

**THE DESIGN OF OVARY-SPECIFIC LRBP KNOCKOUT MICE
AND THE DESIGN AND AMPLIFICATION OF PROBES FOR THE
TARGETING VECTOR**

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ABSTRACT

Mevalonate kinase (MvK) is a metabolic enzyme involved in cholesterol biosynthesis. It also serves as an RNA binding protein in luteinizing hormone receptor (LHR) down-regulation that occurs after the LH surge. Because of this property, the enzyme is referred to as LHR mRNA binding protein (LRBP). The mechanism of the LHR down-regulation pathway and the role of LRBP have been extensively studied by the Menon laboratory. What has yet to be investigated is what occurs in the ovarian cycle when LRBP is completely absent. In this paper, a knockout mouse model is outlined that selectively knocks out the gene coding for LRBP in the ovaries. In order for the Transgenic Animal Model Core (TAMC) to carry out the creation of the LRBP knockout mouse model, it is imperative to develop a Southern blot assay that will be able to detect the presence of the LRBP knockout targeting vector in embryonic stem (ES) cells. To conduct the Southern blot assay, radioactively labeled probes must be prepared that will bind the targeting vector, revealing its presence in the ES cells. In addition to discussing the strategy used in the design of the targeting vector and the creation of the knockout mice, this paper also explains the steps involved in the refinement and amplification of the probes for Southern blot analysis.

INTRODUCTION

The ovarian cycle in most mammals involves follicle recruitment, maturation, ovulation, luteinization, and luteolysis [1]. These processes are regulated by two anterior pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH regulates follicle recruitment and maturation whereas LH controls pre-ovulatory

steroidogenesis, ovulation, and corpus luteum function. FSH has an important role in follicle development and estrogen production by the ovary. During follicle maturation, estradiol, produced by the pre-ovulatory follicles in response to FSH, plays a synergistic role with FSH in promoting the formation of Graafian follicles. The expression of LH receptors increase in response to FSH action, and the LH receptors are responsible for responding to the pre-ovulatory LH surge that results in the release of the ovum for fertilization [2]. LH action is mediated by a membrane receptor family of G-protein-coupled receptors (GPCRs). The expression of LH receptors shows dynamic changes during the ovarian cycle. For example, its expression increases during follicle maturation in response to FSH and undergoes down-regulation in response to the pre-ovulatory LH surge in order to keep the ovary in a transient quiescent state during the differentiation of granulosa cells to luteal cells. The LH receptor expression then increases to support progesterone synthesis by the corpus luteum. In the absence of fertilization, LH receptor expression declines coincident with the regression of the corpus luteum leading to the onset of the next cycle.

Menon Lab has shown that the pre-ovulatory LH surge triggers the down-regulation of LH receptor expression through targeted degradation of LH receptor mRNA rather than the result of decreased transcription [3]. Both in rat and human ovaries, an LH receptor mRNA binding protein (LRBP) has been discovered, and this protein mediates LH receptor mRNA degradation through a post-transcriptional mechanism [4]. Menon lab also showed that LRBP selectively binds to the coding region of LH receptor mRNA and acts as a *trans* factor to target the mRNA for degradation. Furthermore, by altering the cellular levels of LRBP, ovarian LH receptor mRNA expression is regulated by

controlling its degradation [4,5]. In addition, the identity of LRBP was established as being mevalonate kinase (MvK)[4]. MvK is a cholesterol biosynthetic enzyme, and the identification of MvK as LRBP has suggested a link between LH receptor mRNA expression and sterol metabolism in ovarian tissue [4].

Since the discovery of LRBP and its identity, our lab has focused on identifying the signaling pathways involved in LH receptor down-regulation. Similar to other GPCRs, an increase in cAMP levels results from LH or hCG binding to the LH receptor [5]. The increased concentration of cAMP in ovarian cells then leads to the activation of cAMP-dependent protein kinase levels [5]. Increase in intracellular cyclic AMP levels were shown to decrease the expression of LH receptor mRNA while simultaneously increasing LRBP binding activity [6]. In addition, the well-known enzymes protein kinase A (PKA) and extracellular signal-regulating kinases 1 and 2 (ERK1/2) were found to have a vital role in the signaling pathways that leads to LRBP binding and subsequent LH receptor down-regulation [5]. PKA mediates LH receptor mRNA degradation through the activation of ERK1/2 signaling that leads to an increase in the levels of LRBP mRNA and LRBP protein [5]. ERKs, when activated, are known to phosphorylate many targets, which leads to the regulation of cell processes such as transcription, translation, and apoptosis [5]. Menon lab studied ERK activity in response to hCG stimulation and found that after treatment with hCG, ERK translocates to the nucleus and initiates events that result in the increased expression of LRBP [5]. This shows that it is likely that LRBP levels are regulated by transcriptional activation [5].

