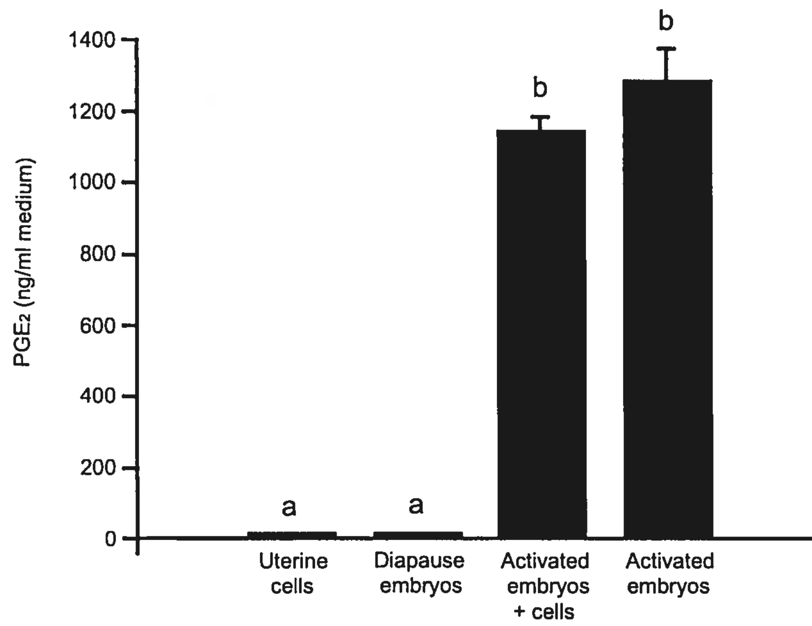
Oligo name	Sequence 5' – 3'
PGEs forward	GCTGCGGAAGAAGGCTTTTG
PGEs reverse	AGGTAGGCCACGGTGTGTAC
EP1 forward	GGCGGCTGCATGGTCTTCTT
EP1 reverse	CAGCAGATGCACGACACCAC
EP2 forward	GCCACGATGCTCATGCTCTT
EP2 reverse	GAATGAGGTGGTCCGTCTCC
EP3 forward	GGAGAGCAAGCGCAAGAAGT
EP3 reverse	CTGATGAAGCACCACGTCC
EP4 forward	ATCTTCGGGGTGGTGGGCAA
EP4 reverse	TTGATGGCCAGGTAGCGCTC

Table 1. Sequences of oligonucleotides used to amplify PGE synthase and the EP receptors.

Oligo name	Sequence 5' – 3'
5'RACE-Sp1	TTGACCCTGTCCCTGTCGTTGC
5'RACE-Sp2	CTCTGACCCCGTCTCTCTCTCT
5'RACE-Sp3	GGGGAAGTAAAGGAGCGATCTC
ChIP-forward	CAGGGGTCACGCCAGTATTCCA
ChIP-reverse	CCTCTGCGCTCCCTACCACTA

Table 2. Oligonucleotides employed for 5' RACE and ChIP.

A.



B.

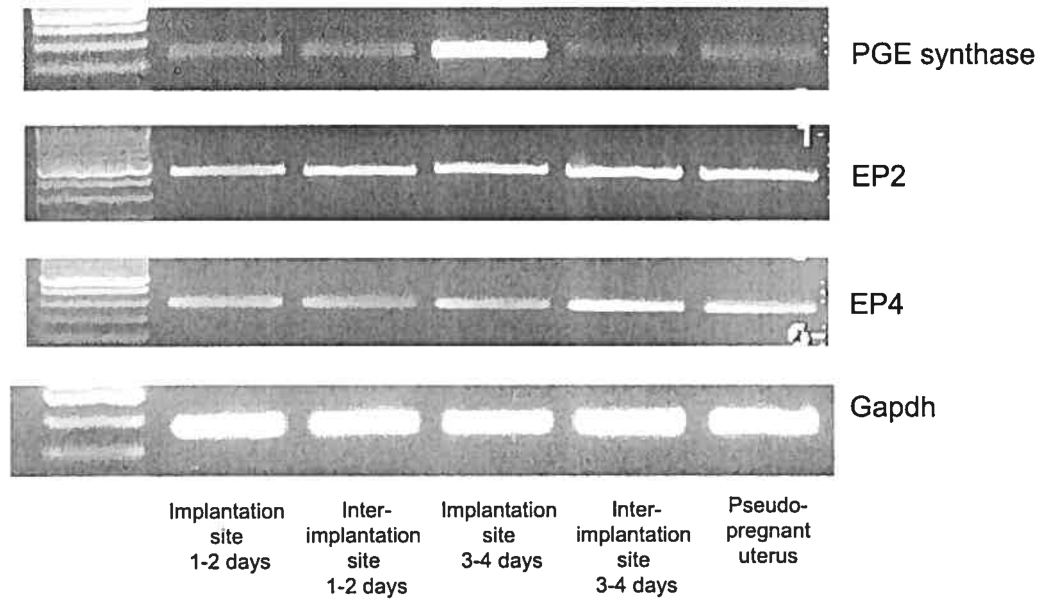


Fig. 1. Activated mink embryos produce PGE_2 and PGE synthase is up-regulated in implanted uteri. A, Diapause and re-activated embryos were flushed from the uterus and placed in culture with or without the presence of mink uterine cells. After 48 h in culture, medium was collected and assayed for PGE_2 by RIA. B, Expression of mRNA of PGE synthase and the receptors EP2 and EP4 were verified by PCR in mink uterine samples collected during early implantation stages (1-2 and 3-4 days following implantation) and from pseudopregnant females.

