

ABSTRACT

SUDI KSHYA PAUDEL. Temporal and Spatial Expression of Adrenomedullin and Its Receptors in the Porcine Uterus and Peri-implantation Conceptuses. (Under the direction of Dr. Xiaoqiu Wang).

Adrenomedullin (ADM) is an evolutionarily conserved multi-functional peptide hormone that regulates implantation, embryo spacing and placentation in humans and rodents. However, the potential roles of ADM in implantation and placentation of domestic animals, particularly pigs, as a litter-bearing species, are not known. This study investigated the mRNA and protein expressions of ADM and its receptor components that include calcitonin receptor-like receptor (CALCRL; G protein-coupled receptor bound by ADM), receptor activity modifying proteins (RAMP2 and RAMP3; dimerized with CALCRL, i.e., CALCRL/RAMP2 or CALCRL/RAMP3) and atypical chemokine receptor 3 (ACKR3; a decoy receptor that serves as a cell-autonomous molecular rheostat to dampen ADM signaling) in uteri from cyclic and pregnant gilts (Days 10-16) as well as conceptuses (embryonic/fetus and its extra-embryonic membranes) during the peri-implantation period of pregnancy (Days 10-16) when 30-40% of embryonic death loss occurs. Gilts (n=42) exhibiting at least two normal estrous cycles were bred via artificial insemination twice and then assigned randomly to be ovariectomized on Day 10, 11, 12, 13, 14, 15, or 16 of pregnancy (n=6 gilts/day; Day 0 is day of onset of estrus). Pregnancy was confirmed by the presence of morphologically normal conceptuses. Each uterine horn was flushed with 20 ml sterile PBS (pH 7.2). Conceptuses had expected morphological features, from spherical (Days 10 and 11), ovoid and/or tubular (Day 12) to filamentous forms (Days 13 to 16 of pregnancy). Based on the RNA sequencing data analysis the expression of ADM and its receptors CALCRL and RAMP2 were highly expressed on Day 12 of pregnant uterus. However, the expression of ACKR3 was decreased in the pregnant uterus as compared to cyclic uterus at Day 12. Immunohistochemical analyses revealed that the localization of ADM was only observed in the uterine LE of pregnant gilts

between Days 12 and 16, whereas CALCRL and RAMP2 were expressed in the uterine LE, glandular epithelia (GE) and stroma between Days 12 and 16 of pregnancy in gilts. In porcine conceptuses, expression of ADM, CALCRL and RAMP2 proteins increased in trophoctoderm cells between Days 12 and 16 of pregnancy. Further *in situ* hybridization showed that mRNA expression of *ADM*, *CALCRL*, *RAMP2* and *RAMP3* genes increased in the porcine conceptus trophoctoderm between Days 12 and 16 of pregnancy; whereas *ACKR3* mRNA increased between Days 13 and 14 of pregnancy but decreased thereafter in the conceptus trophoctoderm. These results indicate that ADM may play functional roles in uterine receptivity as well as survival, growth, and development of the porcine conceptus during the peri-implantation period of pregnancy. This research was supported by the Hatch project 1020014 from the USDA National Institute of Food and Agriculture.

© Copyright 2022 by Sudikshya Paudel

All Rights Reserved

Temporal and Spatial Expression of Adrenomedullin and Its Receptors in the Porcine Uterus and
Peri-implantation Conceptuses

by
Sudikshya Paudel

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Animal Science

Raleigh, North Carolina

2022

APPROVED BY:

Dr. Xiaoqiu Wang
Committee Chair

Dr. Daniel H. Poole

Dr. John Gadsby

DEDICATION

To my parents who showed me the value in education.

BIOGRAPHY

Sudikshya Paudel was born and raised on Chitwan, Nepal. After graduating from high school, Sudikshya started her undergraduate degree in Bachelor of Veterinary Science and Animal Husbandry (B.V.Sc. & A. H.) in Himalayan Institute of Agricultural Sciences and Technology, Kalanki, Kathmandu at Purbanchal University. In fall of 2018, Sudikshya came to United States to pursue her master's degree in Animal Science at North Carolina State University. Since then, she has been working on her research project under the supervision of Dr. Xiaoqiu Wang in the area of Physiology of Reproduction in pigs. The thesis involves the temporospatial expression patterns of Adrenomedullin, an evolutionarily conserved peptide hormone, in porcine uterus and conceptuses.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Xiaoqiu Wang for his guidance and professional advises during my academic program. Through his guidance I have learned the skills necessary to conduct the research in professional manner. This thesis would have not been possible without his continuous support.

I thank my co-workers Mr. Bangmin Liu and Ms. Magdalina Cummings for their help and support during my master's study. I appreciate the help and support that I received from my colleagues in the Department of Animal Science at North Carolina State University. I also thank the faculty and staff in the Department of Animal Science for all the help and for making this time memorable in my life.

At last, I would like to thank my husband, parents, brother, and Sister-in-law Sangeeta Adhikari for being a sister I never had, inspiration, and moral support to complete my master's degree.

TABLE OF CONTENTS

DEDICATION	ii
BIOGRAPHY	iii
ACKNOWLEDGMENTS	iv
CHAPTER 1 – LITERATURE REVIEW	1
Introduction	1
Physiology of Early Pregnancy in Gilts/Sows	2
Hormonal Patterns during Pregnancy of Gilts/Sows.....	4
Uterine Histotroph and Pregnancy	5
Adrenomedullin (ADM).....	6
Localization of ADM	6
Receptors of ADM	8
Expression of ADM in reproductive organs	9
Role of ADM in regulation of cell growth.....	11
Role of ADM as antiapoptotic factor	12
Role of ADM in nervous system.....	13
Role of ADM in female reproductive system	13
Role of ADM in male reproductive system	15
Role of ADM on fetal growth and development.....	16
Role of ADM in fertility and implantation.....	17
Role of ADM in placentation and maternal-fetal circulation.....	18
Role of ADM in pregnancy related complications.....	19
<i>ADM in gestational diabetes</i>	20
<i>ADM in preeclampsia</i>	21
<i>ADM and preterm birth</i>	22
<i>ADM and intrauterine growth restriction</i>	23
Conclusion.....	24
REFERENCES	25
CHAPTER 2 - DATA MINING REVEALS THAT THE EXPRESSION OF ADM IS INCREASED IN PORCINE ENDOMETRIUM DURING PREGNANCY	53
Abstract	53
Introduction	54
Materials and Methods	55

Results	59
Discussion	60
REFERENCES	62
CHAPTER 3 – TEMPORAL AND SPATIAL EXPRESSION OF ADRENOMEDULLIN AND ITS RECEPTORS IN PORCINE UTERUS AND PERI-IMPLANTATION CONCEPTUSES.	65
Abstract	65
Introduction	67
Material and Methods.....	68
Results	71
Discussion	73
REFERENCES	76
CHAPTER 4 – CONCLUSIONS AND FUTURE DIRECTIONS	86
REFERENCES	89

LIST OF FIGURES

Figure 1.	The amino acid sequence of ADM in different species, aligned for comparison.	6
Figure 2.	Gene expression of <i>ADM</i> and its receptors in pig endometrium based on RNA-seq data.	64
Figure 3.1	Localization of <i>ADM</i> mRNA in pregnant and cyclic porcine endometrium and conceptuses.	78
Figure 3.2	Localization of ADM protein in pregnant and cyclic porcine endometrium and conceptuses.	79
Figure 3.3	Localization of <i>CALCRL</i> mRNA in pregnant and cyclic porcine endometrium and conceptuses.	80
Figure 3.4	Localization of CALCRL protein in pregnant and cyclic porcine endometrium and conceptuses.	81
Figure 3.5	Localization of <i>RAMP2</i> mRNA in pregnant and cyclic porcine endometrium and conceptuses.	82
Figure 3.6	Localization of RAMP2 protein in pregnant and cyclic porcine endometrium and conceptuses.	83
Figure 3.7	Localization of <i>RAMP3</i> mRNA in pregnant and cyclic porcine endometrium and conceptuses.	84
Figure 3.8	Localization of <i>ACKR3</i> mRNA in pregnant and cyclic porcine endometrium and conceptuses.	85

CHAPTER 1 – LITERATURE REVIEW

Introduction

Embryonic mortality is a major constraint to reproductive performance in all mammalian species (Bazer, 1975). Estimated embryonic death loss in pigs, ruminants, and most mammals are 20-40%, with two-thirds of those losses occurring during peri-implantation period of pregnancy (Nancarrow, 1994; Spencer & Bazer, 2004). The successful establishment and maintenance of pregnancy requires appropriate development of the conceptus (embryonic/fetus and its extra-embryonic membranes) for pregnancy recognition signaling, which is critical for maintenance of the corpora lutea (CL) and their continued secretion of progesterone (P4) during pregnancy (Bazer, 1975; Spencer & Bazer, 2004). P4 is required for the development of an intrauterine environment that supports implantation, placentation and uterine functions essential for birth of healthy offspring (Spencer, Johnson, Bazer, et al., 2004). During the peri-implantation period of gestation in pigs and ruminants, conceptuses undergo dramatic morphological transitions from spherical to tubular to filamentous forms prior to implantation (Spencer & Bazer, 2004). Interactions among the conceptus and various uterine cell types, especially luminal (LE), superficial glandular (sGE) and glandular (GE) epithelia and stroma cells, are essential to conceptus development, signaling for maternal recognition of pregnancy, maintenance of uterine blood flow, water and electrolyte movement, transport of sugars and amino acids into the uterine lumen, as well as secretion of proteins by uterine epithelial cells. This orchestrated process is highly dependent on the composition of histotroph (Bazer et al., 2012; Bazer, Wang, et al., 2015; Bazer et al., 2014; Gao, Wu, et al., 2009a, 2009b, 2009c, 2009d, 2009e; Gao, Wu, Spencer, Johnson, Li, et al., 2009; J. Kim et al., 2011; J. Y. Kim et al., 2011a, 2011b; Kong et al., 2014; Lenis et al., 2018; Lenis et al., 2016; Wang, Burghardt, et al., 2015; Wang, Frank, Little, et al., 2014; Wang, Frank, Xu, et al.,

2014; Wang, Johnson, et al., 2015, 2016; Wang, Li, et al., 2016; Wang, Ying, et al., 2014), i.e., secretions from uterine LE, sGE, and GE as well as selective transport of nutrients into the uterine lumen, including enzymes, growth factors, adhesion proteins, cytokines, hormones, amino acids, glucose and fructose (Spencer, Johnson, Burghardt, et al., 2004). Individual conceptuses may fail to develop or not develop properly due to failure to respond to various components of histotroph that orchestrate events of successful growth and development of the conceptus, during the peri-implantation (Bazer, Wang, et al., 2015; Wang, Burghardt, et al., 2015; Wang, Frank, Little, et al., 2014; Wang, Frank, Xu, et al., 2014; Wang, Johnson, et al., 2015, 2016; Wang, Li, et al., 2016; Wang, Ying, et al., 2014). However, the underlying mechanisms for embryonic mortality are not clearly understood.

Physiology of Early Pregnancy in Gilts/Sows

Gilts (nulliparous females) and sows (parous females) are receptive for mating during estrus, which lasts for 24 to 72 h (Bazer & First, 1983). Ovulation occurs approximately 44 h after onset of estrus, and, if the female becomes pregnant, a gestation period of 114 to 115 days follows. After fertilization, porcine embryos are near the ampullary-isthmic junction of the oviduct. The four-cell stage embryos enter the uterus on Day 3 (60 to 72 h after onset of estrus) and reach the blastocyst stage by Day 5 of pregnancy (Geisert, Brookbank, et al., 1982; Geisert, Renegar, et al., 1982). The spherical blastocyst (0.5-1 mm in diameter) sheds the zona pellucida between Days 6 and 7 and further expands from 2-6 mm in diameter (early spherical blastocyst) to 9-10 mm in diameter (late spherical blastocyst) between Days 8 and 10. After this time, the development of porcine embryos diverges dramatically from that of rodents and primates, and the presumptive placental membranes (trophectoderm and extra-embryonic endoderm) elongate rapidly to a filamentous form by Day 16; i.e. the blastocyst transforms from late spherical (Day 10, 9-10 mm),

to tubular (Day 11, 11-50 mm), to early filamentous (Day 12, 100 mm), mid filamentous (Days 13 to 14, 100-200 mm) and late filamentous (Days 14 to 16, 800-1000 mm) form. It is during this period of morphological and functional transition that 30 to 40% of the conceptuses die due to a failure to elongate and establish extensive contact between trophoctoderm and uterine LE, that is required for uptake of secretions from uterine glands needed for survival of the conceptus (Bazer & Johnson, 2014).

After hatching from the zona pellucida, blastocysts undergo an initial expansion at about 0.25 mm/h between the early (Day 8, < 7 mm) and late (Day 10, 9-10 mm) spherical stages (Bazer & Johnson, 2014). Following that, there is a remarkable increase in the rate of elongation to 30-45 mm/h between late spherical (Day 10, 9-10 mm) and early filamentous (Day 12, 100 mm) stages, that occurs within a few hours, primarily due to an increase in cellular remodeling and hypertrophy (Geisert, Brookbank, et al., 1982). No differences in total DNA and a 40% reduction in mitotic index was observed in porcine conceptuses between Days 10 and 12, indicating that cellular hyperplasia does not account for initial elongation of pig blastocysts (Bazer & First, 1983; Bazer & Johnson, 2014). However, total DNA and RNA in pig conceptuses increase exponentially from Days 12 through 16, as blastocysts elongate from 100 to 1000 mm in length (Bazer & First, 1983; Bazer & Johnson, 2014; Geisert, Brookbank, et al., 1982). Coincident with initiation of blastocyst elongation is the initiation of estrogen production by porcine conceptuses with the peak production on Days 10 to 12 (Knight et al., 1977; Robertson & King, 1974; Stoner et al., 1986) that results in a marked increase in uterine secretions from both uterine GE and conceptus trophoctoderm, including but not limited to arginine (Arg), glutamine (Gln), glutamate (Glu) leucine (Leu), citrulline (Cit), ornithine (Orn), glycine (Gly), proline (Pro), SPP1, PGF2 α , PGE2, and

plasminogen activator (Bazer, Johnson, et al., 2015; Fazleabas et al., 1982; Geisert, Renegar, et al., 1982; Geisert, Thatcher, et al., 1982).

Hormonal Patterns during Pregnancy of Gilts/Sows

Hormonal events associated with the first 14 days of the estrous cycle and pregnancy are essentially identical in pigs. After that time, however, functional CL must be maintained for the duration of pregnancy. Loss of CL function at any stage of gestation leads to abortion within 24 to 36 h (Belt et al., 1971). Concentrations of progesterone (P4) in plasma are 30 to 40 ng/ml between Days 12 and 14 of pregnancy, decrease to 10 to 25 ng/ml by Day 25 of pregnancy (Guthrie et al., 1974; Knight et al., 1977; Robertson & King, 1974), and then remain fairly constant until about Day 100 of gestation. Then, P4 levels decline slowly to parturition (Day 114 to 115), when they decrease abruptly to less than 1 ng/ml. The aromatase activity to produce estradiol increases during Day 14 and 18 in porcine conceptus during pregnancy (Gadsby et al., 1980). Concentrations of estradiol (E2) and estrone (E1), both free and conjugated forms, in plasma change during pregnancy in pigs; however, E₁SO₄ is the predominant form of estrogens in plasma during pregnancy. Porcine blastocysts begin to produce estrogens at the 10 mm spherical stage (Bazer & First, 1983; Geisert, Renegar, et al., 1982). Estrone sulfate (E₁SO₄) in maternal plasma reflects secretion of estrogens by pig conceptuses that is triphasic with peaks on Day 10 (0.8 ng in uterine flushing) to 12 (3.4 ng in uterine flushing) (Geisert, Renegar, et al., 1982), 16 (60 pg/ml) to 30 (3 ng/ml), and 60 (35 pg/ml) to term (114 days; 3 ng/ml) (Knight et al., 1977; Robertson & King, 1974; Stoner et al., 1986). Those high concentrations of E₁SO₄ are coincidental with initiation of blastocyst elongation, expansion and development of the allantois, and rapid fetal growth, and preparation for parturition, respectively (Bazer & First, 1983; Bazer & Johnson, 2014).

Uterine Histotroph and Pregnancy

All mammalian uteri contain uterine glands that produce or selectively transport a complex array of proteins and other molecules known collectively as histotroph. Uterine glands are required for normal conceptus development (Bazer et al., 2008; Bazer et al., 2009; Spencer et al., 2007) as they are highly secretory during the luteal phase of the estrous cycle and throughout pregnancy in livestock species (Bazer & First, 1983; Bazer et al., 2009; Guillomot, 1995; Perry, 1981; Roberts & Bazer, 1988). Components of histotroph necessary for elongation and development of conceptuses are transported into the uterine lumen via specific transmembrane transporters, receptors, or by pinocytosis, to be taken up by conceptus trophoderm (Bazer et al., 2008; Bazer et al., 2009). In the Uterine Gland Knockout ewe model that lacks uterine glands and histotroph production, these animals fail to exhibit normal estrous cycles or maintain pregnancy beyond Day 14 (Gray et al., 2002; Kelleher et al., 2019). Female mice lacking uterine glands are infertile whereby the blastocysts fail to implant due to the interference of uterine secretions (Kelleher et al., 2017). Similarly in pigs, estrogen exposure for 2 weeks after birth, inhibits neonatal development of uterine glands (adenogenesis), thereby disrupting adult uterine function permanently with respect to reproductive performance (Bartol et al., 1993; Chen et al., 2010; Cooke et al., 2013; Tarleton et al., 2003). Because placentation is superficial in the pig, as it is in the cow, ewe, and mare, the direct transfer of nutrients or histotroph from endometrial surface and glandular epithelium to the chorionallantois occurs at least through the second trimester of gestation. Therefore, it is necessary to study the functional roles of histotroph including, but not limited to enzymes, growth factors, adhesion proteins, cytokines, hormones, amino acids, glucose and fructose, on growth and development of embryos/fetuses during implantation and throughout the placentation period of pregnancy.

Adrenomedullin (ADM)

Adrenomedullin (ADM) was first discovered in the human pheochromocytoma, a novel hypotensive peptide with a potent and long-lasting hypotensive effect (Kitamura et al., 1993). Distribution of adrenomedullin was examined with highest concentration on the pheochromocytoma with $1,900 \pm 450$ fmol/mg wet weigh, compared with that of normal adrenal medulla which contained 150 ± 24 fmol/mg. The concentration of adrenomedullin in lung and kidney is less than 1% of that of normal adrenal medulla but the total amount of adrenomedullin in lungs and kidney is considerably larger than the total amount in adrenal medulla (Kitamura et al., 1993). The protein encoding gene *ADM* encodes for the precursor preprohormone, preproadrenomedullin, which is 185 amino acids (AAs) long in human and mice (Kitamura et al., 1993; Sakata et al., 1993), and 188 AAs long in pigs (Kitamura et al., 1994). The matured product of ADM is composed of 52 amino acids in humans and pig (with only one amino acid difference), and 50 amino acids in mouse (**Figure 1**).

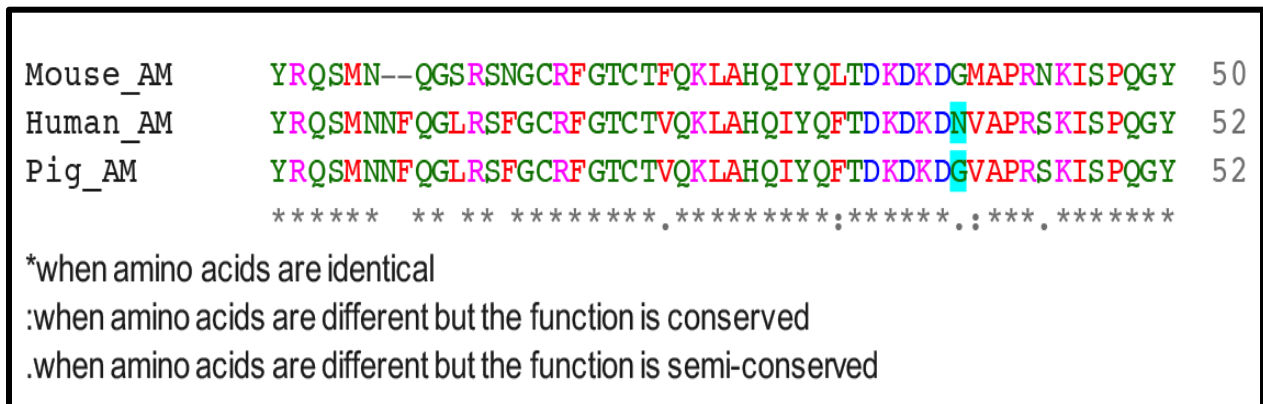


Figure 1. The amino acid sequence of ADM in different species, aligned for comparison.

Localization of ADM

Since ADM was first discovered, it has been identified in number of organ systems. ADM in human blood plasma serves as a circulating hormone that regulates the systemic blood pressure

(Ichiki et al., 1995; Kitamura et al., 1993). ADM is also found to be released from vascular endothelial cells and to act directly on the vascular smooth muscle cells in rats, humans, porcine, and bovine (Sugo et al., 1994). Expression of ADM is correlated directly with the increase in vascular density and endothelial cell proliferation in the leiomyomas of myometrium in humans (Hague et al., 2000), which may account for the vascular proliferation during the development of these structures. The *ADM* gene is also expressed in the human placenta and leukocytes (Trollmann et al., 2002). The upregulation of ADM is associated with hypoxic-ischemic complications of the fetus, suggesting that ADM may be a potential marker of asphyxia or hypoxia (Trollmann et al., 2002). The oviduct in rat produces large amounts of ADM which is believed to result in increased ciliary beat frequency, and decreased muscular contraction (Liao et al., 2010). ADM is highly expressed in the dental pulp stem cells during G2/S/M phase of cell division, and promotes the proliferation, and inhibits apoptosis, of dental pulp stem cells in humans (Zhu et al., 2016).

The localization of ADM and its receptors suggests that it has autocrine or paracrine modes of action (Montuenga et al., 1997). Signal transduction pathway for ADM's actions occurs via its interaction with its receptors (e.g. Calcitonin Receptor Like Receptor (CALCRL)), and Receptor Activity Modifying Proteins (RAMPs), which are coupled with G-proteins that activates the adenylate cyclase and increases the intracellular level of Cyclic adenosine monophosphate (cAMP) (McLatchie et al., 1998). It has been demonstrated that ADM elevates c-fos (a proto-oncogene protein product) expression and Activator Protein 1 (AP-1) binding activity in smooth muscle cells and myocytes (Sato & Autelitano, 1995). These effects could have been achieved via regulation of Protein Kinase A (PKA), Protein Kinase C (PKC) or Mitogen-Activated Protein Kinase (MAPK) signaling cascades.