The most recent studies being conducted in Menon laboratory investigate the roles of microRNA miR-122 and sterol regulatory element-binding proteins (SREBPs) in LRBP

expression. Recently, microRNAs have been found to be increasingly important in gene expression and regulation [7]. They commonly perform their function by binding to the 3'-untranslated region of protein-coding mRNA transcripts that either block translation or target the mRNA for degradation [7]. Our lab hypothesized that miR-122 could regulate LRBP expression based on previous studies which showed that the inhibition of miR-122 in mouse liver also inhibits the expression of MvK [8]. Since MvK also functions as LRBP, Menon lab tested whether LRBP expression was affected by the presence of miR-122 in rat ovaries [7]. Using *in situ* hybridization, we analyzed the localization of miR-122 in rat ovaries and examined the changes in miR-122 expression during hCG-induced LH receptor down-regulation [7].

Using real-time PCR to observe the changing levels of miR-122 during hCG-induced LH receptor down-regulation, it was found that there was a substantial increase of miR-122 expression 30 minutes after the rats were treated with hCG, and this increase peaked around 1-2 hours [7]. The presence of miR-122 was confirmed by fluorescent *in situ* hybridization (FISH) of the rat ovary [7]. In additional experiments, the increase in miR-122 expression was found to be followed by an increase in LRBP mRNA, which, in turn, was followed by an increase in LRBP protein levels [7]. This correlation implies a role of miR-122 in the regulation of LRBP in LH receptor mRNA down-regulation [7]. Furthermore, when PKA and ERK1/2 (which were shown in previous experiments to be included in the signaling cascade that leads to LH receptor down-regulation [5]), were suppressed, the expression of miR-122 was blocked, and hCG-mediated LH receptor mRNA down-regulation was abrogated [7]. This indicates that the cAMP/PKA and ERK1/2 pathways mediate miR-122, which, in turn, plays an integral role in LH receptor

mRNA down-regulation [7]. The final aspect of this pathway that was tested in the most recent study was the time course of SREBP activation in relation to miR-122-mediated LRBP induction [7]. In the time scale of LH receptor down-regulation, the activation of SREBP-2 and SREBP-1a occurred 1-4 hours after hCG treatment, which occurs between the peaks of miR-122 expression and LRBP expression [7]. Menon Lab also suppressed the effect of miR-122 on LH receptor down-regulation by injecting a locked nucleic acid (LNA) conjugated miR-122 antagomir directly into the bursa of the rat ovary [7]. This experiment, in its entirety, showed an important role for miR-122 on the LH receptor mRNA degradation pathway [7].

While many of the relationships between LRBP and the major players discovered to be involved in LHR mRNA down-regulation have been investigated quite extensively as described above, our lab would now propose to investigate the physiological significance of LHR mRNA down-regulation in otherwise normal physiologic conditions. As indicated earlier, down-regulation of LHR mRNA in response to the pre-ovulatory LH surge to allow differentiation of granulosa cells to luteal cells and to suppress premature onset of vascular endothelial growth factor (VEGF) production to pressure normal ovarian function. However, availability of a model where LHR down-regulation is eliminated would reveal even potentially more interesting roles of LHR down-regulation in reproductive physiology. In order to conduct these experiments, we must engineer a genetically mutated “knockout mouse” that deletes the LRBP gene. Removing the gene that codes for LRBP, *Mevk1*, will allow our lab to study the impact of regulation of ovarian function by LHR, in the absence of LRBP. Additionally, the examination of LHR, LHR mRNA, miR-122, SREBP 1/2, and ERK 1/2 levels in the granulosa cells of