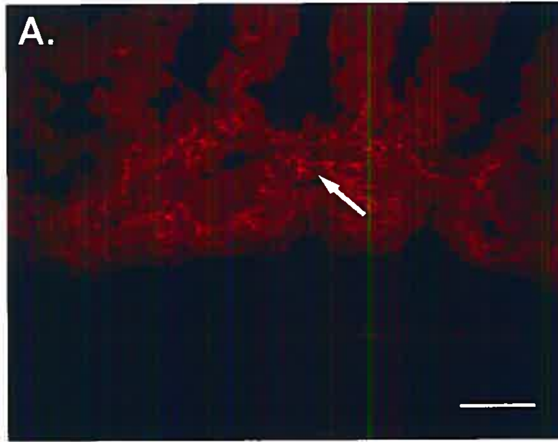
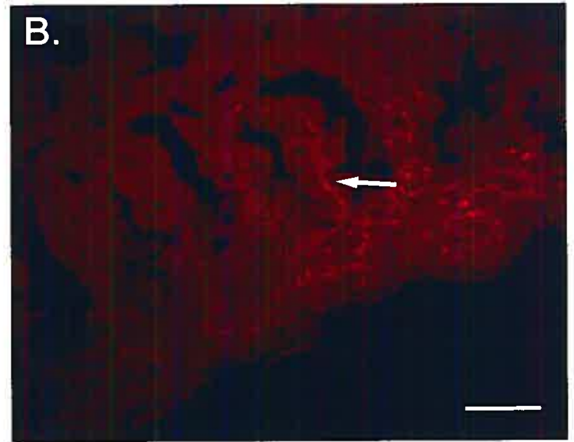
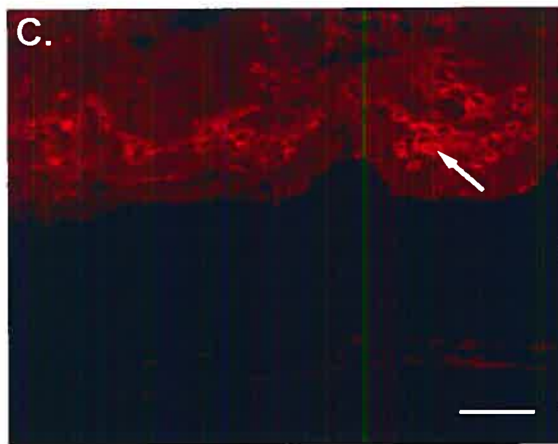
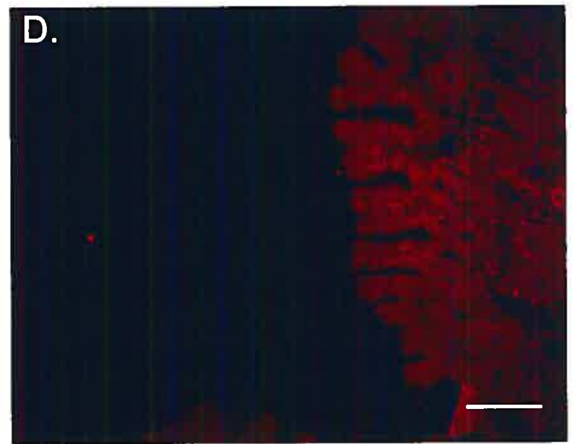
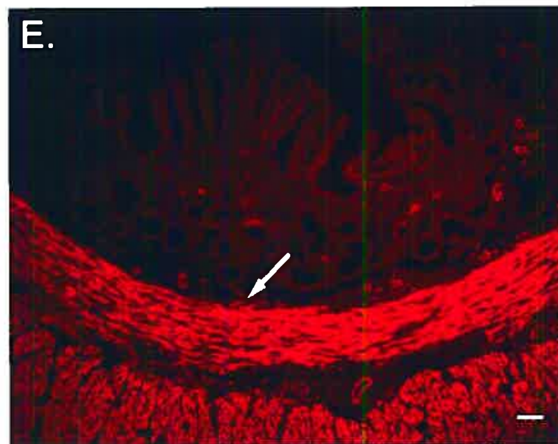
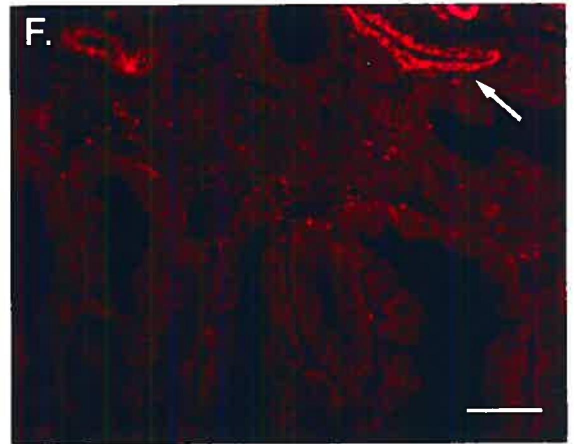
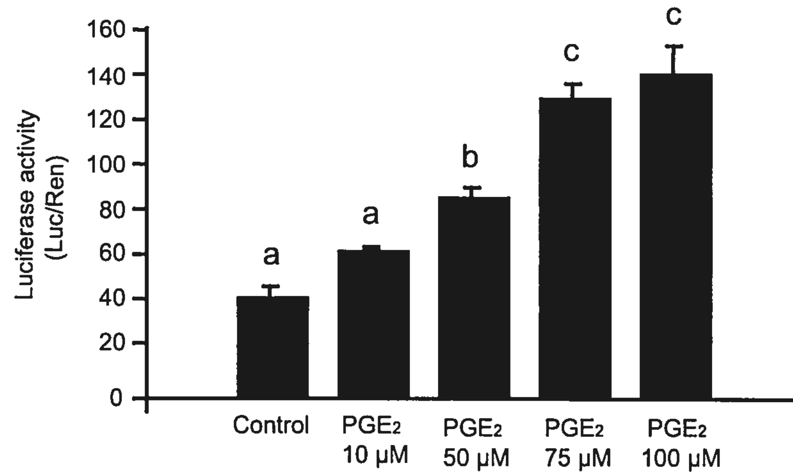
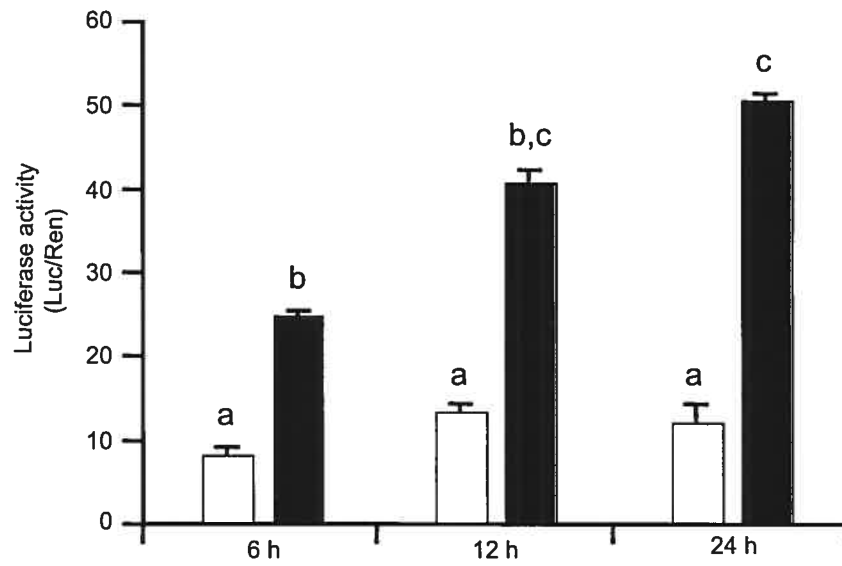
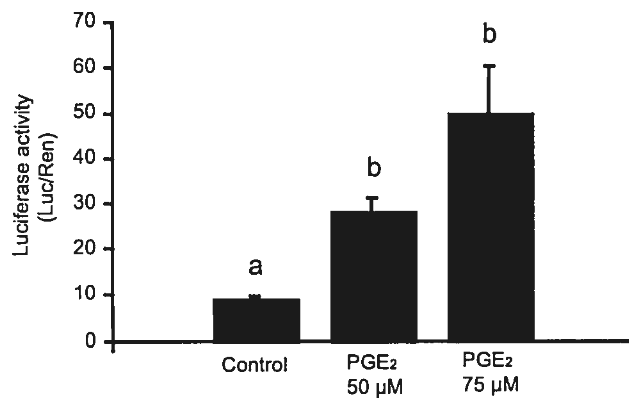

A.

B.

C.

D.

E.

F.

Fig. 2. Immunohistochemical characterization of PGE synthase in the mink uterus. A, B, C, PGE synthase is localizes principally to the stromal cells surrounding the implanting trophoblast cells, as demonstrated by the white arrows. D, Lack of PGE synthase localization in the uterine tissue on the mesometrial (opposite) side of the uterus to the invading embryo. E, F, VEGF localizes at the vessels and the myometrium (white arrows), but not in the endomtrial cells, in samples collected from inter-implantation sites. Bars = 500 μm


A.
Luciferase activity
(Luc/Ren)
160
140
120
100
80
60
40
20
0
a
a
b
c
c
Control
PGE2
10 µM
PGE2
50 µM
PGE2
75 µM
PGE2
100 µM


B.
Luciferase activity
(Luc/Ren)
60
50
40
30
20
10
0
a
b
a
b,c
a
c
6 h
12 h
24 h


C.
Luciferase activity
(Luc/Ren)
70
60
50
40
30
20
10
0
a
b
b
Control
PGE2
50 µM
PGE2
75 µM

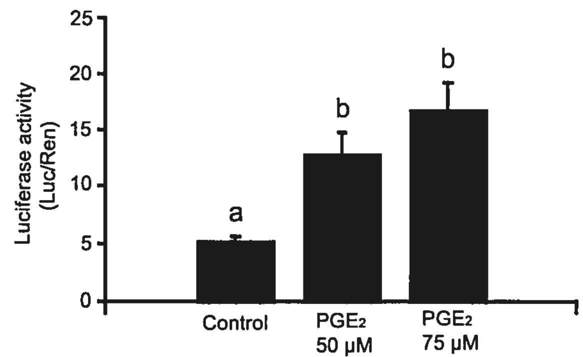
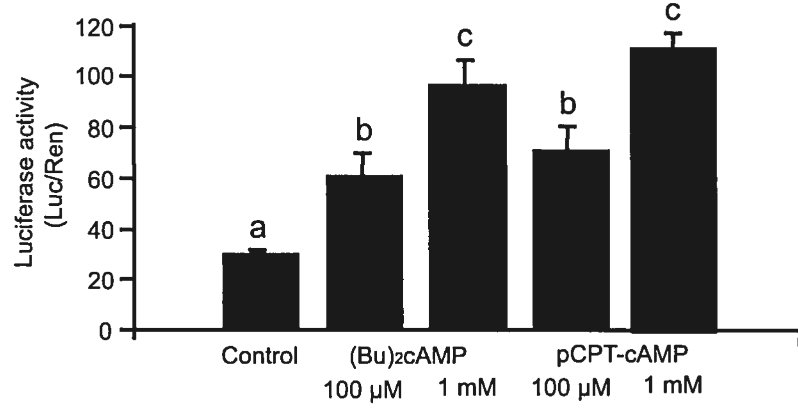

D.
Luciferase activity
(Luc/Ren)
25
20
15
10
5
0
a
b
b
Control
PGE2
50 µM
PGE2
75 µM

Fig. 3. VEGF promoter activity is increased following PGE_2 treatment in different cell types. Cells were transfected with 1.5kb of the mink VEGF promoter driving the luciferase reporter gene. A, Transfected mink stromal cells were treated for 12 h with different doses of PGE_2 (10, 50, 75 and 100 μM). B, Cells were treated with 75 μM of PGE_2 for different times (6, 12 and 24 h). C, Transfected mink ovarian tumor cells, and D, MCF-7 cells were treated for 12 h with 75 μM of PGE_2. The quantification represents mean ± SEM of triplicate transfection experiments. Different superscripts represent significant differences in means ($P < 0.05$).

A.



B.