Receptors of ADM

ADM belongs to calcitonin gene related peptides (CGRP) family. Other family members include calcitonin, amylin, intermedin (i.e., adrenomedullin 2) and calcitonin-receptor-stimulating peptide. Human ADM is composed of 52 AAs with a disulfide bond that forms a six membered ring structure similar to that found in CGRP and pancreatic amylin (Ichiki et al., 1995; Kitamura et al., 1993). ADM initiates signal transduction through the receptor complexes consisting of G-protein coupled receptor Calcitonin Receptor Like Receptor (CALCRL) and Receptor Activity Modifying Proteins (RAMPs), a family comprised of RAMP1, RAMP2 and RAMP3. Inactive CALCRL are cytosolic and the heterodimerization of the RAMP2 or RAMP3 with CALCRL results in activation and translocation of specific ADM receptor 1 (ADM₁: CALCRL/RAMP2) and/or ADM receptor 2 (ADM₂: CALCRL/RAMP3) to the plasma membrane (Hay et al., 2004). Heterodimerization of RAMP1 with CALCRL forms a CGRP receptor complex which can also bind to ADM, but with much lower affinity than that to AM1 and AM2 (Hay et al., 2004; McLatchie et al., 1998).

Another ADM receptor, ACKR3, was initially described as a regulator of the vasodilator peptides, calcitonin gene-related peptides (CGRP) and ADM (Kapas & Clark, 1995). ADM binds to ACKR3 with high affinity which suggested that ACKR3 could be an additional CGRP and ADM receptor (Quinn et al., 2018). A study by Autelitano and Tango determined that all of the ADM receptors were found to be expressed in rat lungs, although only ACKR3 was detectable in vascular smooth muscle cells (VCMC), suggesting that ACKR3 may be an important modulator of ADM function in VSMC (AUTELITANO & Tang, 1999). ACKR3 knock-out mice (*Ackr3*^{-/-}) mice had ventricular septal defects and semi-lunar heart valve malformations (Sierro et al., 2007). The haploinsufficiency of ADM in *Ackr3*^{-/-} mice reversed cardiac and lymphatic

hyperproliferation, demonstrating that ACKR3 could act as a decoy receptor, and is required as a molecular rheostat for controlling ADM ligand during embryonic development (Klein et al., 2014). Overexpression of the ADM ligand in *Adm^{hi/hi}* mice results in gross cardiac enlargement due to cardiac hyperplasia during embryogenesis, which closely phenocopies the dysmorphic cardiac hyperplasia of *Ackr3^{-/-}* mice. Moreover, although there are no changes in survival rates between wildtype and *Ackr3^{+/-}* mice, *Ackr3^{+/-}* mice with genetic *Adm* overexpression exhibit increased lethality further illustrating the critical importance of ACKR3 in titrating ADM levels. (Klein et al., 2014)

Expression of ADM in reproductive organs

In the normal human female reproductive tract, mRNA and protein levels of ADM and its receptors have been localized throughout all structures of the female reproductive system with marked expression in the epithelial cells of the uterus, fallopian tubes, and blood vessels (Nikitenko et al., 2000). The localization of ADM immunoreactivity in the uterus appears closely related to the menstrual phases in humans (Asada et al., 1999; Elkas et al., 2000; Michishita et al., 1999). During the proliferative phase there is a more intense cytoplasmic staining of the epithelial lining and the glandular endometrial cells than the stromal and myometrial elements (Cameron & Fleming, 1998; Jacobs et al., 1998; Laoag-Fernandez et al., 2000). Conversely, during the secretory phase, ADM staining increases in the stromal cells and decreases in the epithelial component, including the secreting uterine glands. The myometrium exhibits cytoplasmic immunoreactivity in the proliferative phase that becomes nuclear staining in the secretory phase (Jacobs et al., 1998). However, Asada and co-workers (1999) did not observe ADM immunoreactivity in the myometrium, but did find prominent ADM immunostaining in both the

glands and the epithelium of the endocervix. The Fallopian tube epithelium and stroma also demonstrated cyclic regulation of ADM expression (Asada et al., 1999; Jacobs et al., 1998).

In addition, ADM expression increases in uterus of rat during normal pregnancy (Upton et al., 1997). In the rat ovary, many cell types show ADM positive staining, including the granulosa and thecal cells, cells of the corpus luteum, and the germinal epithelium (Abe et al., 1998). ADM expression in the granulosa lutein-containing cells increases from mid-luteal phase to early pregnancy in human (Abe et al., 2000; Moriyama et al., 2000). ADM expression has also been found highly expressed in human and rat placenta, particularly in the cytotrophoblast and syncytiotrophoblast cells (Marinoni et al., 1998; Montuenga et al., 1998; Montuenga et al., 1997; Morrish et al., 1996). Hayashi et al reported mRNA and protein expression of ADM and its receptor-related genes CALCRL, RAMP2 and RAMP3 in the bovine uteroplacental tissues between days 25 and 250 of pregnancy. ADM and its receptors are expressed in uterine caruncles, inter-caruncular endometrium, extra embryonic membrane including cotyledonary villi and inter-cotyledonary chorion throughout the pregnancy with two peaks of expression on days 60 and 200 of pregnancy (Hayashi et al., 2013).

ADM is also present in the mammary glands, particularly in the epithelia of small and large ducts and in the terminal end buds of glands (Asada et al., 1999; Jahnke et al., 1997). ADM immunoreactivity was also found in the milk present in the ducts, suggesting a secretion of the peptide into the milk. This observation was later confirmed by Western blotting and RIA (Jahnke et al., 1997; Pio et al., 2000). ADM receptors have been identified in almost all regions of the female reproductive system (Jacobs et al., 1998), particularly in follicular and corpus luteum cells (Abe et al., 2000). CALCRL and RAMP proteins have been reported in human endometrial endothelial cells (Nikitenko et al., 2001) and in rat uterus (Yanagita et al., 2000), and the number

of ADM receptors has been shown to increase in pregnancy (Upton et al., 1997). ADM receptors are also expressed in placental and umbilical arteries in human (Apodaca et al., 2000) and in the mammary glands in mouse (Jahnke et al., 1997).

The presence of ADM in the male reproductive system seems to be less conspicuous than in the female reproductive system. ADM immunoreactivity was reported in testis of rats in some studies (Montuenga et al., 1998; Sakata et al., 1994; Shoji et al., 1995), but was not found in other studies (Cameron & Fleming, 1998). According to Asada et al. (1999) ADM immunoreactivity was present in ductal epithelia of epididymis but was absent from the seminiferous tubules. ADM and ADM receptors have been described in testicular peritubular myoid cells (Rossi et al., 2000; Santiemma et al., 2001) and ADM receptors have been found in vas deferens of guinea pig (Poyner et al., 1999). The highest expression of ADM in the male reproductive tract occurs in the prostate gland (Asada et al., 1999; Jiménez et al., 1999). ADM protein and mRNA are widespread in prostatic glandular epithelia of human and rats, whereas proadrenomedullin N-terminal 20 peptide (PAMP) immunoreactivity is restricted to neuroendocrine cells of the utriculus, where they colocalize with chromogranin and serotonin (Jiménez et al., 1999; Pewitt et al., 1999). ADM expression in prostatic glands in rats is induced by androgens (Pewitt et al., 1999).

Role of ADM in regulation of cell growth

Both stimulation and inhibition of cell proliferation have been reported upon exposure to synthetic ADM. This differential behavior may be cell context dependent. For instance, ADM stimulates proliferation of the zona glomerulosa cells of the adrenal cortex through CGRP1 receptors and activation of MAPK (Andreis et al., 2000; Nussdorfer, 2001; Semplicini et al., 2001), whereas PAMP did not have any effect (Nussdorfer, 2001). ADM also acts as a growth factor for normal skin (Martínez et al., 1997) and human oral keratinocytes (Kapas et al., 1997). ADM, but

not PAMP, is involved in C6 glioma cell proliferation (Moody et al., 1997) and has proliferative effects in the Swiss 3T3 fibroblast cell line (Isumi et al., 1998; Withers et al., 1996) and in many tumor cells lines (Miller et al., 1996). ADM induces osteoblast proliferation, probably through an interaction with amylin receptors (Cornish et al., 2001; Cornish et al., 1997; Naot et al., 2001). However, the antiproliferative action of ADM has also been found in rat cardiac fibroblasts (Horio et al., 1999; Tsuruda et al., 1999) and in mesangial cells (Plank et al., 2005). ADM is also involved in proliferation reduction of vascular smooth muscle cells (Dunzendorfer et al., 2000; Horio et al., 1999; Kano et al., 1996; Tsuruda et al., 1999) cultured cardiomyocytes (Tsuruda et al., 1998), and human teratoblastoma cells (Moody et al., 2000). The growth effects of PAMP are poorly understood because few studies have focused on this peptide. PAMP reduces growth of human neuroblastoma cells, TGW by inhibiting N-type Ca²⁺ channels through a pertussis toxin-sensitive G-protein (Ando et al., 1997). Although ADM-induced inhibition of endothelial cell growth was reported in a single case (Michibata et al., 1998), ADM potential for inducing growth in endothelial cells has been reported (Zhao et al., 1998). The second possibility of inducing growth seems to be more relevant in light of the many reports on the angiogenic abilities of ADM in both normal and pathological situations, especially as studied in endometrial tissues (Hague et al., 2000; Kim, 1999; Nikitenko et al., 2000; Oehler et al., 2001; Tomikawa et al., 1999; Zhao et al., 1998).

Role of ADM as antiapoptotic factor

ADM is also an antiapoptotic survival factor. This was first observed in rat endothelial cells, where ADM reduces serum deprivation-induced apoptosis via a cAMP independent mechanism (Kato et al., 1997). It was later reported that this action was also cGMP independent (Sata et al., 2000). The up regulation of the Max gene product in an autocrine or paracrine manner is the mechanism proposed to explain the antiapoptotic activity of ADM (Shichiri et al., 1999). In

addition, ADM increased survival of hypoxia-induced apoptosis in Ishikawa cells (a cell line established from human endometrial adenocarcinoma) overexpressing this regulatory peptide, and this effect was concomitant with an elevation in B-cell lymphoma 2 (Bcl-2) levels (Oehler et al., 2001).

Role of ADM in nervous system

There are several studies that reports the presence of ADM in the pituitary (Cameron & Fleming, 1998; Ichiki et al., 1995; Nussdorfer, 2001; Nussdorfer et al., 1997; Sakata et al., 1994). ADM concentration in pituitary extracts was higher than in the brain (Hwang & Tang, 1999; Takahashi, 2001; Takahashi et al., 1997). Immunocytochemical studies in several mammalian species, including humans, have shown a widespread expression of ADM in the adenohypophysis and the neural lobe, whereas the intermediate lobe showed a lower amount (Asada et al., 1999; Montuenga-Badia et al., 2000). ADM is also present in the organ, colocalizing with follicle-stimulating hormone (FSH) in the gonadotrophs of mammals (Montuenga-Badia et al., 2000). In addition, ADM-positive nerve fibers have been observed in the hypothalamus-neurohypophyseal tract (Ueta et al., 1999).

Role of ADM in female reproductive system

A study in rats by Li et al., has shown increase of *ADM* gene expression during the development of ovarian follicles from small antral follicle, large antral follicle and Corpus Luteum (CL) (Li et al., 2011). ADM also regulated the production of progesterone in CL. The regulation was dependent on the stage of pregnancy, inhibitory at early and late pregnancy but stimulatory during the mid-pregnancy. ADM increased the ciliary beat frequency in the oviduct of rats and relaxes the basal tone of the oviduct (Liao et al., 2010). The haploinsufficiency of the ADM and its related genes leads to reduced fertility, defects in implantation, placentation, and fetal growth.

The *Adm*^{+/-} female mice showed reduced pinopode numbers on the uterine luminal epithelial surface of mice (Li et al., 2008), which might be the cause of poor uterine receptivity. While studying the knockdown of *Adm* gene in mice, homozygous *Adm*-null embryos of the mice die in mid gestation with extreme hydrops fetalis and cardiovascular abnormalities (Caron & Smithies, 2001). The functional role of adrenomedullin was studied in genetically reduced ADM expression heterozygous *Adm*^{+/-} mice. The heterozygous *Adm*^{+/-} mice had reduced fertility, with the *Adm*^{-/-} embryos were more affected than those of *Adm*^{+/-} and *Adm*^{+/+} embryos (Li et al., 2006). Embryos of the most affected genotypes, had defects in the trophoblast cell invasion and leading to impaired placentation and fetal growth and fetal spacing leading to decreased fertility.

By prenatal Day 8 (E8) of mouse development, ADM was intensively expressed in the decidual cells and chorionic plate of the placenta and in the endodermal cells of the yolk sac, whereas the embryonic tissues were almost totally devoid of immunoreactive ADM. *ADM* mRNAs was particularly abundant in trophoblastic giant cells (Montuenga et al., 1998; Montuenga et al., 1997). The developing heart was the first organ that expressed ADM in the embryo proper. In addition, it was the organ that showed higher levels of immunoreactivity through all stages of mouse (Montuenga et al., 1998) and rat (Montuenga et al., 1997). ADM is indispensable for normal heart development, and its absence results in serious cardiovascular abnormalities causing embryos to die in mice (Caron & Smithies, 2001).

A strong expression of ADM was found in uterus, ovary, and anterior pituitary and some fluctuations of the peptide along the menstrual cycle have been described, pointing to important roles for ADM in female reproductive physiology (Cameron & Fleming, 1998; Marinoni et al., 2000; Montuenga-Badia et al., 2000). In in vitro fertilization studies, elevations of ADM content in follicular fluid have been found during the late follicular phase and have been suggested to be a

potential marker of decreased ovarian response (Manau et al., 1999; Manau et al., 2000). These results have also been interpreted as being part of a hypothalamus-pituitary-ovary feedback mechanism (Marinoni et al., 2000). PAMP was localized together with FSH in the secretory granules of pituitary gonadotropic cells, suggesting a role for this peptide in regulation of folliculogenesis (Montuenga-Badia et al., 2000). Effects of ADM on the uterus include vasodilatation of local vessels, uterine smooth muscle relaxation, angiogenesis, antiapoptotic actions, and antimicrobial activities (Hague et al., 2000; Nikitenko et al., 2000; Oehler et al., 2001). During a later period of human pregnancy, ADM contributes to the maintenance of uterine quiescence through CGRP receptors in myometrial cells (Casey et al., 1997; Di Iorio et al., 1998). ADM plasma levels increase dramatically during normal pregnancy (Marinoni et al., 1997; Martinez et al., 1999). In pregnant rats, ADM levels are elevated by progesterone and they seem to contribute to the maintenance of a vasodilatation state as an adaptation to the needs of pregnancy (Jerat & Kaufman, 1998). These elevated levels of ADM expression can also be appreciated in fetoplacental tissues, amniotic fluid, and umbilical plasma during normal pregnancy (Marinoni et al., 1998). ADM could also be responsible for the reduction in stretch-induced ANF release observed during pregnancy (Kaufman & Deng, 1998).

Role of ADM in male reproductive system

The unique function of ADM in the male reproductive system, which has been well documented, concerns penile erection. Intracavernosal injection of ADM induces an increase of penile blood flow and penile erection in normal cat specimens (BIVALACQUA et al., 1998; Ozbek et al., 2000). ADM potency on penile erection is lower than the potency exhibited by VIP, but higher than that exhibited by nociceptin, CGRP, and several other substances (Champion, Wang, Hellstrom, et al., 1997). This ADM action is performed through CGRP receptors and is not

mediated by nitric oxide or opening of K⁺-ATP channels (Champion, Wang, Shenassa, et al., 1997). ADM, PAMP, and their receptors have been found in rat and human prostate glands (Jiménez et al., 1999).

Role of ADM on fetal growth and development

Three independent groups have published data on ADM knockout mice (Caron & Smithies, 2001; Shimosawa et al., 2002; Shindo et al., 2001). In all cases, the embryos of animals homozygous for the deletion died in mid-gestation, indicating the indispensable role of ADM in fetal morphogenesis and maintenance of pregnancy. The first report came from the University of North Carolina at Chapel Hill (Caron & Smithies, 2001) and these mice were obtained by replacing the ADM gene-coding sequence with a DNA fragment encoding enhanced green fluorescence protein. The homozygous null animals developed extreme hydrops fetalis and cardiovascular abnormalities before dying. Some of these modifications included overdeveloped ventricular trabeculae and thinner arterial walls. In a second knockout experiment, Shindo et al. (2001) reported similar findings regarding the lethality of the deletion of the ADM gene. Nevertheless, in this case, the most striking abnormality in the knockout mice was the presence of severe hemorrhage, both under the skin and in internal organs. Electron microscopy studies of the blood vessels of young embryos showed a defect in the basement membrane of the capillaries that allowed efflux of blood cells to the interstitial space. Interestingly, heterozygous animals were able to survive to adulthood. These mice exhibited elevated blood pressure and lower nitric oxide levels than normal animals. In both these studies the whole gene was knocked out and therefore production of both ADM and PAMP was interrupted. In a very recent study, only ADM was deleted, allowing normal production of PAMP (Shimosawa et al., 2002). Here, again, the homozygous null animals were not viable whereas the heterozygous mice survived. When the

heterozygous animals were subjected to stressful conditions including treatment with angiotensin II or a high-salt diet, they developed perivascular fibrosis and intimal hyperplasia in the coronary arteries. These data strongly support the hypothesis that ADM acts as a protective agent against cardiovascular damage.

Role of ADM in fertility and implantation

An important role of ADM in fertility and implantation has come from well-characterized animal models. Findings have implicated ADM in even the earliest stages of pregnancy. Li et al., showed that in a rat model, ovarian ADM expression increases from small antral follicles to large antral follicles to the formation of the corpus luteum, and ADM appears to be involved in the regulation of progesterone production from the corpus luteum (Li et al., 2001). ADM also increases ciliary beat frequency and reduces contraction in the rat oviduct, pointing to a role for ADM-mediated regulation of embryo transport to the uterus (Liao et al., 2010). Expression of ADM and its receptor components are induced in the luminal epithelium of the murine uterus as early as pregnancy Day 0.5. By the peri-implantation period, ADM is expressed both by the blastocyst trophectoderm and the uterine luminal epithelium and stroma at the implantation site (Li et al., 2008). Therefore, the peptide is abundantly expressed throughout the female reproductive tract from the earliest stages of pregnancy. Homozygous deletion of *Adm* results in embryonic lethality with abnormal development of the heart and lymphatic vascular system (Fritz-Six et al., 2008). However, female mice heterozygous for *Adm* (50% ADM expression) survive and are a very useful model for the study of haploinsufficiency of ADM during pregnancy. *Adm*^{+/-} female mice have a significantly reduced pregnancy success rate compared to wild type females, even though ovulation and fertilization occurs normally in these mice. This reduced pregnancy rate persists even when wild type blastocysts are transferred to the *Adm*^{+/-} female, indicating that reduced

maternal ADM is responsible for the uterine receptivity defects in this model (Li et al., 2006). Furthermore, *Adm*^{+/-} female mice have reduced numbers of uterine pinopodes (referred to as uterodomes in humans), which are plasma membrane extravasations of the uterine luminal epithelium that faithfully mark the window of uterine receptivity (Li et al., 2008). Even when implantation is successful in the *Adm*^{+/-} female, fertility defects persist. The litter sizes of *Adm*^{+/-} female mice are reduced when mated to wild type males, while normal litter sizes are born to wild type females mated to *Adm*^{+/-} males. The implantation sites in pregnant *Adm*^{+/-} females are abnormally spaced and overcrowded, resulting in increased rates of embryo loss and remarkable subfertility (Li et al., 2006). Therefore, even a modest 50% reduction in maternal expression of ADM is sufficient to cause major implantation and fertility complications in genetic mouse models. This strong genetic and physiological evidence from rodent models is beginning to be translated to clinical medicine as well. For example, Marinoni et al. has found that elevated ADM levels in follicular fluid is associated with negative outcomes in in vitro fertilization patients (Marinoni et al., 2010).

Role of ADM in placentation and maternal-fetal circulation

One of the most essential maternal responses to pregnancy is the vascular remodeling of uterine spiral arteries, which ensures adequate blood flow to the developing fetus. The development of the placenta is central to this process. The earliest stages of placental development in humans and rodents occur during implantation, when trophoctoderm cells from the blastocyst attach and invade into the wall of the receptive uterus. These trophoctoderm cells differentiate into multinucleate trophoblast cells termed extravillous cytotrophoblasts in humans and giant trophoblast cells in rats and mice, which invade the uterine lining and establish the vascular connection between fetal placental tissue and the maternal blood supply (Lee & DeMayo, 2004).

High ADM expression is present in the trophoctoderm cells and persists in trophoblast giant cells in the mouse (Li et al., 2006), and positive staining for ADM expression in the extravillous cytotrophoblast lineage has been shown in the normal term placenta in humans (Nikitenko et al., 2001). Support for a role for ADM in trophoblast invasion has come from in vitro studies. Zhang et. al. showed that ADM induces proliferation and invasion in JAr cells, a choriocarcinoma cell line, and in HTR-8/SV neo cells, a first trimester cytotrophoblast cell line (Zhang et al., 2005). In isolated fetoplacental vascular beds and stem villous arteries previously constricted with a thromboxane sympathomimetic, ADM infusion induces a dose-dependent vasodilation, suggesting that ADM may help maintain low placental vascular resistance (Hoeldtke et al., 2000). Ross et al., found that ADM treatment in rats induces relaxation of the uterine artery and this effect is enhanced in pregnancy or with estradiol treatment, providing further evidence for a functional role for ADM in maintaining vascular tone in pregnancy (Ross et al., 2010). In women with unexplained recurrent pregnancy loss, high plasma ADM was associated with increased uterine artery pulsatility index and an increased number of previous miscarriages, from which the authors suggest that increased ADM may be acting in a compensatory role (Senna et al., 2008).

Role of ADM in pregnancy related complications

Pre-eclampsia is a disease characterized by hypertension in the placenta. There are several studies on the variations of ADM in this disease, but a great deal of controversy remains as to whether ADM levels increase or decrease in these patients. One study based on samples taken from amniotic fluid and umbilical vein plasma reported that ADM seems to be increase locally in fetal fluid of pre-eclamptic patients. It was concluded that ADM is necessary to maintain placental (LOW?) vascular resistance and fetal circulation at physiological levels (Di Iorio et al., 1998). In contrast, another study found decreased ADM synthesis in syncytiotrophoblasts and no differences

in the amnion and extravillous trophoblast cells (Kanenishi et al., 2000). There is also variability in the results of experiments that studied ADM plasma levels. Again, some studies reported elevations (Lauria et al., 1999) or diminutions (Hata et al., 1997) in the levels of ADM in pre-eclamptic patients; however, other reports showed no differences in ADM plasma levels between women with pre-eclamptic and normal pregnancies (Jerat et al., 2001; Minegishi et al., 1999). In animal models of preeclampsia, there was an increase in ADM plasma levels. These levels increased as well in fetal membranes and in blood taken from the umbilical artery (Lü et al., 1999; I. Makino et al., 1999; Y. Makino et al., 1999). ADM has also been associated with some fetal disorders. ADM and its receptors have been implicated in pregnancies complicated by oligohydramnios, a fetoplacental response to vascular compromise (Apodaca et al., 2000). In addition, fetuses with intrauterine growth restriction and abnormal umbilical artery function have also shown a marked elevation of ADM plasma levels as a response to reduced utero-placental blood flow (Di Iorio et al., 2000). Also, there are some indications that absence of ADM may induce nonimmune hydrops fetalis (Caron & Smithies, 2001).

Defects in the ability of trophoblast cells to fully invade the maternal uterine wall and remodel vessels are thought to underlie many serious reproductive conditions (Chaddha et al., 2004; Lala & Chakraborty, 2003). Given that female mice heterozygous for *Adm* exhibit marked subfertility, and that homozygous deletion of *Adm* causes embryonic lethality, it is not surprising that altered ADM expression has been associated with several of these pregnancy complications.