these mice will further elucidate, not only the role of LRBP in post-LH surge LHR down-regulation, but the physiological importance of LHR down-regulation in general. There is a problem, however, with the universal knockout of *Mevk1* that requires additional selection and cell differentiation. In addition its role as LRBP, it is well known that MvK catalyzes the phosphoryltransfer from a molecule of ATP to (R)-mevalonate to produce (R)-5-phosphomevalonate and ADP [9]. This reaction is a crucial step in isoprenoid and sterol biosynthesis pathways, and the systematic absence of expression of the enzyme is lethal [9]. Therefore, the design of a *Mevk1* knockout mouse must include the excision *Mevk1* exclusively in the ovaries.

This thesis discusses the long-range plans to genetically engineer a knockout mouse model (MvK cKO) that will selectively eliminate expression of mevalonate kinase (LRBP) in the ovaries of the mice. In this paper, multiple strategies are described, which are used to properly insert the knockout targeting vector, to excise it in the appropriate cell type, and to select for the mice with the desired knocked-out genotype. The knockout and selection tools that are discussed in this paper are the Cre-loxP approach using a *Cyp19* primer, the PGK-Neo cassette used for embryonic stem cell selection, and the FLP-FRT knockout approach.

Our lab is fortunate enough to have a central Transgenic Animal Model Core (TAMC) facility of the medical center for the creation of these mice [10]. Although the core facility will carry out most of the important steps, such as the microinjection of the targeting vector into embryonic stem (ES) cells for homologous recombination, the responsibility for screening the ES cells for the production of recombined LRBP is the responsibility of our laboratory [11]. TAMC's protocol necessitates the completion of

certain tasks that ensures that our lab is capable of performing a Southern blot assay that detects the presence of the desired targeting vector [11]. To complete this requirement, we constructed two probes that bind complementarily with sequences flanking our targeting vector that, when used in a Southern blot assay, would identify the presence of the targeting vector and confirm the success of proper homologous recombination in the ES cells. In addition to discussing the design behind our MvK cKO targeting vector, this paper will also demonstrate the process performed and results obtained from the amplification of these probes. My specific task in this process was to design primers for the probes and amplify them sufficiently for use in the crucial Southern blot verification assay. I accomplished this by first amplifying the probes from bacterial artificial chromosomes (BACs) using PCR and then through molecular cloning.

MATERIALS AND METHODS

Materials

Dr. Michael Zeidler (Transgenic core, The University of Michigan) engineered the BAC containing the targeting vector. In the first intron of *Mevkl*, a loxP site was introduced, and in the third intron, another loxP site and an FRT-flanked PGK-Neo cassette were inserted. The 3' and 5' custom primers, and the competent DH5 α cells were purchased from Thermo Fisher Scientific (Waltham, MA). The pBluescript II KS(-) plasmid was acquired from Agilent Technologies (Santa Clara, CA). The HindIII restriction enzyme, XhoI restriction enzyme, and T4 DNA Ligase were purchased from New England BioLabs (Ipswich, MA). The Zymo Research Quick-gDNA Miniprep was acquired from Zymo Research (Irvine, CA). The QIAquick Nucleotide Removal Kit, QIAprep Spin

Miniprep Kit, and the QIAGEN Plasmid Maxi Kit were purchased from QIAGEN (Venlo, Netherlands).

PCR Amplification

3'-sense and 5'-sense primers were designed with XhoI restriction sites on the 5' ends, and 3'-antisense and 5'-antisense primers were designed with HindIII restriction sites on the 5' ends. The primers with 5' restriction sites were used in the amplification of the probes using PCR and in the introduction of the XhoI and HindIII restriction sites flanking the probes. PCR mixture contained (one for each probe) 45 μ L PCR Platinum Supermix, 2 μ L DNA (BAC), 2 μ L DepC H₂O, 0.5 μ L 3'/5'-sense primer (40 pmol/ μ L), and 0.5 μ L 3'/5'-antisense primer (40 pmol/ μ L). The PCR cycle started with denaturation at 94 °C for 2 minutes and then repeated the following cycle 35 times: 94 °C for 30 seconds, 65 °C for 30 seconds, 72 °C for 18 seconds. This was followed by 10 minutes at 72 °C and finally cooling at 4 °C. The PCR products (5 μ L sample + 2 μ L 6X loading dye) were run on a 1.2% agarose gel in 1X TBE to confirm the success of the amplification (specifics about gel electrophoresis are enumerated in the Agarose Gel Electrophoresis section of Materials and Methods). The remaining 45 μ L were purified using QIAquick Nucleotide Removal Kit as follows: First, 10 volumes of Buffer PN1 (add 19 mL of 100% isopropanol prior to use) were added to 1 volume of the reaction samples and mixed. The mixtures were applied to QIAquick columns and centrifuged for 1 minute at 6000 rpm at room temperature (RT). The flow-through was discarded and the QIAquick columns were washed using 750 μ L of Buffer PE (add 24 mL of 100% ethanol prior to use) for 1 min. at 6000 rpm at RT. The DNA was eluted using 50 μ L of