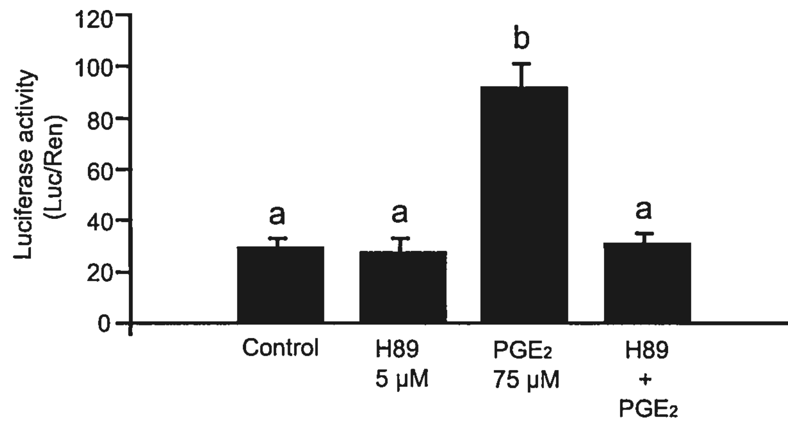
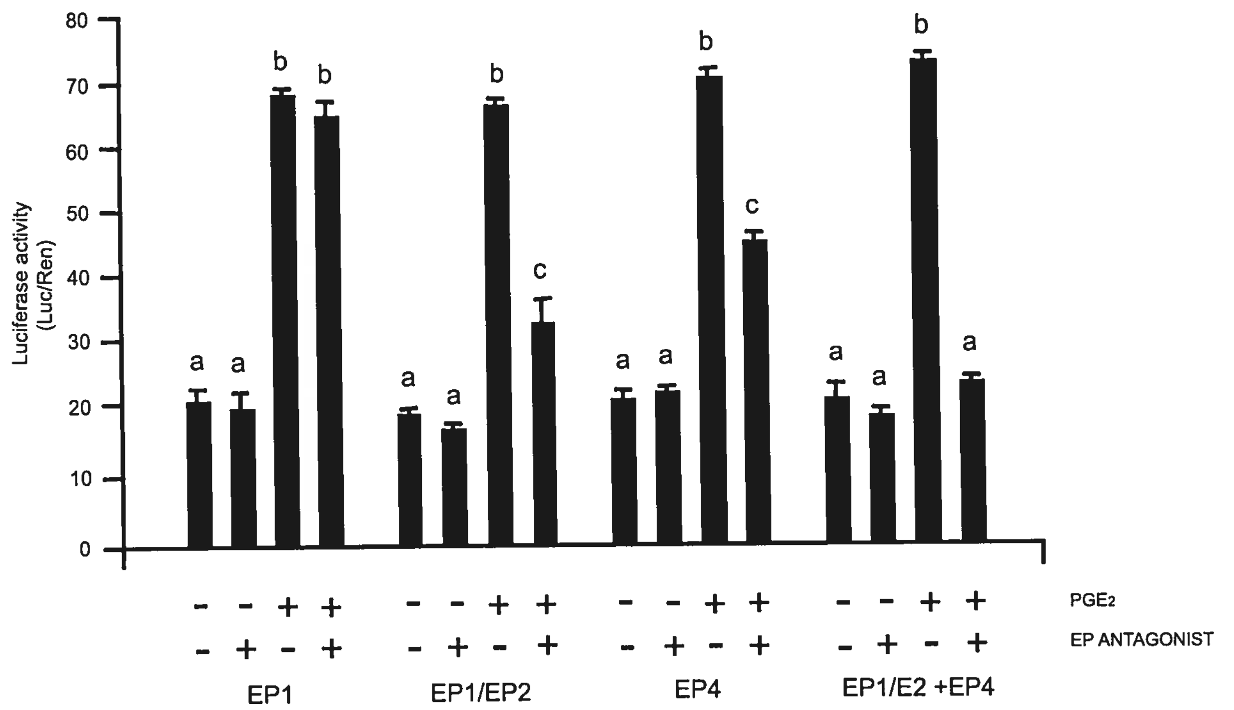


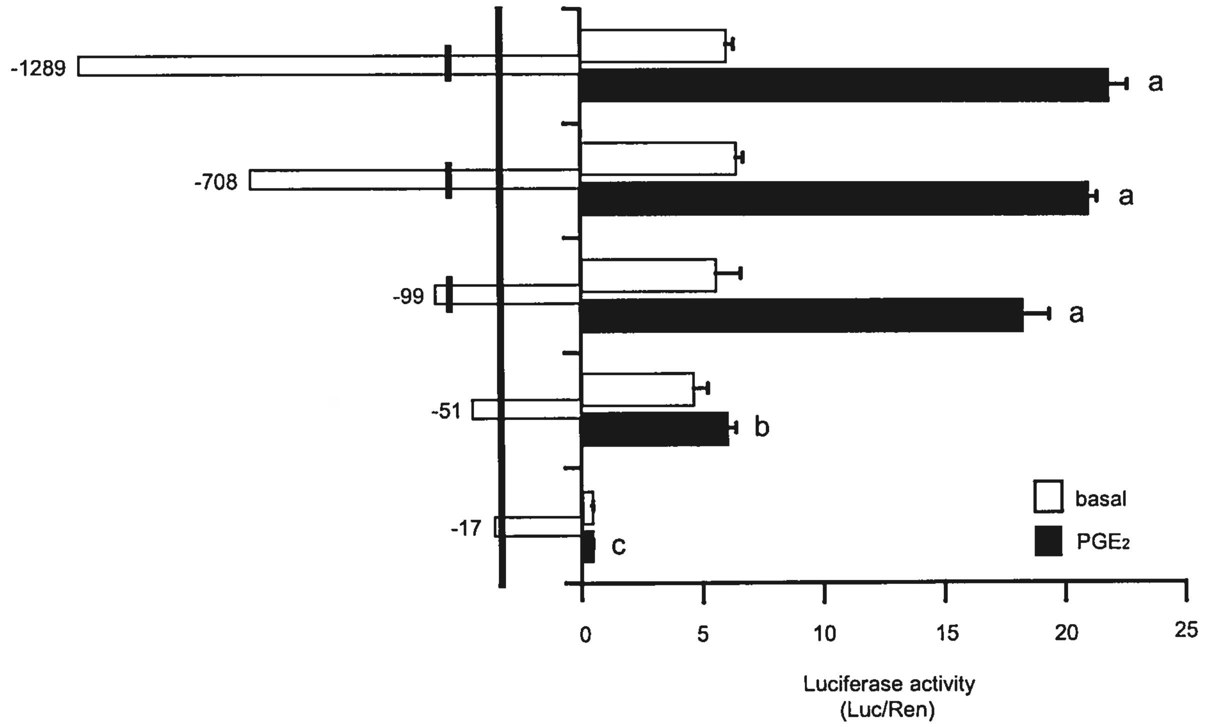
Fig. 4. VEGF promoter activity is stimulated by PGE_2 through a PKA dependent mechanism. Mink stromal cells were transfected with 1.5 kb of the proximal mink VEGF promoter. A, Transfected cells were treated with dibutyryl cAMP (Bu_2cAMP; 100 μM and 1mM) and chlorophenylthio cAMP (pCPT cAMP; 100 μM and 1 mM) for 12 h and promoter activity was measured by luciferase assay. B, Transfected cells were pre-treated with H89 (10 μM) and then treated with PGE_2 (75 μM) for 12 h. Promoter activity was measured by luciferase assay. The quantification represents mean ± SEM of triplicate transfection experiments. Different superscripts represent significant differences in means ($P < 0.05$).



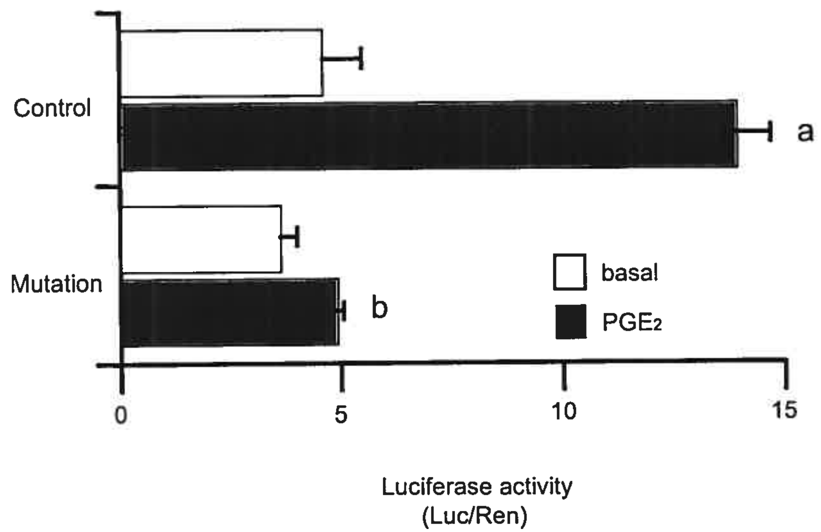
Luciferase activity
(Luc/Ren)
80
70
60
50
40
30
20
10
0
a a b b
a a b c
a a b c
a a b a
PGE2
EP ANTAGONIST
EP1
EP1/EP2
EP4
EP1/E2 +EP4

Fig. 5. Transactivation of the VEGF promoter by PGE_2 is dependent upon binding to the PKA related receptors EP2/EP4. Transfected cells were pre-treated for 1 h with the antagonists for EP1+EP2 (AH6809; 20 μM), for EP1 (SC19220; 10 μM) alone, for EP4 (AH2384B; 30 μM) and a combination of both the EP1+EP2 and EP4. PGE_2 treatment was then added and luciferase activity was assayed after 12 h of PGE_2 treatment. The quantification represents mean ± SEM of triplicate transfection experiments. Different superscripts represent significant differences in means ($P < 0.05$).