ADM in gestational diabetes

To date, there is relatively limited data available on whether ADM levels are altered in gestational diabetes. Martinez et al. showed no change in plasma ADM levels in pregnant women with gestational diabetes, compared to pregnant women without gestational diabetes (Martinez et

al., 1999). Di Iorio et al. also found that ADM levels were not changed in the maternal circulation, but found higher ADM levels in the amniotic fluid of pregnant diabetic women (Di Iorio et al., 2001). Plasma ADM was found to be unchanged in pregnant women with type I diabetes mellitus (T1DM) (Loukovaara et al., 2005), and in a subsequent study ADM was found to be unchanged in the plasma of women with gestational diabetes (Pöyhönen - Alho et al., 2010). These data suggest that circulating ADM may not be altered in pregnant women with gestational diabetes, but fetal or placental ADM production may be elevated, potentially resulting in the observed increased amniotic fluid ADM concentration.

ADM in preeclampsia

Based on the known roles for ADM in trophoblast invasion and vascular adaptation to pregnancy, there is significant interest in determining whether changes in ADM peptide or expression levels contributes to the pathogenesis of preeclampsia (PE). Results from an experimental rat model of maternal hypertension further piqued this interest. In this model, whereby administration of the inhibitor of nitric oxide synthases L-NAME (nitro-L-arginine methyl ester) during gestation, results in hypertension and pup mortality, maternal infusion of ADM attenuated the hypertensive phenotype (I. Makino et al., 1999). However, results from human studies have been highly variable and controversial. One of the earliest studies examining ADM levels in PE, by Hata et al. found that circulating ADM levels were reduced in women with PE (Hata et al., 1997). But a multitude of other studies have found markedly variable results. For example, studies have shown either decreased (Boć-Zalewska et al., 2011; Kanenishi et al., 2000; Knerr et al., 2002) or increased (Gratton et al., 2003) ADM mRNA levels in placental tissues of preeclamptic (PE) patients. Attempts to look at ADM protein levels have been equally variable. ADM peptide production from purified cytotrophoblast cultures of preeclamptic patients was

shown to be decreased (Li et al., 2003), and the majority of studies looking at plasma levels of ADM have been inconsistent, showing both increased (Boć-Zalewska et al., 2011; Di Iorio et al., 1998; Lauria et al., 1999; Senna et al., 2008), decreased (Dikensoy et al., 2009), and unchanged (Jerat et al., 2001; Minegishi et al., 1999) ADM levels in PE. Al-Ghafra et al. attempted to clarify the role of ADM in PE by limiting their study to patients with severe PE and by separating patients by term versus preterm delivery; they found that ADM protein levels were increased in fetal membranes in PE patients with both term and preterm gestation, and ADM mRNA levels were also increased in preterm choriodecidual tissue in PE patients (Al-Ghafra et al., 2006). Though there is strong evidence for altered ADM levels being either a cause or a secondary effect in PE, it is clearer that more controlled experiments to address the direction of the change and the exact role of ADM PE are needed.

ADM and preterm birth

It has long been thought that altered ADM levels may be present in cases of preterm birth. Human studies dating back over 10 years ago have suggested that ADM protein may be elevated in patients with preterm birth. The Di Iorio laboratory has published several studies on this subject, finding increased amniotic ADM levels in cases of preterm premature rupture of membranes (PPROM) (Di Iorio et al., 1999), and increased amniotic fluid ADM in patients with preterm labor (Di Iorio et al., 2001). Elevated second trimester amniotic fluid levels of ADM have also been reported in patients that go on to preterm deliveries (Yamashiro et al., 2002). Glucocorticoids may be involved in the regulation of ADM levels in pregnancy, as administration of betamethasone to stimulate fetal lung maturity in patients at risk for preterm birth resulted in significantly increased maternal and fetal plasma ADM levels (Marinoni et al., 2006). However, a 2009 study by Iavazzo et al. found that there was no change in ADM levels in spontaneous preterm delivery or preterm

premature rupture of membrane (PPROM) (Iavazzo et al., 2009), suggesting that further studies may still be needed in this area. In addition to genetic and environmental causes, it is interesting to speculate as to whether ADM may play a role in another major cause of preterm birth – ascending intrauterine bacterial infections, which are thought to account for 25–40% of preterm births (Goldenberg et al., 2008). The spread of microorganisms to the amniotic cavity causes the activation of toll-like receptors and the release of proinflammatory cytokines, leading to increased prostaglandins and the stimulation of premature uterine contractions and degradation of fetal membranes (Challis et al., 2009; Romero et al., 2007). There are multiple characteristics of ADM that implicate it as a regulator of innate immunity and host defense (Zudaire et al., 2006). These include its six-member cysteine ring structure which is a characteristic of both human and murine beta-defensins (Bauer et al., 2001) and which allows these molecules to penetrate bacterial cell membranes, and the known anti-inflammatory effects of ADM (Christiaens et al., 2008). The potent bactericidal properties of ADM (Allaker et al., 2006) and its high level of expression in the skin, oral cavity cervical mucosa, fetal membranes, and breast milk (Allaker et al., 1999; Trollmann et al., 2002) also support its role as a possible mediator of host defense.

ADM and intrauterine growth restriction

Numerous rodent models have provided evidence for the necessity of ADM in normal fetal growth. Yallampalli's laboratory found that antagonism of ADM during pregnancy resulted in intrauterine growth restriction (IUGR), abnormal placental vascularization, and increased fetal resorption, in the rat (Witlin, 2002). Similar studies in the mouse have shown that *Adm*^{+/-} mothers have a high rate of fetal growth restriction, which occurs in all fetal genotypes. The incidence of fetal growth restriction was highest among *Adm*^{-/-} embryos, indicating that both maternal and fetal ADM may contribute to normal fetal growth (Li et al., 2006). Results from human studies have

not been as consistent. Two early studies found that elevated ADM levels in the umbilical plasma and amniotic fluid, respectively, were associated with reduced fetal growth (Di Iorio et al., 2000; Yamashiro et al., 2002), which could be a compensatory effect. However, a 2001 study by Yamashiro et al. and a more recent study by Akturk et al. showed no difference in ADM concentrations between small for gestational age and appropriate for gestational age infants (Akturk et al., 2007; Yamashiro et al., 2001). Based on animal studies, it is likely that altered ADM levels may contribute to either the development of IUGR or the resulting adaptive compensation to other primary causes of IUGR. However, the inconsistencies between studies in the human population points to the necessity of further studies to determine with certainty how changes in ADM levels may be involved in the pathogenesis of growth restricted pregnancies.

Conclusion

Given that ADM is critical for implantation, placentation and spacing of blastocysts in human and rodents, it is imperative that the function and regulation of ADM as related to uterine receptivity, as well as growth and development of conceptus during the pregnancy in all domestic animals be established. This is particularly relevant for pigs as they are the litter bearing species with high incidence of early embryonic death (30-40%), naturally occurring intrauterine growth restriction, and frequent occurrences of stillborn piglets (3-9%) (Bazer et al., 2014).

REFERENCES

- Abe, K., Minegishi, T., Ibuki, Y., Kojima, M., & Kangawa, K. (2000). Expression of adrenomedullin in the human corpus luteum. *Fertility and sterility*, *74*(1), 141-145.
- Abe, K., Minegishi, T., Tano, M., Hirakawa, T., Tsuchiya, M., Kangawa, K., Kojima, M., & Ibuki, Y. (1998). Expression and effect of adrenomedullin on rat granulosa cell. *Endocrinology*, *139*(12), 5263-5266.
- Akturk, A. m., Onal, E. E., Atalay, Y., Yurekli, M. t. n., Erbas, D. z., Okumus, N., Turkyilmaz, C., Unal, S., Ergenekon, E., & Koc, E. n. (2007). Maternal and umbilical venous adrenomedullin and nitric oxide levels in intrauterine growth restriction. *The Journal of Maternal-Fetal & Neonatal Medicine*, *20*(7), 521-525.
- Al-Ghafra, A., Gude, N. M., Brennecke, S. P., & King, R. G. (2006). Increased adrenomedullin protein content and mRNA expression in human fetal membranes but not placental tissue in pre-eclampsia. *Molecular human reproduction*, *12*(3), 181-186.
- Allaker, R. P., Grosvenor, P. W., McAnerney, D. C., Sheehan, B. E., Srikanta, B. H., Pell, K., & Kapas, S. (2006). Mechanisms of adrenomedullin antimicrobial action. *Peptides*, *27*(4), 661-666.
- Allaker, R. P., Zihni, C., & Kapas, S. (1999). An investigation into the antimicrobial effects of adrenomedullin on members of the skin, oral, respiratory tract and gut microflora. *FEMS Immunology & Medical Microbiology*, *23*(4), 289-293.
- Ando, K., Omi, N., Shimosawa, T., & Fujita, T. (1997). Proadrenomedullin N-terminal 20 peptide (PAMP) inhibits proliferation of human neuroblastoma TGW cells. *FEBS letters*, *413*(3), 462-466.

- Andreis, P. G., Markowska, A., Champion, H. C., Mazzocchi, G., Malendowicz, L. K., & Nussdorfer, G. G. (2000). Adrenomedullin enhances cell proliferation and deoxyribonucleic acid synthesis in rat adrenal zona glomerulosa: receptor subtype involved and signaling mechanism. *Endocrinology*, *141*(6), 2098-2104.
- Angel, P., & Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, *1072*(2-3), 129-157.
- Apodaca, C. C., Moore, K. H., Rossignol, T. M., Pierce, B., Matej, L. A., Hume Jr, R. F., & Calhoun, B. C. (2000). Localization of messenger ribonucleic acid for adrenomedullin and adrenomedullin receptor in the human placenta in normal pregnancies and pregnancies complicated by oligohydramnios. *American journal of obstetrics and gynecology*, *183*(5), 1213-1219.
- Asada, Y., Hara, S., Marutsuka, K., Kitamura, K., Tsuji, T., Sakata, J., Sato, Y., Kisanuki, A., Eto, T., & Sumiyoshi, A. (1999). Novel distribution of adrenomedullin-immunoreactive cells in human tissues. *Histochemistry and cell biology*, *112*(3), 185-191.
- AUTELITANO, D. J., & Tang, F. (1999). Co-expression of prepro-adrenomedullin with a putative adrenomedullin receptor gene in vascular smooth muscle. *Clinical Science*, *96*(5), 493-498.
- Bartol, F. F., Wiley, A. A., Spencer, T. E., Vallet, J. L., & Christenson, R. K. (1993). Early uterine development in pigs. *J Reprod Fertil Suppl*, *48*, 99-116.
<https://www.ncbi.nlm.nih.gov/pubmed/8145217>
- Bauer, F., Schweimer, K., Klüver, E., Conejo - Garcia, J. R., Forssmann, W. G., Rösch, P., Adermann, K., & Sticht, H. (2001). Structure determination of human and murine β -

- defensins reveals structural conservation in the absence of significant sequence similarity. *Protein Science*, 10(12), 2470-2479.
- Bazer, F. W. (1975). Uterine protein secretions: Relationship to development of the conceptus. *J Anim Sci*, 41(5), 1376-1382. <http://www.ncbi.nlm.nih.gov/pubmed/1104552>
- Bazer, F. W., Burghardt, R. C., Johnson, G. A., Spencer, T. E., & Wu, G. (2008). Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. *Reprod Biol*, 8(3), 179-211. <http://www.ncbi.nlm.nih.gov/pubmed/19092983>
- Bazer, F. W., & First, N. L. (1983). Pregnancy and parturition. *J Anim Sci*, 57 Suppl 2, 425-460. <http://www.ncbi.nlm.nih.gov/pubmed/6352591>
- Bazer, F. W., & Johnson, G. A. (2014). Pig blastocyst-uterine interactions. *Differentiation*, 87(1-2), 52-65. <https://doi.org/10.1016/j.diff.2013.11.005>
- Bazer, F. W., Johnson, G. A., & Wu, G. (2015). Amino acids and conceptus development during the peri-implantation period of pregnancy. *Adv Exp Med Biol*, 843, 23-52. https://doi.org/10.1007/978-1-4939-2480-6_2
- Bazer, F. W., Kim, J., Ka, H., Johnson, G. A., Wu, G., & Song, G. (2012). Select nutrients in the uterine lumen of sheep and pigs affect conceptus development. *J Reprod Dev*, 58(2), 180-188. <http://www.ncbi.nlm.nih.gov/pubmed/22738901>
- Bazer, F. W., Spencer, T. E., & Johnson, G. A. (2009). Interferons and uterine receptivity. *Semin Reprod Med*, 27(1), 90-102. <https://doi.org/10.1055/s-0028-1108013>
- Bazer, F. W., Wang, X., Johnson, G. A., & Wu, G. (2015). Select nutrients and their effects on conceptus development in mammals. *Animal Nutrition*, 1(3), 85-95. <https://doi.org/http://dx.doi.org/10.1016/j.aninu.2015.07.005>

- Bazer, F. W., Wu, G., Johnson, G. A., & Wang, X. (2014). Environmental factors affecting pregnancy: endocrine disrupters, nutrients and metabolic pathways. *Mol Cell Endocrinol*, 398(1-2), 53-68. <https://doi.org/10.1016/j.mce.2014.09.007>
- Belt, W. D., Anderson, L. L., Cavazos, L. F., & Melampy, R. M. (1971). Cytoplasmic granules and relaxin levels in porcine corpora lutea. *Endocrinology*, 89(1), 1-10. <https://doi.org/10.1210/endo-89-1-1>
- BIVALACQUA, T. J., Rajasekaran, M., Champion, H. C., Wang, R., Sikka, S. C., KADOWTTZ, P. J., & Hellstrom, W. J. (1998). The influence of castration on pharmacologically induced penile erection in the cat. *Journal of andrology*, 19(5), 551-557.
- Boć-Zalewska, A., Seremak-Mrozikiewicz, A., Barlik, M., Bogacz, A., Mrozikiewicz, P. M., Grześkowiak, E., & Drews, K. (2011). Adrenomedullin mRNA expression in placenta of preeclamptic women. *Ginekologia polska*, 82(8).
- Cameron, V. A., & Fleming, A. M. (1998). Novel sites of adrenomedullin gene expression in mouse and rat tissues. *Endocrinology*, 139(5), 2253-2264.
- Caron, K. M., & Smithies, O. (2001). Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. *Proceedings of the National Academy of Sciences*, 98(2), 615-619. <https://doi.org/10.1073/pnas.98.2.615>
- Casey, M. L., Smith, J., Alsabrook, G., & MacDonald, P. C. (1997). Activation of adenylyl cyclase in human myometrial smooth muscle cells by neuropeptides. *The Journal of Clinical Endocrinology & Metabolism*, 82(9), 3087-3092.
- Chaddha, V., Viero, S., Huppertz, B., & Kingdom, J. (2004). Developmental biology of the placenta and the origins of placental insufficiency. *Seminars in Fetal and Neonatal Medicine*,

- Challis, J. R., Lockwood, C. J., Myatt, L., Norman, J. E., Strauss, J. F., & Petraglia, F. (2009). Inflammation and pregnancy. *Reproductive sciences*, *16*(2), 206-215.
- Champion, H. C., Wang, R., Hellstrom, W., & Kadowitz, P. (1997). Nociceptin, a novel endogenous ligand for the ORL1 receptor, has potent erectile activity in the cat. *American Journal of Physiology-Endocrinology and Metabolism*, *273*(1), E214-E219.
- Champion, H. C., Wang, R., Shenassa, B. B., Murphy, W. A., Coy, D. H., Hellstrom, W. J., & Kadowitz, P. J. (1997). Adrenomedullin induces penile erection in the cat. *European journal of pharmacology*, *319*(1), 71-75.
- Chen, J. C., Wiley, A. A., Ho, T. Y., Frankshun, A. L., Hord, K. M., Bartol, F. F., & Bagnell, C. A. (2010). Transient estrogen exposure from birth affects uterine expression of developmental markers in neonatal gilts with lasting consequences in pregnant adults. *Reproduction*, *139*(3), 623-630. <https://doi.org/10.1530/REP-09-0454>
- Christiaens, I., Zaragoza, D. B., Guilbert, L., Robertson, S. A., Mitchell, B. F., & Olson, D. M. (2008). Inflammatory processes in preterm and term parturition. *Journal of reproductive immunology*, *79*(1), 50-57.
- Cooke, P. S., Spencer, T. E., Bartol, F. F., & Hayashi, K. (2013). Uterine glands: development, function and experimental model systems. *Mol Hum Reprod*, *19*(9), 547-558. <https://doi.org/10.1093/molehr/gat031>
- Cornish, J., Callon, K., Bava, U., Coy, D., Mulyey, T., Murray, M., Cooper, G., & Reid, I. (2001). Systemic administration of adrenomedullin (27-52) increases bone volume and strength in male mice. *Journal of endocrinology*, *170*(1), 251-257.

- Cornish, J., Callon, K. E., Coy, D. H., Jiang, N.-Y., Xiao, L., Cooper, G. J., & Reid, I. R. (1997). Adrenomedullin is a potent stimulator of osteoblastic activity in vitro and in vivo. *American Journal of Physiology-Endocrinology and Metabolism*, 273(6), E1113-E1120.
- Di Iorio, R., Marinoni, E., Letizia, C., Alo, P., Villaccio, B., & Cosmi, E. V. (1998). Adrenomedullin, a new vasoactive peptide, is increased in preeclampsia. *Hypertension*, 32(4), 758-763.
- Di Iorio, R., Marinoni, E., Letizia, C., Alò, P., Villaccio, B., Poverini, R., & Cosmi, E. V. (2001). Influence of labor on fetoplacental adrenomedullin concentrations. *American journal of obstetrics and gynecology*, 185(3), 697-702.
- Di Iorio, R., Marinoni, E., Letizia, C., Gazzolo, D., Lucchini, C., & Cosmi, E. V. (2000). Adrenomedullin is increased in the fetoplacental circulation in intrauterine growth restriction with abnormal umbilical artery waveforms. *American journal of obstetrics and gynecology*, 182(3), 650-654.
- Di Iorio, R., Marinoni, E., Letizia, C., Villaccio, B., Alberini, A., & Cosmi, E. V. (1999). Adrenomedullin production is increased in normal human pregnancy. *European journal of endocrinology*, 140(3), 201-206.
- Dikensoy, E., Balat, O., Pence, S., Balat, A., Cekmen, M., & Yurekli, M. (2009). The changes of plasma malondialdehyde, nitric oxide, and adrenomedullin levels in patients with preeclampsia. *Hypertension in Pregnancy*, 28(4), 383-389.
- Dunzendorfer, S., Meierhofer, C., Xu, Q., & Wiedermann, C. J. (2000). Pentoxifylline - augmented antiproliferative effects of adrenomedullin on vascular smooth muscle cells. *European journal of heart failure*, 2(3), 257-260.

- Elkas, J., Armstrong, A., Pohl, J., Cuttitta, F., Martínez, A., & Gray, K. (2000). Modulation of endometrial steroid receptors and growth regulatory genes by tamoxifen. *Obstetrics & Gynecology*, *95*(5), 697-703.
- Fazleabas, A. T., Bazer, F. W., & Roberts, R. M. (1982). Purification and properties of a progesterone-induced plasmin/trypsin inhibitor from uterine secretions of pigs and its immunocytochemical localization in the pregnant uterus. *J Biol Chem*, *257*(12), 6886-6897. <https://www.ncbi.nlm.nih.gov/pubmed/6211438>
- Fritz-Six, K. L., Dunworth, W. P., Li, M., & Caron, K. M. (2008). Adrenomedullin signaling is necessary for murine lymphatic vascular development. *The Journal of clinical investigation*, *118*(1), 40-50.
- Gadsby, J., Heap, R., & Burton, R. (1980). Oestrogen production by blastocyst and early embryonic tissue of various species. *Reproduction*, *60*(2), 409-417.
- Gao, H., Wu, G., Spencer, T. E., Johnson, G. A., & Bazer, F. W. (2009a). Select nutrients in the ovine uterine lumen. ii. glucose transporters in the uterus and peri-implantation conceptuses. *Biol Reprod*, *80*(1), 94-104. <https://doi.org/10.1095/biolreprod.108.071654>
- Gao, H., Wu, G., Spencer, T. E., Johnson, G. A., & Bazer, F. W. (2009b). Select nutrients in the ovine uterine lumen. III. Cationic amino acid transporters in the ovine uterus and peri-implantation conceptuses. *Biol Reprod*, *80*(3), 602-609. <https://doi.org/10.1095/biolreprod.108.073890>
- Gao, H., Wu, G., Spencer, T. E., Johnson, G. A., & Bazer, F. W. (2009c). Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses. *Biol Reprod*, *80*(6), 1196-1208. <https://doi.org/10.1095/biolreprod.108.075440>

- Gao, H., Wu, G., Spencer, T. E., Johnson, G. A., & Bazer, F. W. (2009d). Select nutrients in the ovine uterine lumen. V. Nitric oxide synthase, GTP cyclohydrolase, and ornithine decarboxylase in ovine uteri and peri-implantation conceptuses. *Biol Reprod*, *81*(1), 67-76. <https://doi.org/10.1095/biolreprod.108.075473>
- Gao, H., Wu, G., Spencer, T. E., Johnson, G. A., & Bazer, F. W. (2009e). Select nutrients in the ovine uterine lumen. VI. Expression of FK506-binding protein 12-rapamycin complex-associated protein 1 (FRAP1) and regulators and effectors of mTORC1 and mTORC2 complexes in ovine uteri and conceptuses. *Biol Reprod*, *81*(1), 87-100. <https://doi.org/10.1095/biolreprod.109.076257>
- Gao, H., Wu, G., Spencer, T. E., Johnson, G. A., Li, X., & Bazer, F. W. (2009). Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine luminal flushings of cyclic and pregnant ewes. *Biol Reprod*, *80*(1), 86-93. <https://doi.org/10.1095/biolreprod.108.071597>
- Geisert, R. D., Brookbank, J. W., Roberts, R. M., & Bazer, F. W. (1982). Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. *Biol Reprod*, *27*(4), 941-955. <https://doi.org/10.1095/biolreprod27.4.941>
- Geisert, R. D., Renegar, R. H., Thatcher, W. W., Roberts, R. M., & Bazer, F. W. (1982). Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. *Biol Reprod*, *27*(4), 925-939. <https://doi.org/10.1095/biolreprod27.4.925>
- Geisert, R. D., Thatcher, W. W., Roberts, R. M., & Bazer, F. W. (1982). Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered

- on day 11 of the estrous cycle. *Biol Reprod*, 27(4), 957-965.
<https://doi.org/10.1095/biolreprod27.4.957>
- Goldenberg, R. L., Culhane, J. F., Iams, J. D., & Romero, R. (2008). Epidemiology and causes of preterm birth. *The Lancet*, 371(9606), 75-84.
- Gratton, R., Gluszynski, M., Mazzuca, D., Nygard, K., & Han, V. (2003). Adrenomedullin messenger ribonucleic acid expression in the placentae of normal and preeclamptic pregnancies. *The Journal of Clinical Endocrinology & Metabolism*, 88(12), 6048-6055.
- Gray, C. A., Burghardt, R. C., Johnson, G. A., Bazer, F. W., & Spencer, T. E. (2002). Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction*, 124(2), 289-300.
<https://www.ncbi.nlm.nih.gov/pubmed/12141942>
- Guillomot, M. (1995). Cellular interactions during implantation in domestic ruminants. *J Reprod Fertil Suppl*, 49, 39-51. <http://www.ncbi.nlm.nih.gov/pubmed/7623329>
- Guthrie, H. D., Henricks, D. M., & Handlin, D. L. (1974). Plasma hormone levels and fertility in pigs induced to superovulate with PMSG. *J Reprod Fertil*, 41(2), 361-370.
<https://www.ncbi.nlm.nih.gov/pubmed/4452979>
- Hague, S., Zhang, L., Oehler, M. K., Manek, S., MacKenzie, I. Z., Bicknell, R., & Rees, M. C. (2000). Expression of the hypoxically regulated angiogenic factor adrenomedullin correlates with uterine leiomyoma vascular density. *Clinical cancer research*, 6(7), 2808-2814.
- Hata, T., Miyazaki, K., & Matsui, K. (1997). Decreased circulating adrenomedullin in pre-eclampsia. *The Lancet*, 350(9091), 1600.

