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Antisense Regulation and Tissue-specific Roles of Peg3 in Lactation and Maternal Care

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A Dissertation

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in

The Department of Biological Sciences

by

Wesley D. Frey
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<td>Peg3</td>
<td>Paternally Expressed Gene 3</td>
</tr>
<tr>
<td>APeg3</td>
<td>Antisense Paternally Expressed Gene 3</td>
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<tr>
<td>Cre</td>
<td>Cre-recombinase</td>
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<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
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<td>Zona Pellucida Glycoprotein 3</td>
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<td>RNAi</td>
<td>RNA Interference</td>
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<tr>
<td>PEG</td>
<td>Paternally Expressed Gene</td>
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<tr>
<td>MEG</td>
<td>Maternally Expressed Gene</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-Guanine</td>
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<tr>
<td>DMR</td>
<td>Differentially Methylated Region</td>
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<tr>
<td>ICR</td>
<td>Imprinting Control Region</td>
</tr>
<tr>
<td>P1</td>
<td>Postnatal Day 1</td>
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<tr>
<td>P21</td>
<td>Postnatal Day 21</td>
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<tr>
<td>EUCOMM</td>
<td>European Conditional Mouse Mutagenesis</td>
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<tr>
<td>dpp</td>
<td>Day Post-partum</td>
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<tr>
<td>PBS</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse-Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered Saline</td>
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<tr>
<td>Peg3\textsuperscript{CoKO}</td>
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B6  C57BL/6J
ANOVA  One-way Analysis of Variance
T0  Time of Reintroduction
Δ  Change
TSS  Transcriptional Start Site
MFE  Minimal Fold Energy
DNA  Deoxyribonucleic Acid
RNA  Ribonucleic Acid
ncRNA  Non-coding RNA
IncRNA  Long Non-coding RNA
mRNA  Messenger RNA
smRNA  Small RNA
miRNA  Micro-RNA
snoRNA  Small Nucleolar RNA
piRNA  Piwi-interacting RNA
siRNA  Small Interfering RNA
OxtR  Oxytocin Receptor
EMSA  Electrophoretic Mobility Shift Assay
BS  Binding Site
ChIP  Chromatin Immunoprecipitation
IgG  Immunoglobulin Gamma
ABSTRACT

Genomic imprinting is characterized by the restrictive expression of a gene from one of the two inherited parental alleles. Many of these genes have been implicated in neonatal growth and mammalian reproduction. The two studies discussed in this dissertation focus on the tissue-specific contributions of Paternally Expressed Gene 3 (Peg3) on lactation and maternal care as well as the regulation of this gene through an antisense RNA (APeg3). In the first study, a conditional knockout allele has been developed to further characterize these known functions of Peg3 in a tissue-specific manner. The mutant line was first crossed with germline Cre, producing a progeny that displayed growth retardation phenotypes consistent with previous reports. The mutant line was then crossed individually with MMTV- and Nkx2.1-Cre lines to test Peg3’s roles in the mammary gland and hypothalamus, respectively. The results indicate that the milk letdown process was impaired in the nursing females with the Peg3 mutation in the mammary gland, but not in the hypothalamus. In contrast, one of the maternal-caring behaviors, nest-building, was interrupted in the females with the mutation in both MMTV- and Nkx2.1-driven lines. Overall, this is the first study to introduce a conditional knockout allele of Peg3 and to further dissect its contribution to mammalian reproduction in a tissue-specific manner.

In the second study, we investigate the functions of APeg3, a gene that lies antisense of the Peg3 3’-untranslated region, with comparative genomics and cell line-based approaches. APeg3 transcript displays a high degree of sequence conservation among placental mammals, but without any obvious open reading frame, suggesting APeg3 may have been selected as a ncRNA gene during eutherian evolution. RNA secondary structure analyses also support a ncRNA
function. The results from cell line-based transfection experiments demonstrate $A Peg3$ has the potential to down-regulate the mRNA and protein levels of $Peg3$. Overall, these results suggest that $A Peg3$ has evolved as a ncRNA gene and controls the function of its sense gene $Peg3$. When combined, the two studies in this dissertation offer new insight into the regulation and function of the imprinted transcription factor $Peg3$. 
CHAPTER ONE
INTRODUCTION

1.1 Epigenetics
The beauty of our genome lies in its complexity. Where computers are able to code in binary, a series of zero’s and one’s; our genome has a quaternary system, as A’s, G’s, C’s and T’s. It is incredible to think that the addition of two extra variables can give rise to the immense extra-computational processes of individuals, such as the development of organ systems, logic and even thought. In fact, it is impossible to describe the complexities found in nature when limited to the primary sequence of a stretch of DNA in a genome. We know now that these extra dimensions of processing are due, in large part, to the coding that occurs just outside and around the DNA.

Epigenetics is the study of heritable changes in gene expression that are not due to a change in the sequence of the DNA. Epigenetics is fascinating because it has the power to explain the changes that occur during development, temporally and spatially, as well as the changes that are taken in as a result of environmental influences. For example, any given mammal has two sets of each chromosome. This is all the genetic information they are given from the beginning of their life, when a sperm fertilizes an egg. The development of the hundreds of cell types that make up all the organ systems in the body cannot be explained by this inheritance alone. To understand what regulates complexity of this magnitude, it was necessary to come up with an entirely new field, epigenetics (1).
When the term epigenetics was first used, the aim was to describe the interplay between the genetic content and the surrounding environment that gives rise to fully developed multicellular organisms. Over the years this definition has been reshaped by the findings within the field to give rise to the definition scientists use today (2). The dynamic landscape that surrounds and interacts with the DNA has the ability to be condensed into fundamental changes responsible for the considerable plasticity of the various cells in our body. In fact, these epigenetic mechanisms can be broken down into three distinct layers of encoding that are responsible for the expression and repression of the information contained within the DNA: DNA methylation, Histone modification and RNA interference (RNAi).

1.2 Genomic Imprinting
Diploid cells contain two copies of each autosomal chromosome, inheriting one from each parent. If the two parental chromosomes are identical, transcription of genes from these chromosomes is also identical. However, in mammals, there exists a subset of genes whose expression is allowed from only one of the two chromosomal copies and is dependent on which parent the allele is inherited from (3,4). The nomenclature of these genes indicates their origin of expression. Paternally-expressed genes (PEGs) are maternally silenced, owing expression solely to the paternal copy, while maternally-expressed genes (MEGs) are paternally silenced and are expressed from the maternal allele. When these genes were discovered, it was understood that the silenced copy of these genes must carry a mark, or “imprint” with them in the germline that is transmitted through fertilization, this spawned the term “Genomic imprinting” (5,6).
Genomic imprinting is a phenomenon which results in parent-of-origin specific monoallelic expression of a gene (7). As such, these genes are functionally hemizygous, having no functional back-up copy on which to rely. This results in a set of genes, which comprise less than 1% of the human genome, with a substantial susceptibility to genetic disorders (8). Several of these genes have been implicated in the regulation of growth and prenatal development.

As more imprinted genes were identified through nuclear transplantation studies, patterns in their location and regulation began to emerge. First, most imprinted genes were found to be clustered within the genome. Greater than 80% of imprinted genes are found near another imprinted gene. The human genome contains 16 of these imprinted clusters (9). Second, the parent-of-origin specific repression of these genes was found to be dependent on DNA methylation at spans of Cytosine-phosphate-Guanines (CpGs) that are differentially methylated between the maternal and paternal copy. The Differentially Methylated Regions (DMRs) that contain spans of CpGs, termed “CpG Islands”, could control imprinting of the entire imprinted cluster. DMRs that were seen to have this function were termed Imprinting Control Regions (ICRs). Finally, each imprinted domain was seen to contain at least one imprinted non-coding RNA. In some instances, these imprinted ncRNAs have been implicated in the control of the whole imprinted domain (9,10)

1.3 Paternally Expressed Gene 3
Paternally Expressed Gene 3 (Peg3) is an imprinted gene located on human chromosome 19q13.4, mouse chromosome 7qA1 and encodes a 12-finger kruppel-type zinc finger containing
protein (11,12). Peg3 has previously been localized to the nuclei of neuronal cells and has been shown to have DNA-binding capabilities (13,14). This implicates Peg3 as a transcription factor and suggests that the expression of multiple target genes could change from a deficit in Peg3 (15). Peg3 expression is most dominant in the ovary, placenta and brain as observed in murine models (12,16). PEG3 is conserved among placental mammals, and has been shown to be important for a range of processes in eutherians from tumor suppression in breast, ovarian and glial cancer to maternal-caring behavior (16,17,18,19). The inherent monoallelic expression of Peg3 makes this gene an easy target for the effects of deleterious mutations. As a result, Peg3 has been implicated in many diseases and phenotypes, highlighting the importance of PEG3 expression to human health.

The potential roles of Peg3 in disease have triggered the production of Peg3 mutant mouse lines which have been studied for multiple characteristics. However, the most striking and consistent characteristics observed in a Peg3 mutant line is an inability for the mothers to take care of their young, as well as a growth defect in neonatal mice (19,20). A more recent mouse line with a deletion in Peg3 promoter-region as well as a strain carrying a transcriptional truncation were reported to have similar effects in Peg3-deficient neonates by our group (15,21). Peg3-deficient neonates in these studies have shown a 10% deficit in growth at postnatal day 1 (P1) and up to a 30% deficit in growth at P21 (15,20,21). Furthermore, Peg3-deficient mothers have poor maternal-caring behavior which often results in a mortality rate of 80% in neonates (22). While imprinted genes account for <1% of all transcriptional units, a disproportionate fraction of them, including Peg3, are involved in growth and prenatal development.
The effects of Peg3-deficient mothers have been linked to inadequate milk letdown as well as deficits in maternal-caring behaviors (22). However, the process of milk letdown is complex requiring a stimulus and response from both the neonate and the mother in multiple organs. In response to a baby suckling on a mother’s teat, the hypothalamus of the mother is triggered to release oxytocin into the bloodstream. Oxytocin then signals myoepithelial cells in the mammary gland to contract and then release milk through ducts in the teat (26). It has been seen that oxytocin release in milk ejection has a pro social effect on the mother as well as a calming, analgesic-like effect on the pup tying the two systems together in synchrony and producing positive reinforcement cues for the mother as well as the offspring (27,28).

The impacts of maternal-caring behavior in Peg3 mice have been linked closely to behaviors seen in Oxytocin-deficient mice described in previous literature, but have yet to be explained (30). Furthermore, in Peg3-deficient mice, oxytocinergic neuron density is lower than Wild-type littermates (22). This suggests Peg3 may be acting upstream of the neuropeptide, but does not specify the tissues in which Peg3 is necessary for proper milk letdown. The studies also suggest Peg3 may be regulating oxytocin, but do not propose a molecular model for this hypothesis.

1.4 Antisense Paternally Expressed Gene 3
Recent progresses in high-throughput sequencing has allowed for the rapid advancement of many consortiums, such as ENCODE, to harvest massive amounts of data pertaining to human genomic and transcriptomic profiles. The ENCODE project was able to detect an average of three transcripts for every one of the ~25,000 genes found in the human genome. Antisense
RNAs are well known for their ability to silence the expression of the sense counterpart, either through transcriptional inhibition or post-transcriptional inhibition of the sense transcript (30). One of the most plausible theories of antisense gene function is their use in transcriptional regulation of their corresponding sense mRNAs through transcriptional interference (31). However, antisense genes can also function in post-transcriptional regulation through RNA degradation, RNA-initiated transcriptional suppression and translational inhibition. Each mechanism could be acting through its own complex inter-regulatory network. Although many of these genes have been identified through transcriptional profiling, the function of each of these genes has yet to be characterized.