nuclease-free H₂O by centrifugation for 1 minute. Purified samples were stored at -20 °C.

Restriction Digestion

Restriction digestion was performed on the 3' BAC PCR product, 5' BAC PCR product, and the pBluescript II KS(-) plasmid. The digestion solutions for the PCR products contained 45 µL of the purified 3'/5' PCR product, 5 µL NEB 2.1 Buffer, 1 µL HindIII restriction enzyme (10 ng/µL), and 1 µL XhoI restriction enzyme (10 ng/µL). The pBluescript digestion solution contained 1 µL pBluescript plasmid (1 µg/µL), 2 µL NEB 2.1 Buffer, 1 µL HindIII, 1 µL XhoI, and 15 µL nuclease-free H₂O. Digested the three solutions at 37 °C for 3 hours, followed by purification using QIAquick Nucleotide Removal Kit (same protocol as explained in the PCR section of Materials and Methods). The samples (5 µL sample + 2 µL 6X loading dye) were then run on 1.2% agarose gel in 1X TBE buffer (specifics about gel electrophoresis are enumerated in the Agarose Gel Electrophoresis section of Materials and Methods). The digested and purified 5' probe, 3' probe, and pBluescript were located using a 1 kB DNA marker, and the fragments were cut out using a razor with the gel under a U.V. light (cutting out as little agarose as possible). The nucleotides were purified using the Zymo Research Quick-gel DNA kit as follows: 3 volumes of agarose dissolving buffer (ADB) were added to each vial of recovered DNA-containing gel and incubated at 37 °C for 10 minutes. The dissolved solutions were loaded into Zymo-Spin Columns that filter into 1.7 mL collection tubes and centrifuged for 30 seconds. DNA was washed twice by adding 200 µL of DNA Wash

Buffer and centrifuged at full speed for 30 seconds. DNA eluted in 50 μL of nuclease-free H_2O . Samples were stored at $-20\text{ }^\circ\text{C}$.

Ligation

Digested and purified pBluescript was dephosphorylated by adding 40 μL of the digested plasmid, 4 μL dephosphorylation buffer, and 4 μL of alkaline phosphatase and incubating at $37\text{ }^\circ\text{C}$ for 30 minutes. QIAquick Nucleotide Removal Kit was used to purify the dephosphorylated pBluescript (same protocol as explained in the PCR section of Materials and Methods). Two separate ligation mixtures were created – one for each of the probes. Both the 5' and 3' solutions contained a 1:10 ratio of pBluescript plasmid to digested probe. To each ligation solution, 100 ng of pBluescript (digested, dephosphorylated, and purified), 1000 ng of 3'/5' probe (digested and purified), 2 μL of Ligation Buffer, and 1 μL of T4 DNA ligase were added. Nuclease-free H_2O was added to each solution to bring the total volume to 20 μL . The two ligation mixtures were incubated at $16\text{ }^\circ\text{C}$ overnight and stored at $-20\text{ }^\circ\text{C}$.