- Hay, D. L., Conner, A. C., Howitt, S. G., Smith, D. M., & Poyner, D. R. (2004). The pharmacology of adrenomedullin receptors and their relationship to CGRP receptors. *Journal of Molecular Neuroscience*, 22(1), 105-113.
- Hayashi, K.-G., Hosoe, M., Sakumoto, R., & Takahashi, T. (2013). Temporo-spatial expression of adrenomedullin and its receptors in the bovine placenta. *Reproductive Biology and Endocrinology*, 11(1), 1-14.
- Hoeldtke, N. J., Wagner, R. K., Calhoun, B. C., & Hume Jr, R. F. (2000). Vasodilatory response of fetoplacental vasculature to adrenomedullin after constriction with the thromboxane sympathomimetic U46619. *American journal of obstetrics and gynecology*, 183(6), 1573-1578.
- Horio, T., Nishikimi, T., Yoshihara, F., Matsuo, H., Takishita, S., & Kangawa, K. (1999). Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts. *European journal of pharmacology*, 382(1), 1-9.
- Hwang, I., & Tang, F. (1999). The distribution and gene expression of adrenomedullin in the rat brain: peptide/mRNA and precursor/active peptide relationships. *Neuroscience letters*, 267(2), 85-88.
- Iavazzo, C., Tassis, K., Gourgiotis, D., Boutsikou, M., Baka, S., Hassiakos, D., Hadjithomas, A., Vrachnis, N., & Malamitsi-Puchner, A. (2009). Adrenomedullin concentration in second trimester amniotic fluid cannot be used as a predictor of preterm delivery. *in vivo*, 23(6), 1021-1026.
- Ichiki, Y., Kitamura, K., Kangawa, K., Kawamoto, M., Matsuo, H., & Eto, T. (1995). Distribution and characterization of immunoreactive adrenomedullin in porcine tissue, and isolation of

- adrenomedullin [26–52] and adrenomedullin [34–52] from porcine duodenum. *The Journal of Biochemistry*, 118(4), 765-770.
- Isumi, Y., Minamino, N., Katafuchi, T., Yoshioka, M., Tsuji, T., Kangawa, K., & Matsuo, H. (1998). Adrenomedullin production in fibroblasts: its possible function as a growth regulator of Swiss 3T3 cells. *Endocrinology*, 139(5), 2552-2563.
- Jacobs, R., Bales, L., Sundborg, M., Armstrong, A., Gehlbach, D., & Mitchell, A. (1998). Adrenomedullin Is Widely Expressed throughout Normal and Abnormal Reproductive Tissues of Women. *Adrenomedullin*, 207.
- Jahnke, G., Miller, M. J., Martinez, A., Montuenga-Badia, L. M., & Cuttitta, F. (1997). Adrenomedullin expression in the mouse mammary gland: evidence for the mature form in milk.
- Jerat, S., & Kaufman, S. (1998). Effect of pregnancy and steroid hormones on plasma adrenomedullin levels in the rat. *Canadian journal of physiology and pharmacology*, 76(4), 463-466.
- Jerat, S., Morrish, D. W., Davidge, S. T., & Kaufman, S. (2001). Effect of adrenomedullin on placental arteries in normal and preeclamptic pregnancies. *Hypertension*, 37(2), 227-231.
- Jiménez, N., Calvo, A., Martínez, A., Rosell, D., Cuttitta, F., & Montuenga, L. M. (1999). Expression of adrenomedullin and proadrenomedullin N-terminal 20 peptide in human and rat prostate. *Journal of Histochemistry & Cytochemistry*, 47(9), 1167-1177.
- Kanenishi, K., Kuwabara, H., Ueno, M., Sakamoto, H., & Hata, T. (2000). Immunohistochemical adrenomedullin expression is decreased in the placenta from pregnancies with pre-eclampsia. *Pathology international*, 50(7), 536-540.

- Kano, H., Kohno, M., Yasunari, K., Yokokawa, K., Horio, T., Ikeda, M., Minami, M., Hanehira, T., Takeda, T., & Yoshikawa, J. (1996). Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. *Journal of hypertension*, *14*(2), 209-213.
- Kapas, S., Brown, D. W., Farthing, P. M., & Hagi-Pavli, E. (1997). Adrenomedullin has mitogenic effects on human oral keratinocytes: involvement of cyclic AMP. *FEBS letters*, *418*(3), 287-290.
- Kapas, S., & Clark, A. J. (1995). Identification of an orphan receptor gene as a type 1 calcitonin gene-related peptide receptor. *Biochemical and biophysical research communications*, *217*(3), 832-838.
- Kato, H., Shichiri, M., Marumo, F., & Hirata, Y. (1997). Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology*, *138*(6), 2615-2620.
- Kaufman, S., & Deng, Y. (1998). Adrenomedullin suppresses atrial natriuretic factor (ANF) secretion from isolated atrium. *Life sciences*, *63*(12), 1017-1022.
- Kelleher, A. M., DeMayo, F. J., & Spencer, T. E. (2019). Uterine Glands: Developmental Biology and Functional Roles in Pregnancy. *Endocr Rev*. <https://doi.org/10.1210/er.2018-00281>
- Kelleher, A. M., Peng, W., Pru, J. K., Pru, C. A., DeMayo, F. J., & Spencer, T. E. (2017). Forkhead box a2 (FOXA2) is essential for uterine function and fertility. *Proc Natl Acad Sci U S A*, *114*(6), E1018-E1026. <https://doi.org/10.1073/pnas.1618433114>
- Kim, J., Burghardt, R. C., Wu, G., Johnson, G. A., Spencer, T. E., & Bazer, F. W. (2011). Select nutrients in the ovine uterine lumen. IX. Differential effects of arginine, leucine, glutamine, and glucose on interferon tau, ornithine decarboxylase, and nitric oxide synthase in the ovine conceptus. *Biol Reprod*, *84*(6), 1139-1147. <https://doi.org/10.1095/biolreprod.110.088153>

- Kim, J. Y., Burghardt, R. C., Wu, G., Johnson, G. A., Spencer, T. E., & Bazer, F. W. (2011a). Select nutrients in the ovine uterine lumen. VII. Effects of arginine, leucine, glutamine, and glucose on trophectoderm cell signaling, proliferation, and migration. *Biol Reprod*, 84(1), 62-69. <https://doi.org/10.1095/biolreprod.110.085738>
- Kim, J. Y., Burghardt, R. C., Wu, G., Johnson, G. A., Spencer, T. E., & Bazer, F. W. (2011b). Select nutrients in the ovine uterine lumen. VIII. Arginine stimulates proliferation of ovine trophectoderm cells through MTOR-RPS6K-RPS6 signaling cascade and synthesis of nitric oxide and polyamines. *Biol Reprod*, 84(1), 70-78. <https://doi.org/10.1095/biolreprod.110.085753>
- Kim, S. (1999). Vasoactive substance and vascular remodeling. *Nihon rinsho. Japanese journal of clinical medicine*, 57(7), 1508-1513.
- Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., & Eto, T. (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochemical and biophysical research communications*, 192(2), 553-560.
- Kitamura, K., Kangawa, K., Kojima, M., Ichiki, Y., Matsuo, H., & Eto, T. (1994). Complete amino acid sequence of porcine adrenomedullin and cloning of cDNA encoding its precursor. *FEBS letters*, 338(3), 306-310.
- Klein, K. R., Karpinich, N. O., Espenschied, S. T., Willcockson, H. H., Dunworth, W. P., Hoopes, S. L., Kushner, E. J., Bautch, V. L., & Caron, K. M. (2014). Decoy receptor CXCR7 modulates adrenomedullin-mediated cardiac and lymphatic vascular development. *Developmental cell*, 30(5), 528-540.

- Knerr, I., Dachert, C., Beinder, E., Metzler, M., Dötsch, J., Repp, R., & Rascher, W. (2002). Adrenomedullin, calcitonin gene-related peptide and their receptors: evidence for a decreased placental mRNA content in preeclampsia and HELLP syndrome. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *101*(1), 47-53.
- Knight, J. W., Bazer, F. W., Thatcher, W. W., Franke, D. E., & Wallace, H. D. (1977). Conceptus development in intact and unilaterally hysterectomized-ovariectomized gilts: interrelations among hormonal status, placental development, fetal fluids and fetal growth. *J Anim Sci*, *44*(4), 620-637. <https://doi.org/10.2527/jas1977.444620x>
- Kong, X., Wang, X., Yin, Y., Li, X., Gao, H., Bazer, F. W., & Wu, G. (2014). Putrescine stimulates the mTOR signaling pathway and protein synthesis in porcine trophoblast cells. *Biol Reprod*, *91*(5), 106. <https://doi.org/10.1095/biolreprod.113.113977>
- Lala, P., & Chakraborty, C. (2003). Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. *Placenta*, *24*(6), 575-587.
- Laoag-Fernandez, J. B., Otani, T., & Maruo, T. (2000). Adrenomedullin expression in the human endometrium. *Endocrine*, *12*(1), 15-19.
- Lauria, M. R., Standley, C. A., Sorokin, Y., Yelian, F. D., & Cotton, D. B. (1999). Adrenomedullin levels in normal and preeclamptic pregnancy at term. *The Journal of the Society for Gynecologic Investigation: JSGI*, *6*(6), 318-321.
- Lee, K. Y., & DeMayo, F. J. (2004). Animal models of implantation. *Reproduction*, *128*(6), 679-695.
- Lenis, Y. Y., Johnson, G. A., Wang, X., Tang, W. W., Dunlap, K. A., Satterfield, M. C., Wu, G., Hansen, T. R., & Bazer, F. W. (2018). Functional roles of ornithine decarboxylase and

- arginine decarboxylase during the peri-implantation period of pregnancy in sheep. *J Anim Sci Biotechnol*, 9, 10. <https://doi.org/10.1186/s40104-017-0225-x>
- Lenis, Y. Y., Wang, X., Tang, W., Wu, G., & Bazer, F. W. (2016). Effects of agmatine on secretion of interferon tau and catecholamines and expression of genes related to production of polyamines by ovine trophoctoderm cells. *Amino Acids*, 48(10), 2389-2399. <https://doi.org/10.1007/s00726-016-2216-1>
- Li, H., Dakour, J., Kaufman, S., Guilbert, L. J., Winkler-Lowen, B., & Morrish, D. W. (2003). Adrenomedullin is decreased in preeclampsia because of failed response to epidermal growth factor and impaired syncytialization. *Hypertension*, 42(5), 895-900.
- Li, L., Wai-Sum, O., & Tang, F. (2011). Adrenomedullin in rat follicles and corpora lutea: expression, functions and interaction with endothelin-1. *Reproductive Biology and Endocrinology*, 9(1), 1-12.
- Li, M., Wu, Y., & Caron, K. M. (2008). Haploinsufficiency for adrenomedullin reduces pinopodes and diminishes uterine receptivity in mice. *Biology of reproduction*, 79(6), 1169-1175.
- Li, M., Yee, D., Magnuson, T. R., Smithies, O., & Caron, K. M. (2006). Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. *The Journal of clinical investigation*, 116(10), 2653-2662.
- Li, Z., Takeuchi, S., Otani, T., & Maruo, T. (2001). Implications of adrenomedullin expression in the invasion of squamous cell carcinoma of the uterine cervix. *International journal of clinical oncology*, 6(6), 263-270.
- Liao, S., Ho, J., Tang, F., & WS, O. (2010). Adrenomedullin increases ciliary beat frequency and decreases muscular contraction in the rat oviduct. *Reproduction (Cambridge, England)*, 141(3), 367-372.

- Loukovaara, S., Immonen, I. J., Yandle, T. G., Nicholls, G., Hiilesmaa, V. K., & Kaaja, R. J. (2005). Vasoactive mediators and retinopathy during type 1 diabetic pregnancy. *Acta Ophthalmologica Scandinavica*, 83(1), 57-62.
- Lü, B., Wang, A., & Fei, Y. (1999). Study of the plasma adrenomedullin value in pregnancy induced hypertension patients. *Zhonghua fu Chan ke za zhi*, 34(1), 17-19.
- Makino, I., Shibata, K., Makino, Y., Kangawa, K., & Kawarabayashi, T. (1999). Adrenomedullin attenuates the hypertension in hypertensive pregnant rats induced by NG-nitro-L-arginine methyl ester. *European journal of pharmacology*, 371(2-3), 159-167.
- Makino, Y., Shibata, K., Makino, I., Ono, Y., Kangawa, K., & Kawarabayashi, T. (1999). Expression of adrenomedullin in feto-placental circulation of human normotensive pregnant women and pregnancy-induced hypertensive women. *Endocrinology*, 140(11), 5439-5442.
- Manau, D., Balasch, J., Jiménez, W., Fábregues, F., Casamitjana, R., Creus, M., Arroyo, V., & Vanrell, J. (1999). Adrenomedullin and nitric oxide in menstrual and in vitro fertilization cycles. Relationship to estradiol. *Acta obstetricia et gynecologica Scandinavica*, 78(7), 626-631.
- Manau, D., Balasch, J., Jiménez, W., Fábregues, F., Civico, S., Casamitjana, R., Creus, M., & Vanrell, J. A. (2000). Follicular fluid concentrations of adrenomedullin, vascular endothelial growth factor and nitric oxide in IVF cycles: relationship to ovarian response. *Human Reproduction*, 15(6), 1295-1299.
- Marinoni, E., Di Iorio, R., Letizia, C., Lucchini, C., Alò, P., & Cosmi, E. V. (2000). Changes in plasma adrenomedullin levels during the menstrual cycle. *Regulatory peptides*, 87(1-3), 15-18.

- Marinoni, E., Di Iorio, R., Letizia, C., Villaccio, B., Scucchi, L., & Cosmi, E. V. (1998). Immunoreactive adrenomedullin in human fetoplacental tissues. *American journal of obstetrics and gynecology*, 179(3), 784-787.
- Marinoni, E., Feliciani, E., Muzzonigro, F., Letizia, C., Tranquilli, A., Tranquilli, D., Aragona, C., Moscarini, M., & Di Iorio, R. (2010). Intrafollicular concentration of adrenomedullin is associated with IVF outcome. *Gynecological Endocrinology*, 26(6), 435-439.
- Marinoni, E., Scavo, D., Letizia, C., & Cosmi, E. V. (1997). Adrenomedullin in pregnancy. *The Lancet*, 349(9048), 328.
- Marinoni, E., Zacharopoulou, C., Di Rocco, A., Letizia, C., Moscarini, M., & Di Iorio, R. (2006). Effect of betamethasone in vivo on placental adrenomedullin in human pregnancy. *Journal of the Society for Gynecologic Investigation*, 13(6), 418-424.
- Martinez, A., Elsasser, T., Bhatena, S., Pio, R., Buchanan, T., Macri, C., & Cuttitta, F. (1999). Is adrenomedullin a causal agent in some cases of type 2 diabetes? *Peptides*, 20(12), 1471-1478.
- Martínez, A., Elsasser, T. H., Muro-Cacho, C., Moody, T. W., Miller, M. J., Macri, C. J., & Cuttitta, F. (1997). Expression of adrenomedullin and its receptor in normal and malignant human skin: a potential pluripotent role in the integument. *Endocrinology*, 138(12), 5597-5604.
- McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., & Foord, S. M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature*, 393(6683), 333-339.

- Michibata, H., Mukoyama, M., Tanaka, I., Suga, S.-i., Nakagawa, M., Ishibashi, R., Goto, M., Akaji, K., Fujiwara, Y., & Kiso, Y. (1998). Autocrine/paracrine role of adrenomedullin in cultured endothelial and mesangial cells. *Kidney international*, 53(4), 979-985.
- Michishita, M., Minegishi, T., Abe, K., Kangawa, K., Kojima, M., & Ibuki, Y. (1999). Expression of adrenomedullin in the endometrium of the human uterus. *Obstetrics & Gynecology*, 93(1), 66-70.
- Miller, M. J., Martínez, A., Unsworth, E. J., Thiele, C. J., Moody, T. W., Elsasser, T., & Cuttitta, F. (1996). Adrenomedullin expression in human tumor cell lines Its potential role as an autocrine growth factor. *Journal of Biological Chemistry*, 271(38), 23345-23351.
- Minegishi, T., Nakamura, M., Abe, K., Tano, M., Andoh, A., Yoshida, M., Takagi, T., Nishikimi, T., Kojima, M., & Kangawa, K. (1999). Adrenomedullin and atrial natriuretic peptide concentrations in normal pregnancy and pre-eclampsia. *Molecular human reproduction*, 5(8), 767-770.
- Montuenga-Badia, L. M., Burrell, M., Garayoa, M., Llopiz, D., Vos, M., Moody, T., Garcia-Ros, D., Martinez, A., Villaro, A. C., & Elssasser, T. (2000). Expression of proadrenomedullin derived peptides in the mammalian pituitary: co-localization of follicle stimulating hormone and proadrenomedullin N-20 terminal peptide-like peptide in the same secretory granules of the gonadotropes.
- Montuenga, L. M., Martínez, A., Miller, M. J., Garayoa, M., Elsasser, T., & Cuttitta, F. (1998). Expression of AM and PAMP in normal adult and developing mammals. *Adrenomedullin. Amsterdam, IOS Press and Ohmsha*, 49-68.

- Montuenga, L. M., Martínez, A., Miller, M. J., Unsworth, E. J., & Cuttitta, F. (1997). Expression of adrenomedullin and its receptor during embryogenesis suggests autocrine or paracrine modes of action. *Endocrinology*, *138*(1), 440-451.
- Moody, T., Coy, D., Cuttitta, F., & Montuenga, L. (2000). Proadrenomedullin NH₂-terminal 20 peptide (PAMP) and adrenomedullin bind to teratocarcinoma cells☆. *Peptides*, *21*(1), 101-107.
- Moody, T., Miller, M., Martinez, A., Unsworth, E., & Cuttitta, F. (1997). Adrenomedullin binds with high affinity, elevates cyclic AMP, and stimulates c-fos mRNA in C6 glioma cells. *Peptides*, *18*(8), 1111-1115.
- Moriyama, T., Otani, T., & Maruo, T. (2000). Expression of adrenomedullin by human granulosa lutein cells and its effect on progesterone production. *European journal of endocrinology*, *142*(6), 671-676.
- Morrish, D., Linetsky, E., Bhardwaj, D., Li, H., Dakour, J., Marsha, R., Paterson, M., & Godbout, R. (1996). Identification by subtractive hybridization of a spectrum of novel and unexpected genes associated with in vitro differentiation of human cytotrophoblast cells. *Placenta*, *17*(7), 431-441.
- Nancarrow, C. D. (1994). Embryonic mortality in the ewe and doe. In M. T. Zavy & R. D. Geisert (Eds.), *Embryonic mortality in domestic species*. (pp. (Ch.4): 79-97). CRC Press.
- Naot, D., Callon, K. E., Grey, A., Cooper, G. J., Reid, I. R., & Cornish, J. (2001). A potential role for adrenomedullin as a local regulator of bone growth. *Endocrinology*, *142*(5), 1849-1857.
- Nikitenko, L., Brown, N., Smith, D., MacKenzie, I., Bicknell, R., & Rees, M. (2001). Differential and cell-specific expression of calcitonin receptor-like receptor and receptor activity modifying proteins in the human uterus. *Molecular human reproduction*, *7*(7), 655-664.

- Nikitenko, L., MacKenzie, I., Rees, M., & Bicknell, R. (2000). Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. *Molecular human reproduction*, 6(9), 811-819.
- Nussdorfer, G. G. (2001). Proadrenomedullin-derived peptides in the paracrine control of the hypothalamo-pituitary-adrenal axis. *International review of cytology*, 206, 249-284.
- Nussdorfer, G. G., Rossi, G. P., & Mazzocchi, G. (1997). Role of adrenomedullin and related peptides in the regulation of the hypothalamo-pituitary-adrenal axis. *Peptides*, 18(7), 1079-1089.
- Oehler, M., Norbury, C., Hague, S., Rees, M. C., & Bicknell, R. (2001). Adrenomedullin inhibits hypoxic cell death by upregulation of Bcl-2 in endometrial cancer cells: a possible promotion mechanism for tumour growth. *Oncogene*, 20(23), 2937-2945.
- Ozbek, E., Yurekli, M., Soylu, A., Davarci, M., & Balbay, M. (2000). The role of adrenomedullin in varicocele and impotence. *BJU international*, 86(6), 694-698.
- Perry, J. S. (1981). The mammalian fetal membranes. *J Reprod Fertil*, 62(2), 321-335.
<https://www.ncbi.nlm.nih.gov/pubmed/7252917>
- Pewitt, E. B., Haleem, R., & Wang, Z. (1999). Adrenomedullin gene is abundantly expressed and directly regulated by androgen in the rat ventral prostate. *Endocrinology*, 140(5), 2382-2386.
- Pio, R., Martínez, A., Elsasser, T. H., & Cuttitta, F. (2000). Presence of immunoreactive adrenomedullin in human and bovine milk. *Peptides*, 21(12), 1859-1863.
- Plank, C., Hartner, A., Klanke, B., Geissler, B., Porst, M., Amann, K., Hilgers, K. F., Rascher, W., & Datsch, J. (2005). Adrenomedullin reduces mesangial cell number and glomerular

- inflammation in experimental mesangioproliferative glomerulonephritis. *Kidney international*, 68(3), 1086-1095.
- Pöyhönen - Alho, M., Viitasalo, M., Nicholls, M., Lindström, B. M., Väänänen, H., & Kaaja, R. (2010). Imbalance of the autonomic nervous system at night in women with gestational diabetes. *Diabetic Medicine*, 27(9), 988-994.
- Poyner, D. R., Taylor, G. M., Tomlinson, A. E., Richardson, A. G., & Smith, D. M. (1999). Characterization of receptors for calcitonin gene - related peptide and adrenomedullin on the guinea - pig vas deferens. *British journal of pharmacology*, 126(5), 1276-1282.
- Quinn, K., Mackie, D., & Caron, K. (2018). Emerging roles of atypical chemokine receptor 3 (ACKR3) in normal development and physiology. *Cytokine*, 109, 17-23.
- Roberts, R. M., & Bazer, F. W. (1988). The functions of uterine secretions. *J Reprod Fertil*, 82(2), 875-892. <http://www.ncbi.nlm.nih.gov/pubmed/3283351>
- Robertson, H. A., & King, G. J. (1974). Plasma concentrations of progesterone, oestrone, oestradiol-17beta and of oestrone sulphate in the pig at implantation, during pregnancy and at parturition. *J Reprod Fertil*, 40(1), 133-141. <https://www.ncbi.nlm.nih.gov/pubmed/4415090>
- Romero, R., Espinoza, J., Gonçalves, L. F., Kusanovic, J. P., Friel, L., & Hassan, S. (2007). The role of inflammation and infection in preterm birth. *Seminars in reproductive medicine*,
- Ross, G. R., Yallampalli, U., Gangula, P. R., Reed, L., Sathishkumar, K., Gao, H., Chauhan, M., & Yallampalli, C. (2010). Adrenomedullin relaxes rat uterine artery: mechanisms and influence of pregnancy and estradiol. *Endocrinology*, 151(9), 4485-4493.