Imprinted non-coding RNAs are one of the most uncharacterized subsets of genes in the mammalian genome. Many of these genes are shown to be required for regulatory maintenance of entire imprinted gene clusters and have strong implications in neuroendocrine function (10, 32). A Peg3, an imprinted gene lying antisense of Peg3, has been identified as one of the major genes up-regulated in response to hyperosmotic stress in vasopressinergic neurons of the hypothalamus (33,34,35). This implicates the gene in such functions as regulation of blood pressure and bodily fluid retention. However, the function of A Peg3 on a cellular level has yet to be revealed.

In the following studies, we characterize the regulation of Peg3 by A Peg3 as well as the tissue-specific functions of Peg3. The first of the following studies utilizes Peg3 mutant mice and suggests that Peg3 serves specific roles in different tissues, each centered on the aspect of
offspring growth and development. The second study uses comparative genomics and molecular techniques to investigate the functional role of APeg3 and show its potential to downregulate Peg3 expression in neuronal cells. When combined, the studies illustrate the complex regulation and function of the imprinted transcription factor Peg3.

The notable results and contributions presented in this work are: 1) The generation and characterization of a Peg3 conditional knockout mouse line, which is defective in PEG3 expression at the protein level; 2) The examination of the tissue-specific functions of Peg3; 3) The implication of these tissue-specific functions for maternal care and reproduction; 4) The molecular link between Peg3 cellular function and the phenotype observed through binding and regulation of oxytocin receptor; 5) The cell-type specific regulation of Peg3 through the non-coding RNA, APeg3; 6) The potential impact of regions containing conserved secondary structures in APeg3 on the regulation of Peg3.

1.5 References


CHAPTER TWO
TISSUE-SPECIFIC CONTRIBUTIONS OF PEG3 IN LACTATION AND MATERNAL CARE

2.1 Introduction

Paternally Expressed Gene 3 (Peg3) is an imprinted gene located on human chromosome 19q13.4 and mouse chromosome 7qA1 (1,2). Peg3 encodes a protein with DNA-binding capabilities that is localized to the nucleus of neuronal cells (3,4). Expression of Peg3 is most dominant in the ovary, placenta and hypothalamus as observed in murine models (2,5). The potential roles of Peg3 in diseases ranging from low birth-weight to breast and cervical cancer have triggered the production of mutant mouse lines, which have been studied for multiple characteristics. The most striking and consistent characteristics observed in a previous Peg3 knock-in line is a maternal caring problem in Peg3-deficient dams and a growth defect in neonatal pups (6,7,8). More recently, our group has generated a mouse line with a deletion in the promoter region as well as a strain carrying a transcriptional truncation, which were both reported to have similar effects (9,10).

Peg3-deficient dams have been linked to inadequate milk letdown as well as problems with maternal-caring behaviors (7). However, the process of milk letdown is complex, requiring a stimulus and response from both the neonate and the mother in multiple organs. In response to a pup suckling on a dam's teat, the hypothalamus of the dam is triggered to release oxytocin into the bloodstream. Oxytocin then signals myoepithelial cells of the mammary gland to contract, releasing milk through ducts in the teat (11). It has been shown that oxytocin release in milk ejection has a pro-social effect on the dam as well as a calming effect on the pup, tying the two
systems in synchrony and producing positive reinforcement cues for the mother as well as the offspring (6,10).

The neonatal growth, lactation and maternal care effects observed in Peg3 mutant mouse lines are similar to the effects seen in oxytocin and oxytocin receptor-deficient mice in previous literature (13,14,15). Furthermore, in Peg3-deficient mice, oxytocin neuron density is lower than wild-type littermates (7). This suggests that Peg3 may be acting upstream of the neuropeptide or the receptor. However, the neuropeptide/receptor interplay between organ systems is a circuit. Often, the shutdown of a single component in the system results in a more dramatic effect than shutting down the entire system. In that regard, we wanted to test the individual tissues responsible for the phenotypes observed in Peg3-deficient mice. To narrow down the systems responsible for the developmental, physiological and behavioral roles of Peg3, we generated and characterized a line that allows for conditional deletion of the gene in vivo. Using this conditional knockout line, we deleted Peg3 in the hypothalamus and mammary gland to observe defects in neonatal growth, lactation and maternal care.

2.2 Materials and Methods

2.2.1 Generation of Peg3FlpKO and Peg3DelKO strains

The Peg3CoKO strain has been generated using a targeted ES cell, Peg3tm1a (EUCCOMM) Hmgu, from the EUCCOMM (European Conditional Mouse Mutagenesis) consortium, and has been maintained in the lab through maternal transmission for multiple generations (10). The Peg3FlpKO line has been derived by crossing Peg3CoKO with the commercially available Rosa26-FLP line (Jackson Lab, Stock No. 009086, B6.129S4-Gt (ROSA)26Sortm1(FLP1)Dym/RainJ). Progeny were tested for the
excision of β-Galactosidase and Neomycin resistance markers by PCR using the set of the following primers: Peg3-5ARM (5'-CCCTCAGCAGAGCTGTTTCCTGCG-3') and Peg3-3ARM (5'-AAGCTACCTGGGAAATGAGTGGG-3'). These generated mice were further screened to remove the Rosa26 Flippase allele by PCR using the following set of primers: NEW-ROSA26-FlippaseF (5'-TAAGTGAGGGTGAAAGCATCTGGG-3') and NEW-ROSA26 FlippaseR (5'-ACTCGTTTTAGGACTGGTTCAGA-3'). The identified mice heterozygous for the Peg3<sup>FlpKO</sup> allele without the ROSA26-Flippase allele have been maintained with the mutant allele being transmitted as a maternal allele. This Peg3<sup>FlpKO</sup> line has been maintained through backcrossing with the C57BL/6J strain for greater than 5 generations. For conditional knockout experiments, the Peg3<sup>FlpKO</sup> mouse line was then crossed with commercially available MMTV-Cre (Stock # 003553, B6129-Tgn(MMTV-Cre)4Mam-LineD) and Nkx2.1-Cre (Stock # 008661 C57BL/6J-Tg(Nkx2.1-cre)2Sand/J) lines from Jackson lab (18,19). MMTV-Cre line D was used for its restricted expression in the brain (<10% recombination) and its inability to affect mammary development (20). Primers used to test recombination in tissues from the conditional knockout breeding are: Pre-LoxF (5'-TGGACTTGGGAGATACTGTATTTATCAGCA-3'), LoxR1 (5'-TGCCTTCTCTTTTAGAAGTGACTGAGG-3') and PostLoxR2 (TCTGACTTCCTGGGAGCCAGTAAGA-3').

The Peg3<sup>DelKO</sup> line has been derived through the crossing of female Peg3<sup>WT/FlpKO</sup> with male Zp3-Cre (Jackson Lab, Stock No. 003651, C57BL/6-Tg (Zp3-cre) 93Knw/J). Female heterozygous for the Peg3<sup>DelKO</sup> allele were screened with the following set of primers: Peg3-5ARM and Peg3-LoxR (5'-TGAACTGATGGCGAGCTCAGACC-3'). These F1 mice were further tested for the absence of the Cre recombinase cassette by PCR with the following set of primers: Zp3-CreF
(5’-TAGGAATCAGTGGAGTGTCT-3’) and oIMR1085 (5’-
GTGAAACAGCATTGCTGTCACTT-3’). This Peg3DelKO line was similarly backcrossed with male C57BL/6J for greater than 4 generations. These mice were then used for testing the neonatal growth experiments. For genotyping, ear snips were incubated overnight in the lysis buffer (50 mM Tris-Cl at pH 8.0, 100 mM EDTA at pH 8.0, 250 mM NaCl, 1% SDS, along with 20 μg/mL Proteinase K) at 65°C. The 60-fold diluents of these lysed samples were used as a template for PCR amplification using the PCR premix kit (iNtRON Biotech) at the following conditions (Step 1, 95°C-30 sec; Step 2, 95°C-30 sec, 60°C-30 sec, 72°C-60 sec for 35 cycles; Step 3, 72°C-10 min).

2.2.2 Milk retention and maternal care experiments
As displayed in the conditional knockout breeding scheme (Figure 2.1), four different types of females were bred with male B6 to observe maternal care and lactation phenotypes. Beginning one hour after the start of the dark cycle, dams, their litters and bedding material were weighed and pups were separated from their dams. Pup/mother separation lasted for 2 hours and was followed by weight measurements of the same materials and a reintroduction to the mother. Every two hours after reintroduction to the mothers, their litters and bedding material were monitored for weight gain/loss as well as whether or not the mother was crouching over the pups at the time of observation. This experimental setup was tested on 2 litters for each dam (5 Nkx-Cre/Wt, 5 Nkx-Cre/FlpKO, 7 MMTV-Cre/Wt, 7 MMTV-Cre/FlpKO) at three different time points (5 dpp, 10 dpp, and 15 dpp). To measure the amount of nutrient transfer from the dams to the pups, we subtracted the amount of weight gain/loss of the litter from the amount of gain/loss of the dam every two hours.
Figure 2.1. Breeding scheme for conditional knockouts. (Cross A) Peg3\textsuperscript{FlpKO/WT} males were crossed with Nkx2.1-Cre females to produce offspring with paternally-transmitted deletions of Peg3. These pups were tested for lethality and growth defects that resulted in Figure 2.4. Female Nkx2.1-Cre/WT and Nkx2.1-Cre/FlpKO from these experiments were then used for (Cross B), where they were bred with B6 males to be used for testing lactation and maternal caring behaviors. A similar breeding scheme was used for (Cross C) and (Cross D). However, MMTV-Cre females in (Cross C) were available as MMTV-Cre\textsuperscript{tg/tg}, which allowed for a simpler breeding scheme.

The nest-building behavior was monitored with a modified protocol\textsuperscript{20}. Modifications to the original protocol are as follows: 1) The weight of the untorn bedding material was measured every two hours to determine the dams with exceptional nest-building behavior. 2) The time course was extended from 30 minutes to 14 hours to account for any dams with slow nest-
building behavior. 3) The nest-building behavior was tested at three developmental stages: 5 dpp, 10 dpp and 15 dpp.

2.2.3 Mammary oxytocin response experiments
Oxytocin response experiments were completed using the modified protocol\textsuperscript{21}. We determined the optimal period of latency for the Nkx2.1-Cre mice to be 40 minutes, and for the MMTV-Cre mice to be 20 minutes to build up milk. Following CO2-mediated sacrifice of the conditional knockout and wild-type dams, mammary glands were exposed. Pictures of the mammary glands were taken prior to addition of any solutions. Phosphate-buffered saline (PBS) was added to both sides of the mouse mammary glands, then syphoned off and the mammary glands were imaged. Then, Oxytocin (Sigma Cat No O4375), re-suspended in 1x PBS at a concentration of 1 mg/mL, was added to the mammary gland and allowed to incubate for 1 minute before the solution was syphoned off. The mammary gland was then imaged. This protocol was performed for three of each conditional knockout and corresponding WT littermates.