Transformation

Four separate transformation mixtures were created – one positive control, one negative control, one mixture for 3' probe + plasmid ligated product, and one mixture for 5' probe + plasmid ligated product. The positive control mixture contained 50 μL of competent DH5 α cells and 1 μL of pure pBluescript (10 ng/ μL). The negative control contained 50 μL of competent DH5 α cells and 1 μL of nuclease-free H_2O , and each of the 3'/5' probe

mixtures contained 50 μL of competent DH5 α cells and 5 μL of the probe + plasmid ligated products (10 ng/ μL). The tubes were placed first on ice for 3 hours and then in a 42 $^{\circ}\text{C}$ water bath for 60 seconds, and then quickly transferred back to ice for 5 minutes. 800 μL of S.O.C. medium (without ampicillin) was added to each vial, and the mixtures were shaken in a 37 $^{\circ}\text{C}$ incubator for 1 hour. After the samples settled down, 600 μL of solution was removed from the top. 10 μL of ampicillin (50 $\mu\text{g}/\mu\text{L}$) was added to each vial. For each of the four transformation mixtures, three 25 mL plates were made, each containing 25 mL of LB Agar (32 mg/mL) and 2 μL ampicillin (50 $\mu\text{g}/\mu\text{L}$). The transformation mixtures were plated using sterile spreaders in volumes of 100 μL , 50 μL , and 25 μL for each mixture on the LB Agar + ampicillin plates for a total of 12 plates. The plates were left at RT until the liquid was absorbed. Then, they were placed in the inverted position and incubated at 37 $^{\circ}\text{C}$ overnight. 3 colonies were selected from each plate and grown in LB Broth containing ampicillin (from 100 mL stock solution of 20 mg/mL LB Broth and 50 $\mu\text{g}/\text{mL}$ ampicillin) by incubating (shaking) at 37 $^{\circ}\text{C}$ overnight. The negative control did not have any colonies. , DNA was isolated from a small aliquot of the culture using QIAGEN Plasmid Miniprep Kit as follows: After centrifugation, the supernatants were poured off and the pellets were resuspended in 250 μL Buffer P1. 250 μL Buffer P2 was added and mixed followed by the addition of 350 μL Buffer N3 and subsequent mixing. The samples were centrifuged, the resulting supernatants removed, and applied to the QIAprep Spin Columns, leaving the white pellet in the discarded tubes. The columns were put over new 1.7 mL microcentrifuge tubes and centrifuged for 30-60 seconds. The flow-through was discarded. The columns were washed with 0.75 Buffer PE and centrifuged for 30-60 seconds. The flow-through was discarded and the columns

were centrifuged again to fully remove the remaining wash buffer. The columns were put in fresh 1.7 mL centrifuge tubes, and the purified DNA was eluted with 50 μ L nuclease-free H₂O and centrifugation. Isolated DNA samples were stored at -20 °C.

Agarose Gel Electrophoresis

In order to verify whether the probes were properly ligated into the pBluescript and transformed into the DH5 α cells, the isolated DNA samples from the QIAprep Spin Miniprep Kit were run on an agarose gel. The DNA concentration of each sample was calculated by measuring the OD at 280 nm. For running the gel, two of the positive controls, seven 3' probe DNA samples, and five 5' probe DNA samples were selected based on DNA concentrations. The samples were then digested as explained in the Restriction Digestion section of Materials and Methods. These digestion mixtures, however, contained 1 μ g of the purified 3'/5'/control DNA samples, 5 μ L NEB 2.1 Buffer, 1 μ L HindIII restriction enzyme (10 ng/ μ L), and 1 μ L XhoI restriction enzyme (10 ng/ μ L). The total volume of each solution was brought to 20 μ L by adding the appropriate volume of nuclease-free H₂O (depending on each individual sample concentration). After digestion, a 1.2% agarose gel in 1X TBE was loaded with 10 μ L of the 1 kB DNA marker and 5 μ L of the digested samples mixed with 2 μ L of 6X loading dye. The gel ran for approximately 1 hour at 100V and was inspected under the U.V. light. DNA samples containing the 3' and 5' probes were identified using the DNA ladder. The two positive cultures that displayed the strongest fluorescent bands for each of the probes were then prepared to be used in QIAGEN Plasmid Maxi Kit to obtain larger quantities of the probe DNA necessary for nucleotide sequencing and in the