- Rossi, F., Guerrini, L., Pasimeni, G., Markouizou, A., Fabbrini, A., & Santiemma, V. (2000). Testicular peritubular myoid cells are a target for adrenomedullin. *Archives of andrology*, 44(2), 103-107.
- Sakata, J., Shimokubo, T., Kitamura, K., Nakamura, S., Kangawa, K., Matsuo, H., & Eto, T. (1993). Molecular cloning and biological activities of rat adrenomedullin, a hypotensive peptide. *Biochemical and biophysical research communications*, 195(2), 921-927.
- Sakata, J., Shimokubo, T., Kitamura, K., Nishizono, M., Iehiki, Y., Kangawa, K., Matsuo, H., & Eto, T. (1994). Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. *FEBS letters*, 352(2), 105-108.
- Santiemma, V., Rossi, F., Guerrini, L., Markouizou, A., Pasimeni, G., Palleschi, S., & Fabbrini, A. (2001). Adrenomedullin inhibits the contraction of cultured rat testicular peritubular myoid cells induced by endothelin-1. *Biology of reproduction*, 64(2), 619-624.
- Sata, M., Kakoki, M., Nagata, D., Nishimatsu, H., Suzuki, E., Aoyagi, T., Sugiura, S., Kojima, H., Nagano, T., & Kangawa, K. (2000). Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis via a cyclic GMP-independent mechanism. *Hypertension*, 36(1), 83-88.
- Sato, A., & Autelitano, D. J. (1995). Adrenomedullin induces expression of c-fos and AP-1 activity in rat vascular smooth muscle cells and cardiomyocytes. *Biochemical and biophysical research communications*, 217(1), 211-216.
- Semplicini, A., Ceolotto, G., Baritono, E., Malendowicz, L. K., Andreis, P. G., Sartori, M., Rossi, G. P., & Nussdorfer, G. G. (2001). Adrenomedullin stimulates DNA synthesis of rat adrenal zona glomerulosa cells through activation of the mitogen-activated protein kinase-dependent cascade. *Journal of hypertension*, 19(3), 599-602.

- Senna, A. A., Zedan, M., Abd El Salam, G. E., & El Mashad, A. I. (2008). Study of plasma adrenomedullin level in normal pregnancy and preclampsia. *The Medscape Journal of Medicine*, *10*(2), 29.
- Shichiri, M., Kato, H., Doi, M., Marumo, F., & Hirata, Y. (1999). Induction of max by adrenomedullin and calcitonin gene-related peptide antagonizes endothelial apoptosis. *Molecular Endocrinology*, *13*(8), 1353-1363.
- Shimosawa, T., Shibagaki, Y., Ishibashi, K., Kitamura, K., Kangawa, K., Kato, S., Ando, K., & Fujita, T. (2002). Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. *Circulation*, *105*(1), 106-111.
- Shindo, T., Kurihara, Y., Nishimatsu, H., Moriyama, N., Kakoki, M., Wang, Y., Imai, Y., Ebihara, A., Kuwaki, T., & Ju, K.-H. (2001). Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation*, *104*(16), 1964-1971.
- Shoji, H., Minamino, N., Kangawa, K., & Matsuo, H. (1995). Endotoxin markedly elevates plasma concentration and gene transcription of adrenomedullin in rat. *Biochemical and biophysical research communications*, *215*(2), 531-537.
- Sierro, F., Biben, C., Martínez-Muñoz, L., Mellado, M., Ransohoff, R. M., Li, M., Woehl, B., Leung, H., Groom, J., & Batten, M. (2007). Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proceedings of the National Academy of Sciences*, *104*(37), 14759-14764.
- Spencer, T. E., & Bazer, F. W. (2004). Uterine and placental factors regulating conceptus growth in domestic animals. *J Anim Sci*, *82 E-Suppl*, E4-13.
<http://www.ncbi.nlm.nih.gov/pubmed/15471813>

- Spencer, T. E., Johnson, G. A., Bazer, F. W., & Burghardt, R. C. (2004). Implantation mechanisms: insights from the sheep. *Reproduction*, *128*(6), 657-668.
<https://doi.org/10.1530/rep.1.00398>
- Spencer, T. E., Johnson, G. A., Bazer, F. W., Burghardt, R. C., & Palmarini, M. (2007). Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. *Reprod Fertil Dev*, *19*(1), 65-78.
<http://www.ncbi.nlm.nih.gov/pubmed/17389136>
- Spencer, T. E., Johnson, G. A., Burghardt, R. C., & Bazer, F. W. (2004). Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biol Reprod*, *71*(1), 2-10. <https://doi.org/10.1095/biolreprod.103.024133>
- Stoner, C. S., Bazer, F. W., Thatcher, W. W., Wilcox, C. J., Combs, G. E., Knight, J. W., Wettemann, R. P., & White, C. E. (1986). Relationship between estrone sulfate in plasma and litter size at farrowing for sows and gilts. *Theriogenology*, *25*(5), 709-720.
<https://www.ncbi.nlm.nih.gov/pubmed/16726162>
- Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T., & Matsuo, H. (1994). Endothelial cells actively synthesize and secrete adrenomedullin. *Biochemical and biophysical research communications*, *201*(3), 1160-1166.
- Takahashi, K. (2001). Adrenomedullin from a pheochromocytoma to the eye: implications of the adrenomedullin research for endocrinology in the 21st century. *The Tohoku journal of experimental medicine*, *193*(2), 79-114.
- Takahashi, K., Satoh, F., Sone, M., Murakami, O., Sasano, H., Mouri, T., & Shibahara, S. (1997). Expression of adrenomedullin mRNA in the human brain and pituitary. *Peptides*, *18*(7), 1051-1053.

- Tarleton, B. J., Braden, T. D., Wiley, A. A., & Bartol, F. F. (2003). Estrogen-induced disruption of neonatal porcine uterine development alters adult uterine function. *Biol Reprod*, *68*(4), 1387-1393. <https://doi.org/10.1095/biolreprod.102.011346>
- Tomikawa, M., Wang, H., Jones, M. K., Sugimachi, K., Sarfeh, I. J., & Tarnawski, A. S. (1999). Reduced adrenomedullin expression in gastric mucosa of portal hypertensive rats after ethanol-induced injury. *Annals of surgery*, *230*(1), 38.
- Trollmann, R., Schoof, E., Beinder, E., Wenzel, D., Rascher, W., & Dotsch, J. (2002). Adrenomedullin gene expression in human placental tissue and leukocytes: a potential marker of severe tissue hypoxia in neonates with birth asphyxia. *European journal of endocrinology*, *147*(5), 711-716.
- Tsuruda, T., Kato, J., Kitamura, K., Kawamoto, M., Kuwasako, K., Imamura, T., Koiwaya, Y., Tsuji, T., Kangawa, K., & Eto, T. (1999). An autocrine or a paracrine role of adrenomedullin in modulating cardiac fibroblast growth. *Cardiovascular research*, *43*(4), 958-967.
- Tsuruda, T., Kato, J., Kitamura, K., Kuwasako, K., Imamura, T., Koiwaya, Y., Tsuji, T., Kangawa, K., & Eto, T. (1998). Adrenomedullin: a possible autocrine or paracrine inhibitor of hypertrophy of cardiomyocytes. *Hypertension*, *31*(1), 505-510.
- Ueta, Y., Hara, Y., Setiadji, V. S., Isse, T., Shibuya, I., Kitamura, K., Kangawa, K., Matsuo, H., Eto, T., & Hattori, Y. (1999). Adrenomedullin-like immunoreactivity in the rat hypothalamo-neurohypophysial tract. *Peptides*, *20*(2), 199-204.
- Upton, P. D., Austin, C., Taylor, G. M., Nandha, K. A., Clark, A. J., Ghatei, M. A., Bloom, S. R., & Smith, D. M. (1997). Expression of adrenomedullin (ADM) and its binding sites in the

- rat uterus: increased number of binding sites and ADM messenger ribonucleic acid in 20-day pregnant rats compared with nonpregnant rats. *Endocrinology*, 138(6), 2508-2514.
- Wang, X., Burghardt, R. C., Romero, J. J., Hansen, T. R., Wu, G., & Bazer, F. W. (2015). Functional roles of arginine during the peri-implantation period of pregnancy. III. Arginine stimulates proliferation and interferon tau production by ovine trophoblast cells via nitric oxide and polyamine-TSC2-MTOR signaling pathways. *Biol Reprod*, 92(3), 75. <https://doi.org/10.1095/biolreprod.114.125989>
- Wang, X., Frank, J. W., Little, D. R., Dunlap, K. A., Satterfield, M. C., Burghardt, R. C., Hansen, T. R., Wu, G., & Bazer, F. W. (2014). Functional role of arginine during the peri-implantation period of pregnancy. I. Consequences of loss of function of arginine transporter SLC7A1 mRNA in ovine conceptus trophoblast. *FASEB J*, 28(7), 2852-2863. <https://doi.org/10.1096/fj.13-248757>
- Wang, X., Frank, J. W., Xu, J., Dunlap, K. A., Satterfield, M. C., Burghardt, R. C., Romero, J. J., Hansen, T. R., Wu, G., & Bazer, F. W. (2014). Functional role of arginine during the peri-implantation period of pregnancy. II. Consequences of loss of function of nitric oxide synthase NOS3 mRNA in ovine conceptus trophoblast. *Biol Reprod*, 91(3), 59. <https://doi.org/10.1095/biolreprod.114.121202>
- Wang, X., Johnson, G. A., Burghardt, R. C., Wu, G., & Bazer, F. W. (2015). Uterine histotroph and conceptus development. I. cooperative effects of arginine and secreted phosphoprotein 1 on proliferation of ovine trophoblast cells via activation of the PDK1-Akt/PKB-TSC2-MTORC1 signaling cascade. *Biol Reprod*, 92(2), 51. <https://doi.org/10.1095/biolreprod.114.125971>

- Wang, X., Johnson, G. A., Burghardt, R. C., Wu, G., & Bazer, F. W. (2016). Uterine Histotroph and Conceptus Development. II. Arginine and Secreted Phosphoprotein 1 Cooperatively Stimulate Migration and Adhesion of Ovine Trophectoderm Cells via Focal Adhesion-MTORC2 Mediated Cytoskeleton Reorganization. *Biol Reprod*, 95(3), 71. <https://doi.org/10.1095/biolreprod.115.137943>
- Wang, X., Li, D., Wu, G., & Bazer, F. W. (2016). Functional Roles of Fructose: Crosstalk between O-Linked Glycosylation and Phosphorylation of Akt-TSC2-MTOR Cell Signaling Cascade in Ovine Trophectoderm Cells. *Biol Reprod*, 95(5), 102. <https://doi.org/10.1095/biolreprod.116.142281>
- Wang, X., Ying, W., Dunlap, K. A., Lin, G., Satterfield, M. C., Burghardt, R. C., Wu, G., & Bazer, F. W. (2014). Arginine decarboxylase and agmatinase: an alternative pathway for de novo biosynthesis of polyamines for development of mammalian conceptuses. *Biol Reprod*, 90(4), 84. <https://doi.org/10.1095/biolreprod.113.114637>
- Withers, D. J., Coppock, H. A., Seufferlein, T., Smith, D. M., Bloom, S. R., & Rozengurt, E. (1996). Adrenomedullin stimulates DNA synthesis and cell proliferation via elevation of cAMP in Swiss 3T3 cells. *FEBS letters*, 378(1), 83-87.
- Witlin, A. G. (2002). *The role of adrenomedullin in placental and fetal growth and development in rat pregnancy* [The University of Texas Medical Branch Graduate School of Biomedical Sciences].
- Yamashiro, C., Hayashi, K., Yanagihara, T., & Hata, T. (2001). Plasma adrenomedullin levels in pregnancies with appropriate for gestational age and small for gestational age infants.

- Yamashiro, C., Kanenishi, K., Akiyama, M., Tanaka, H., Shiota, A., & Hata, T. (2002). Adrenomedullin concentrations in early 2nd-trimester amniotic fluid: relation to preterm delivery and fetal growth at birth. *Gynecologic and obstetric investigation*, *54*(2), 99-104.
- Yanagita, T., Yamamoto, R., Sugano, T., Kobayashi, H., Uezono, Y., Yokoo, H., Shiraishi, S., Minami, S. i., & Wada, A. (2000). Adrenomedullin inhibits spontaneous and bradykinin - induced but not oxytocin - or prostaglandin F₂ α - induced periodic contraction of rat uterus. *British journal of pharmacology*, *130*(8), 1727-1730.
- Zhang, X., Green, K. E., Yallampalli, C., & Dong, Y. L. (2005). Adrenomedullin enhances invasion by trophoblast cell lines. *Biology of reproduction*, *73*(4), 619-626.
- Zhao, Y., Hague, S., Manek, S., Zhang, L., Bicknell, R., & Rees, M. C. (1998). PCR display identifies tamoxifen induction of the novel angiogenic factor adrenomedullin by a non estrogenic mechanism in the human endometrium. *Oncogene*, *16*(3), 409-415.
- Zhu, Q., Tian, G., Tang, Z., Gao, J., & Tan, Y. (2016). Adrenomedullin promotes the proliferation and inhibits apoptosis of dental pulp stem cells involved in divergence pathways. *Journal of endodontics*, *42*(9), 1347-1354.
- Zudaire, E., Portal - Núñez, S., & Cuttitta, F. (2006). The central role of adrenomedullin in host defense. *Journal of leukocyte biology*, *80*(2), 237-244.

CHAPTER 2 - DATA MINING REVEALS THAT THE EXPRESSION OF ADM IS INCREASED IN PORCINE ENDOMETRIUM DURING PREGNANCY

Abstract

Adrenomedullin (ADM) is an evolutionarily conserved multi-functional peptide hormone that regulates implantation, embryo spacing and placentation in humans and rodents. However, the potential roles of ADM in implantation and placentation in pigs are not known. In this Chapter, we performed a data-mining based analysis to investigate the expression patterns of ADM and its receptors components (*CALCRL*, *RAMP1*, *RAMP2*, *RAMP3*, and *ACKR3*) in uteri from cyclic and pregnant gilts as well as the uterine compartments within pregnant uterine endometrium i.e., Luminal epithelium (LE), Glandular epithelium (GE) and Stromal cells (S). The mRNA levels of *ADM*, *CALCRL*, *RAMP2*, *RAMP3* and *ACKR3* were increased ($P < 0.05$) in porcine endometrium between days 6 and 18 of estrous cycle; whereas *RAMP1* mRNA remained unchanged. Re-analysis of Laser Capture Microdissection (LCM)-based RNA-Seq data further revealed that *ADM* mRNA was expressed in endometrial LE and S, but not GE, at both cyclic (CD) and gestational (GD) day 12; whereas *CALCRL*, *RAMP2* and *ACKR3* mRNAs were detected in all three compartments. Moreover, in pregnancy, expression of *ADM* in LE and S were stimulated, while that of *ACKR3* in LE, GE and S, were inhibited. The mRNA levels of *CALCRL* and *RAMP2* were not affected by pregnancy in LE, GE and S. Together, these results provided the rationale to further investigate the temporal and cell-specific expressions of ADM and its receptor components in porcine endometrium and conceptuses during peri-implantation period of pregnancy.

Introduction

The endometrium is the inner layer of the uterus and is composed of epithelial cells, stromal cells, immune cells, and endothelial cells which contribute to the vasculature of the uterus. The epithelial cells form a single layer of columnar epithelium (luminal epithelial cells; LE) that faces the lumen of the uterus. Epithelial cells (glandular epithelium; GE) which invaginate within the endometrial stroma (S), form distinct structures with a secretory function named endometrial glands. These glands are branched-tubular structures that develop through the endometrial stroma reaching the myometrium. The structure and function of these glands dynamically change throughout the estrous cycle and during pregnancy. During the peri-implantation period of pregnancy, glands achieve their peak functional role, together with LE, providing histotroph to facilitate the embryo-endometrial crosstalk. The endometrial stroma, on the other hand, responds to ovarian progesterone and/or conceptus signals, and actively communicates with LE and GE to further support the establishment and maintenance of pregnancy (Ashary et al., 2018; Mazur et al., 2015).

Within the first week of pregnancy, the mammalian embryo undergoes sequences of developmental changes, that includes cleavage, morula and blastocyst stages, which is similar in all mammalian species. After hatching from the zona pellucida, pig blastocysts undergo rapid morphological transitions from large spheres of 10 to 15 mm diameter, to tubular (15 mm by 50mm) to filamentous (1 mm by 100 to 200 mm) forms between Days 10 and 12 of pregnancy and achieve a final length of 800 to 1000 mm between Days 12 and 15 of pregnancy (Bazer et al., 1982). During this peri-implantation period of pregnancy, endometrial secretions driven by ovarian progesterone and conceptus signals, which are essential for conceptus growth and development.

To date, a number of studies have been performed to analyze transcriptomic alterations in the porcine endometrium in response to various embryonic signals and different pregnant states, generating a large amount of high content datasets. Thus, in this Chapter, we performed a data mining-based analysis of two publicly available porcine endometrial RNA-Seq datasets and identified mRNA levels of *ADM* and its receptor components in a timely and cell-specific manner.

Materials and Methods

The porcine endometrial transcriptome datasets GSE108570 and GSE109539 were obtained from the Gene Expression Omnibus (GEO) repository (<https://www.ncbi.nlm.nih.gov/gds>).

Experimental design for GSE108570

Twenty-one crossbred gilts (Landrace X Yorkshire) gilts of similar age (6-8 months) and weight (100-200kg) were utilized in this study after experiencing at least two normal estrous cycle of 18-22 days. Gilts were observed daily for estrous behavior in the presence of boars. The day on which estrous behavior was first exhibited was designated as Day 0. The ovary, endometrium and oviduct tissues of the gilts were collected on Days 0, 3, 6, 9, 12, 15, and 18 of estrous cycle (n=3 gilts/day) by hysterectomy to encompass the whole stages of the estrous cycle. Endometrial tissues were dissected free of myometrium from the middle portion of the uterine horns. Tissues were snap-frozen in liquid nitrogen and stored at -80 degrees for RNA extraction (Kim et al., 2018).

The total RNA was extracted from the endometrium using TRIzol reagent (Invitrogen, Life Technology, Carlsbad, CA) according to the manufacturer's recommendations. The quality of RNA was assessed by spectrophotometry and integrity of RNA was validated by electrophoresis in 1% agarose gel. The mRNA of 1µg of total RNA was converted into a library of template

molecules suitable for subsequent cluster generation using the reagents provided in the Illumina® TruSeq™ RNA Sample Preparation Kit. Firstly, the poly-A containing mRNA molecules were purified using poly-T oligo attached magnetic beads. Following the purification, the mRNA was fragmented into small pieces using divalent cations under elevated temperature. The cleaved mRNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments then underwent an end repair process, the addition of a single ‘A’ base, then ligation with adaptors. The products were then purified and enriched with PCR to create the final cDNA library. The libraries were then quantified using qPCR according to the qPCR quantification guide by the manufacturer and quantified using an Agilent Technologies 2100 Bioanalyzer. The cDNA libraries were sequenced using the paired-end sequencing by Illumina HiSeq 2000.

Experimental design for GSE109539

Eight prepuberal gilts were bought from a livestock trader (crossbreeds of German Landrace and Piétrain) and received a single injection of 750 IU PMSG (Intergonan®, MSD Animal Health Innovation GmbH, Schwabenheim, Germany) and 72 h later 750 IU hCG (Ovogest®, MSD Animal Health Innovation GmbH) to synchronize ovulation. Gilts of the “pregnant” group (n = 4) were inseminated twice (24 h and 36 h after hCG) with a standard dose of German Landrace semen whereas gilts of the “non-pregnant” control group (n = 4) were inseminated with the supernatant of centrifuged (10 min, 3000 rpm) semen of the same boar. Gilts were slaughtered on Day 12 after insemination. Endometrial samples (medial part of the uterine horns) were collected and snap-frozen in liquid nitrogen and stored at – 80° until preparation for

Laser capture microdissection (LCM). Pregnancy was confirmed by the presence of filamentous conceptuses in the flush of the uterine horns. Briefly, frozen endometrium samples were cut in 10µm thick sections with a Leica CM1950 clinical cryostat (Leica Biosystems, Germany), mounted onto membrane slides (MembraneSlide NF 1.0 PEN, Zeiss, Germany), and stained using a modified, rapid Cresyl violet staining protocol to identify LE, GE, and stromal cells. Briefly, the slides were first fixed with 70% ethanol (Sigma), and quickly washed in 50% ethanol. Cresyl violet was used to stain for 3 min, and then the stained slides were washed again with 50%, 70%, 100% ethanol, respectively (dip slides two/three times into each solution). Finally, the slides were dried at room temperature. All solutions were prepared with RNase-free water. The target cells from the sections were captured using an LCM Zeiss 200 M (inverse) microscope (Zeiss PALM Microsystems, Germany). When satisfactory cutting was achieved, the target tissue was lifted to the LCM cap (AdhesiveCap 200 clear, Zeiss, Germany) and 50 µl extraction buffer was used to incubate the LCM samples at 42 °C for 30 min to lyse the cells.

Total RNA was isolated from luminal epithelium, glandular epithelium, and stromal samples of each pig using the PicoPure RNA Isolation Kit (Applied Biosystems™, Vilnius, Lithuania) following the manufacturer's instructions. Integrity and quantity of the RNA were assessed using the Agilent RNA 6000 Pico assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The quality of the isolated total RNA extracted from LE, GE, and S ranged from 6.6 to 9.4 (RNA integrity number, RIN). Most of the samples which had a RIN around 8 were used to prepare a total of 24 RNA-seq libraries for biological replicates per group (n=4 gilts) and cell type. The Ovation SoLo Single Cell RNA-Seq System (NuGen Technologies, San Carlos, USA) was used for preparing RNA-Seq libraries starting from 500 pg of total RNA (corresponding to no more than 50 cells) according to the manufacturer's

recommendations. The number of PCR cycles for the first amplification step (determined by qPCR according to the manual) was between 15 and 20, and for most of the samples was 17. The 24 libraries were prepared from each individual sample for the three cell types of each replicate of both experimental groups. Then, all individual barcoded libraries were pooled for sequencing on two lanes of a single-read flow cell on an Illumina HiSeq 2500 instrument. Sequencing and demultiplexing were performed at the Functional Genomics Center Zurich (FGCZ).