2.2.4 cDNA synthesis and Reverse-Transcription PCR (RT-PCR)
Total RNA was isolated from mouse organs using Trizol (Life Technologies Cat No 10296028) according to the manufacturer’s protocol. The isolated RNA was first reverse-transcribed using the M-MLVRT First-Strand Synthesis System (Invitrogen Cat No. 28025-013) in accordance with manufacturer’s protocol with random hexamer and oligo-dT primers. Subsequent cDNA was used as a template for RT-PCR. All RT-PCR reactions were carried out for 33 cycles under standard PCR conditions. The primers for Peg3 and \( \beta\)-Actin were as follows: Peg3-RT-Exon3F2 (5’-ATCCCTGAAACGCTCAAGCCTT-3’), Peg3-Ex7R (5’-
2.2.5 Protein isolations and western blots

Mice were sacrificed by cervical dislocation in accordance with Institutional Animal Care and Use Committee (IUCAC) guidelines. Whole heads were harvested and immediately homogenized in lysis buffer (0.25M Tris–HCl, pH 7.8, plus 0.1% NP-40) with Proteinase Inhibitor Cocktail (EMD-Millipore Cat No 539131) at 1x concentration. The cellular debris was removed by centrifugation for 10 minutes at 4°C. Protein concentrations were determined by the Bradford assay kit (Pierce), using diluted BSA as protein standards. Sixty micrograms of each lysate were separated on 10% SDS–PAGE gels and transferred to PVDF membranes (Hybond-P, Amersham) using a Mini Trans-Blot wet transfer cell (Bio-Rad). Membranes were blocked for 1 hour in the Tris-buffered saline (TBS) containing 1% skim milk and 0.05% Tween 20, and incubated overnight at 4°C with a custom-made purified anti-PEG3 antibody (10). These blots were incubated for an additional 1 hour with the secondary antibody linked to horseradish peroxidase (Sigma Cat No A6154). The blots were developed using the Western blot detection system according to the manufacturer’s protocol (Thermo Scientific Cat No 17295).

2.3 Results

2.3.1 Molecular characterization of Peg3^{FlpKO} and Peg3^{DelKO} mouse lines

In order to test when and where Peg3 expression is required for proper growth and development, two new mouse lines have been generated, Peg3^{FlpKO} and Peg3^{DelKO} (Figure 2.2, a.).
Figure 2.2. Molecular characterization of Peg3<sup>FlpKO</sup> and Peg3<sup>DelKO</sup> mouse lines. (a) Schematic representation of Peg3 alleles. Arrows above each allele indicate transcriptional direction and length. Exons are indicated by boxes, with Exon 6 denoted as a white “6”. Flippase recognition target (FRT) sites are shown as green triangles. LoxP sites are indicated by red triangles. In the Peg3<sup>CoKO</sup> allele, we inserted a cassette containing a splice acceptor (SA) sequence, an internal ribosomal entry site (IRES) and a β-Galactosidase (β-Gal) reporter gene, followed by a polyadenylation signal (pA). Neomycin Resistance gene (Neo<sup>R</sup>) is followed by another pA. Crossing Peg3<sup>CoKO</sup> mice with a Flp-expressing line results in the Peg3<sup>FlpKO</sup> allele. Successive crossing of Peg3<sup>FlpKO</sup> mice with Cre-expressing lines results in the Peg3<sup>DelKO</sup> allele. (b) RT-PCR of Peg3 from various tissues in Peg3<sup>FlpKO</sup> line. β-Actin was used as an internal control. (c) RT-PCR of Peg3 from various tissues in Peg3<sup>DelKO</sup>. Cre-mediated recombination of Exon 6 results in the smaller amplicon size (346 bp in length) as compared to the wild-type product (453 bp in length). (d) Western blots from the 1-day-old heads of Peg3<sup>DelKO</sup>. To visualize expression of PEG3 protein, western blots were probed for Peg3, stripped and then probed for β-Actin.

The first line was generated by recombination of a knock-in allele (Peg3<sup>CoKO</sup>) of Peg3 by Flp recombinase (10). Upon recombination, two polyadenylation sites were excised, recovering the full-length transcription of Peg3 (Peg3<sup>FlpKO</sup>). In this line, Exon 6 is flanked by two LoxP sites. This results in a conditional knockout-ready line, which can be bred with Cre-recombinase-
expressing (Cre) lines to produce a deletion of Exon 6. The deletion of Exon 6 generates the second mouse line, Peg3\(^{\text{DelKO}}\) (Figure 2.2, a.). This deletion causes a frameshift and subsequent translational truncation of the Peg3 ORF (Open Reading Frame), which starts at Exon 3 and ends at Exon 9. We used three Cre-expressing lines in this study (Zp3-Cre, MMTV-Cre and Nkx2.1-Cre). Zp3-Cre was used to make the Peg3\(^{\text{DelKO}}\) line through germline recombination, whereas MMTV and Nkx2.1-Cre were used for the tissue-specific deletion of Exon 6 in Peg3.

Proper splicing in the Peg3\(^{\text{FlpKO}}\) line was first tested with RT-PCR using the total RNA isolated from the brain, testes and heart of the wild-type (WT) and heterozygote (FlpKO) mice carrying the mutant allele paternally (Figure 2.2, b.). The results indicated no abnormal splicing between exons 3-7 of the Peg3\(^{\text{FlpKO}}\) line based on detection of the same-size product (453 bp in length) between the WT and FlpKO samples (Figure 2.2, b.). Furthermore, a series of RT-PCR testing the different parts of the 9.0-kb transcript of Peg3 also confirmed the full-length transcription of Peg3 in the Peg3\(^{\text{FlpKO}}\) line (data not shown). In the Peg3\(^{\text{DelKO}}\) line, the proper expression of Peg3 transcript lacking Exon 6 was also tested by RT-PCR in a similar scheme as the Peg3\(^{\text{FlpKO}}\) line (Figure 2.2, c.). According to the results, RT-PCR generated the expected 346-bp product from the multiple tissues of Peg3\(^{\text{DelKO}}\) line, which is lacking the 107-bp-long Exon 6. On the other hand, the same PCR generated the 453-bp product from WT tissues. However, the brain and testes also produced additional products besides the 346-bp products. In the case of testis, the upper band (453 bp) corresponds to the product from the maternal allele due to its derepression in germ cells. In the case of the brain, sequencing of the 410-bp minor product revealed the presence of a small exon, 64 bp in length, between Exons 4 and 5. More detailed analysis with the total RNA from WT confirmed that this is a minor exon that was previously undetected but
usually included as part of the normal transcript of Peg3 (data not shown). Proper protein expression in the Peg3\textsuperscript{DelKO} line was tested by western blot using the protein extracts from the brains of one-day-old pups. The results indicated a complete abrogation of the protein PEG3, confirming that the deletion of Exon 6 causes the frameshift and subsequent truncation in the ORF of Peg3 (Figure 2.2, d.). Taken together, these results confirm the successful generation of the Peg3\textsuperscript{FlpKO} and Peg3\textsuperscript{DelKO} mouse lines. Since the Cre lines used for the current study have the mixed background of C57BL/6J (B6) and 129/SvJ, the females from the Peg3\textsuperscript{FlpKO} and Peg3\textsuperscript{DelKO} lines have been backcrossed with B6 males for two years, thus deriving these lines with the relatively pure B6 background.

2.3.2 Growth retardation in Peg3\textsuperscript{DelKO} pups

To examine whether the Peg3\textsuperscript{DelKO} line also displayed growth effects consistent with the previous studies (7,9,10), we performed breeding experiments measuring body weights at two different time points: 1 and 21 days postpartum (dpp). In these experiments, we crossed male Peg3\textsuperscript{DelKO}/WT with C57BL/6J females. Their progeny were weighed and genotyped. Their whole-body weights were then converted into a percentile relative to the average weight of the litter. According to the results, at 1 dpp (Figure 2.3, a.), the average litter size was 8.5, which is smaller than that of B6 mice (~10). The ratio between the heterozygous Peg3\textsuperscript{WT/DelKO} and Peg3\textsuperscript{WT/WT} was 0.795:1, and did not vary significantly from the expected mendelian ratio (P=0.3390). These results suggest that Peg3\textsuperscript{WT/DelKO} mice had not been selected against during fertilization or during embryogenesis. The breeding results at 21 dpp (weaning age) also derived similar outcomes (Figure 2.3, b.). The average litter size was 9.0 and the transmission ratio was
also similar to the mendelian ratio \(\text{Peg3}^{\text{WT/WT}}:\text{Peg3}^{\text{WT/DelKO}} = 0.85:1\), again indicating that no selection had occurred against the \(\text{Peg3}^{\text{WT/DelKO}}\) pups during the postnatal stages \((p=0.5287)\). 

**Figure 2.3.** Growth effects of Peg3\(^{\text{DelKO}}\) allele in neonates.  (a) Male Peg3\(^{\text{DelKO/WT}}\) were bred with female wild-type littermates to generate Peg3\(^{\text{WT/DelKO}}\) pups with the paternal transmission of the mutant allele. Pups were genotyped and weighed at 1 day postpartum (dpp), then compared to the average weight of the litter. The number of pups belonging to each percentile weight range was then graphed to visualize the weight distribution of Peg3\(^{\text{DelKO}}\) pups in comparison to their wild-type littermates. (b) The same series of analyses were repeated with the pups at the weaning age (21 dpp). (c) and (d) display representative wild-type (WT) and Peg3\(^{\text{DelKO}}\) littermates exhibiting weight differences.

Although significant levels of lethality were not observed in the Peg3\(^{\text{WT/DelKO}}\) pups, major growth retardation was observed at both 1- and 21-dpp time points (Figure 2.3, a. b.). At 1 dpp, the average weight difference was 6.99% between Peg3\(^{\text{WT/DelKO}}\) pups and their WT littermates.
(p=0.0028). A more dramatic growth effect was observed at 21 dpp, which displayed 20% weight difference between Peg3WT/DelKO pups and their WT littermates (p=0.0001). Taken together, these results confirm the critical role of Peg3 in controlling growth rates. The results also highlight a more significant role of Peg3 during postnatal stages than during gestation in the newly derived Peg3WT/DelKO mutant line.

2.3.3 Peg3 conditional knockout pups: growth effects

The floxed allele Peg3WT/FlpKO was crossed with two Cre lines to test Peg3’s tissue-specific roles in controlling growth rates (Figure 2.4).

![Graphs showing growth effects in conditional knockout pups.](image)

Figure 2.4. Peg3 conditional knockout pups: impact on growth. Pups with conditional Peg3 knockouts were weighed and compared to the average weight within the litter. Percentile weights within litter were then separated into 10% ranges. The numbers of pups in each percentile range are shown for MMTV-driven deletion at 1 dpp (a) and 21 dpp (b) and for Nkx2.1-driven deletion at 1 dpp (c) and 21 dpp (d).
MMTV-Cre and Nkx2.1-Cre lines were employed to abolish Peg3 expression in the mammary gland and hypothalamus, respectively. Recombination in these target organs was verified by PCR with primers flanking the inserted cassette (Figure 2.5).

**a) Placenta**

![Placenta image](image)

**b) Hypothalamus**

![Hypothalamus image](image)

Figure 2.5. Peg3 recombination in tissues correlated with growth and lactation phenotype. Genomic DNA was isolated from tissues known to have high expression of Peg3 and have implications in reproduction. Mammary and hypothalamic were isolated from dams who inherited the Peg3\(^{FlpKO}\) allele, along with the conditionally-expressing Cre line indicated. Placentas were isolated from earlier experiments, wherein the embryos inherited the Peg3\(^{FlpKO}\) allele and the MMTV-driven Cre allele. The Peg3\(^{FlpKO}\) and Peg3\(^{DelKO}\) alleles were amplified from the mammary and placenta using (5-ARM, LoxR primers) and are indicated by the red labels with arrows (a,b). M-T indicates the top mammary gland from a mouse, while M-B distinguishes the bottom mammary gland. Hypothalamic regions were amplified using (PreLoxF, LoxR1 and PostLoxR2 primers) which display the Peg3\(^{WT}\), Peg3\(^{FlpKO}\) and Peg3\(^{DelKO}\) alleles.

This series of breeding experiments were also analyzed in a similar manner as the Peg3\(^{DelKO}\) line (described above), measuring the average litter size and body weights. For simplicity, the
genotypes of the pups were abbreviated in the following manner: MMTV-Cre^tg/+; Peg3^WT/FlpKO as MMTV-Cre/FlpKO and MMTV-Cre^tg/+; Peg3^WT/WT as MMTV-Cre/WT.