A.

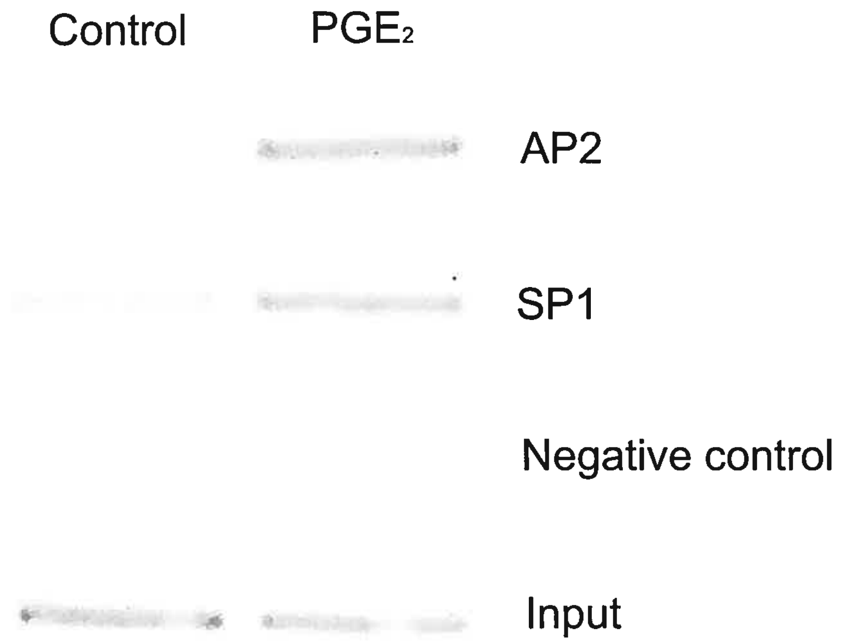


B.



<u>Fig. 6.</u> Deletion constructs of the mink VEGF promoter. A, Fragments of the promoter driving the luciferase reporter gene were transfected into mink uterine stromal cells. Treatment consisted of addition of PGE_2 (75 μM) for 12 h. Approximate location of the AP2 and SP1 sites are indicated by black rectangles. B, Mutation of the AP2 and SP1 sites inhibits the PGE_2 induced VEGF promoter transactivation. Mink stromal cells transfected with constructs containing intact AP2 and SP1 sites or mutated forms of these response elements. Transfected cells were treated for 12 h with PGE_2 and promoter activity was then assessed by luciferase assay. The quantification represents mean ± SEM of triplicate transfection experiments. Different superscripts represent significant differences in means ($P < 0.05$).

A.



B.

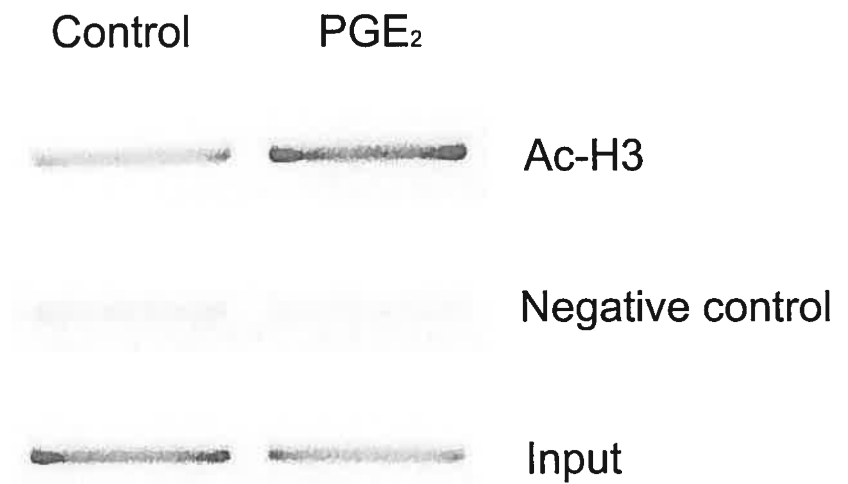


Fig. 7. PGE_2 treatment induces AP2 and SP1 interaction with the VEGF promoter as well as histone modification in mink uterine cells. Mink stromal cells were serum starved for 24 h prior to addition of PGE_2 (75 μM) for 6 h. A, Immunoprecipitation was performed using antibodies against AP2 and SP1 and B, Acetylated histone H3. Following chromatin precipitation, 500 bp of the proximal promoter region of VEGF was amplified by PCR. Input control was established by amplification of an equivalent amount of DNA that had not been previously immunoprecipitated. Control for antibody specificity was established by precipitation with rabbit IgG.

GENERAL DISCUSSION

The American mink is a carnivore with interesting and unique reproductive characteristics. It displays mating-induced ovulation and an embryo that will, upon arrival in the uterus at the blastocyst stage, enter obligate diapause. The embryos remain in this stage of arrested mitotic activity until melatonin levels from the pineal gland are reduced coinciding with the vernal equinox, allowing for prolactin levels to rise and rescue the corpora lutea (CLs). Increased progesterone from the CLs will then re-activate the embryos and this will bring about implantation. This nidatory event in the mink is followed by formation of an endothelial-chorial placenta as reviewed in Chapter I.

Given the peculiar reproductive characteristics of this species, we were interested in understanding the characteristics and regulation of the angiogenic process that takes place during early stages of pregnancy and that is vital for embryo development in other species.

In the studies presented in this thesis, we provide new information on the expression of a major angiogenic factor, VEGF, and its receptors during the peri-implantation period in the mink. We further characterize the presence of PGE_2 in the uterus during this stage and describe the mechanism of regulation of VEGF transcription by this prostaglandin.

As discussed before, the process of formation and development of blood vessels in the placenta and embryo, as well as the increased vascularity in the uterus itself, is indispensable for maintenance of pregnancy. Given the growing need for gas exchange, nutrient supply and waste removal, it is clear that an efficient regulation of vasculogenesis and angiogenesis must be present at this time.

Uterine angiogenesis during early pregnancy

VEGF-A, a homodimeric glycoprotein of 40–45 kDa, was first identified for its induction on vascular permeability (Senger et al., 1983). In 1989, Ferrara and Henzel described this protein as a potent endothelial cell-specific mitogen. VEGF exerts its action through binding to tyrosine kinase receptors, Flt-1 (fms-like tyrosine kinase, also known as VEGFR-1) and KDR (kinase domain region, also known as VEGFR-2) (Jussila e Alitalo, 2002). These two processes that induce development of new vascular system (vasculogenesis), as well as enlargement of the pre-existing one (angiogenesis), are principally directed by VEGF.

Increased uterine vascularity, associated with implantation, is a condition that has been observed in several species to date. In 1973, Psychoyos through the use of a protein-bound blue dye, reported an increase in vascular permeability as the earliest uterine response to the implantation process. This demonstrated that vascular changes are amongst

one of the first uterine modifications occurring during successful embryo implantation and subsequent placentation.

Since then, several researchers have focused on the expression of the major angiogenic regulator, VEGF. In the hamster, VEGF and its receptors are up-regulated in the uterus during early pregnancy (Yi et al., 1999). During the peri-implantation stages, both mice and rabbit present intense uterine expression of VEGF (Chakraborty et al., 1995; Das et al., 1997). In Chapter II, we describe the expression of VEGF and the receptors Flt-1 and KDR in the mink uterus during early pregnancy. We observed that the mRNA levels for the three most common VEGF isoforms, 121, 164 and 189, increase following embryo activation and this increase continues through to implantation. Highest levels were observed during immediate pre-implantation stages (late stages of embryo activation) and in the early implantation sites. Interestingly, VEGF mRNA levels were also up-regulated in pseudopregnant uteri, indicating a maternal rather than embryonic regulation of expression of this growth factor. Amongst the several regulators of VEGF studied to date, progesterone is a likely candidate to be controlling VEGF during pregnancy. In 1993, Cullinan-Bove and Koos reported that progesterone stimulates VEGF expression in the rat uterus. In human breast cancer cells, the PRB receptor appears to be mediating the progesterone induction of VEGF (Wu et al., 2004). The idea of progesterone regulation is corroborated by the low expression of VEGF during diapause in the mink, considering the low levels of progesterone during this stage due to the inactive CL present at this time. As

embryo activation in the mink is dictated by the rescue of the CLs and increase in progesterone levels (Douglas et al., 1997), it is plausible to place progesterone as a major regulator of uterine VEGF at this stage in this species.