Southern Blot assay. To prepare for the Plasmid Maxi Kit, 100 μ L of the chosen 3' and 5' probe were incubated in 200 mL of LB Broth (20 mg/mL) at 37°C. The two cultures, one with DH5 α cells containing the 3' probe DNA ligated into pBluescript and the other with cells containing the 5' probe DNA ligated into pBluescript, were used in the QIAGEN Plasmid Maxi Kit as follows: Both cultures were centrifuged at 6000g at 4 °C for 15 minutes. The supernatants were discarded and the bacterial pellets were resuspended in 10 mL Buffer P1 (RNase A must be added to the buffer prior to use). 10 mL Buffer P2 was added, mixed, and incubated at RT for 5 minutes. 10 mL of pre-chilled Buffer P3 was added, mixed, and incubated on ice for 20 minutes. Next, it was centrifuged at 20,000g at 4 °C for 30 minutes, and the supernatants were isolated (discarding the pellets) and re-centrifuged under the same conditions for 15 minutes. The supernatants were applied (in separate runs) to the QIAGEN-tip and allowed to enter the resin by gravity flow. For each run, the QIAGEN-tip was washed with 30 mL Buffer QC two times, allowing Buffer QC to move through the QIAGEN-tip by gravity flow. The DNA was eluted with 15 mL Buffer QF into two clean 50 mL containers. The DNA was precipitated by adding 10.5 mL of RT isopropanol to the eluted DNA solutions and mixed well. The solutions were then centrifuged for 30 minutes at 4 °C and the supernatants were decanted. The DNA pellets were washed with 5 mL of RT 70% ethanol and centrifuged for 10 minutes. The supernatants were decanted again. The pellets were air-dried for 5-10 minutes and the DNA was resuspended in 5 mL of nuclease-free H₂O. 500 μ L of sodium acetate was added to each sample and mixed. 10.5 mL of cold 100% ethanol was added and placed on ice for 20 minutes. The mixtures were centrifuged for 15 minutes, and the supernatants were decanted. 1 mL of 70% ethanol

was mixed into the samples. Again they were centrifuged, and the supernatants were poured off. The purified DNA pellets were air-dried and resuspended in 1 mL of nuclease-free H₂O. The DNA was stored at -20 °C.

RESULTS

Primer Construction

Sense and antisense primers were designed for the PCR amplification of the 5' probe and the 3' probe. Figures 1 and 2 show the association of the primers with the complimentary probe DNA strands for the 3' probe and 5' probes, respectively.



Figure 1: The sense and antisense primers designed for the 3' probe are shown in red associated with the complimentary bases in the probe shown in bold. During PCR, replication of the BAC proceeds as indicated. During PCR, the palindromic 6 base pair restriction sites for XhoI and HindIII are introduced, flanking the 3' probe in the replicated DNA.

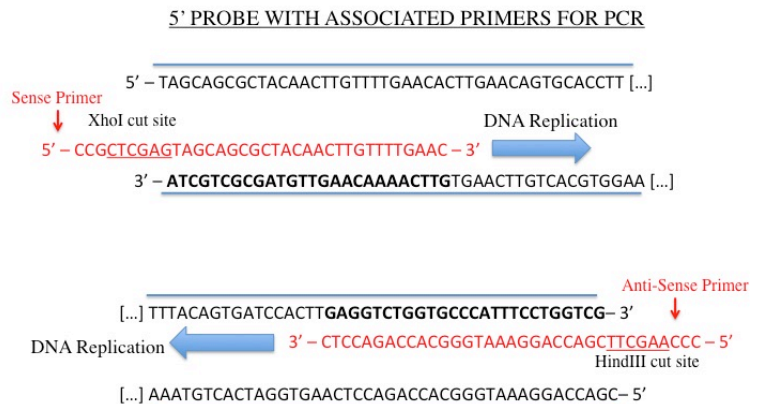


Figure 2: The sense and antisense primers designed for the 5' probe are shown in red associated with the complimentary bases in the probe shown in bold. During PCR, replication of the BAC proceeds as indicated. During PCR, the palindromic 6 base pair restriction sites for XhoI and HindIII are introduced, flanking the 5' probe in the replicated DNA.