The breeding of male Peg3^FlpKO/WT with female MMTV-Cre^tg/tg yielded the following results: At 1 dpp, the average litter size was 7.4 (59 pups/8 litters), which was slightly smaller than expected. The ratio between MMTV-Cre/WT and MMTV-Cre/FlpKO (39:20) deviated significantly from the expected mendelian ratio (1:1), suggesting that potential embryonic lethality is associated with MMTV-Cre/FlpKO pups (p=0.0134). This embryonic lethality may be attributed to the small first litter size in MMTV-Cre mothers or expression of MMTV-Cre in the embryo-driven placenta (Figure 2.5). At 21 dpp, the average litter size was 6.8 (34 pups/5 litters) and the ratio of MMTV-Cre/WT and MMTV-Cre/FlpKO (16:18) was close to the mendelian ratio (p=0.7317). This indicates that no obvious lethality is associated with the MMTV-Cre/FlpKO pups at the 21-dpp time point. The weight profiles also show no major difference between MMTV-Cre/WT and MMTV-Cre/FlpKO at both 1 and 21-dpp time points (p=0.1615 for 1 dpp and p=0.5997; Figure 2.4, a. and Figure 2.4, b.). This suggests that MMTV-driven deletion of Peg3 alone may not have any major impact on growth rates of the pups during gestation and also during postnatal stages.

The second set of breeding experiments were performed through crossing male Peg3^FlpKO/WT with female Nkx2.1-Cre^+/tg. The litter sizes at both 1 and 21-dpp time points were overall normal: 8 at 1 dpp and 7.8 at 21 dpp. The ratios of the pups with 4 genotypes were also close to the mendelian ratio of two independently segregating heterozygous loci (1:1:1:1) (p=0.7698 for 1
dpp and p=0.0649 for 21 dpp). The actual ratios for these breedings are shown in Figure 2.4, c. and 2.4, d. This indicated no obvious lethality in the Nkx2.1-Cre/FlpKO pups from this breeding experiment. The weight profiles for the pups with these four genotypes also displayed no statistically significant variance at both time points, based on the one-way analysis of variance (ANOVA) (p=0.593639 for 1 dpp and p=-0.643121 for 21 dpp). Collectively, the results from the two sets of breeding experiments concluded that MMTV-driven (mammary-specific) deletion of Peg3 may produce lethality when litter size is constrained (as in MMTV-Cre first litters), but did not result in lethality from later litters. Also, MMTV-driven Peg3 deletion did not affect growth rates. Our results also show Nkx2.1-driven (hypothalamus-specific) deletion of Peg3 did not have any significant impact on survival and growth rates of the animals during gestation and postnatal stages.

2.3.4 Peg3 conditional knockout dams: milk letdown
One of the mutant phenotypes associated with Peg3 is a defect in milk letdown in nursing females (7,10). This particular phenotype was detected among the females of the new mutant line Peg3WT/DelKO (data not shown). Four sets of females derived from the previous breeding experiments were further utilized to test this mutant phenotype of Peg3. This series of analyses used 7 MMTV-Cre/FlpKO, 7 MMTV-Cre/WT, 5 Nkx2.1-Cre/FlpKO and 5 Nkx2.1-Cre/WT mice. The test was performed in the following manner. The efficient milk letdown process by nursing females was evaluated through measuring weight (milk) transfer from nursing females to pups (Figure 2.6, a. and 2.6, b.).
Figure 2.6. Peg3 conditional knockout dams: impact on milk letdown. Milk retention at 10 dpp of MMTV-driven (a) and Nkx2.1-driven (b) deletions in Peg3 are shown as a function of the weight change between every two hours in the dam subtracted by the weight change between the same time points in the pups. Therefore, the weight flux shown is a measure of how much nutrient transfer is occurring from the dam to her pups at each given time interval with positive slopes indicating weight gain in the dam that is not transferred over to the pups. Conversely, negative slopes indicate a dam giving all her mass to the pups, without gaining any weight herself. Blue lines indicate individuals inheriting the wild type allele for Peg3 and Red lines indicate dams who have inherited the FlpKO allele. The dashed black box indicates the critical point wherein reintroduction of the dams to their pups has just occurred. (c) Oxytocin-induced milk ejection was surveyed in MMTV-driven Peg3 deleted mice alongside wild-type littermate controls. Black arrows indicate ductal branches in the mammary gland.

A nursing female was first separated from her litter for two hours, and then reintroduced to her pups. From the reintroduction (T0), the weights of the female and the litter were individually measured every two hours (Figure 2.7).
During every two-hour period, the weight change of the female (Δ Dam weight) was further subtracted by that of the litter (Δ Litter weight). For each female, a series of these subtracted weight values were summarized as a line graph to indicate the trend of the weight fluctuation. As shown in Figure 2.6, a., the largest surge of weight (milk) transfer occurred during the Reintroduction to T+4 time period in the majority of the wild-type females (7 MMTV-Cre/WT, blue line). This is visualized by the blue lines with negative slope, indicating that the accumulated weight of nursing females was transferred to the weight of their litters. On the other hand, all of the mutant females (7 MMTV-Cre/FlpKO, red line) displayed positive slopes, indicating that the accumulated weight of the nursing females were not efficiently transferred to their pups during this time period. This indicated that the mutant females lacking Peg3 expression in the mammary gland most likely have a problem in releasing milk to their pups.
This series of experiments was repeated with two litters for each female, and also performed at three different time points, at 5, 10, 15 dpp. Overall, the outcomes were consistent and reproducible among the different experiments. The results from the experiment at 10 dpp are shown as a representative set (Figure 2.6, a.).

This series of experiments was also performed with another set of females (Nkx2.1-Cre/WT and Nkx2.1-Cre/FlpKO, Figure 2.6, b.). The results did not show any major difference between the two groups of females, indicating that the deletion of Peg3 in the hypothalamus did not have any definitive impact on the weight (milk) transfer. To follow up on the results derived from these two series of experiments, we also performed an independent series of experiments testing the response of the mammary gland to the hormone oxytocin. For these experiments, three females from each of the four groups (MMTV-Cre/WT, MMTV-Cre/FlpKO, Nkx2.1-Cre/WT, Nkx2.1-Cre/FlpKO) were sacrificed, and their mammary glands were exposed to a high concentration of oxytocin 1 mg/mL (10x physiological concentration) (Figure 2.6, c.). The milk flow through the mammary ducts was easily detected among the MMTV-Cre/WT females. In contrast, the milk efflux was not obvious among the 3 MMTV-Cre/FlpKO females tested, indicating that the mammary gland without Peg3 expression is not responsive to the high dose of oxytocin. This further suggests potential defects in the oxytocin circuitry as a main cause for the observed defect in the milk letdown process. We also performed the same series of oxytocin-induced milk ejection experiments with the set of Nkx2.1-Cre/WT and Nkx2.1-Cre/FlpKO (Figure 2.8).
Figure 2.8. Oxytocin-induced milk ejection in Nkx2.1-Cre dams. Similar to the experiment observed in Figure 2.6c, 1 mg/mL oxytocin in 1x PBS was dripped onto the mammary glands of an Nkx2.1-Cre/FlpKO dam and WT littermate. Mammary glands were then visualized for movement of milk through the mammary ducts.

However, in the Nkx2.1-Cre lines, there was no observable difference in milk flow between the wild type and conditional knockout females. This set of results is also consistent with the results derived from the weight transfer experiments described above, showing the defects only among the females with the deletion of Peg3 in the mammary gland. In summary, the results demonstrated that conditional knockout of Peg3 in the mammary gland causes a major defect in the milk letdown process, most likely through affecting the oxytocin circuitry. It is also important to note that this defect is more readily detectable through mutating Peg3 in the mammary gland than in the hypothalamus.
2.3.5 Peg3 conditional knockout dams: nest building

While performing the weight transfer experiments, we also observed one behavioral abnormality that differentiated the wild type and mutant females, nest-building behavior. Nursing females usually tear apart solid bedding materials and build nests to provide insulation for their pups. However, both mutant types, MMTV-Cre/FlpKO and Nkx2.1-Cre/FlpKO, were found to be inefficient at nest building (Figure 2.9, a.).

![Figure 2.9](image-url)

**Figure 2.9.** Peg3 conditional knockout mothers: nest-building behavior. (a) Representative conditional knockout dams and wild-type littermates highlight differences observed in the nest-building behavior. (b) A graph shows the number of dams marked as good, mediocre and bad at building nests for their litters. “Good” dams are those who tore 100% of bedding material by 14 hours. “Mediocre” dams were able to use some, but not all of the bedding material by 14 hours. “Bad” dams are those that display no interest in tearing up the bedding material to insulate their pups.
To systematically quantify this behavioral defect, we measured the weight of the untorn bedding materials every two hours. The relative weight of the remaining material to the initial weight was used to measure the efficiency of the nest-building behavior. The nursing females were further ranked from the best to worst nest builder based on their performance, which was judged by the relative weight of the remaining bedding materials (Figure 2.9, b., Figure 2.10, Figure 2.11 and Figure 2.12).

![Figure 2.10. Raw nest-building data from dams with 5 dpp pups. The table displays the percent of the original bedding material that was still left at each time point from all four genotypes tested (MMTV-Cre/WT, MMTV-Cre/FlpKO, Nkx2.1-Cre/WT, Nkx2.1-Cre/FlpKO). Dams with WT genotypes are in black, while dams harboring the FlpKO allele are in Red. Red dashed lines indicate borders used to separate the “Good”, “Mediocre” and “Bad” nesting behaviors in Figure 2.9, b. >100% bedding material remaining indicates the addition of weight to the bedding material by the dam, in most cases by the addition of water.](image)
According to the results, a large fraction of both mutant females, MMTV-Cre/FlpKO and Nkx2.1-Cre/FlpKO, were consistently ranked at the bottom of the list, thus making up the majority of the worst nest builders (Figure 2.9, b., Figure 2.10, Figure 2.11 and Figure 2.12).

Figure 2.11. Raw nest-building data from dams with 10 dpp pups. The table displays the percent of the original bedding material that was still left at each time point from all four genotypes tested (MMTV-Cre/WT, MMTV-Cre/FlpKO, Nkx2.1-Cre/WT, Nkx2.1-Cre/FlpKO). Dams with WT genotypes are in black, while dams harboring the FlpKO allele are in Red. Red dashed lines indicate borders used to separate the “Good”, “Mediocre” and “Bad” nesting behaviors in Figure 2.9, b. >100% bedding material remaining indicates the addition of weight to the bedding material by the dam, in most cases by the addition of water.

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</table>

Ratio WT:KO:

8:2

5:12

1:5
We used the same set of nursing females and litters as the weight transfer experiment (Figure 2.6, a. and 2.6, b.), and also repeated two litters for each female at three different time points, 5, 10, 15 dpp.

<table>
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<th>Bedding Material Remaining (% of Original Mass)</th>
<th>Ratio WT:KO</th>
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<td>100 89 111 106 106 111</td>
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</table>

Figure 2.12. Raw nest-building data from dams with 15 dpp pups. The table displays the percent of the original bedding material that was still left at each time point from all four genotypes tested (MMTV-Cre/WT, MMTV-Cre/FlpKO, Nkx2.1-Cre/WT, Nkx2.1-Cre/FlpKO). Dams with WT genotypes are in black, while dams harboring the FlpKO allele are in Red. Red dashed lines indicate borders used to separate the “Good”, “Mediocre” and “Bad” nesting behaviors in Figure 2.9, b. >100% bedding material remaining indicates the addition of weight to the bedding material by the dam, in most cases by the addition of water.
Overall, the outcomes were consistent and reproducible among individual sets of experiments. Regardless of genotype, we observed that the nest-building behavior of the animals was the most obvious during the early stage of nursing, at 5 dpp. Once the pups have fur (around 10 dpp), this behavior of the nursing female slowly diminished (Figure 2.9, b., Figure 2.11 and Figure 2.12). Nest building behavior was also the most obvious in the first litter for each female. In summation, the results from this series of experiments indicated that females with the mutation of Peg3 in both the MMTV-driven and Nkx2.1-driven Peg3 mutation have a problem in the nest-building behavior compared to their littermates. This nest-building behavior appears to be a subtle behavior of the mouse that is very closely associated with the dosage and functional sites of Peg3, since a deletion of Peg3 in both MMTV and Nkx2.1-driven tissue affects the trait.