Estrogen is also a powerful inducer of VEGF, and in species like mice, where there is an estrogen requirement for initiation of implantation (reviewed by Paria et al., 2001), it likely plays a major role in controlling VEGF regulation. However, in the mink, the pre-implantation rise in estradiol while it may occurr, has not been demonstrated, thus we can only speculate that this might account for the rise in VEGF seen during embryo activation.

The expression of the receptors for VEGF was also increased leading to implantation, but interestingly, unlike VEGF, mRNA for both receptors was low in pseudopregnant uteri, indicating embryonic control over the expression of the receptors. Less is currently known about regulation of the angiogenic receptors in the context of implantation, but an embryonic factor has been shown to participate in this regulation. In humans, human chorionic gonadotropin (hCG) was found to induce Flt-1 mRNA expression in cultured oviductal cells (Lam et al., 2004). A gonadotropin similar to hCG has not yet been described in mink, and it is unlikely that it exists, as there appears to be no uterine-derived control of the CL (Douglas et al., 1997). Identification of the factor(s), originating from the embryos, and responsible for regulation of the angiogenic receptors represents an exciting future direction for this research.

VEGF regulation in the mink uterus

In general, regulation of VEGF and, consequently, angiogenesis is mainly driven by locally produced growth factors (Goldman et al., 1993; Yen et al., 2002; Petit et al., 1997; Sugano et al. 2003; Deroanne et al., 1997), cyclic hormones, such as estradiol and progesterone (reviewed by Loureiro e D'Amore, 2005) and hypoxia (Liu et al., 2002). Such control assures that vessel development and/or growth is initiated and proceeds in a localized and temporally controlled manner.

While we cannot eliminate the possibility of strict and sole control over VEGF transcription by steroids (in fact the increase in progesterone levels fits nicely with the increase in VEGF), there are other potential candidates to VEGF control, such as prolactin, given that its rise is also temporally associated with the up-regulation of VEGF. In fact, prolactin has been shown to stimulate VEGF in human macrophages through up-regulation of heme oxygenase-1 (Malaguarnera et al., 2004). Further evidence for this postulate can be found in studies that show that heme oxygenase-1 has the same pattern of expression as VEGF during rat gestation (Kreiser et al., 2003). This attractive and new pathway of uterine regulation of angiogenesis merits further investigation.

In the present study we were interested in placing locally-produced PGE_2 as a regulator of the peri-implantation increase in VEGF. Our laboratory has previously

demonstrated that the rate-limiting enzyme necessary for prostaglandin production, COX-2, is expressed at early stages of implantation and that it is localized in the uterine stromal cells surrounding the invading embryos (Song et al., 1998). We further demonstrated in Chapter III that the activated embryos produce copious amounts of PGE_2 and that PGE synthase is expressed in the same cell type as COX-2, and is also dependent on the presence of the embryo. These results indicate that PGE_2 is most certainly present in the uterus during up-regulation of VEGF. We believe it to be produced by the uterine cells themselves, following implantation, and by the blastocysts following activation.

By utilizing mink uterine cells transfected with the mink VEGF promoter, and performing deletional and mutational studies on the promoter, we were able to verify that PGE_2, through binding to its two receptors EP2 and EP4, activates PKA which in turn leads to AP2/SP1 binding to the proximal promoter region. As mentioned before, this cluster has been previously involved in transactivation of VEGF by other upstream regulators (Milanini et al., 1998; Brenneisen et al., 2003). The region where these response elements were identified, corresponds to a GC rich region, which is also involved in the transcriptional activation of VEGF by AP2 and SP1 in combination or alone, confirming therefore, the importance of this GC rich region to the transactivation of VEGF. Given the fact that the PKA pathway was proven necessary for PGE_2-induced promoter transactivation, it was surprising that we did not find predicted sites for CREB within the smallest fragment that remained responsive to PGE_2. This finding is not unusual, given

that the activation of an AP2/SP1 cluster downstream of PKA has been previously demonstrated (Zhang and Veldhuis, 2004).

Our lab has previously produced immortalized mink uterine stromal cells (Moreau et al., 1995) and the use of this cell line allowed us to test the mink VEGF promoter transactivation in the in vitro model closest to the uterine cells that produce PGE_2, and that also express VEGF only following implantation. An intriguing finding in the current study was the fact that stromal localization of VEGF was observed only following implantation, and temporally coordinated with COX-2 expression (Song et al., 1998) in the mink uterus. We show similar spatial and temporal correlation of PGE synthase with the VEGF in the stroma immediately surrounding the invading embryo. It is tempting to hypothesize that up-regulation of VEGF at the stromal layer and surrounding tissues is dependent upon the presence of PGE_2, whose synthesis is in turn associated with the contact with the embryo.

Transcriptional activation does not depend only upon binding of transcription factors to their response elements. Histone modification, namely that of histones H3 and H4, are processes required for chromatin remodelling into an active form (Struhl, 1998). Amongst the histone modifications associated with permissive chromatin are acetylation and phosphorylation of H3 and H4, which results in loss of stability of the nucleosome making the access to response elements possible (Struhl, 1998). In the work presented in chapter III, we observed by ChIP that treatment of mink uterine cells with PGE_2, induces

acetylation of H3, which in turn is expected to prompt the chromatin modifications required for binding of transcription factors. Further, the promoter fragment that was amplified following immunoprecipitation for the acetylated H3 encompasses the area where we demonstrated the binding of AP2 and SP1 to the endogenous promoter following PGE_2 treatment. We conclude that PGE_2 elicits histone H3 acetylation, resulting in active chromatin at the site required for PGE_2 induced promoter transactivation, and it further induces, through PKA activation, the binding of AP2 and SP2 to the site.

CONCLUSION

During our studies we demonstrated that the angiogenic factor VEGF and its two tyrosine kinase receptors, KDR and Flt-1, are up-regulated during the process of implantation in the mink, indicating their central role in increasing the network of blood vessels in the uterus and placenta in order to meet the growing demands for oxygen and nutrients of the implanting embryo. We concluded that regulation of VEGF is driven in its majority by maternal factors, whereas the increased receptors expression is embryo dependent.

We have shown that PGE_2 is produced by mink embryos following escape from obligate diapause and also by the uterine stromal cells surrounding the implanting embryos. We verified in a mink stromal cell line, that VEGF is stimulated by PGE_2 following binding of this prostaglandin to its receptors EP2 and EP4. This binding induces activation of the PKA pathway leading to downstream promoter transactivation. PGE_2 treatment induced binding of AP2 and SP1 to the proximal promoter region of mink VEGF, and mutation of this specific binding region in the VEGF results in loss of PGE_2 induced response. As promoter transactivation requires remodelling of the chromatin, we also demonstrated that PGE_2 induces histone H3 acetylation, which we expect results in conformational changes rendering the chromatin active. Since this epigenetic modification was observed in the promoter region associated with the response elements for the AP2 and

SP1 factors, we concluded that PGE_2 not only induces binding of these factors to the promoter of VEGF, but also elicits covalent modifications in histone H3 allowing for the transcription factors to bind to the VEGF promoter and initiate transcription.

In summary, our studies demonstrated that in the mink VEGF and receptors are increased during the peri-implantation period and that PGE_2 is a major regulator of VEGF transcription, stimulating binding of transcription factors to the promoter as well as epigenetic changes leading to a permissive chromatin. This places PGE_2 as an important regulator of peri-implantation angiogenesis, a process crucial for successful maintenance of pregnancy.

REFERENCES

Ainsworth, L., Tsang, B. K., Downey, B. R., Baker, R. D., Marcus, G. J., and Armstrong, D. T. (1979). Effects of indomethacin on ovulation and luteal function in gilts. *Biol Reprod* **21,** 401-11.

Akarsu, E. S., and Ayhan, I. H. (1993). Iloprost, a stable analogue of PGI2, potentiates the hyperthermic effect of PGE2 in rats. *Pharmacol Biochem Behav* **46,** 383-9.

Ancelin, M., Buteau-Lozano, H., Meduri, G., Osborne-Pellegrin, M., Sordello, S., Plouet, J., and Perrot-Applanat, M. (2002). A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. *Proc Natl Acad Sci U S A* **99,** 6023-8.

Andersen, N. H., Eggerman, T. L., Harker, L. A., Wilson, C. H., and De, B. (1980). On the multiplicity of platelet prostaglandin receptors. I. Evaluation of competitive antagonism by aggregometry. *Prostaglandins* **19,** 711-35.