Bioinformatics and data analysis

The raw RNA-Seq reads from both GSE108570 and GSE109539 datasets were initially processed by filtering with average quality score greater than 20. Reads which passed the initial processing were aligned to the pig reference genome (Sscrofa 11.1) using TopHat version 2.0.4 (Trapnell et al., 2009) and assembled using Cufflinks version 2.0.2 (Trapnell et al., 2010). BigWig files were generated from normalized bedgraph file of each sample using bedGraphToBigWig. Scores represent normalized mapped read coverage. Expression values of RNA-Seq were expressed as FPKM (fragments per kilobase of exon per million fragments) values. Differential expression was calculated using Cuffdiff (Trapnell et al., 2010). Transcripts with FPKM >1, $q < 0.05$, and at least 1.5-fold change were defined as differentially expressed genes (DEGs). For individual mRNA of interests, the normality of data and homogeneity of variance were tested using the Shapiro-Wilk test and Brown-Forsythe test in Statistical Analysis System, respectively (version 8.1.; SAS Institute). Data were analyzed by least squares one-way analysis of variance and post hoc analysis (the Fisher least significant difference) with each gilt as an experimental unit. Data are expressed as means with SEM. $P < 0.05$ was considered statistically significant.

Results

Effect of estrous cycle on expression of ADM in porcine endometrium

The mRNA Expression was measured in terms of Fragments per Kilobase of transcripts per Million mapped reads (FPKM). In porcine endometrium, expression of *ADM* mRNA increased ($P<0.05$) between days 6 and 18 of estrous cycle with the highest expression at cyclic day 15 (6.8-fold, as compared to cyclic day 6) (**Figure 2a**).

*Effect of estrous cycle on expressions of *CALCRL*, *RAMP2*, *RAMP1*, *RAMP3* and *ACKR3* in porcine endometrium*

As the receptor components of ADM, mRNA expressions of *CALCRL* and *RAMP2* gradually increased ($P<0.05$) in porcine endometrium between days 6 and 18 of estrous cycle (**Figure 2a**). In particular, endometrial *CALCRL* mRNA levels reached the highest on cyclic day 15 with 5.1-fold increases ($P<0.05$, as compared to cyclic day 6) and then dropped between days 15 and 18 of estrous cycle, whereas *RAMP2* mRNA increased by 2.2-fold ($P<0.05$) between days 6 and 18 of estrous cycle (**Figure 2a**). While mRNA levels of *RAMP1* remained unchanged, *RAMP3* mRNA were first increased by 1.6-fold between days 6 and 12 and decreased thereafter (**Figure 2b**). In addition, *ACKR3* mRNA expression was lower between days 6 and 9 of estrous cycle and increased on day 12 of estrous cycle and maintained its high level on days 15 and 18 (**Figure 2b**).

Effect of pregnant status on cell-specific expressions of ADM associated genes in porcine endometrium

After mining the cell-type specific RNA-Seq data, *ADM* mRNA were detected in both LE and S, but not GE, of porcine endometria at day 12 of estrous cycle (CD12) and gestation (GD12); whereas mRNAs of *CALCRL*, *RAMP2* and *ACKR3* genes were expressed in endometrial LE, GE

and S in both cyclic and pregnant pigs (**Figure 2c**). In particular, pregnancy stimulated *ADM* mRNA levels by 10.5- and 2.3-fold ($P<0.05$) in LE and S, respectively; and inhibited *ACKR3* mRNA levels by 83.3%, 48.8% and 66.5%, in LE, GE, and S, respectively. No significant differences in mRNA levels of *CALCRL* and *RAMP2* were detected in endometrial LE, GE or S between cyclic and pregnant animals (**Figure 2c**).

Discussion

Adrenomedullin is a highly conserved peptide hormone required for intra-uterine spacing of blastocysts and angiogenesis during early pregnancy in rodents. This is the first study to investigate the relative mRNA expression of ADM and its related receptors on pig endometrium using a data-mining based analysis of two publicly available RNA-Seq datasets. We determined that the *ADM* and several of its associated receptor mRNA expressions were increased during estrous cycle, and pregnancy, but their expression patterns in endometrial LE, GE and S was differentially regulated.

Previous studies have shown that ADM is highly expressed in the reproductive tissues of humans (Cameron et al., 2002) and rats (Cameron & Fleming, 1998). In the normal human female reproductive tract, mRNA and protein levels of ADM and its receptors have been localized throughout all structures of the female reproductive system with marked expression in the epithelial cells of the uterus, fallopian tube, and blood vessels (Nikitenko et al., 2000). ADM also increased ciliary beat frequency and reduced contraction in rat oviduct, pointing to a possible role for ADM in mediating the regulation of embryo transport to the uterus (Liao et al., 2010). Expression of ADM and its receptor components are induced in the LE of the murine uterus as early as gestational day 0.5, lending support for the notion that ADM is required for embryo implantation and placentation in humans and mice. Insufficient levels of ADM expression in the

murine uterus causes abnormal placentation (Matson & Caron, 2014) and post-parturition complications (Karpinich et al., 2013). ADM administration to the murine endometrium results in increased water transport and formation of pinopodes in epithelia (Brooke C Matson et al., 2017). During the peri-implantation period of pregnancy in rodents, ADM is produced by both blastocyst trophectoderm and the uterine LE and stroma at the implantation site (Manyu Li et al., 2008). In addition, ADM is an angiogenic, anti-inflammatory and vasodilatory protein consistent with its likely role in cardiac, lymphatic and vascular system. (Karpinich et al., 2013). Similar to rodents, pig is also a litter-bearing species that faces early embryonic loss and fetal crowding. Thus, it is imperative that expression patterns of ADM and its receptor components in porcine endometrium be established. This data-mining based analysis of RNA-Seq datasets provided basic evidence of the presence of ADM and its associated receptor components in both cyclic and pregnant porcine endometrium. Thus, we proposed to investigate further, the temporal and cell-specific expressions of ADM and its associated receptor components in the endometrium, as well as in conceptuses, during peri-implantation period of pregnancy in pigs.

REFERENCES

- Ashary N, Tiwari A, Modi D. Embryo implantation: war in times of love. *Endocrinology* 2018;**159**:1188-1198.
- Bazer F, Geisert R, Thatcher W, Roberts R. establishment and maintenance of pregnancy. *Proceedings-Easter School in Agricultural Science, University of Nottingham* 1982.
- Cameron VA, Autelitano DJ, Evans JJ, Ellmers LJ, Espiner EA, Nicholls MG, Richards AM. Adrenomedullin expression in rat uterus is correlated with plasma estradiol. *American Journal of Physiology-Endocrinology and Metabolism* 2002;**282**:E139-E146.
- Cameron VA, Fleming AM. Novel sites of adrenomedullin gene expression in mouse and rat tissues. *Endocrinology* 1998;**139**:2253-2264.
- Karpnich NO, Kechele DO, Espenschied ST, Willcockson HH, Fedoriw Y, Caron KM. Adrenomedullin gene dosage correlates with tumor and lymph node lymphangiogenesis. *The FASEB Journal* 2013;**27**:590-600.
- Kim J-M, Park J-E, Yoo I, Han J, Kim N, Lim W-J, Cho E-S, Choi B, Choi S, Kim T-H. Integrated transcriptomes throughout swine oestrous cycle reveal dynamic changes in reproductive tissues interacting networks. *Scientific reports* 2018;**8**:1-14.
- Li M, Wu Y, Caron KM. Haploinsufficiency for adrenomedullin reduces pinopodes and diminishes uterine receptivity in mice. *Biology of reproduction* 2008;**79**:1169-1175.
- Liao S, Ho J, Tang F, WS O. Adrenomedullin increases ciliary beat frequency and decreases muscular contraction in the rat oviduct. *Reproduction (Cambridge, England)* 2010;**141**:367-372.
- Matson BC, Caron KM. Adrenomedullin and endocrine control of immune cells during pregnancy. *Cellular & molecular immunology* 2014;**11**:456-459.

- Matson BC, Pierce SL, Espenschied ST, Holle E, Sweatt IH, Davis ES, Tarran R, Young SL, Kohout TA, van Duin M. Adrenomedullin improves fertility and promotes pinopodes and cell junctions in the peri-implantation endometrium. *Biology of Reproduction* 2017;**97**:466-477.
- Mazur E, Large M, DeMayo F. Human oviduct and endometrium: changes over the menstrual cycle. *Knobil and Neill's Physiology of Reproduction Elsevier* 2015:1077-1097.
- Nikitenko L, MacKenzie I, Rees M, Bicknell R. Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. *Molecular human reproduction* 2000;**6**:811-819.
- Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 2009;**25**:1105-1111.
- Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 2010;**28**:511-515.

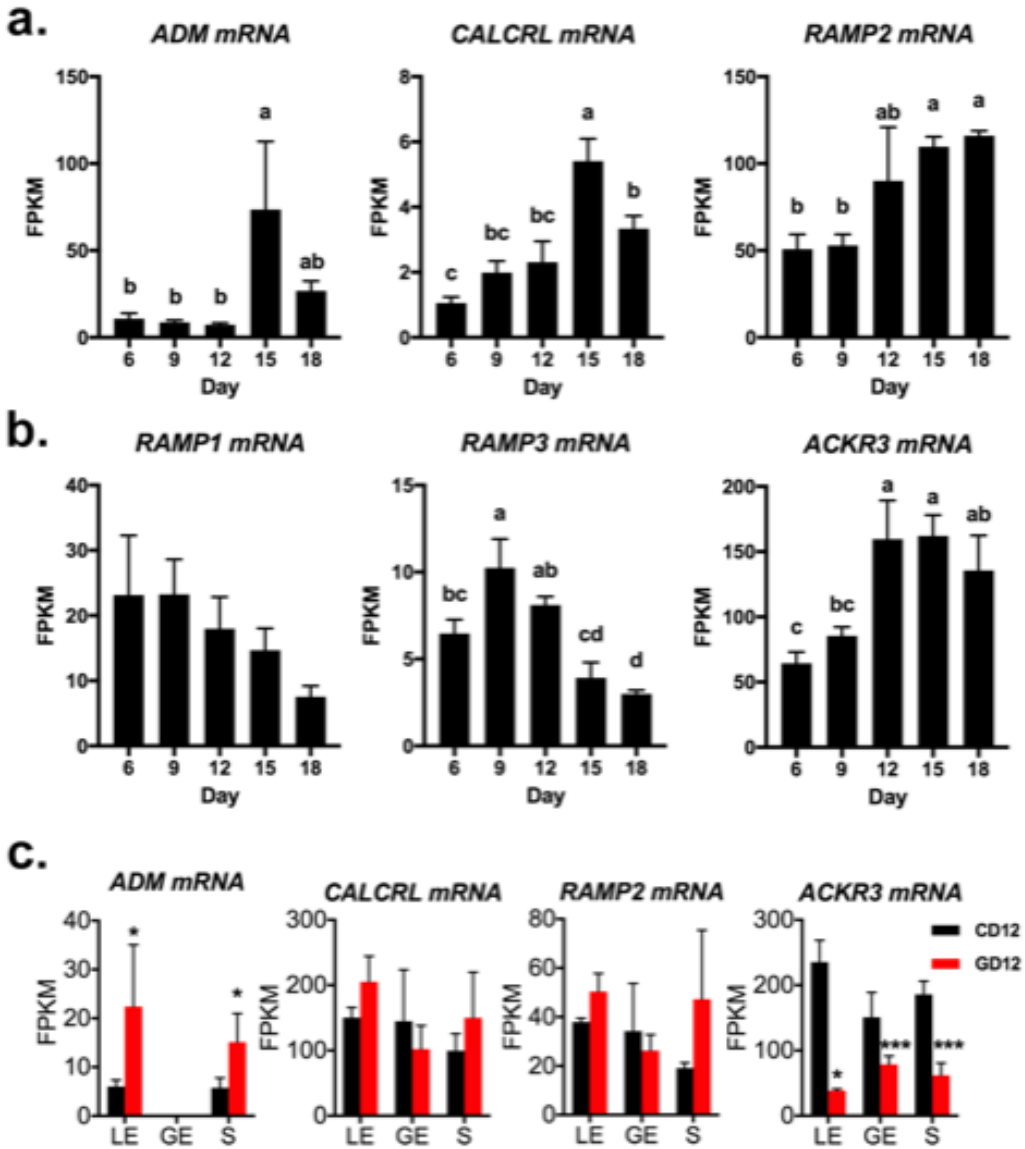


Figure 2. Gene expression of ADM and its receptors in pig endometrium based on RNA-seq data. (a, b) Effects of estrous cycle on ADM associated gene expression in porcine endometrium. n=3. Different superscript letters denote significant differences ($P < 0.05$). (c) Effects of pregnancy on ADM associated gene expression in each compartment of porcine endometrium. N=4. LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; CD, cyclic day; GD, gestational day. * $P < 0.05$; ***, $P < 0.001$. Data are presented as means \pm SEM.

**CHAPTER 3 – TEMPORAL AND SPATIAL EXPRESSION OF ADRENOMEDULLIN
AND ITS RECEPTORS IN PORCINE UTERUS AND PERI-IMPLANTATION
CONCEPTUSES.**

Abstract

Adrenomedullin (ADM) is an evolutionarily conserved multi-functional peptide hormone that regulates implantation, embryo spacing and placentation in humans and rodents. However, the potential roles of ADM in implantation and placentation of domestic animals, particularly pigs, as a litter-bearing species, are not known. This Chapter investigated the expression patterns of *ADM* mRNA and protein as well as its receptor components that include calcitonin receptor-like receptor (CALCRL; G protein-coupled receptor bound by ADM), receptor activity modifying proteins (RAMP2 and RAMP3; dimerized with CALCRL, i.e., CALCRL/RAMP2 or CALCRL/RAMP3) and atypical chemokine receptor 3 (ACKR3; a decoy receptor served as a cell-autonomous molecular rheostat to dampen ADM signaling) in uteri from cyclic and pregnant gilts (Days 10-16) as well as conceptuses (embryonic/fetus and its extra-embryonic membranes) during peri-implantation period of pregnancy when 30-40% of embryonic death loss occurs. Gilts (n=42) exhibiting at least two normal estrous cycles were bred via artificial insemination twice and then assigned randomly to be ovariectomized on Day 10, 11, 12, 13, 14, 15, or 16 of pregnancy (n=6 gilts/day; Day 0 is day of onset of estrus). Pregnancy was confirmed by the presence of morphologically normal conceptuses. Each uterine horn was flushed with 20 ml sterile PBS (pH 7.2). Conceptuses had expected morphological features, from spherical (Days 10 and 11), ovoid and/or tubular (Day 12) to filamentous forms (Days 13 to 16 of pregnancy). Immunohistochemical analyses revealed localization of ADM only in the uterine luminal epithelia (LE) of pregnant gilts between Days 12 and 16; whereas CALCRL and RAMP2 were expressed in the LE, glandular

epithelia (GE) and stroma of endometrium between Days 12 and 16 of pregnancy in gilts. In porcine conceptuses, expression of ADM, CALCRL and RAMP2 proteins increased in trophoctoderm cells between Days 12 and 16 of pregnancy. Further *in situ* hybridization have shown that mRNA expression of *ADM*, *CALCRL*, *RAMP2* and *RAMP3* genes increased in the porcine conceptus trophoctoderm between Days 12 and 16 of pregnancy; whereas *ACKR3* mRNA increased between Days 13 and 14 of pregnancy but decreased thereafter in the conceptus trophoctoderm. These results indicate that ADM may play functional roles in uterine receptivity as well as survival, growth, and development of the porcine conceptus during the peri-implantation period of pregnancy.

Introduction

Embryonic mortality is a major constraint to reproductive performance in all mammalian species. Estimated embryonic death loss in pigs, ruminants, and most mammals are 20-40%, with two-thirds of those losses occurring during peri-implantation period of pregnancy. The successful establishment and maintenance of pregnancy requires appropriate development of the conceptus (embryonic/fetus and its extra-embryonic membranes) for pregnancy recognition signaling which is critical for maintenance of the corpus luteum (CL) that secrete progesterone (P4). P4 is required for an intrauterine environment that support implantation, placentation, and uterine functions essential for birth of healthy offspring. During the peri-implantation period of pigs and ruminants, conceptus undergoes dramatic morphological transition from spherical to tubular to filamentous forms prior to implantation. Interactions among the conceptus and various uterine cell types, especially luminal (LE), superficial glandular epithelium (sGE) and glandular (GE) and stroma cells, are essential to conceptus development, transport of sugars and amino acids into the uterine lumen, as well as secretion of protein by uterine epithelial cells. This orchestrated process is highly dependent upon the composition of histotroph, i.e., secretions from uterine LE, sGE, and GE as well selective transport of nutrient into uterine lumen, including enzymes, growth factors, adhesion proteins, cytokines, hormones, amino acids, glucose, and fructose. Individual conceptus may fail to develop or not develop properly due to failure to respond to various components of histotroph that orchestrate events during the peri-implantation period of successful growth and development of the conceptus. While the underlying mechanism of embryonic mortality is not clearly understood. Expression of adrenomedullin (ADM), an evolutionary conserved peptide hormone required for implantation and embryo spacing in humans and rodents could have a potential role in implantation and placentation during pregnancy in pig. Thus, this Chapter investigated the

temporal and spatial expressions of ADM and its receptor components in porcine uterus and peri-implantation conceptuses.

Material and Methods

All experimental and surgical procedures were in compliance with the Guide for Care and Use of Agriculture Animals in Research and Teaching and approved by Institutional Animal Care and Use Committee of North Carolina State University.

Animal model and sample collection

Sexually mature gilts (F1 cross of Yorkshire X Landrace sows and Duroc boars) were maintained at the North Carolina State University Educational Swine Unit. Gilts were observed daily for signs of estrus (Day 0) and exhibited at least two estrous cycles of normal duration (18-21 days) prior to being used in experiments. Cyclic gilts were assigned randomly and hysterectomized on either Day 10, 11, 12, 13, 14 or 15 of the estrous cycle (n=3-6 gilts/day), while pregnant gilts, they were bred via artificial insemination (AI) at 12 and 24 h after detection of estrus and assigned randomly to be hysterectomized on either day 10, 11, 12, 13, 14, 15, or 16 of pregnancy (n=6 gilts/day). After hysterectomy, each uterine horn of all gilts was flushed with 20 ml sterile phosphate buffered saline (PBS, pH7.2). Pregnancy was confirmed by the presence of morphologically normal conceptuses in the uterine flushing. After recovering the conceptuses from the uterine flushings with transfer pipette, the recoverable volume of uterine flushings were recorded. Conceptuses were fixed in fresh 4% paraformaldehyde (PFA; prepared in PBS, pH 7.2) for 48 h and then in 70% ethanol for 24 h. The fixed tissues were dehydrated through a graded series of alcohol to xylene and embedded in Paraplast-Plus (Sigma-Aldrich, St. Louis, MO). Sections (~1 cm) of uterine wall of the cyclic and pregnant uterine horn were fixed in fresh 4% PFA for eventual embedding in Paraplast-Plus as described above. The remaining endometrium

from the uterine horn were dissected from myometrium, frozen in liquid nitrogen and stored in -80°C for future use. Uterine flushings were clarified by centrifugation (5,000 x g for 15 min at 4°C), aliquoted and stored in -80°C for future studies.

RNA in situ hybridization analyses

RNAscope *in situ* hybridization (ISH) (Advanced Cell Diagnostic, Newark, CA, USA) was performed according to the manufacturer's instructions using paraformaldehyde-fixed uterine and conceptus tissues (~5 µm) as well as customized probes Ss-ADM (819541), Ss-CALCRL (819561), Ss-RAMP2 (819551), Ss-RAMP3 (857371), and Ss-ACKR3 (819571) for porcine ADM, CALCRL, RAMP2, RAMP3 and ACKR3, respectively. A porcine peptidylprolyl isomerase B probe (Ss-PPIB; 428591) and a bacterial DapB gene probe (310043) were used as positive and negative controls, respectively. The HD 2.5 Red Detection Kit (322360-USM; Advanced Cell Diagnostic) was used for visualization of probe binding. Briefly, the sections were deparaffinized in Citrisolve (14201213, Decon Labs) 3 times for 5 minutes followed by incubation in 100% ethanol (CAS# 64-17-5) 2 times for 3 minutes then let it air dry for 5 minutes. The tissues were pretreated with hydrogen peroxide for 10 min at room temperature. The target retrieval was performed by placing the tissue rack in boiling target retrieval solution then treated with protease at 40°C for 30 minutes. Samples were circled with Hydrophobic Barrier Pen (H-4000; Vector Laboratories). For hybridization, the slides were then incubated in the order of the following solution: target probes (including customized probes, and probes for positive and negative control) for 2 h at 40°C, Amplifier-1 for 30 min at 40°C, Amplifier-2 for 15 min at 40°C, Amplifier-3 for 30 min at 40°C, Amplifier-4 for 15 min at 40°C, Amplifier 5 and 6 for 30 and 15 min respectively at room temperature. The signal detection was done using the label probe RED-A and RED-B at the ratio of 1:60 for 10 minutes at room temperature. The slides were counterstained with 50%

hematoxylin (MHS32-1L, Sigma aldrich) for 1 minutes followed by 40 seconds of saturated lithium carbonate (1.54g lithium carbonate in 100ml of ddH₂O). The slides were then washed in water 5-6 times and airdried for 30 min and fixed with coverslip.

Immunohistochemical analyses

Immunohistochemical (IHC) localization of ADM, CALCRL and RAMP2 protein in porcine uterine and conceptus tissues (~5µm) was performed as described previously (Wang, Frank, Little, et al., 2014; Wang, Frank, Xu, et al., 2014; Wang et al., 2018). Goat anti-ADM polyclonal immunoglobulin G (IgG; AF6108; R&D systems, Minneapolis, MN, USA), rabbit anti-CALCRL polyclonal IgG (NLS6731; Thermo Fisher, Waltham, MA, USA) and rabbit anti-RAMP2 polyclonal IgG (PA5-21953; Invitrogen, Waltham, MA, USA) were used at dilutions of 1:200, 1:500, 1:500, respectively. Antigen retrieval was performed using antigen unmasking solution (H-3300; Vector Laboratories, Burlingame, CA, USA) for ADM, CALCRL and RAMP2. Purified nonrelevant goat or mouse IgG was used as a negative control to replace the primary antibody at the same final concentration. For ADM, ADM-overexpressed ($ADM^{OE/OE}$) mouse uterine tissue at Day 4.5 (PPD 4.5) of pseudopregnancy and ADM-deleted ($ADM^{-/-}$) mouse placenta were used as positive and negative controls, respectively. Immunoreactive proteins were visualized in sections using the Vectastain ABC Kit (PK-6100; Vector Laboratories), following the manufacturer's instructions, and 3,3'-diaminobenzidine tetrahydrochloride (D8001; Sigma-Aldrich) was used as the color substrate. Sections were counterstained with hematoxylin before dehydrating and affixing coverslips with Permount. Digital images of uteri and conceptuses were captured using an Axioplan 2 microscope with an Axiocam HR camera and Axiovision 4 software (Carl Zeiss, Thornwood, NY, USA).

Results

Localization of ADM mRNA and protein in porcine endometria and conceptuses

ISH analyses was used to detect *ADM* mRNA in a cell-specific manner in uteri of cyclic and pregnant gilts, as well as conceptuses (**Figure 3.1**). In cyclic gilts, *ADM* mRNA was weakly detectable in uterine LE and GE on days 10, 11, 14 and 15 of the estrous cycle (**Figure 3.1**, left column). In pregnant gilts, *ADM* mRNA in uterine LE and GE was detectable between days 10 and 12, and visually abundant only in uterine LE between days 13 and 16 with the strongest expression on day 14 of pregnancy (**Figure 3.1**, mid column). In porcine conceptuses, *ADM* mRNA abundance was weak on days 10 and 11, but visually stronger in trophoctoderm (Tr) and extraembryonic endoderm (En) between days 12 and 16 of pregnancy (**Figure 3.1**, right column).