2.4 Discussion
In the current study, two new mouse lines targeting the Peg3 locus, Peg3\(^{FlpKO}\) and Peg3\(^{DelKO}\), have been derived and subsequently characterized at the molecular and organismal levels. The Peg3\(^{DelKO}\) line, lacking Exon 6, displayed a set of phenotypes that are consistent with those observed from previous mutant alleles targeting Peg3. The Peg3\(^{FlpKO}\) line, containing a floxed allele, was also utilized to further characterize the two well-known phenotypes of Peg3, a defect in the milk letdown process and in the nest-building behavior. According to the results, Peg3’s role in the mammary gland appears to be critical for the milk letdown process. In contrast, Peg3’s role in both the MMTV- and Nkx2.1-expressing tissues is important for the nest-building behavior. Overall, the current study introduced two additional mutant lines targeting Peg3, and also further characterized the mutant phenotypes of Peg3 in terms of its tissue specific contribution.
The new mutant line Peg3\textsuperscript{DelKO} displayed a similar set of phenotypes that have been seen from the other mutant alleles of Peg3 (Figure 2.3). It is worthwhile to compare this new allele with the other existing mutant alleles of Peg3. This new mutant allele lacks only a small portion of the genomic region, a 600-bp region containing Exon 6 (Figure 2.2, a.), whereas the other alleles have an insertion of large expression cassettes in either Exon 5 or Intron 5 (7,10). The mode of operation in these loss-of-function mutant alleles is also quite different. In the case of the Peg3\textsuperscript{DelKO} allele, the transcription of Peg3 is not affected at all, but the translation of the mutant transcript is affected, thus causing a complete abrogation of the PEG3 protein (Figure 2.2, d.). By contrast, the two existing models truncate the transcription of Peg3 through the Poly-A signals that have been included as part of the expression cassettes. Thus, the transcription of Peg3 is affected in both mutant models. This could have potential side effects since the transcription and/or transcript of an imprinted gene is known to influence the function of the adjacent genes. In that regard, it is important to note that the 3’-UTR of Peg3 contains another imprinted gene, A\textit{Peg3} (Antisense Peg3) (16,17). The transcriptional truncation of Peg3 (sense gene) might have unknown consequences on the transcription and thus function of A\textit{Peg3} (antisense gene). Besides these differences, the two existing mutant models have an insertion of relatively large genomic fragments, spanning 5 to 7 kb in length, which might also have unknown effects on the locus itself and/or the adjacent imprinted genes. Nevertheless, all three models seem to have a common phenotype, delayed growth rate, although the newest model Peg3\textsuperscript{DelKO} appears to have a more dramatic effect during the postnatal stage than during early embryogenesis (Figure 2.3). Given this observation, we can safely conclude that the observed phenotype is most likely caused by the lack of the gene product, the PEG3 protein, rather than the other defects potentially present in each of these mutant models. Furthermore, it is also
important to note that the observed phenotype is also derived from mutant mice with the pure genetic background, C57BL/6, ruling out any possible complications that might originate from differing genetic backgrounds.

The Peg3$^{FlpKO}$ line was utilized for a series of conditional knockout experiments to further dissect Peg3’s role in the milk letdown process (Figure 2.6). According to the results, the mutation of Peg3 in the mammary gland seems to provide a more readily detectable defect than the mutation in the hypothalamus. This is quite intriguing since it has been believed that the main cause for this defect may be due to the reduced number of oxytocin-producing neurons in the Peg3 mutant mice (7). Although we have not thoroughly analyzed this aspect of the mutant mice that have been used for the current study, it is difficult to envision that the MMTV-driven deletion of Peg3 could have a drastic effect on the number of these specific neurons in the hypothalamus. Thus, we believe that this mutant phenotype of Peg3 is most likely caused by a defect at a more global level than at a local level. One possibility would be that this might be caused by a lost connection in the oxytocin circuitry of the mutant mice, but not simply by a defect at one tissue. For the milk letdown process, the oxytocin circuitry requires the proper communication between the hypothalamus and the mammary gland through its molecular ligand and receptor. Any defect in this circuitry could easily cause a problem in the milk letdown process. It is important to note that Peg3 is highly expressed in the tissues that are known to respond to the ligand, oxytocin, such as the ovary, uterus and placenta (2). Thus, it is also possible that Peg3 might be involved in the proper function of the receptor, oxytocin receptor. This possibility should be particularly interesting to pursue since Peg3 has been recently identified as a DNA-binding transcription factor$^4$. It is reasonable to predict that Peg3 could
modulate both the ligand and receptor for this circuitry. Thus, it would be of great interest to further narrow down the actual cause of this mutant phenotype in the near future.

The nest-building behavior of nursing females is shown to be affected by the mutation of Peg3 in both MMTV- and Nkx2.1-driven lines (Figure 2.9). In both cases, the mutant females were either slow or inefficient at nest-building for their pups. This maternal caring defect is consistent with a previous Peg3 knockout line that was characterized for maternal care phenotypes (7). However, the data collected in this study also displays the limitations of these conditional knockout lines. First, inherent variability in the efficiency of Cre will result in a variable degree of Peg3 deletion among different individuals (even among littermates with the same genotype, Figure 2.5, Figure 2.10, Figure 2.11 and Figure 2.12). Second, as the conditional deletions of Peg3 rarely result in 100% deletion in the tissues targeted, this limits our ability to understand the full contribution of Peg3 in these tissues. Despite these two limitations, these results help us understand the functions of Peg3 in regards to expression sites and dosage, as two general patterns also emerge from our data. First, only the MMTV-driven deletion of Peg3 impacted lactation, while either the Nkx2.1- or the MMTV-driven deletion of Peg3 resulted in behavioral effects. This suggests that the behavioral phenotype is more sensitive to dosage changes than the more physiological, lactation phenotype. Second, there was no significant difference in growth of the Peg3 conditional knockouts, yet effects on lactation and maternal care were still observed (Figure 2.4, Figure 2.6 and Figure 2.9). This suggests the growth effects may require the mutation of Peg3 at a more global level than the lactation and maternal care phenotypes. Overall, the results displayed a hierarchy of phenotypes in response to a loss of Peg3, the germline deletion exhibited all phenotypic effects, while both the MMTV and Nkx2.1-driven
deletion presented nest-building effects and only the MMTV-driven deletion demonstrated lactation effects.

2.5 References


3.1 Introduction

APeg3 is an antisense gene that is located within the 3’-untranslated region of an imprinted gene, Peg3 (Paternally expressed gene 3). The expression of APeg3 is detected only in vasopressinergic neurons of the hypothalamus, suggesting a very specialized role in the neuronal cells controlling blood pressure and the volume of bodily fluid in mammals (1). In fact, APeg3 was initially identified as a gene that is highly up-regulated in response to osmotic challenges in rat brains (1). Studies on Peg3 also demonstrate up-regulation against osmotic stress in this cell type, suggesting both APeg3 and Peg3 may play important roles in the functions of vasopressin-expressing neurons (2). Earlier studies from rat brain suggest APeg3 might code for a small-sized Open Reading Frame (ORF), but were not substantiated by later studies as the observed ORF is not conserved in other mammals, such as humans and mice. Interestingly, APeg3 is also maternally imprinted as seen in Peg3: only the paternal allele is expressed while the maternal allele is repressed (3). Nevertheless, the functional impetus for APeg3 imprinting is currently not well understood.

APeg3 is one of several ncRNA genes that have been identified from mammalian imprinted domains. The list of the imprinted ncRNA genes includes H19, IPW, Kcnq1ot1, Nespas, Airn, Copg2, and Gtl2 (4). APeg3 is very unique compared to the other imprinted ncRNA genes in the

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following aspects. First and foremost, APeg3 is the only imprinted antisense transcript expressed from the same (paternal) allele as its sense counterpart. Similar antisense ncRNA counterparts to imprinted genes, such as anti-Rtl1 and Copg2-AS, are selectively expressed from the opposite allele as the sense genes (5, 6). Secondly, the size of APeg3 is relatively small, 1.5 kb in length, whereas the size of the other imprinted non-coding genes range up to several hundreds of kb in length (1, 4). H19 is the only other imprinted ncRNA of similar length at ~1.9 kb (7). Thirdly, APeg3 lies antisense to the protein-coding gene, Peg3. Furthermore, the transcribed region overlaps with the 3’UTR of Peg3. This is quite different from other imprinted ncRNA genes, such as Gtl2, which tend to overlap with the entire imprinted region. Finally, the transcript of APeg3 is detected as an intronless mRNA with poly-A tails in vivo, suggesting that APeg3’s mRNA goes through normal processes as Pol II transcripts (3). By contrast, other long ncRNA genes, such as Airn, Kcnqot1, and Rian reside inside the nucleus and do not go through normal processes as Pol II transcripts. Despite these unique features, the biological roles of APeg3 are currently unknown. However, given the antisense/sense relationship between APeg3 and Peg3, it is reasonable to predict the main function of APeg3 may be controlling the transcription and protein levels of Peg3. To investigate this possibility, we performed a series of comparative genomics and cell-line based functional assays in the current study. The results suggest APeg3 may have evolved as a ncRNA gene controlling Peg3 mRNA and protein levels.

3.2 Materials and Methods

3.2.1 Database search and gene prediction

The intronless human antisense transcript, PEG3-AS1 (NR_023847.2), was used as a reference sequence in the BLAST program offered through UCSC genome browser.
Using this sequence, homologous nucleotide sequences were obtained from the available genome sequences of multiple mammalian species. After our initial inspection of the retrieved sequences, one representative sequence from each order of placental mammals was used for sequence alignment with the ClustalW multiple sequence alignment (www.genome.jp/tools/clustalw/) as well as CLC Bio Workbench. The 1.5-kb genomic region for each species’ $APeg3$ gene is as follows:

*Mus musculus* for Rodentia (GRCm38/mm10 chr7: 6,706,295-6,707,624), *Homo sapiens* for Primates (GRCh37/hg19 chr19:57,323,893-57,325,161), *Equus caballus* for Perissodactyla (Broad/EquCab2 chr10: 25,780,355-25,781,817), *Oryctolagus cuniculus* for Lagomorpha (Broad/oryCun2 chrUn0113: 622,308-623,587), *Loxodonta africana* for Proboscidea (Broad/loxAfr3 scaffold_4: 18,505,516-18,506,810), *Tursiops truncatus* for Cetacea (Baylor Ttru_1.4/turTru2 JH478484: 13,610-14,893), *Myotis lucifugus* for Chiroptera (Broad Institute Myoluc2.0/myoluc2 GL430552: 92,812-94,034), *Dasypus novemcinctus* for Cingulata (Baylor/dasNov3 JH562679: 85,036-86,329) and *Trichechus manatus* for Sirenia (Broad v1.0/triMan1 JH594782: 4,734,304-4,735,606). These sequences were further analyzed to identify several sequence features for the transcribed region of $APeg3$. Transcription Start Site (TSS) and Poly-A site were identified based on switchgear analyses. A potential TATA box was identified 25-bp upstream of the TSS site as an evolutionarily conserved element. A target site for miR-124 was also identified based on TargetScan (http://www.targetscan.org/) data. The results of these analyses are readily available on the UCSC genome browser.
3.2.2 Secondary structure prediction of *APeg3*

The primary sequence alignment of *APeg3* was used as an input for covariant and minimal fold energy (MFE) analysis using Vienna Suite software (8). RNA folding was predicted using the following two programs: RNA AliFold (http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi) to predict the macro-secondary structures (global folds) and RNAz (http://www.tbi.univie.ac.at/~wash/RNAz/) to predict the micro-secondary structures (local folds) of *APeg3* (8, 9). The programs used the primary sequence alignments to generate a consensus fold structure for *APeg3*. This series of analyses used two controls. The sequence of the H19 gene was included as a positive control, which has been previously shown to have significantly conserved secondary structures important for its function as a lncRNA (11), and a shuffled sequence of *APeg3* was also included as a negative control.