Benckert, C., Jonas, S., Cramer, T., Von Marschall, Z., Schafer, G., Peters, M., Wagner, K., Radke, C., Wiedenmann, B., Neuhaus, P., Hocker, M., and Rosewicz, S. (2003). Transforming growth factor beta 1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells. *Cancer Res* **63,** 1083-92.

Bergstrom, S., and Samuelsson, B. (1962). Isolation of prostaglandin E1 from human seminal plasma. Prostaglandins and related factors. 11. *J Biol Chem* **237,** 3005-6.

Bermont, L., Lamielle, F., Lorchel, F., Fauconnet, S., Esumi, H., Weisz, A., and Adessi, G. L. (2001). Insulin up-regulates vascular endothelial growth factor and stabilizes its messengers in endometrial adenocarcinoma cells. *J Clin Endocrinol Metab* **86,** 363-8.

Boie, Y., Sawyer, N., Slipetz, D. M., Metters, K. M., and Abramovitz, M. (1995). Molecular cloning and characterization of the human prostanoid DP receptor. *J Biol Chem* **270,** 18910-6.

Bradbury, D., Clarke, D., Seedhouse, C., Corbett, L., Stocks, J., and Knox, A. (2005). Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1 transcription factor binding sites. *J Biol Chem* **280,** 29993-30000.

Brenneisen, P., Blaudschun, R., Gille, J., Schneider, L., Hinrichs, R., Wlaschek, M., Eming, S., and Scharffetter-Kochanek, K. (2003). Essential role of an activator protein-2 (AP-2)/specificity protein 1 (Sp1) cluster in the UVB-mediated induction of the human vascular endothelial growth factor in HaCaT keratinocytes. *Biochem J* **369,** 341-9.

Buteau-Lozano, H., Ancelin, M., Lardeux, B., Milanini, J., and Perrot-Applanat, M. (2002). Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: a complex interplay between estrogen receptors alpha and beta. *Cancer Res* **62,** 4977-84.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nat Med* **6,** 389-95.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435-9.

Casibang, M., Purdom, S., Jakowlew, S., Neckers, L., Zia, F., Ben-Av, P., Hla, T., You, L., Jablons, D. M., and Moody, T. W. (2001). Prostaglandin E2 and vasoactive intestinal peptide increase vascular endothelial cell growth factor mRNAs in lung cancer cells. *Lung Cancer* **31,** 203-12.

Chakraborty, I., Das, S. K., and Dey, S. K. (1995). Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation. *J Endocrinol* **147,** 339-52.

Chang, M. C. (1968). Reciprocal insemination and egg transfer between ferrets and mink. *J Exp Zool* **168,** 49-59.

Chang, S. H., Liu, C. H., Wu, M. T., and Hla, T. (2005). Regulation of vascular endothelial cell growth factor expression in mouse mammary tumor cells by the EP2 subtype of the prostaglandin E2 receptor. *Prostaglandins Other Lipid Mediat* **76,** 48-58.

Clyman, R. I., Mauray, F., Roman, C., and Rudolph, A. M. (1978). PGE2 is a more potent vasodilator of the lamb ductus arteriosus than is either PGI2 or 6 keto PGF1alpha. *Prostaglandins* **16,** 259-64.

Coleman, R. A., and Sheldrick, R. L. (1989). Prostanoid-induced contraction of human bronchial smooth muscle is mediated by TP-receptors. *Br J Pharmacol* **96,** 688-92.

Cullinan-Bove, K., and Koos, R. D. (1993). Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology* **133,** 829-37.

Das, S. K., Chakraborty, I., Wang, J., Dey, S. K., and Hoffman, L. H. (1997). Expression of vascular endothelial growth factor (VEGF) and VEGF-receptor messenger ribonucleic acids in the peri-implantation rabbit uterus. *Biol Reprod* **56,** 1390-9.

Davis, B. J., Lennard, D. E., Lee, C. A., Tiano, H. F., Morham, S. G., Wetsel, W. C., and Langenbach, R. (1999). Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. *Endocrinology* **140,** 2685-95.

Dennis, E. A. (1994). Diversity of group types, regulation, and function of phospholipase A2. *J Biol Chem* **269,** 13057-60.

Deroanne, C. F., Hajitou, A., Calberg-Bacq, C. M., Nusgens, B. V., and Lapiere, C. M. (1997). Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. *Cancer Res* **57,** 5590-7.

Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B., Contel, N. R., Eng, V. M., Collins, R. J., Czerniak, P. M., and et al. (1995). Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* **378,** 406-9.

Dorn, G. W., 2nd, Becker, M. W., and Davis, M. G. (1992). Dissociation of the contractile and hypertrophic effects of vasoconstrictor prostanoids in vascular smooth muscle. *J Biol Chem* **267,** 24897-905.

Douglas, D. A., Song, J. H., Houde, A., Cooke, G. M., and Murphy, B. D. (1997). Luteal and placental characteristics of carnivore gestation: expression of genes for luteotrophic receptors and steroidogenic enzymes. *J Reprod Fertil Suppl* **51,** 153-66.

Eibl, G., Bruemmer, D., Okada, Y., Duffy, J. P., Law, R. E., Reber, H. A., and Hines, O. J. (2003). PGE(2) is generated by specific COX-2 activity and increases VEGF

production in COX-2-expressing human pancreatic cancer cells. *Biochem Biophys Res Commun* **306,** 887-97.

Enders, A. C. (1957). Histological observations on the chorio-allantoic placenta of the mink. *Anat Rec* **127,** 231-45.

Ferrara, N., and Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* **161,** 851-8.

Filion, F., Bouchard, N., Goff, A. K., Lussier, J. G., and Sirois, J. (2001). Molecular cloning and induction of bovine prostaglandin E synthase by gonadotropins in ovarian follicles prior to ovulation in vivo. *J Biol Chem* **276,** 34323-30.

Fong, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376,** 66-70.

Fukuda, R., Kelly, B., and Semenza, G. L. (2003). Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1. *Cancer Res* **63,** 2330-4.

Ghosh, D., Sharkey, A. M., Charnock-Jones, D. S., Dhawan, L., Dhara, S., Smith, S. K., and Sengupta, J. (2000). Expression of vascular endothelial growth factor (VEGF)

and placental growth factor (PlGF) in conceptus and endometrium during implantation in the rhesus monkey. *Mol Hum Reprod* **6,** 935-41.

Ginther, O. J. (1974). Internal regulation of physiological processes through local venoarterial pathways: a review. *J Anim Sci* **39,** 550-64.

Goldman, C. K., Kim, J., Wong, W. L., King, V., Brock, T., and Gillespie, G. Y. (1993). Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell* **4,** 121-33.

Grinwich, D. L., Kennedy, T. G., and Armstrong, D. T. (1972). Dissociation of ovulatory and steroidogenic actions of luteinizing hormone in rabbits with indomethacin, an inhibitor of prostaglandin biosynthesis. *Prostaglandins* **1,** 89-96.

Halder, J. B., Zhao, X., Soker, S., Paria, B. C., Klagsbrun, M., Das, S. K., and Dey, S. K. (2000). Differential expression of VEGF isoforms and VEGF(164)-specific receptor neuropilin-1 in the mouse uterus suggests a role for VEGF(164) in vascular permeability and angiogenesis during implantation. *Genesis* **26,** 213-24.

Hansson, A. (1947). The physiology of reproduction in mink (Mustela vison) with special reference to delayed implantation. *Acta Zoll Stockh* **28,** 1-136.

Hayaishi, O. (1991). Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D2 and E2. *Faseb J* **5,** 2575-81.

He, Y., Smith, S. K., Day, K. A., Clark, D. E., Licence, D. R., and Charnock-Jones, D. S. (1999). Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* **13,** 537-45.

Hirashima, M., Lu, Y., Byers, L., and Rossant, J. (2003). Trophoblast expression of fms-like tyrosine kinase 1 is not required for the establishment of the maternal-fetal interface in the mouse placenta. *Proc Natl Acad Sci U S A* **100,** 15637-42.

Hirata, H., Nagakura, T., Tsujii, M., Morita, A., Fujisawa, K., and Uchida, A. (2004). The relationship of VEGF and PGE2 expression to extracellular matrix remodelling of the tenosynovium in the carpal tunnel syndrome. *J Pathol* **204,** 605-12.

Hirata, T., Ushikubi, F., Kakizuka, A., Okuma, M., and Narumiya, S. (1996). Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J Clin Invest* **97,** 949-56.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. (1998). Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A* **95,** 9349-54.

Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuoka, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S., and Ichikawa, A. (1999). Abortive

expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). *Proc Natl Acad Sci U S A* **96,** 10501-6.

Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991). The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* **5,** 1806-14.

Houck, K. A., Leung, D. W., Rowland, A. M., Winer, J., and Ferrara, N. (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* **267,** 26031-7.

Huang, S. P., Wu, M. S., Shun, C. T., Wang, H. P., Lin, M. T., Kuo, M. L., and Lin, J. T. (2004). Interleukin-6 increases vascular endothelial growth factor and angiogenesis in gastric carcinoma. *J Biomed Sci* **11,** 517-27.