The lengths of the complimentary portion of the primers were chosen in order to maximize GC content. The lengths (not including the 5' non-complimentary restriction sites) of the 3'-sense, 3'-antisense, 5'-sense, and 5'-antisense primers were determined to be 24 bp, 27 bp, 26 bp, and 26 bp for a total (including the 5' restriction sites) of 33 bp, 36 bp, 35 bp, and 35 bp, respectively. These primers have 73%, 60%, 51%, and 56% GC

content, respectively, which each fall above the recommended 50% threshold. For the 3' probe, the melting temperatures (T_m) for the primers were 110 °C and 104 °C. For the 5' probe, the melting temperatures (T_m) for the primers were 99 °C and 105 °C.

In order to introduce the XhoI and HindIII restriction sites flanking the probes, a sequence corresponding to the 6 bp restriction sites were put on the 5' ends of the sense and antisense primers. 3 bp 5' extensions were added to the ends of the non-complimentary restriction sites because they have been shown to increase cleavage efficiencies of many restriction enzymes [12]. The most effective 5' extension for the 5'-CTGGAG-3' XhoI cut site is 5'-CCG-3', and the most effective 5' extension for the 5'-AAGCTT-3' HindIII cut site is 5'-CCC-3' [12]. So the 9 bp non-complimentary sequence chosen for both the 3'-sense and 5'-sense primers was 5'-CCGCTGGAG-3', which introduced the XhoI restriction site. The 9 bp non-complimentary sequence chosen for both the 3'-antisense and 5'-antisense primers was 5'-CCCAAGCTT-3', which introduced the HindIII restriction site. These aspects of the primers are shown in figures 1 and 2, which show the complimentary portion of the primers annealed with the probe DNA strands and the 9 bp non-complimentary overhang to be integrated on either side of the probe DNA through PCR.

PCR Amplification of Probes

The 5' and 3' probe PCR mixtures included PCR Platinum Supermix, the sense primer, the antisense primer, water, and varying volumes of DNA (BAC). Volumes of 2 μ L (samples 2 and 6), 3.5 μ L (samples 3 and 7), and 5 μ L (samples 4 and 8) were used in the PCR mixtures. In order to determine whether the PCR was successful, the samples

were run on a 1.2% agarose gel with a 1 kB DNA marker and both 5' and 3' probe controls. Prior to running the gel, the replicated BACs are digested with XhoI and HindIII. The gel is shown in figure 3.