3.2.3 Construction and transfection of *APeg3* overexpression vectors

To construct the Short- and Full-length *APeg3* expression vectors, the cDNA prepared from adult mouse brains were amplified with artificial *BamHI* and *NotI* sites using the following primers:

*APeg3* Full-length -

\[
\text{APeg3Full\_BamHI CGCGGATCCCGGAATTAAGTCTGGAGACACAAAGATCTAAGG and APeg3Short\_NotI ATAAGAATGCGGCCGCACCAGTGCAGGTGGTGCGGA. APeg3Short\_BamHI CGCGGATCCCAATCAGTCTCAAGGGGTCTGGGT and APeg3Short\_NotI ATAAGAATGCGGCCGCACCAGTGCAGGTGGTGCGGA. (-)APeg3 Full-length -}
\]
(-)APeg3Short_BamHI CGCGGATCCGCACCAGTGCAGGTGGTGCGGA and (-)APeg3Full_NotI
ATAGAATGCGGCCGCGGAATTAAGTCTGGAGACACAAAGATCTAAGG

(-)APeg3 Short-length –

(-)APeg3Short_NotI ATAGAATGCGGCCGCGGGAATTAAGTCTGGAGACACAAAGATCTAAGG

The amplified products were digested with BamHI and NotI and cloned into pCDNA3.1Hygro(-) vector (Invitrogen). In this vector system, the expression of APeg3 was driven by the CMV promoter and terminated by the Poly-A signal of BGH. The constructed vectors were transfected into Neuro2A cells with each well containing 0.15x10^6 cells using 4 μL lipofectamine 2000 (Invitrogen). Transfections were carried out in triplicate with 3 wells designated for subsequent RNA isolation and 3 wells for protein extraction. The transfected cells were selected using 500 μg/mL hygromycin B 24 hours post-transfection. The cells were harvested for RNA isolation using TRIzol (Invitrogen) 48 hours post-transfection, and also for protein extraction in lysis buffer (0.25M Tris–HCl, pH 7.8, plus 0.1% NP-40) supplemented with fresh β-mercaptoethanol and the proteinase inhibitor cocktail (Calbiotech).

3.2.4 cDNA synthesis and quantitative Reverse-Transcription PCR (qRT-PCR)
Total RNA was isolated from transfected Neuro2A cells using a commercial kit (Trizol, Invitrogen). The isolated RNA was first reverse-transcribed using the MLV First-Strand Synthesis System (Invitrogen) with random and oligo dT primers. Subsequent cDNA was used as a template for qRT-PCR. This analysis was performed with the iQ SYBR green supermix
(Bio-Rad) using the iCycler iQTM multicolor real-time detection system (Bio-Rad). All qRT-PCR reactions were carried out for 40 cycles under standard PCR conditions. The expression levels of Peg3 were first normalized to those of β-Actin, then compared to those of the empty vector (pCDNA) controls. The primers for Peg3 and β-Actin were as follows: Peg3-1a (5’-GGTTCAATGGGTCAGTACTAGACT-3’), Peg3-1b.1 (5’-GCTCACACCCAGGGCTTGAGGT-3’), Peg3-1b.3 (5’-TCCCTAGTGTGCATGATCTGGT-3’), bactin-1a (5’-GAGCACCCCTGTGCTGCTCAG-3’) and bactin-1b (5’-CTCTTTGATGTCACGCAGATTTC-3’).

3.2.5 Protein isolations and western blots
The transfected Neuro2A cells were first rinsed with 1X PBS, and lysed with the lysis buffer (0.25M Tris–HCl, pH 7.8, plus 0.1% NP-40). The subsequent crude lysates were collected and snap-frozen in liquid nitrogen. Later, the lysate were then thawed at 4°C to prevent protein degradation. The cellular debris was removed by centrifugation for 10 minutes at 4°C. Protein concentrations were determined by the Bradford assay kit (Pierce), using diluted BSA as protein standards. Sixty micrograms of each lysate were separated on 10% SDS–PAGE gels and transferred to PVDF membranes (Hybond-P, Amersham) using a Mini Trans-Blot wet transfer cell (Bio-Rad). Membranes were blocked for 1 hour in the Tris-buffered saline containing 1% skim milk and 0.05% Tween100, and incubated overnight at 4°C with the custom-made anti-PEG3 antibody (12). These blots were incubated for an additional 1 hour with the secondary antibody linked to horseradish peroxidase (Sigma). The blots were developed using the Western blot detection system according to the manufacturer’s protocol (Intron Biotech). Densitometry was measured using Imol: an open-source Java viewer for chemical structures in 3D.
3.3 Results

3.3.1 \textit{APeg3} as an evolutionarily conserved ncRNA in placental mammals

To analyze the evolutionary conservation of \textit{APeg3}, human \textit{PEG3-AS1} sequence (NR 023847.2) was used as a probe to search the UCSC genome browser, yielding genomic sequences for each of the 9 eutherian orders. Multiple sequence alignments were performed using the 9 representative sequences, and the results were visualized as a graph in the bottom panel of Figure 3.1. The UCSC PhyloP-derived placental mammal sequences used for the zoomed-out conservation includes multiple species corresponding mainly to primate lineages as these are the most complete sequenced genomes and does not account for gaps in sequences. In this way, the conservation analysis could be biased. However, our custom zoomed-in conservation was derived from only one representative species of each order within the class eutheria, offering an unbiased characterization of the region. Differences in conservation level between the zoomed-out view and the zoomed-in view are the result of hand-picking genomic regions of \textit{APeg3} to enhance the accuracy of the initial PhyloP conservation. High levels of sequence conservation are again observed throughout the 1.5-kb region with many small-size insertions/deletions specific to individual species, which can be seen as sudden drop-offs in the graph. Several sequence motifs, important for Pol II transcription, are also found within or around this 1.5-kb genomic region, including a TATA box, Transcription Start Site (TSS) and two Poly-Adenylation sites. However, there are no ORFs or Kozak sequences that are conserved among placental mammals. These results suggest the 1.5-kb genomic region of \textit{APeg3} is a gene template for a poly-adenylated non-coding RNA (ncRNA).
Figure 3.1. Evolutionary conservation of APEG3 among placental mammals. UCSC Genome Browser Mus musculus assembly 9 (mm9) displays the 100-kb genomic region surrounding mouse APEG3. The transcribed and exon regions of Peg3 and Zim1 are also presented along with the transcribed region of APEG3. Evolutionary conservation of this genomic interval among placental mammals is shown with a graphical output of the PhyloP analysis. PhyloP conservation analysis of Zim1 3’ UTR is designated in a red box. In a zoom-in view, two black boxes represent the different-length known cDNAs for APEG3. APEG3-Full is based on the deposited cDNA sequences from human and rat, while APEG3-Short is based on the cDNA sequences from mouse. The evolutionary conservation of APEG3 was further analyzed using the genomic sequences obtained from 9 representative placental mammals (Supplemental Figure 3.1). This analysis identified a highly conserved TATA box (TATA), transcriptional start site (TSS) and a poly-adenylation signal (pA1). This analysis also confirmed the conservation of a potential target site for miRNA (miRNA-124). Areas of high conservation within these regions are underscored in red.

Using these sequences, pairwise sequence comparison analyses were also conducted, and the results indicated high levels of sequence conservation between different mammals: 76 to 84% nucleotide sequence identity in the 1.5-kb transcribed region of APEG3 (Table 3.1).
<table>
<thead>
<tr>
<th>Organism</th>
<th>E. caballus</th>
<th>T. truncatus</th>
<th>M. lucifugus</th>
<th>L. africana</th>
<th>T. manatus</th>
<th>D. novemcinctus</th>
<th>M. musculus</th>
<th>O. cuniculus</th>
<th>H. Sapiens</th>
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<td>75.12</td>
<td>76.47</td>
<td>75.23</td>
<td>71.66</td>
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<td>80.99</td>
</tr>
<tr>
<td>Myotis lucifugus</td>
<td>82.41</td>
<td>79.78</td>
<td>100</td>
<td>74.03</td>
<td>74.92</td>
<td>73.96</td>
<td>72.64</td>
<td>77.18</td>
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</tr>
<tr>
<td>Loxodonta africana</td>
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<td>75.12</td>
<td>74.03</td>
<td>100</td>
<td>89.01</td>
<td>77.34</td>
<td>68.61</td>
<td>73.15</td>
<td>75.53</td>
</tr>
<tr>
<td>Trichechus manatus</td>
<td>79.82</td>
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<td>Homo Sapiens</td>
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<td>76.42</td>
<td>77.33</td>
<td>83.84</td>
<td>100</td>
</tr>
</tbody>
</table>
The high levels of sequence conservation detected within the 1.5-kb region are also unusual, and can be contrasted with the 3’-UTR of an adjacent gene, Zim1, which shows no sequence conservation (Mammalian Cons Plot in Figure 3.1). Overall, the high level of primary sequence conservation further supports the region has not evolved simply as an UTR for the sense gene, Peg3. Instead, this region demonstrates signs of selection as a ncRNA gene during mammalian evolution.

3.3.2 Global and local secondary structure of APeg3 with evolutionary conservation

To determine potential functions of APeg3 as a structural ncRNA, secondary structure predictions were performed using RNAalifold and RNAz programs. First, RNAalifold uses sequence alignments and determines folding free energies of relatively large RNA sequences (>200 bp) (13). This program produces a consensus fold structure based on the lowest folding energy modeled and weights the conservation of folded structures among individual sequences. Both APeg3 and the corresponding reverse complement (-)APeg3 alignment were analyzed using RNAalifold to detect potential large-scale secondary structures in both orientations. This series of analyses also used two controls: H19 as a positive control, an imprinted ncRNA previously seen to harbor functional characteristics of similar length to APeg3, and a shuffled APeg3 alignment as a negative control. According to the outcomes of the prediction (Figure 3.2), the sense and antisense APeg3 exhibit similar conservation levels of secondary structure with different overall structures.
Figure 3.2. Predicted secondary structures of APeg3 mRNA. The 1.5-kb nucleotide sequences from 9 representative mammals were used to predict the global secondary structures of APeg3 using the Alifold prediction program: sense (A) and antisense (B) strand of APeg3. Conserved bases are color-coded based on their conservation level (legend inset). Local secondary structures were also modeled using the lowest fold energy as well as covariant analysis on small (100 bp) windows within APeg3. The RNAz program predicted a single conserved hairpin-loop structure within the sense (P=0.91) and antisense (P=0.99) APeg3 transcripts (C, D) with the 98.93% folding accuracy. The predicted hairpin-loop structure is located in the 5’ side of the APeg3 transcript (nucleotide positions 145-185 in mouse APeg3).

Second, the RNAz program was used to detect any conserved micro-secondary structures within APeg3. The program analyzes multiple sequence alignments and recognizes conserved fold structures in small (200 bp) sliding-windows (9). These folds are based on the lowest MFE of each short sequence. Covariant analysis was then performed on each fold and the resulting folds were scored by the program. In this program, a class probability (P) is used to measure the
likelihood of a fold. The program uses a cutoff of $P=0.9$, which can accurately predict 75% of known ncRNAs and classify folds with 99% specificity (8). According to the outcomes, a single fold was predicted for $APeg3$ in the 1.5-kb interval (Figure 3.2, C). The fold was conserved at the 5'-side of the transcript (nucleotide position 145-185 in NR 023846.1) ($P=0.99$).