Inoue, H., Takamori, M., Shimoyama, Y., Ishibashi, H., Yamamoto, S., and Koshihara, Y. (2002). Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. *Br J Pharmacol* **136,** 287-95.

Irvine, R. F. (1982). How is the level of free arachidonic acid controlled in mammalian cells? *Biochem J* **204,** 3-16.

Ito, S., Sakamoto, K., Mochizuki-Oda, N., Ezashi, T., Miwa, K., Okuda-Ashitaka, E., Shevchenko, V. I., Kiso, Y., and Hayaishi, O. (1994). Prostaglandin F2 alpha receptor is coupled to Gq in cDNA-transfected Chinese hamster ovary cells. *Biochem Biophys Res Commun* **200,** 756-62.

Jakobsson, P. J., Thoren, S., Morgenstern, R., and Samuelsson, B. (1999). Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci U S A* **96,** 7220-5.

Jiang, B. H., Zheng, J. Z., Leung, S. W., Roe, R., and Semenza, G. L. (1997). Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. *J Biol Chem* **272,** 19253-60.

Josko, J., and Mazurek, M. (2004). Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis. *Med Sci Monit* **10,** RA89-98.

Jussila, L., and Alitalo, K. (2002). Vascular growth factors and lymphangiogenesis. *Physiol Rev* **82,** 673-700.

Kawaguchi, M., Akagi, M., Gray, M. J., Liu, W., Fan, F., and Ellis, L. M. (2004). Regulation of vascular endothelial growth factor expression in human gastric cancer cells by interleukin-1beta. *Surgery* **136,** 686-92.

Kendall, R. L., and Thomas, K. A. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A* **90,** 10705-9.

Kennedy, C. R., Zhang, Y., Brandon, S., Guan, Y., Coffee, K., Funk, C. D., Magnuson, M. A., Oates, J. A., Breyer, M. D., and Breyer, R. M. (1999). Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP2 receptor. *Nat Med* **5,** 217-20.

Keyt, B. A., Berleau, L. T., Nguyen, H. V., Chen, H., Heinsohn, H., Vandlen, R., and Ferrara, N. (1996). The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. *J Biol Chem* **271,** 7788-95.

Kimura, M., Osumi, S., and Ogihara, M. (2001). Prostaglandin E(2) (EP(1)) receptor agonist-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes: the involvement of TGF-alpha. *Endocrinology* **142,** 4428-40.

Kreiser, D., Kelly, D. K., Seidman, D. S., Stevenson, D. K., Baum, M., and Dennery, P. A. (2003). Gestational pattern of heme oxygenase expression in the rat. *Pediatr Res* **54,** 172-8.

Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V., and Alitalo, K. (1996). VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* **122,** 3829-37.

Lam, P. M., Briton-Jones, C., Cheung, C. K., Cheung, L. P., and Haines, C. (2004). Upregulation of mRNA expression of vascular endothelial growth factor and its receptors by exogenous human chorionic gonadotropin in cultured oviduct mucosal cells. *Fertil Steril* **82,** 1708-10.

Lim, H., and Dey, S. K. (1997). Prostaglandin E2 receptor subtype EP2 gene expression in the mouse uterus coincides with differentiation of the luminal epithelium for implantation. *Endocrinology* **138,** 4599-606.

Lim, H., Gupta, R. A., Ma, W. G., Paria, B. C., Moller, D. E., Morrow, J. D., DuBois, R. N., Trzaskos, J. M., and Dey, S. K. (1999). Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. *Genes Dev* **13,** 1561-74.

Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997). Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91,** 197-208.

Liu, L. X., Lu, H., Luo, Y., Date, T., Belanger, A. J., Vincent, K. A., Akita, G. Y., Goldberg, M., Cheng, S. H., Gregory, R. J., and Jiang, C. (2002). Stabilization of

vascular endothelial growth factor mRNA by hypoxia-inducible factor 1. *Biochem Biophys Res Commun* **291,** 908-14.

Loureiro, R. M., and D'Amore, P. A. (2005). Transcriptional regulation of vascular endothelial growth factor in cancer. *Cytokine Growth Factor Rev* **16,** 77-89.

Ma, W., Tan, J., Matsumoto, H., Robert, B., Abrahamson, D. R., Das, S. K., and Dey, S. K. (2001). Adult tissue angiogenesis: evidence for negative regulation by estrogen in the uterus. *Mol Endocrinol* **15,** 1983-92.

Malaguarnera, L., Imbesi, R. M., Scuto, A., D'Amico, F., Licata, F., Messina, A., and Sanfilippo, S. (2004). Prolactin increases HO-1 expression and induces VEGF production in human macrophages. *J Cell Biochem* **93,** 197-206.

Martinet, L., Allais, C., and Allain, D. (1981). The role of prolactin and LH in luteal function and blastocyst growth in mink (Mustela vison). *J Reprod Fertil Suppl* **29,** 119-30.

Matlhagela, K., Borsick, M., Rajkhowa, T., and Taub, M. (2005). Identification of a prostaglandin-responsive element in the Na,K-ATPase beta 1 promoter that is regulated by cAMP and Ca2+. Evidence for an interactive role of cAMP regulatory element-binding protein and Sp1. *J Biol Chem* **280,** 334-46.

Meyer, M., Clauss, M., Lepple-Wienhues, A., Waltenberger, J., Augustin, H. G., Ziche, M., Lanz, C., Buttner, M., Rziha, H. J., and Dehio, C. (1999). A novel vascular

endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *Embo J* **18,** 363-74.

Milanini, J., Vinals, F., Pouyssegur, J., and Pages, G. (1998). p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. *J Biol Chem* **273,** 18165-72.

Miquerol, L., Gertsenstein, M., Harpal, K., Rossant, J., and Nagy, A. (1999). Multiple developmental roles of VEGF suggested by a LacZ-tagged allele. *Dev Biol* **212,** 307-22.

Miquerol, L., Langille, B. L., and Nagy, A. (2000). Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development* **127,** 3941-6.

Moreau, G. M., Arslan, A., Douglas, D. A., Song, J., Smith, L. C., and Murphy, B. D. (1995). Development of immortalized endometrial epithelial and stromal cell lines from the mink (Mustela vison) uterus and their effects on the survival in vitro of mink blastocysts in obligate diapause. *Biol Reprod* **53,** 511-8.

Morita, I., Schindler, M., Regier, M. K., Otto, J. C., Hori, T., DeWitt, D. L., and Smith, W. L. (1995). Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *J Biol Chem* **270,** 10902-8.

Mueller, M. D., Vigne, J. L., Minchenko, A., Lebovic, D. I., Leitman, D. C., and Taylor, R. N. (2000). Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors alpha and beta. *Proc Natl Acad Sci U S A* **97,** 10972-7.

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh, S., and Kudo, I. (2000). Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem* **275,** 32783-92.

Murdoch, W. J., and Dunn, T. G. (1983). Luteal function after ovulation blockade by intrafollicular injection of indomethacin in the ewe. *J Reprod Fertil* **69,** 671-5.

Murphy, B. D., Concannon, P. W., Travis, H. F., and Hansel, W. (1981). Prolactin: the hypophyseal factor that terminates embryonic diapause in mink. *Biol Reprod* **25,** 487-91.

Murphy, B. D., DiGregorio, G. B., Douglas, D. A., and Gonzalez-Reyna, A. (1990). Interactions between melatonin and prolactin during gestation in mink (Mustela vison). *J Reprod Fertil* **89,** 423-9.

Namba, T., Oida, H., Sugimoto, Y., Kakizuka, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1994). cDNA cloning of a mouse prostacyclin receptor. Multiple signaling pathways and expression in thymic medulla. *J Biol Chem* **269,** 9986-92.

Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993). Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature* **365,** 166-70.

Nancarrow, C. D., Buckmaster, J., Chamley, W., Cox, R. I., Cumming, I. A., Cummins, L., Drinan, J. P., Findlay, J. K., Goding, J. R., Restall, B. J., Schneider, W., and Thorburn, G. D. (1973). Hormonal changes around oestrus in the cow. *J Reprod Fertil* **32,** 320-1.

Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999). Prostanoid receptors: structures, properties, and functions. *Physiol Rev* **79,** 1193-226.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J* **13,** 9-22.

Ni, H., Sun, T., Ding, N. Z., Ma, X. H., and Yang, Z. M. (2002). Differential expression of microsomal prostaglandin e synthase at implantation sites and in decidual cells of mouse uterus. *Biol Reprod* **67,** 351-8.

Olofsson, B., Pajusola, K., von Euler, G., Chilov, D., Alitalo, K., and Eriksson, U. (1996). Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. *J Biol Chem* **271,** 19310-7.

Orczyk, G. P., and Behrman, H. R. (1972). Ovulation blockade by aspirin or indomethacin--in vivo evidence for a role of prostaglandin in gonadotrophin secretion. *Prostaglandins* **1,** 3-20.