Unlike mRNA levels, immunoreactive ADM protein was detectable by IHC analyses in uterine LE and GE, as well as stromal cells of cyclic and pregnant gilts (**Figure 3.2**). However, ADM protein in uterine LE was visually greater for pregnant than cyclic gilts between days 12 and 16 (**Figure 3.2**, left and mid columns). In addition, ADM protein was detectable in conceptuses between days 10 and 11, but visually abundant in conceptus Tr and En between days 12 and 16 of pregnancy (**Figure 3.2**, right column). Together, the expression of *ADM* gene increased significantly in cells of the uterus (particularly in LE) and in conceptus tissue during early pregnancy.

Localization of CALCRL, RAMP2, RAMP3, and ACKR3 mRNAs and/or proteins in porcine endometria and conceptuses

Next, we investigated the localization of ADM receptors ADM₁ (CALCRL/RAMP2) and ADM₂ (CALCRL/RAMP3), and associated component ACKR3 at mRNA and/or protein in uteri of cyclic and pregnant gilts and in conceptuses (**Figures 3.3-3.8**).

CALCRL mRNA and protein were detectable in uterine LE, GE, and stromal cells of cyclic and pregnant gilts, as well as Tr and En of conceptuses (**Figures 3.3 and 3.4**). In cyclic gilts, *CALCRL* mRNA and protein were expressed weakly in uterine LE, GE and stromal cells between Days 10 and 11, undetectable in uteri between Days 12 and 13, and then visually abundant in the uteri between Days 14 and 15. On the other hand, *CALCRL* mRNA and protein were strongly expressed in superficial GE (sGE) and GE between Days 10 and 11, and in uterine LE between Days 12 and 16 of pregnancy. In peri-implantation conceptuses, *CALCRL* mRNA and protein were weakly expressed in Tr between Days 10 and 11, but visually abundant in Tr and En between Days 12 and 16 of pregnancy.

Both *RAMP2* mRNA and protein were expressed weakly in uterine LE, GE and stromal cells on Days 10, 11, 14 and 15, but were undetectable between Days 12 and 13 of the estrous cycle in gilts (**Figures 3.5 and 3.6**). In contrast, expression of *RAMP2* mRNA and protein was visually abundant in uterine LE, GE and stromal cells between Days 10 and 16 of pregnancy. Furthermore, *RAMP2* mRNA and protein were expressed in Tr and En of conceptuses and visually abundant between Days 12 and 16 of pregnancy.

Due to the limited sources of antibodies, localization of *RAMP3* and *ACKR3* genes were only detected at the mRNA level via *in situ* hybridization (**Figures 3.7 and 3.8**). *RAMP3* mRNA in uterine LE, GE and stroma was detectable on Days 11, 14 and 15 of the estrous cycle and visually abundant between Days 13 and 16 of pregnancy (**Figure 3.7**). In porcine conceptuses, *RAMP3* mRNA was expressed in Tr and En and visually abundant between Days 12 and 16 of pregnancy (**Figure 3.7**). In addition, *ACKR3* mRNA was expressed weakly in uterine LE and GE between Days 12 and 13, but visually abundant at Days 10, 11, 14 and 15 of the estrous cycle in gilts (**Figure 3.8**). In pregnant gilts, *ACKR3* mRNA in uterine LE was abundant on Day 10 and

expressed weakly between Days 11 and 16 of gestation; whereas expression of *ACKR3* mRNA in uterine GE and stromal cells was strong on Day 10, remained visually elevated through Day 15, and undetectable to Day 16 (**Figure 3.8**). In porcine conceptuses, *ACKR3* mRNA was detectable in Tr between Days 14 and 16 of pregnancy.

Discussion

Adrenomedullin (ADM) is a highly conserved peptide hormone required for intra-uterine spacing of blastocyst and angiogenesis during early pregnancy in rodents (Kathleen M. Caron & Oliver Smithies, 2001; Manyu Li et al., 2008). ADM levels in human amniotic fluid are also inversely correlated with birth weight and length of babies (Patricia M Lenhart & Kathleen M Caron, 2012). However, little is known about the expression patterns of ADM and its receptors, as well as the significance of ADM in the porcine uterus and conceptuses during early pregnancy. This is the first report of changes in ADM and its associated components (*CALCRL*, *RAMP2*, *RAMP3* and *ACKR3*) during the estrous cycle and peri-implantation period of pregnancy in pigs.

We investigated the sources of ADM using ISH and IHC analysis. *ADM* mRNA was expressed mainly by uterine LE and sporadically by uterine GE, but not by uterine stromal cells during early pregnancy. However, at the protein level, ADM was detectable in stromal cells, indicating the transport of ADM from maternal blood into the uterine tissue cells. Even though the expression of *ADM* mRNA increased significantly in both cyclic and pregnant porcine uteri between Days 10 and 16, the increase was greater during pregnancy. This suggests that the uterine expression may be progesterone (P4)-induced and estradiol (E2)- stimulated as conceptus E2 is the pregnancy recognition signaling in pigs. On the other hand, ADM mRNA and protein were also expressed in the porcine conceptus Tr and En and most abundant between Days 12 and 16 of pregnancy, with greatest expression on Days 12 and 15 respectively. This also suggests a

significant role for ADM in the initiation of conceptus elongation and implantation as porcine conceptuses elongate most rapidly from Day 12 to Day 16 and undergo implantation.

ADM signals through its receptor complexes ADM1 (CALCRL/RAMP2) and/or ADM2 (CALCRL/RAMP3) on cells (Hay et al., 2004; Kuwasako et al., 2011). CALCRL is a G-protein coupled receptors whereas RAMPs contribute to CALCRL translocation towards the plasma membrane. Unlike ADM that is expressed primarily by uterine LE, expression of CALCRL, RAMP2 and RAMP3 was greatest in uterine LE, GE, and stromal cells of pregnant gilts; since they are a prerequisite for ADM to exert its functional roles during early pregnancy, perhaps this pattern of expression is understandable. Meanwhile, as a decoy receptor to dampen the ADM signaling (Kuwasako et al., 2011), expression of ACKR3 was not affected by days of estrous cycle but decreased in endometria of pregnant gilts between Days 10 and 16 of pregnancy. The decrease in expression of the decoy receptors ACKR3 was most notable in uterine LE and is consistent with the cellular mechanisms to increase ADM signaling as part of enhancing the window of receptivity to implantation.

Notably, the components of ADM receptors, i.e., CALCRL, RAMP2 and RAMP3 were expressed in the porcine conceptus Tr and En during the peri-implantation period of pregnancy, suggesting both paracrine and autocrine effects of ADM on growth and development of porcine conceptuses. Meanwhile, the significant increase in CALCRL and RAMP2, but not RAMP3, particularly between Days 14 and 16 of pregnancy further suggest that: 1) ADM1 (CALCRL/RAMP2) is the regulatory receptor of ADM in the conceptuses in response to pregnancy; and 2) there is a positive correlation between ADM signaling and conceptus behavior of Tr in terms of elongation, migration and adhesion as porcine conceptus elongates from mid(100-200mm) to late-filamentous (800-1000mm) forms, and begins to attach between Days 14 and 16

of pregnancy (Fuller W Bazer & Gregory A Johnson, 2014; Rodney D Geisert et al., 1982). Interestingly, ACKR3 was also detectable in Tr between Days 14 and 16 of pregnancy. ACKR3 is a cell-autonomous molecular rheostat to dampen ADM signaling (Klein et al., 2014). Recent reports indicate that RAMP3 can mediate rapid recycling of ACKR3 and enhance angiogenesis in the retina postnatally (Mackie et al., 2019). Understanding the precise mechanisms by which expression of ACKR3 serves as non-signaling receptors to control the functional dosage of ADM in growth and development of porcine conceptuses in future research.

REFERENCES

- Bazer, F. W., & Johnson, G. A. (2014). Pig blastocyst–uterine interactions. *Differentiation*, 87(1-2), 52-65.
- Caron, K. M., & Smithies, O. (2001). Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. *Proceedings of the National Academy of Sciences*, 98(2), 615-619. <https://doi.org/10.1073/pnas.98.2.615>
- Geisert, R. D., Brookbank, J. W., Michael Roberts, R., & Bazer, F. W. (1982). Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. *Biology of reproduction*, 27(4), 941-955.
- Hay, D. L., Conner, A. C., Howitt, S. G., Smith, D. M., & Poyner, D. R. (2004). The pharmacology of adrenomedullin receptors and their relationship to CGRP receptors. *Journal of Molecular Neuroscience*, 22(1), 105-113.
- Klein, K. R., Karpinich, N. O., Espenschied, S. T., Willcockson, H. H., Dunworth, W. P., Hoopes, S. L., Kushner, E. J., Bautch, V. L., & Caron, K. M. (2014). Decoy receptor CXCR7 modulates adrenomedullin-mediated cardiac and lymphatic vascular development. *Developmental cell*, 30(5), 528-540.
- Kuwasako, K., Kitamura, K., Nagata, S., Hikosaka, T., Takei, Y., & Kato, J. (2011). Shared and separate functions of the RAMP-based adrenomedullin receptors. *Peptides*, 32(7), 1540-1550.
- Lenhart, P. M., & Caron, K. M. (2012). Adrenomedullin and pregnancy: perspectives from animal models to humans. *Trends in Endocrinology & Metabolism*, 23(10), 524-532.
- Li, M., Wu, Y., & Caron, K. M. (2008). Haploinsufficiency for adrenomedullin reduces pinopodes and diminishes uterine receptivity in mice. *Biology of reproduction*, 79(6), 1169-1175.

- Mackie, D. I., Nielsen, N. R., Harris, M., Singh, S., Davis, R. B., Dy, D., Ladds, G., & Caron, K. M. (2019). RAMP3 determines rapid recycling of atypical chemokine receptor-3 for guided angiogenesis. *Proceedings of the National Academy of Sciences*, *116*(48), 24093-24099.
- Wang, X., Frank, J. W., Little, D. R., Dunlap, K. A., Satterfield, M. C., Burghardt, R. C., Hansen, T. R., Wu, G., & Bazer, F. W. (2014). Functional role of arginine during the peri-implantation period of pregnancy. I. Consequences of loss of function of arginine transporter SLC7A1 mRNA in ovine conceptus trophoctoderm. *FASEB J*, *28*(7), 2852-2863. <https://doi.org/10.1096/fj.13-248757>
- Wang, X., Frank, J. W., Xu, J., Dunlap, K. A., Satterfield, M. C., Burghardt, R. C., Romero, J. J., Hansen, T. R., Wu, G., & Bazer, F. W. (2014). Functional role of arginine during the peri-implantation period of pregnancy. II. Consequences of loss of function of nitric oxide synthase NOS3 mRNA in ovine conceptus trophoctoderm. *Biol Reprod*, *91*(3), 59. <https://doi.org/10.1095/biolreprod.114.121202>
- Wang, X., Li, X., Wang, T., Wu, S. P., Jeong, J. W., Kim, T. H., Young, S. L., Lessey, B. A., Lanz, R. B., Lydon, J. P., & DeMayo, F. J. (2018). SOX17 regulates uterine epithelial-stromal cross-talk acting via a distal enhancer upstream of *Ihh*. *Nat Commun*, *9*(1), 4421. <https://doi.org/10.1038/s41467-018-06652-w>

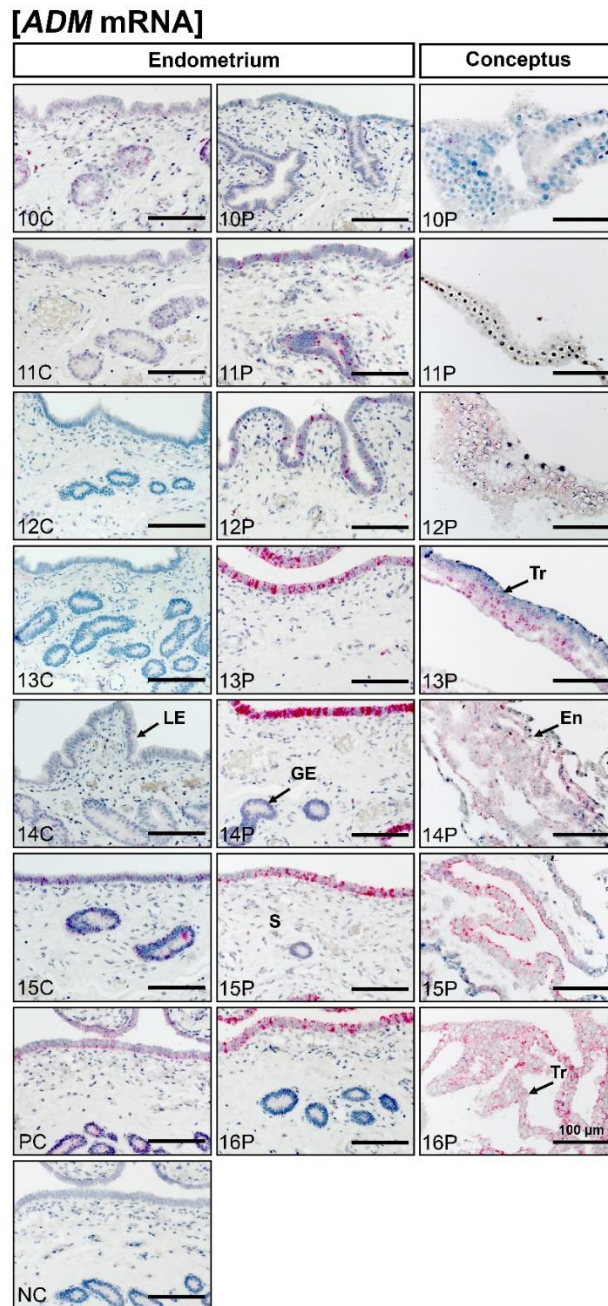


Figure 3.1. Localization of ADM mRNA in pregnant and cyclic porcine endometrium and conceptuses. Adrenomedullin expression in the cyclic porcine Uterus was very low. ADM was upregulated in the pregnant uterus and conceptuses in early pregnancy days starting from day 12. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophoblast cells; En, extraembryonic endoderm; NC, Negative Control; PC, Positive Control.

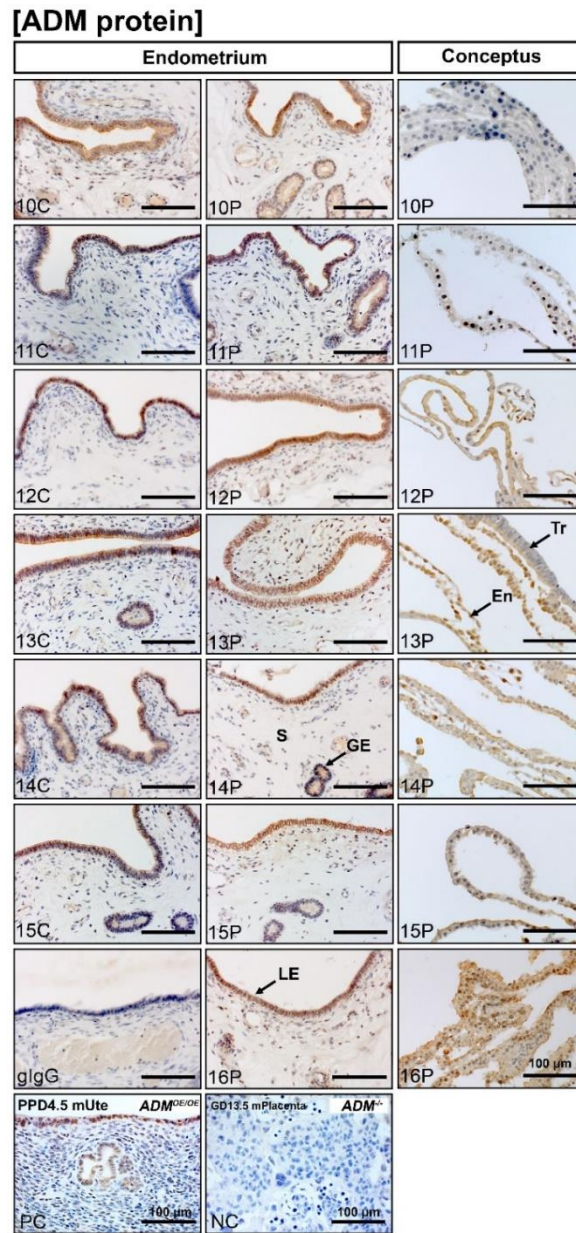


Figure 3.2. Localization of ADM protein in pregnant and cyclic porcine endometrium and conceptuses. Adrenomedullin expression in the cyclic porcine uteri was very low. ADM was upregulated in the pregnant uterus in early pregnancy days starting from day 12. ADM was upregulated on trophoctoderm cells from day 12 porcine conceptuses. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophoctoderm cells; En, extraembryonic endoderm; IgG, goat immunoglobulin-G; NC, Negative Control; PC, Positive Control.

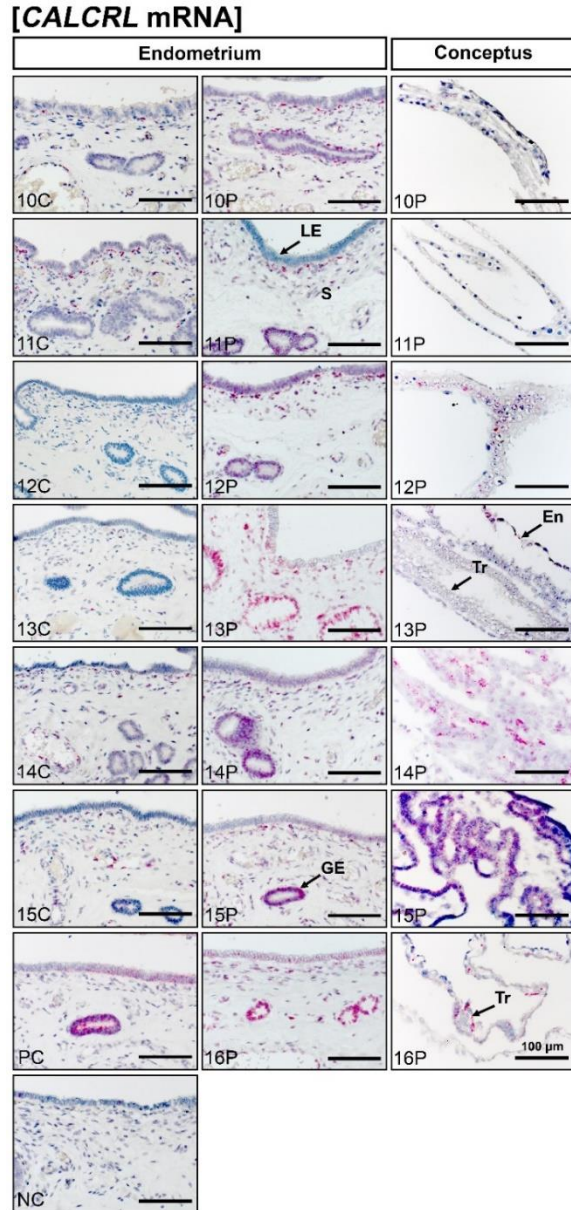


Figure 3.3. Localization of *CALCRL* mRNA in pregnant and cyclic porcine endometrium and conceptuses. *CALCRL* mRNA expression in the cyclic porcine uteri was very low. *CALCRL* was upregulated in the pregnant uterus in early pregnancy days starting from day 12. *CALCRL* mRNA was upregulated in Trophectoderm cells from day 12 of porcine conceptuses. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophectoderm cells; En, extraembryonic endoderm; NC, Negative Control; PC, Positive Control.

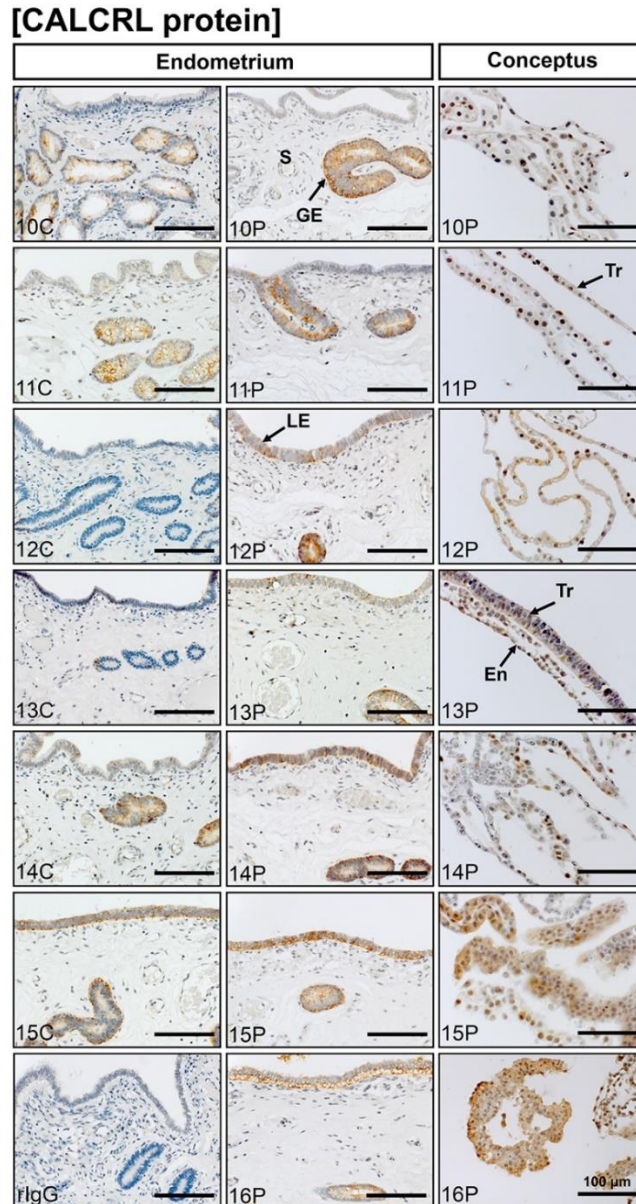


Figure 3.4. Localization of CALCRL protein in pregnant and cyclic porcine endometrium and conceptuses. CALCRL expression in the cyclic porcine uteri was very low. CALCRL was upregulated in the pregnant uterus in early pregnancy days starting from day 12. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophectoderm cells; En, extraembryonic endoderm; rIgG, rabbit immunoglobulin-G as negative control.