Interestingly, the antisense of $APeg3$ also displayed one conserved stem-loop structure, located at the 3'-side of the transcript ($P=0.91$) (Figure 3.2, D). Overall, these secondary structure fold predictions indicate the presence of a conserved hairpin loop structure in both sense and antisense orientations of $APeg3$. A series of predictions were also conducted using two controls, $H19$ and the shuffled $APeg3$. This series of analyses predicted a known small fold, miR675, within $H19$ ($P=1.0$), and only one insignificant fold within the shuffled $APeg3$ ($P=0.76$); confirming the sensitivity of the RNAz program.

3.3.3 $APeg3$ down-regulates $Peg3$ mRNA
As seen in many antisense transcripts (14, 15), the potential function of $APeg3$ may be to regulate the expression of the sense gene, $Peg3$. To determine whether $APeg3$ has the potential to regulate $Peg3$, we performed a series of in vitro experiments as described below. We have constructed 4 expression vectors containing the different portions of mouse $APeg3$: $APeg3$-Full and -Short in both sense and antisense directions (the extent of $APeg3$-Short is indicated in Figure 3.1). After transfections of these vectors to Neuro2A cells, we measured $Peg3$ mRNA levels using qRT-PCR (Figure 3.3).
Figure 3.3. Effects of APeg3 overexpression on the transcription levels of Peg3. A series of constructs transcribing APeg3 were transfected into Neuro2A cells. APeg3 transcription was driven by the Cytomegalovirus (CMV) promoter, and terminated and polyadenylated by a Bovine Growth Hormone (BGH) terminator. Transfected cells were selected with hygromycin antibiotic (500 mg/mL). Potential effects of APeg3 overexpression were measured by determining the transcription levels of Peg3 using the total RNA isolated from the transfected cells with qRT-PCR. The transcription levels of Peg3 was first normalized to β-Actin, then compared to a control (pCDNA, empty vector). The results derived from multiple transfections (n=6) with each trial representing three individual wells were summarized and presented as graphs with standard deviations. Asterisks represent statistically significant changes in the transcription levels of Peg3 between two constructs.

This series of expression analyses used an empty vector as a control (pCDNA). Overexpression of APeg3-Short resulted in down-regulation of Peg3 by 57.60 ± 0.4% (P=0.0034), whereas overexpression of (-)APeg3-Short resulted in a much smaller change in Peg3 expression (27.80 ±
0.16% (P=0.0536). On the other hand, overexpression of APeg3-Full caused a much greater down-regulation of Peg3 (81.32 ± 0.03%) (P=0.0008), which was ~20% lower expression than those from APeg3-Short. Interestingly, however, overexpression of (-)APeg3-Full also caused down-regulation of Peg3 (49.30 ± 0.054%) (P=0.0271). Given the results from the both directions of APeg3-Full, the observed down-regulation by APeg3-Full may be orientation-independent. Furthermore, it is possible that the potential region causing this down-regulation might be located within the 5’-side of the 1.5-kb region of APeg3 since this down-regulation is much more obvious in the Full-length constructs than in the Short-length constructs lacking the 5’-side of APeg3. To account for any minor transcriptional variants of Peg3, we also performed the previous qRT-PCR with primers amplifying the entire Peg3 transcript (Peg3-1a, Peg3-1b.3) and observed a similar trend (Figure 3.4). Peg3 mRNA levels were decreased in transfections of APeg3-Short (71.43 ± 0.056%) (P=0.0118), APeg3-Full (11.13 ± 0.0635%) (P=0.0229) and (-)APeg3-Full (24.61 ± 0.237%) (P=0.0332), while changes in Peg3 mRNA were not statistically significant in the (-)APeg3-Short (54.18 ± 0.425%) (P=0.2135) transfections. However, these alternative forms of Peg3 have minimal contribution as compared to the major Peg3 transcript (16). Taken together, these results demonstrate that APeg3 has the potential to down-regulate the level of Peg3 mRNA *in vitro*. 
Figure 3.4. qRT-PCR amplifying larger portion of the Peg3 transcript. Representative qRT-PCR of Peg3 from Neuro2A transfected with the respective overexpression constructs. Results are representative of multiple individual transfections (n=3) with at least 3 individual wells transfected in each trial. Standard deviations are derived from fluctuations in individual qRT-PCR threshold cycle values for each transfection with at least 3 quantifications run for each well. Peg3 expression is normalized to β-Actin expression values, then compared to empty vector (pCDNA-transfected) cells. Asterisks represent statistically significant changes in mRNA expression.

3.3.4 *APeg3* down-regulates the protein levels of PEG3

To confirm whether the observed down-regulation of Peg3 transcript resulted in reduced levels of the PEG3 protein, we performed western blots with protein extracts prepared from the transfected Neuro2A cells (Figure 3.5).
Figure 3.5. Effects of APEG3 overexpression on the protein levels of PEG3. A set of transfected Neuro2A were further analyzed using Western blotting to measure the protein levels of PEG3. The density of the detected PEG3 bands by anti-PEG3 antibodies was normalized to that of the β-ACTIN band, then compared with serial dilutions of pCDNA-transfected Neuro2A as standards. Numbers below the bands describe the protein levels of PEG3 and β-ACTIN. Numbers within the red box indicate protein levels PEG3 relative to β-ACTIN.

The protein extracts from the two samples transfected with the sense and antisense APEG3-Full, respectively, were analyzed and compared with those from the sample transfected with the empty vector (pCDNA). The protein levels of PEG3 in each sample were first quantified using densitometric assays through Jmol application http://www.jmol.org, and later normalized with those of β-Actin. The normalized values were finally compared with serial dilutions (100-40%) of the control sample transfected with pCDNA. According to the results, the protein levels of
PEG3 were lowered by 85% in the transfectants with APEG3-Full, and by 74% in the transfectant with (−)APEG3-Full (lane 2 and 3 in Figure 3.5). The reduced protein levels observed from these transfections with APEG3-Full are consistent with the down-regulation observed in Peg3 mRNA (Figure 3.3). In conclusion, the results from both qRT-PCR and western blotting demonstrate that the overexpression of APEG3 indeed results in reduction in the mRNA and protein levels of Peg3.

3.4 Discussion

In the current study, the unknown functions of APEG3 have been investigated using two different approaches, comparative genomic and cell line-based functional analyses. First, comparison of the sequences from different mammals revealed unusually high levels of conservation, but without any obvious ORF in the transcribed region of APEG3, suggesting APEG3 may have been selected as a ncRNA gene during mammalian evolution (Figure 3.1). This has been further supported by the detection of a conserved RNA secondary structure within APEG3 (Figure 3.2). Second, the results from cell line-based analyses further demonstrated that APEG3 has the potential to down-regulate the transcription and protein levels of Peg3 (Figure 3.3 and 3.5). Thus, these results suggest APEG3 has likely evolved as a ncRNA gene controlling the function of the sense gene, Peg3.

The high level of sequence conservation within the transcribed region of APEG3 is very unusual given the following reasoning (Figure 3.1): The transcribed region of APEG3 does not harbor any obvious ORF and Kozak consensus motifs that are conserved among mammals, thus suggesting
APeg3 is a ncRNA gene. This is further supported by the presence of many insertions/deletions in the transcribed region of APeg3 between different mammals. At the same time, the sense direction of APeg3 is part of the 3’UTR of Peg3. In terms of functional selection during evolution, both ncRNA and 3’UTRs are expected to have much less constraints than the protein-coding region of individual genes (17). As a result, these non-coding regions should have much less sequence conservation than the coding regions of genes. This can be easily observed in the 3’UTR of many genes, including Zim1, which shows no conservation at all (Figure 3.1). Nevertheless, the transcribed region of APeg3 has maintained high levels of sequence identity during mammalian evolution (Table 3.1), which is quite comparable to the conservation levels of protein-coding regions. This might be related to the fact that this region harbors sequence elements for both genes, APeg3 and Peg3 with opposite transcriptional orientations. Given this special situation, this is the most likely explanation for the unusual sequence conservation observed from APeg3, which needs further investigation in the near future.

According to the results derived from in vitro transfection experiments, APeg3 has the potential to down-regulate expression of Peg3 (Figure 3.3 and 3.5). The actual mechanism by which APeg3 down-regulates Peg3 is currently unknown, but the following scenario can be envisioned with the limited evidence presented in the current study. The most dramatic down-regulation by APeg3 was observed with the full-length APeg3 constructs, yet this down-regulation appears to be somewhat orientation-independent. The complementarity between Peg3 and APeg3 could allow for RNA:RNA interactions at the proposed hairpin-loop which could be causing transcriptional or translational interference of Peg3 via APeg3 regardless of APeg3 orientation.
However, *in silico* predictions do not correlate this interaction to be the most likely at the 5’ hairpin-loop structure (18).

Another possibility for the orientation-independent down-regulation of Peg3 is through small RNA-mediated silencing. There are currently four known mechanisms for small RNA to down-regulate protein-coding mRNA, miRNA, snoRNA, pi-RNA and siRNA (20). Of these four, the miRNA-mediated mechanism is mainly known to be associated with imprinted domains, and functions independent of orientation (19, 20). Thus, the orientation-independent down-regulation by *APeg3* might be mediated through some unknown miRNA embedded within the *APeg3* locus. In that regard, it is relevant to point out the fact that the 5’-side of *APeg3* contains a potential target site for miR-124 (Figure 3.1), further supporting this possibility.

If this is the case, is this potential miRNA from *APeg3* mainly targeting the transcript from Peg3? Given the similar expression profiles in vasopressin-expressing neurons, we believe Peg3 is most likely the main target of *APeg3*. However, we cannot rule out the possibility that other genes in those specific neuronal cells could be targeted by a potential miRNA from *APeg3*. In conclusion, the observed down-regulation of Peg3 by *APeg3* is very intriguing given the crucial roles played by Peg3 in eutherians, thus it would be of great interest to further investigate this observed down-regulation by *APeg3* in terms of molecular mechanisms as well as potential targets.
3.5 References


CHAPTER FOUR
SUMMARY AND FUTURE DIRECTIONS

It is estimated that less than 1% of all genes found in the human genome are imprinted. Yet, this small portion of the genome can have wide-ranging detrimental impacts on the growth and development of an organism when not properly regulated. Furthermore, these genes are highly susceptible to environmental impacts, a direct result of lacking a functional copy in the genome. An understanding of the function and regulation of these imprinted genes in a tissue-specific context allows for a more finite definition of their roles in vivo.

In this dissertation, I have characterized the imprinted transcription factor gene Peg3, known for its impacts on growth and reproduction. Our data introduces the first genetically-engineered mouse line to abolish Peg3 activity with temporal and spatial specificity. Using this line, our data reveals Peg3 serves a distinct tissue-specific role in lactation and maternal care. In the second part of this dissertation, we were also able to reveal the potential for APeg3 to regulate the expression of Peg3 as a non-coding RNA, by using comparative genomics and functional cell line studies. Altogether, these studies reveal significant insights into the gene function and regulation of Peg3.

Chapter two discusses the deletion of Peg3 in a mouse line, concentrating on defects in neonatal development and maternal care. While deleting Peg3 in the germline resulted in dramatic neonatal growth deficiencies and the inability to care for pups, often resulting in death of the offspring, we aimed to identify the tissues responsible for these phenotypes. In order to test
these findings, we used conditional knockout lines under the control of the Nkx2.1 and MMTV promoters and novel assays to detect subtle changes in milk letdown and maternal caring behaviors. Our findings show that expression of Peg3 in the mammary gland is essential for proper milk letdown and narrowed the expression site responsible for nest-building to a site of expression common to both Nkx2.1 and MMTV. These results provide a connection between the expression sites of Peg3 and the resulting phenotypes.