Pai, R., Szabo, I. L., Soreghan, B. A., Atay, S., Kawanaka, H., and Tarnawski, A. S. (2001). PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. *Biochem Biophys Res Commun* **286,** 923-8.

Papke, R. L., Concannon, P. W., Travis, H. F., and Hansel, W. (1980). Control of luteal function and implantation in the mink by prolactin. *J Anim Sci* **50,** 1102-7.

Parent, J., Chapdelaine, P., Sirois, J., and Fortier, M. A. (2002). Expression of microsomal prostaglandin E synthase in bovine endometrium: coexpression with cyclooxygenase type 2 and regulation by interferon-tau. *Endocrinology* **143,** 2936-43.

Paria, B. C., Song, H., and Dey, S. K. (2001). Implantation: molecular basis of embryo-uterine dialogue. *Int J Dev Biol* **45,** 597-605.

Park, J. E., Keller, G. A., and Ferrara, N. (1993). The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* **4,** 1317-26.

Patterson, C., Perrella, M. A., Endege, W. O., Yoshizumi, M., Lee, M. E., and Haber, E. (1996). Downregulation of vascular endothelial growth factor receptors by tumor

necrosis factor-alpha in cultured human vascular endothelial cells. *J Clin Invest* **98,** 490-6.

Peters-Golden, M., and Brock, T. G. (2001). Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets. *FEBS Lett* **487,** 323-6.

Peterson, A. J., Fairclough, R. J., Payne, E., and Smith, J. F. (1975). Hormonal changes around bovine luteolysis. *Prostaglandins* **10,** 675-84.

Peterson, A. J., Tervit, H. R., Fairclough, R. J., Havik, P. G., and Smith, J. F. (1976). Jugular levels of 13, 14-dihydro-15-keto-prostaglandin F and progesterone around luteolysis and early pregnancy in the ewe. *Prostaglandins* **12,** 551-8.

Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., and Kerbel, R. S. (1997). Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* **151,** 1523-30.

Phipps, R. P., Stein, S. H., and Roper, R. L. (1991). A new view of prostaglandin E regulation of the immune response. *Immunol Today* **12,** 349-52.

Psychoyos, A. (1973). "Endocrine control of egg implantation." The Williams and Wilkins Co.,

Psychoyos, A. (1986). Uterine receptivity for nidation. *Ann N Y Acad Sci* **476,** 36-42.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* **386,** 671-4.

Risau, W., and Flamme, I. (1995). Vasculogenesis. *Annu Rev Cell Dev Biol* **11,** 73-91.

Robinson, C. J., and Stringer, S. E. (2001). The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J Cell Sci* **114,** 853-65.

Ryuto, M., Ono, M., Izumi, H., Yoshida, S., Weich, H. A., Kohno, K., and Kuwano, M. (1996). Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1. *J Biol Chem* **271,** 28220-8.

Sales, K. J., Maudsley, S., and Jabbour, H. N. (2004). Elevated prostaglandin EP2 receptor in endometrial adenocarcinoma cells promotes vascular endothelial growth factor expression via cyclic 3',5'-adenosine monophosphate-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase 1/2 signaling pathways. *Mol Endocrinol* **18,** 1533-45.

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219,** 983-5.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376,** 62-6.

Shi, J. J., Ma, X. H., Diao, H. L., Ni, H., Xu, L. B., Zhu, H., and Yang, Z. M. (2005). Differential expression of prostaglandin E receptor subtype EP2 in rat uterus during early pregnancy. *Histol Histopathol* **20,** 1021-8.

Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H., and Sato, M. (1990). Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene* **5,** 519-24.

Shie, J. L., Wu, G., Wu, J., Liu, F. F., Laham, R. J., Oettgen, P., and Li, J. (2004). RTEF-1, a novel transcriptional stimulator of vascular endothelial growth factor in hypoxic endothelial cells. *J Biol Chem* **279,** 25010-6.

Shifren, J. L., Tseng, J. F., Zaloudek, C. J., Ryan, I. P., Meng, Y. G., Ferrara, N., Jaffe, R. B., and Taylor, R. N. (1996). Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J Clin Endocrinol Metab* **81,** 3112-8.

Soker, S., Takashima, S., Miao, H. Q., Neufeld, G., and Klagsbrun, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92,** 735-45.

Song, J. H., Sirois, J., Houde, A., and Murphy, B. D. (1998). Cloning, developmental expression, and immunohistochemistry of cyclooxygenase 2 in the endometrium during embryo implantation and gestation in the mink (Mustela vison). *Endocrinology* **139,** 3629-36.

Sonoshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M., and Taketo, M. M. (2001). Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. *Nat Med* **7,** 1048-51.

Steven, D. (1975). "Anatomy of the placental barrier." Academic Press, New York.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* **12,** 599-606.

Sugano, Y., Matsuzaki, K., Tahashi, Y., Furukawa, F., Mori, S., Yamagata, H., Yoshida, K., Matsushita, M., Nishizawa, M., Fujisawa, J., and Inoue, K. (2003). Distortion of autocrine transforming growth factor beta signal accelerates malignant potential by enhancing cell growth as well as PAI-1 and VEGF production in human hepatocellular carcinoma cells. *Oncogene* **22,** 2309-21.

Sugino, N., Kashida, S., Karube-Harada, A., Takiguchi, S., and Kato, H. (2002). Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy. *Reproduction* **123,** 379-87.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87,** 1171-80.

Takagi, S., Kasuya, Y., Shimizu, M., Matsuura, T., Tsuboi, M., Kawakami, A., and Fujisawa, H. (1995). Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. *Dev Biol* **170,** 207-22.

Tanioka, T., Nakatani, Y., Semmyo, N., Murakami, M., and Kudo, I. (2000). Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. *J Biol Chem* **275,** 32775-82.

Tilley, S. L., Audoly, L. P., Hicks, E. H., Kim, H. S., Flannery, P. J., Coffman, T. M., and Koller, B. H. (1999). Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E2 receptor. *J Clin Invest* **103,** 1539-45.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. (1991). The human gene for vascular endothelial growth factor.

Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* **266,** 11947-54.

Van, D., Beerthuis, R. K., Nugteren, D. H., and Vonkeman, H. (1964). The Biosynthesis of Prostaglandins. *Biochim Biophys Acta* **90,** 204-7.

Vassaux, G., Gaillard, D., Ailhaud, G., and Negrel, R. (1992). Prostacyclin is a specific effector of adipose cell differentiation. Its dual role as a cAMP- and Ca(2+)-elevating agent. *J Biol Chem* **267,** 11092-7.

Von Euler, U. S. (1939). [Further studies of prostaglandin, the physically active substance of certain genital glands]. *Skand Arch Physiol* **81,** 65-80.

Wallach, E. E., Cruz, A., Hunt, J., Wright, K. H., and Stevens, V. C. (1975). The effect of indomethacin of HMG-HCG induced ovulation in the phesus monkey. *Prostaglandins* **9,** 645-58.

Wang, X., Su, Y., Deb, K., Raposo, M., Morrow, J. D., Reese, J., and Paria, B. C. (2004). Prostaglandin E2 is a product of induced prostaglandin-endoperoxide synthase 2 and microsomal-type prostaglandin E synthase at the implantation site of the hamster. *J Biol Chem* **279,** 30579-87.

Watabe, A., Sugimoto, Y., Honda, A., Irie, A., Namba, T., Negishi, M., Ito, S., Narumiya, S., and Ichikawa, A. (1993). Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. *J Biol Chem* **268,** 20175-8.

Wu, J., Richer, J., Horwitz, K. B., and Hyder, S. M. (2004). Progestin-dependent induction of vascular endothelial growth factor in human breast cancer cells: preferential regulation by progesterone receptor B. *Cancer Res* **64,** 2238-44.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407,** 242-8.

Yang, Z. M., Das, S. K., Wang, J., Sugimoto, Y., Ichikawa, A., and Dey, S. K. (1997). Potential sites of prostaglandin actions in the periimplantation mouse uterus: differential expression and regulation of prostaglandin receptor genes. *Biol Reprod* **56,** 368-79.

Yen, L., Benlimame, N., Nie, Z. R., Xiao, D., Wang, T., Al Moustafa, A. E., Esumi, H., Milanini, J., Hynes, N. E., Pages, G., and Alaoui-Jamali, M. A. (2002). Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. *Mol Biol Cell* **13,** 4029-44.

Yi, X. J., Jiang, H. Y., Lee, K. K., O, W. S., Tang, P. L., and Chow, P. H. (1999). Expression of vascular endothelial growth factor (VEGF) and its receptors during embryonic implantation in the golden hamster (Mesocricetus auratus). *Cell Tissue Res* **296,** 339-49.

Zhang, G., and Veldhuis, J. D. (2004). Requirement for proximal putative Sp1 and AP-2 cis-deoxyribonucleic acid elements in mediating basal and luteinizing hormone- and insulin-dependent in vitro transcriptional activation of the CYP17 gene in porcine theca cells. *Endocrinology* **145,** 2760-6.