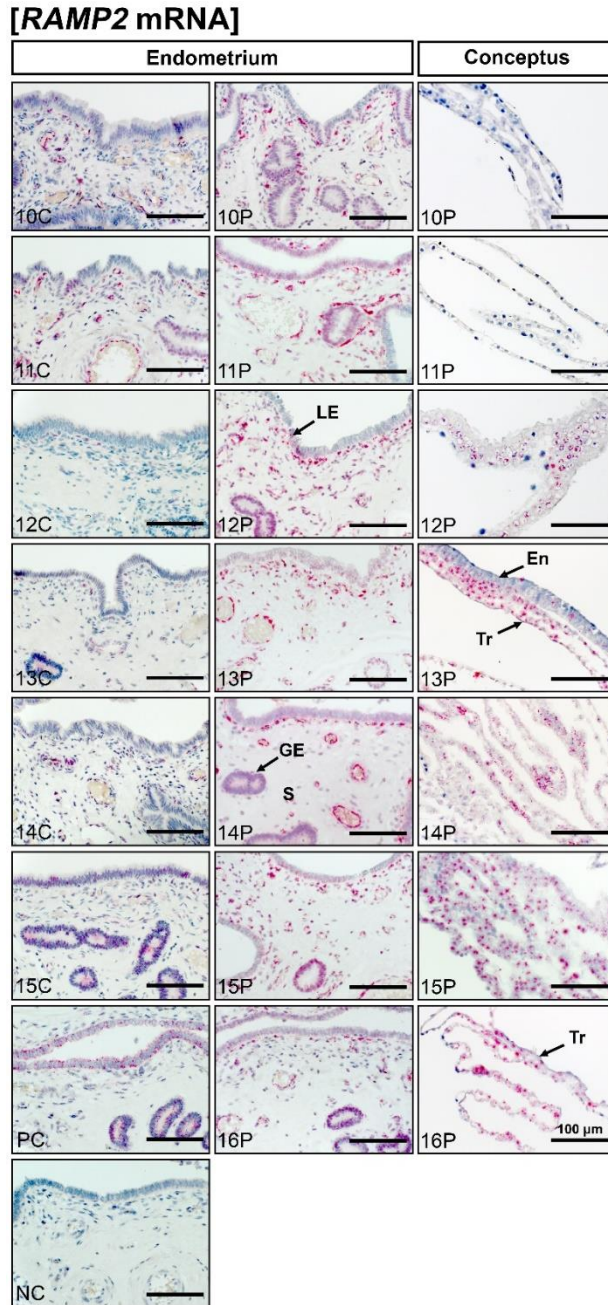


Figure 3.5. Localization of *RAMP2* mRNA in pregnant and cyclic porcine endometrium and conceptuses. *RAMP2* mRNA expression in the cyclic porcine uteri was very low. *RAMP2* was upregulated in the pregnant uterus in early pregnancy days starting from day 10. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophoblast cells; En, extraembryonic endoderm; rIgG, rabbit immunoglobulin-G as negative control.

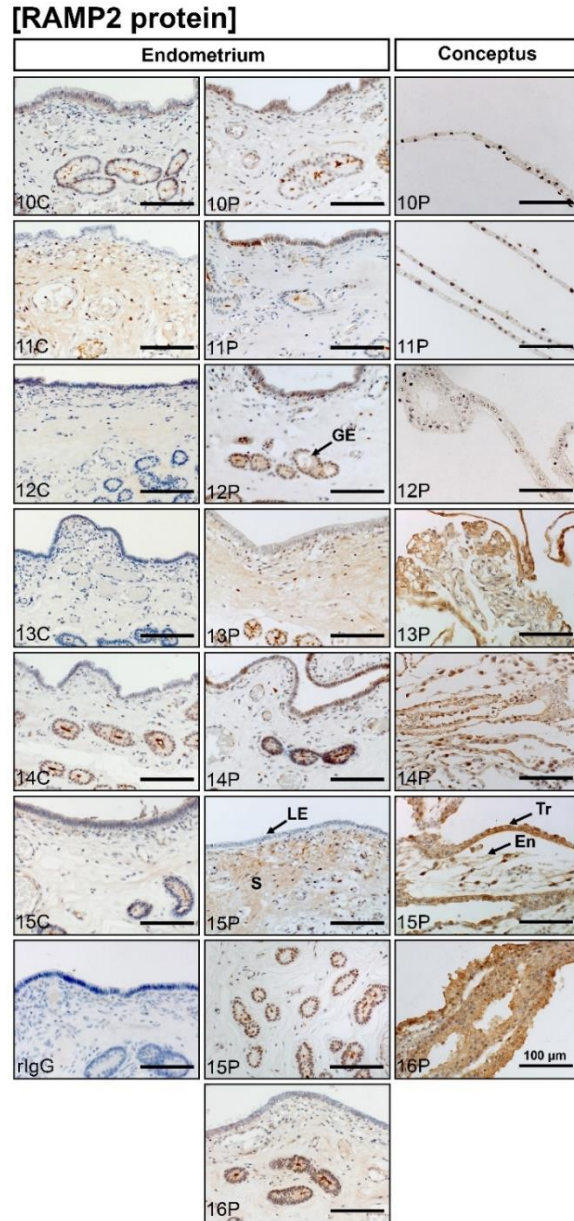


Figure 3.6. Localization of RAMP2 in pregnant and cyclic porcine endometrium and conceptuses. RAMP2 protein expression in the cyclic porcine uteri was very low. CALCRL was upregulated in the pregnant uterus and conceptuses in early pregnancy days starting from day 12 and maintained its high level through day 16. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophoblast cells; En, extraembryonic endoderm; rIgG, rabbit immunoglobulin-G as negative control.

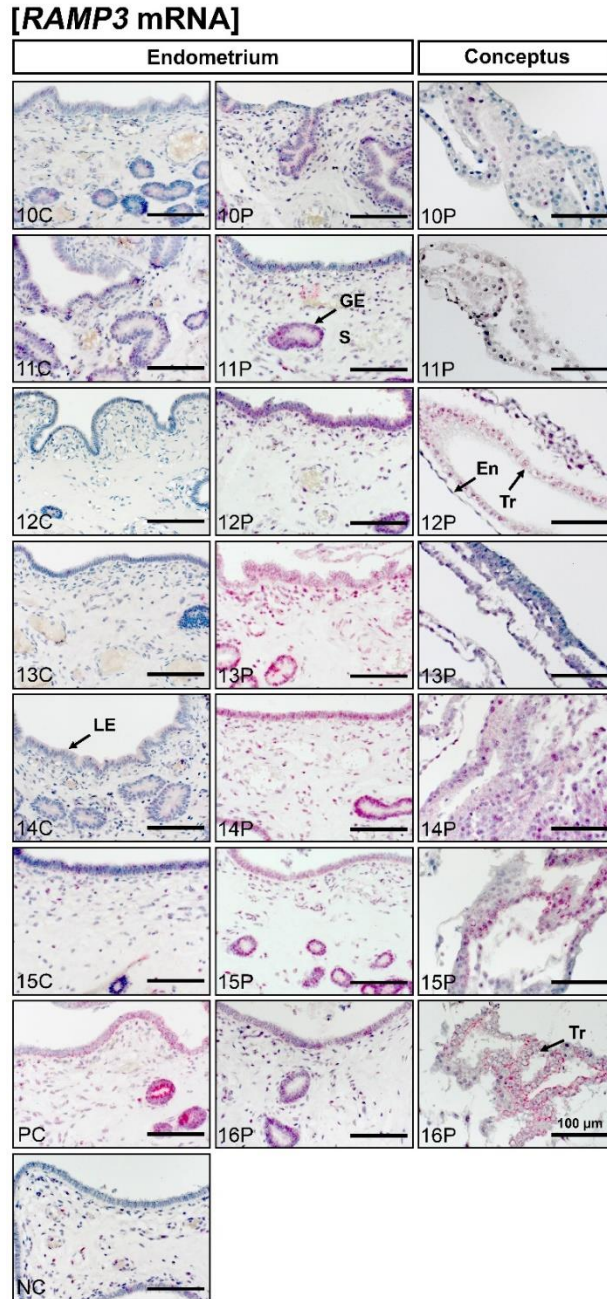


Figure 3.7. Localization of *RAMP3* mRNA in pregnant and cyclic porcine endometrium and conceptuses. *RAMP3* expression in the cyclic porcine uteri was very low. *RAMP3* was upregulated in the pregnant uteri and conceptuses beginning on day 12, and the higher level was maintained throughout early pregnancy. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophectoderm cells; En, extraembryonic endoderm; NC, Negative Control; PC, Positive Control.

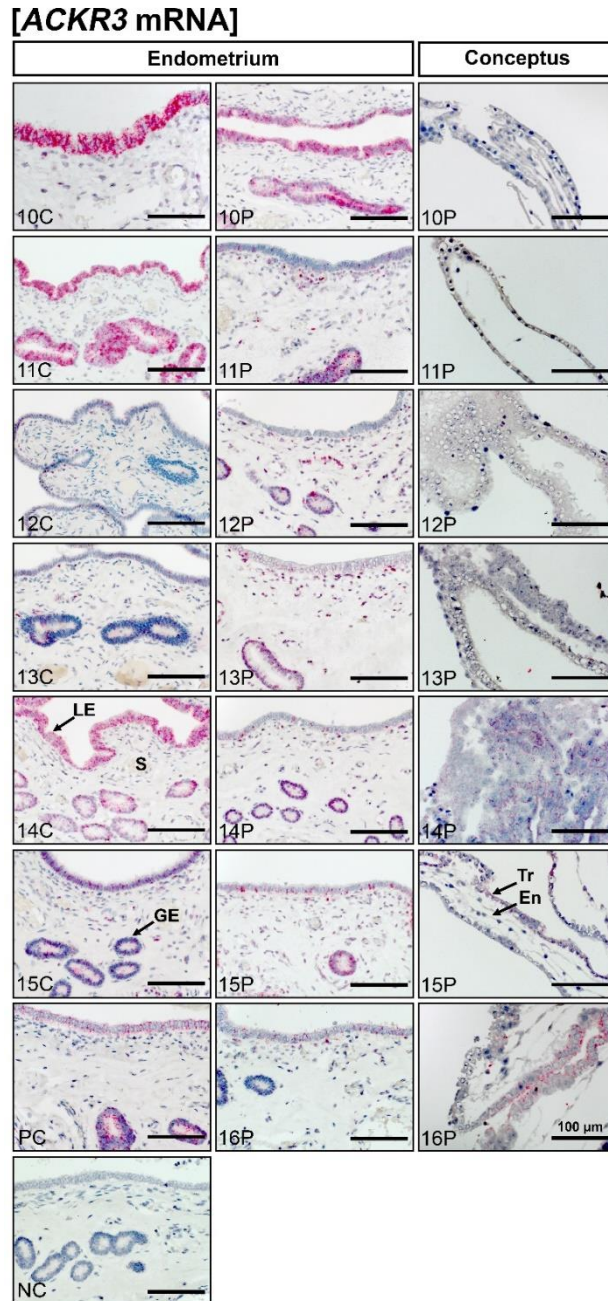


Figure 3.8. Localization of *ACKR3* mRNA in pregnant and cyclic porcine endometrium and conceptuses. *ACKR3* mRNA expression in the cyclic porcine uteri was high. *ACKR3* was downregulated in the pregnant uteri during early pregnancy. N=3. C, cyclic; P, pregnant; LE, luminal epithelium; GE, glandular epithelium; S, stromal cells; Tr, Trophectoderm cells; En, extraembryonic endoderm; NC, Negative Control; PC, Positive Control.

CHAPTER 4 – CONCLUSIONS AND FUTURE DIRECTIONS

Based on the literature outlined in Chapter 1, it is clearly evident that ADM play key roles in the reproductive performance in mammalian species. ADM and its receptors are highly expressed in reproductive tissues of humans and rodents, including the uterine endometrium (Hague et al., 2000), fetal membranes (Trollmann et al., 2002), placenta (Minegishi et al., 1999), stromal macrophages (Zhao et al., 1998), and trophoblast cells (Gratton et al., 2003; Marinoni et al., 1998; Montuenga et al., 1997; Yotsumoto et al., 1998). In rats, ADM increases from small antral follicles to large antral follicles to the formation of the CL, indicating its role in the regulation of P4 production from the CL (Li et al., 2011). ADM also stimulates the frequency of ciliary beats and reduces contraction of the rat oviduct, suggesting its role in regulation of embryo transport to the uterus (Liao et al., 2011). In mice, ADM and its receptors are induced in uterine LE as early as gestational day (GD) 0.5, and AM is expressed by both conceptus trophectoderm and uterine LE and stromal cells at implantation sites during the peri-implantation period (Li et al., 2008). Due to the embryonic lethality in homozygous *Adm*-null mice, the heterozygous *Adm*^{+/-} (50% AM expression) mice have been studied (Caron & Smithies, 2001) and females have a significantly reduced pregnancy success rate compared to wild type females, despite having rates of ovulation and fertilization that are normal (Lenhart & Caron, 2012; Li et al., 2006). The implantation sites in pregnant *Adm*^{+/-} females are abnormally spaced leading to crowding of implantation sites resulting in increased rates of embryo loss and a reduction in prolificacy (Li et al., 2006). This reduced pregnancy rate persists even when wild-type conceptuses are transferred to *Adm*^{+/-} female, suggesting that reduced maternal ADM is responsible for defects in uterine receptivity, implantation and/or placentation (Li et al., 2006). In domestic animals, Hayashi et al. reported expression of ADM system (AM, CALCRL, RAMP2 and RAMP3) genes in bovine utero-

placental tissues between days 25 and 250 of pregnancy (Hayashi et al., 2013). ADM and its receptors are expressed in uterine caruncles, intercaruncular endometrium, extra-embryonic membranes including cotyledonary villi and intercotyledonary chorion throughout pregnancy with the two peaks of expression on days 60 and 200. However, little is known about the ADM system in the porcine reproductive tissue. Given that ADM is critical for implantation, placentation and spacing of blastocysts in humans and rodents (Lenhart & Caron, 2012; Li et al., 2008; Li et al., 2006; Matson et al., 2017), it is imperative that the function and regulation of ADM as related to uterine receptivity, as well as growth and development of conceptus during the pregnancy in all domestic animals be established. This is particularly relevant for pigs as they are the litter-bearing species with a high incidence of early embryonic death (30-40%), naturally occurring intrauterine growth restriction, and frequent occurrences of stillborn piglets (3-9%) (Leenhouders et al., 2001; Mesa et al., 2006; Redmer et al., 2004; Tuchscherer et al., 2000; Vallet et al., 2011; Vallet et al., 2014; Wang et al., 2014; Wang et al., 2010; Wu et al., 2008; Wu et al., 2006).

This is the first report of changes in ADM and its associated components (CALCRL, RAMP2, RAMP3 and ACKR3) in endometrial and/or conceptus tissues during the estrous cycle and peri-implantation period of pregnancy in pigs. In Chapter 2, we first performed a data-mining based analysis on two publicly available RNA-Seq datasets of porcine endometria, investigating the expression patterns of ADM and its receptor components (CALCRL, RAMP1, RAMP2, RAMP3 and ACKR3) in uteri from cyclic and pregnant gilts as well as the specific cell types (LE, GE and S) of pregnant uterine endometrium. Thus, in Chapter 3, ISH and IHC analyses were further used to investigate the temporal and cell-specific expressions of ADM and its receptor components in porcine endometrium and conceptuses during peri-implantation period of pregnancy. In summary, ADM as well as its receptors were highly expressed in both uterine

endometria and conceptuses during peri-implantation period of pregnancy. Because ADM is a multifunctional regulatory peptide hormone known to influence the spacing of blastocysts and angiogenesis, understanding its precise roles and mechanisms of action during early pregnancy in pigs is a prerequisite for improving reproductive efficiency in swine. Therefore, future studies will further dissect the functional role of ADM signaling on peri-implantation conceptus growth and development, as well as pre-implantation spacing of blastocysts and uterine capacity using in vitro and in vivo loss-of-function studies with pigs. The correlation between concentrations of ADM in reproductive fluids (e.g., serum, amniotic and allantoic fluids) and reproductive health status throughout the gestation will also be investigated in pigs.

REFERENCES

- Caron, K. M., & Smithies, O. (2001). Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. *Proc Natl Acad Sci U S A*, 98(2), 615-619. <https://doi.org/10.1073/pnas.021548898>
- Gratton, R. J., Gluszynski, M., Mazzuca, D. M., Nygard, K., & Han, V. K. (2003). Adrenomedullin messenger ribonucleic acid expression in the placentae of normal and preeclamptic pregnancies. *J Clin Endocrinol Metab*, 88(12), 6048-6055. <https://doi.org/10.1210/jc.2003-030323>
- Hague, S., Zhang, L., Oehler, M. K., Manek, S., MacKenzie, I. Z., Bicknell, R., & Rees, M. C. (2000). Expression of the hypoxically regulated angiogenic factor adrenomedullin correlates with uterine leiomyoma vascular density. *Clin Cancer Res*, 6(7), 2808-2814. <https://www.ncbi.nlm.nih.gov/pubmed/10914728>
- Hayashi, K. G., Hosoe, M., Sakumoto, R., & Takahashi, T. (2013). Temporo-spatial expression of adrenomedullin and its receptors in the bovine placenta. *Reprod Biol Endocrinol*, 11, 62. <https://doi.org/10.1186/1477-7827-11-62>
- Leenhouwers, J. I., de Almeida Junior, C. A., Knol, E. F., & van der Lende, T. (2001). Progress of farrowing and early postnatal pig behavior in relation to genetic merit for pig survival. *J Anim Sci*, 79(6), 1416-1422. <https://doi.org/10.2527/2001.7961416x>
- Lenhart, P. M., & Caron, K. M. (2012). Adrenomedullin and pregnancy: perspectives from animal models to humans. *Trends Endocrinol Metab*, 23(10), 524-532. <https://doi.org/10.1016/j.tem.2012.02.007>

- Li, L., O, W. S., & Tang, F. (2011). Adrenomedullin in rat follicles and corpora lutea: expression, functions and interaction with endothelin-1. *Reprod Biol Endocrinol*, 9, 111. <https://doi.org/10.1186/1477-7827-9-111>
- Li, M., Wu, Y., & Caron, K. M. (2008). Haploinsufficiency for adrenomedullin reduces pinopodes and diminishes uterine receptivity in mice. *Biol Reprod*, 79(6), 1169-1175. <https://doi.org/10.1095/biolreprod.108.069336>
- Li, M., Yee, D., Magnuson, T. R., Smithies, O., & Caron, K. M. (2006). Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. *J Clin Invest*, 116(10), 2653-2662. <https://doi.org/10.1172/JCI28462>
- Liao, S. B., Ho, J. C., Tang, F., & O, W. S. (2011). Adrenomedullin increases ciliary beat frequency and decreases muscular contraction in the rat oviduct. *Reproduction*, 141(3), 367-372. <https://doi.org/10.1530/REP-10-0230>
- Marinoni, E., Di Iorio, R., Letizia, C., Villaccio, B., Scucchi, L., & Cosmi, E. V. (1998). Immunoreactive adrenomedullin in human fetoplacental tissues. *Am J Obstet Gynecol*, 179(3 Pt 1), 784-787. <https://www.ncbi.nlm.nih.gov/pubmed/9757990>
- Matson, B. C., Pierce, S. L., Espenschied, S. T., Holle, E., Sweatt, I. H., Davis, E. S., Tarran, R., Young, S. L., Kohout, T. A., van Duin, M., & Caron, K. M. (2017). Adrenomedullin improves fertility and promotes pinopodes and cell junctions in the peri-implantation endometrium. *Biol Reprod*, 97(3), 466-477. <https://doi.org/10.1093/biolre/iox101>
- Mesa, H., Safranski, T. J., Cammack, K. M., Weaber, R. L., & Lamberson, W. R. (2006). Genetic and phenotypic relationships of farrowing and weaning survival to birth and placental weights in pigs. *J Anim Sci*, 84(1), 32-40. <https://doi.org/10.2527/2006.84132x>

- Minegishi, T., Nakamura, M., Abe, K., Tano, M., Andoh, A., Yoshida, M., Takagi, T., Nishikimi, T., Kojima, M., & Kangawa, K. (1999). Adrenomedullin and atrial natriuretic peptide concentrations in normal pregnancy and pre-eclampsia. *Mol Hum Reprod*, 5(8), 767-770. <https://doi.org/10.1093/molehr/5.8.767>
- Montuenga, L. M., Martinez, A., Miller, M. J., Unsworth, E. J., & Cuttitta, F. (1997). Expression of adrenomedullin and its receptor during embryogenesis suggests autocrine or paracrine modes of action. *Endocrinology*, 138(1), 440-451. <https://doi.org/10.1210/endo.138.1.4881>
- Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. *Domest Anim Endocrinol*, 27(3), 199-217. <https://doi.org/10.1016/j.domaniend.2004.06.006>
- Trollmann, R., Schoof, E., Beinder, E., Wenzel, D., Rascher, W., & Dotsch, J. (2002). Adrenomedullin gene expression in human placental tissue and leukocytes: a potential marker of severe tissue hypoxia in neonates with birth asphyxia. *Eur J Endocrinol*, 147(5), 711-716. <https://www.ncbi.nlm.nih.gov/pubmed/12444904>
- Tuchscherer, M., Puppe, B., Tuchscherer, A., & Tiemann, U. (2000). Early identification of neonates at risk: traits of newborn piglets with respect to survival. *Theriogenology*, 54(3), 371-388. [https://doi.org/10.1016/S0093-691X\(00\)00355-1](https://doi.org/10.1016/S0093-691X(00)00355-1)
- Vallet, J. L., Freking, B. A., & Miles, J. R. (2011). Effect of empty uterine space on birth intervals and fetal and placental development in pigs. *Anim Reprod Sci*, 125(1-4), 158-164. <https://doi.org/10.1016/j.anireprosci.2011.03.006>

- Vallet, J. L., McNeel, A. K., Miles, J. R., & Freking, B. A. (2014). Placental accommodations for transport and metabolism during intra-uterine crowding in pigs. *J Anim Sci Biotechnol*, 5(1), 55. <https://doi.org/10.1186/2049-1891-5-55>
- Wang, X., Lin, G., Liu, C., Feng, C., Zhou, H., Wang, T., Li, D., Wu, G., & Wang, J. (2014). Temporal proteomic analysis reveals defects in small-intestinal development of porcine fetuses with intrauterine growth restriction. *J Nutr Biochem*, 25(7), 785-795. <https://doi.org/10.1016/j.jnutbio.2014.03.008>
- Wang, X., Wu, W., Lin, G., Li, D., Wu, G., & Wang, J. (2010). Temporal proteomic analysis reveals continuous impairment of intestinal development in neonatal piglets with intrauterine growth restriction. *J Proteome Res*, 9(2), 924-935. <https://doi.org/10.1021/pr900747d>
- Wu, G., Bazer, F. W., Datta, S., Johnson, G. A., Li, P., Satterfield, M. C., & Spencer, T. E. (2008). Proline metabolism in the conceptus: implications for fetal growth and development. *Amino Acids*, 35(4), 691-702. <https://doi.org/10.1007/s00726-008-0052-7>
- Wu, G., Bazer, F. W., Wallace, J. M., & Spencer, T. E. (2006). Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci*, 84(9), 2316-2337. <https://doi.org/10.2527/jas.2006-156>
- Yotsumoto, S., Shimada, T., Cui, C. Y., Nakashima, H., Fujiwara, H., & Ko, M. S. (1998). Expression of adrenomedullin, a hypotensive peptide, in the trophoblast giant cells at the embryo implantation site in mouse. *Dev Biol*, 203(2), 264-275. <https://doi.org/10.1006/dbio.1998.9073>
- Zhao, Y., Hague, S., Manek, S., Zhang, L., Bicknell, R., & Rees, M. C. (1998). PCR display identifies tamoxifen induction of the novel angiogenic factor adrenomedullin by a non

estrogenic mechanism in the human endometrium. *Oncogene*, 16(3), 409-415.

<https://doi.org/10.1038/sj.onc.1201768>