As a DNA-binding protein, Peg3 is potentially responsible for the regulation of many downstream genes in these tissues. Future experiments will focus on the impact of Peg3 on downstream genes by: performing ChIP-seq to identify all downstream target genes; filtering the genes based on function and known expression site; confirming binding in vitro and in vivo by EMSA and ChIP, respectively; demonstrating transcriptional regulation in vitro; and generating binding site mutants in vivo to confirm a role in transcriptional regulation.

To follow-up our results, we have performed preliminary experiments that suggest a possible downstream gene responsible for the lactation phenotype observed, Oxytocin Receptor (OxtR). To identify genes regulated by Peg3, we have performed RT-PCR in wild-type and knockout (Peg3^CoKO^) mice, focusing on imprinted genes as well as genes involved in neuroendocrine function. Using regions of the fully-developed female brain known to have high expression of Peg3 (the supraoptic nucleus and the pituitary), we were able to assay the expression of many of these genes (Figure 4.1, a). Of the genes tested, OxtR was the most highly affected at the transcriptional level in vivo. Confirmation of this transcriptional change was acquired through
readily available 1dpp (1 day postpartum) neonatal samples, where the expression of OxtR was reduced by 50% through qRT-PCR (Figure 4.1, b).

Figure 4.1 Imprinting and neuroendocrine panel from 6-month-old female brain and 1dpp neonate head. a) RT-PCR from regions surrounding the 6-month-old mouse hypothalamus, including the pituitary and supraoptic nucleus (SON). The left panel includes a number of imprinted genes meant to test the hypothesis that Peg3 is an imprinting hub in these tissues. The right panel displays a number of neuropeptides and receptors which displayed similar expression sites as Peg3. b) RT-PCR and qRT-PCR from Peg3 WT and CoKO pups, displaying a truncation of Peg3 transcript and lessened expression of OxtR. B-Actin serves as the loading control and is used to normalize qRT-PCR.

To assess whether regulation of OxtR was a direct consequence of Peg3’s DNA-binding at promoter/enhancer sites, we performed assays to determine whether Peg3 could directly bind regulatory regions of OxtR. We first identified regions which may be bound by Peg3 based on motifs previously defined in our lab (1). Filtering these sites with upstream regions of OxtR that
show tissue-specific differential methylation, we were able to determine regions expected to be bound by Peg3 that may have regulatory significance (Figure 4.2, a).

![Graphical representation]

**Figure 4.2** Predicted Peg3 binding sites upstream of OxtR. a) Regions used for EMSA and ChIP assays are highlighted on the UCSC genome browser in relation to mouse OxtR gene. Areas deemed positive for Peg3 binding by their respective experiments are color-coded in green, while regions that did not display Peg3 binding in experimental conditions are highlighted in red. Regulatory elements, such as CpG islands and hypomethylated regions (HMRs) are noted below OxtR and are suggestive of tissue-specific sites of regulation. b) Electrophoretic Mobility Shift Assay (EMSA) using duplexes to compete for Peg3 binding. Unlabeled competitor duplexes from OxtR upstream regions were used at [100X] to relieve Peg3 binding from radiolabeled Pgm2l1 probe. Binding site mutants are labeled as 5’ and 3’ or “w”, corresponding to whole binding site mutants. c) ChIP amplifying regions positive for Peg3 binding by EMSA.
Electrophoretic mobility shift assays (EMSAs) were then performed using a radiolabeled probe that corresponds to a region known to recruit Peg3, the promoter of the gene Pgm2l1 (1). Competition assays using the identified regulatory regions of OxtR, denoted as Binding Site # (BS#), were then used to identify the regions that could compete with the Pgm2l1 probe for binding of Peg3. The results of this experiment suggest BS1 and BS3 have the ability to recruit Peg3 in vitro. Once identified, the binding sites were replaced with a poly-T stretch to confirm Peg3 binding at the predicted binding site. These binding sites were then mutated at the 5’ end and 3’ end of the Peg3 binding motif within the competitor duplex to find the bases most essential for binding of Peg3 (Figure 4.2, b). The results indicate recruitment of Peg3 by BS1 requires sequences at the 3’ end of the motif. BS3, on the other hand, requires specific sequences at the 5’ end of the motif to guarantee Peg3 binding. The results also demonstrate the duplexes used for BS4 and BS5 did not compete for Peg3 binding.

To test whether Peg3 is binding these regions in vivo, we performed Chromatin Immunoprecipitations (ChIP) on adult mouse brain chromatin extract using Peg3 antibody. ChIP was performed in wild-type and littermate Peg3CoKO (knockout) brain chromatin extract in order to control for any false positives for Peg3 binding. Input (pre-precipitation DNA) and IgG (pre-immune sera) controls were also used to demonstrate the fold-enrichment of the specified DNA over background pull-down by nonspecific antibody serum. Regions amplified in ChIP experiments were based on EMSA regions, using primers that flanked these regions by ~50 bp. on each side (Figure 4.2, c). Although successful amplification of BS3 could not be achieved, these results indicate BS1 and BS5 are enriched in DNA precipitated with Peg3 antibody. Furthermore, based on our preliminary (semi-quantative) findings, these regions appear to have
high enrichment as compared to a region of Pgm2l1 known to show enrichment for Peg3 binding. Consistent with the \textit{in vitro} EMSA data, BS4 does not show enrichment upon precipitation with Peg3 antibody. BS5, on the other hand, was shown to recruit Peg3 binding \textit{in vivo} by ChIP, but not \textit{in vitro} using EMSA. These results are confounding, but can be explained when considering the regions amplified in ChIP experiments are larger than the potential binding sites tested \textit{in vitro}. The results for BS5 suggest the extra sequence amplified in the ChIP experiments may be responsible for recruitment of Peg3 and will need to be tested in future studies.

Taken together these studies suggest a linkage between Peg3 and OxtR through Peg3’s DNA-binding capability. Furthermore, this connection provides a plausible downstream gene that could be responsible for the lactation defects observed in Peg3 knockout lines. In future studies, it would be beneficial to pursue this angle further. Finding the molecular mechanism responsible for the repression of OxtR in Peg3-deficient mice and further confirming Peg3’s regulation of OxtR \textit{in vivo} are the next steps necessary in characterizing the network from Peg3 to OxtR to lactation.

Chapter three discusses the evolutionary conservation, secondary structure and regulatory function of APeg3 as a non-coding RNA with the potential to suppress the expression of Peg3. Genomic studies revealed the conservation of APeg3 as a ncRNA at the primary (sequence) and secondary (structural) level, identifying a conserved secondary structure at the 5’ end. Functional cell line studies demonstrate the ability of APeg3 to down-regulate Peg3 transcript \textit{in vitro}. This
chapter also identifies a conserved 5’ extension of A Peg3 transcript in mice and shows the region to mediate additional silencing in an orientation independent manner.

Genomic studies show the conservation of a transcriptional start site and poly-adenylation signal for A Peg3 in 9 orders of eutherian mammals. Alongside the absence of a conserved ORF, these genomic studies illustrate the occupation of A Peg3 as a conserved transcriptional element, suggesting its function as a ncRNA. Using this sequence information, we were also able to infer structural characteristics about A Peg3, focusing on the macro-scale long ncRNA as well as the micro-scale small ncRNA folding patterns. The conservation of a small secondary structure at the 5’ end of A Peg3 shows folding and sequence characteristics consistent with those found in previously annotated small RNAs, suggesting the region may function to regulate the sense gene through a small RNA intermediate. Transfection experiments overexpressing the A Peg3 transcript in vitro confirm the potential of A Peg3 to downregulate Peg3 in a neuronal cell line, Neuro2A. These transfection experiments, along with the secondary structure analyses also provide a more in-depth explanation of how this silencing occurs. While Peg3 silencing only occurs when the 3’ region of A Peg3 is overexpressed in the correct orientation, the extended 5’ end confers additional silencing potential in both orientations. The orientation-independent silencing is characteristic of micro-RNA (miRNA), a class of small RNAs which function to regulate their sense counterparts through post-transcriptional gene silencing.

We hypothesize the function of A Peg3 in vivo will be similar to its function in vitro (in cell culture assays), regulation of Peg3. However, unlike Peg3, expression of A Peg3 is limited to
vasopressinergic neurons in the brain, suggesting the transcript’s function will be highly cell-type specific. Future experiments for APeg3 will concentrate on two aspects, the detection of small RNA intermediates from APeg3 transcript and the function of APeg3 in vivo, focusing on its expression and upregulation in salt-dependent, vasopressinergic cells.

The first aspect will be the detection of smRNA originating from the 1.5 Kb, full length APeg3 transcript. Detection of smRNA is historically accomplished through northern blots and smRNA amplification using fluorescently labeled probes (2, 3, 4). However, these assays are prone to low sensitivity and high background, respectively. The best strategy to assay the production of smRNA from a large transcript, such as APeg3, is through radiolabeled in vitro transcription of APeg3, followed by incubation with nuclear extracts from Neuro2A cells. If APeg3 serves as a pri-miRNA, then incubation with nuclear extracts should result in distinguishable smRNA products, which can then be sequenced through conventional cloning and Sanger sequencing. Known precursor RNAs, such as H19, should be used as controls to test the validity of the system (5).

The second aspect of APeg3 centers on the function of APeg3 in vivo. While the best way to examine a gene’s function in vivo is to produce animals that have deletions in the gene, antisense genes have specific caveats that must be taken into account. As regions of APeg3 overlap with the coding region and 3’ UTR of Peg3, deletions of APeg3 will likely produce unintended defects in Peg3 expression. The best option for affecting APeg3 expression without perturbing Peg3 is to delete the promoter of APeg3. Although we have identified a consensus TATA motif
upstream of APeg3, promoter assays must be performed prior to deletion in mice. Once transcriptional regulation by this promoter is confirmed, a mutation/deletion of this region can result in a change of the APeg3 mRNA, without a change in the transcription or translation of Peg3. Another approach could use findings from the characterization of the predicted miRNA to yield sites that could be mutated based on their secondary structure, resulting in an optimal situation for characterizing the potential of this secondary structure in the silencing of Peg3 in vivo. Once these APeg3 mutant mouse lines are created, they can then be crossed with our Peg3^{CoKO} reporter line to assay β–Galactosidase activity in fixed brain tissues under conditions of salt stress. I believe this ncRNA serves a role in regulating expression of Peg3 in vasopressinergic cells when expression of Peg3 is too high. This could be due, in large part, to Peg3’s role in autophagy or apoptosis and could function to modulate the actions of Peg3. In these cells, following salt exposure, Peg3 could be acting to replenish the peptide neurotransmitter, vasopressin, through cycling of cellular proteins. Central nervous cells are irreplaceable, thus APeg3 could be acting as a safeguard, modulating Peg3 expression, to maintain viability of the cell. A proposed model for this relationship is shown (Figure 4.3). This model takes into account the upregulation of Peg3 in response to high salt concentrations in vivo, as well as the recently described role of Peg3 as a master regulator of autophagy.
This dissertation chronicles the function and ncRNA-mediated regulation of an imprinted transcription factor, Peg3. Firstly, the findings illustrate the specific tissues in which Peg3 is necessary for proper physiological and behavioral functions, then links these functions to a possible downstream gene, OxtR. Secondly, the findings show the potential for regulation of Peg3 by A Peg3 in a very specific cell type in the hypothalamus, suggesting a mechanism of smRNA gene silencing may be partly responsible. Our study discusses the critical expression sites and functions of Peg3 in reproduction on the genetic, physiological and behavioral level.
4.1 References


APPENDIX
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