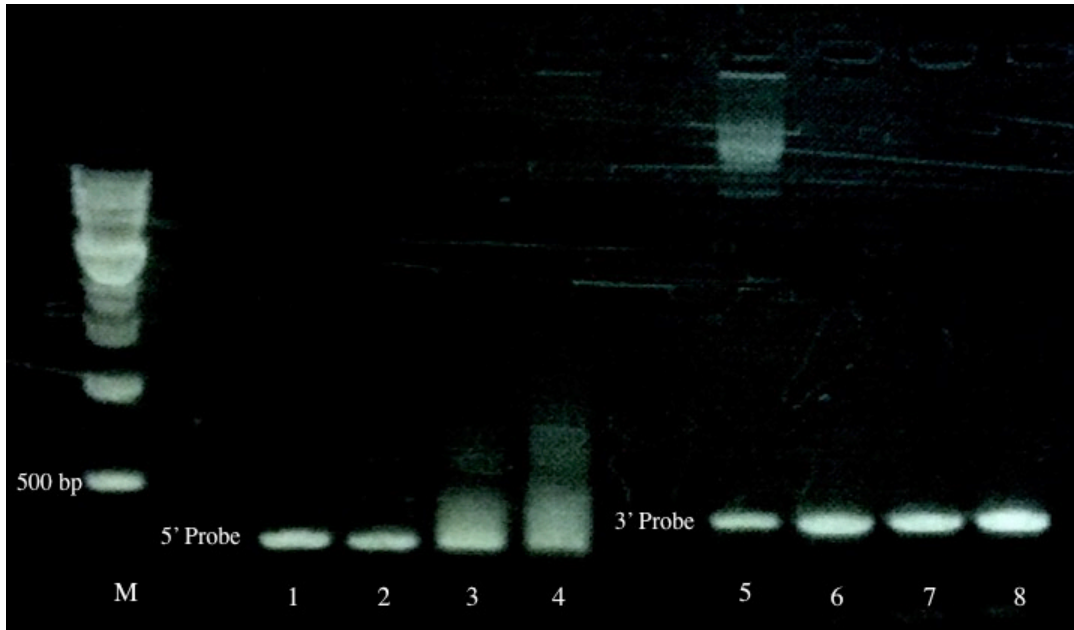


Figure 3: 1.2% agarose gel electrophoresis conducted to confirm the success of PCR. Sample 1 is the 5' probe control and sample 5 is the 3' probe control. Samples 2-4 are digested 5' probe PCR products at varying concentrations and samples 6-8 are digested 3' probe PCR products at varying concentrations. PCRs for samples 2 and 6 were conducted with 2 μ L DNA, samples 3 and 7 were conducted with 3.5 μ L DNA, and samples 4 and 8 were conducted with 5 μ L DNA.

The successful PCR of the 5' probe flanked with XhoI and HindIII restriction sites was confirmed by the alignment of the 5' probe control (sample 1) with the 5' probe PCR product that used 2 μ L of DNA (sample 2). Samples 3 and 4 appeared to also contain some 5' probe; however, the bands were smeared, indicating that these DNA samples were degraded at some point either during or after PCR. All 3 samples of 3' probe PCR product (samples 6-8) showed crisp bands that aligned with the 3' probe control, confirming the successful PCR of the 3' probe flanked with XhoI and HindIII restriction sites. The distance the bands traveled with relation to the marker further authorized the presence of the probes. The 3' probe is approximately 400 bp, so the

location of samples 5-8 slightly below the 500 bp marker band on the gel supported the notion that those bands were the 3' probe. Furthermore, the 5' probe is approximately 350 bp, so the location of samples 1-4 slightly below both the 500 bp marker band and the 400 bp 3' probe bands in samples 5-8 supported the identity of those bands as the 5' probes.

Probe Amplification by Molecular Cloning

In order to amplify the probes in large quantities to be in the Southern Blot assay, I further used molecular cloning technique. For this, the PCR products were digested, and the probes were ligated into a pBluescript II KS(-) plasmid that contained ampicillin resistance genes. The plasmids containing the probes were then transformed into competent DH5 α cells and grown on agar plates containing ampicillin so as to select for colonies that took up the pBluescript during transformation. The molecularly cloned recombinant plasmids were then isolated and digested again. In order to initially verify whether the probes might have successfully inserted into the plasmid and transformed into the bacterial cells, gel electrophoresis on a 1.2% agarose gel was conducted and the results are displayed in figures 4 and 5.

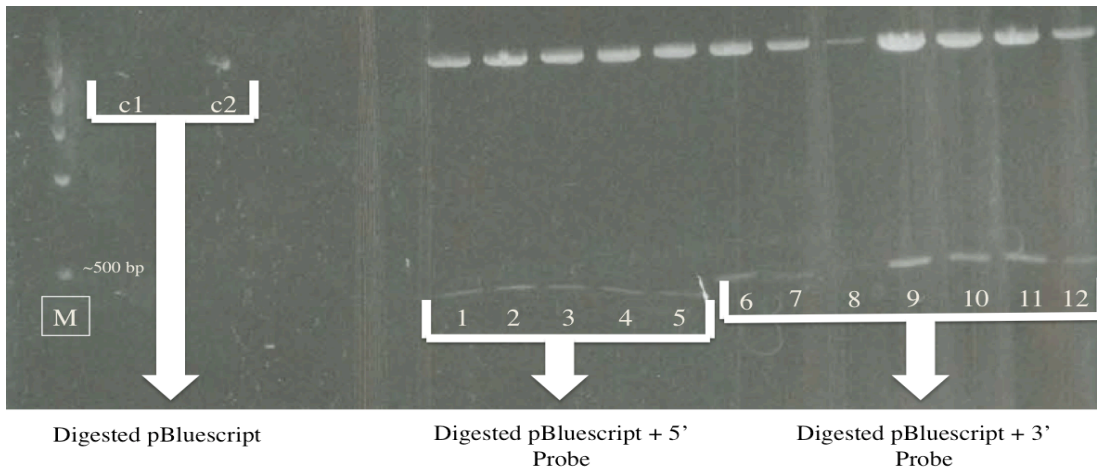


Figure 4: Gel electrophoresis (1.2% agarose) conducted to determine whether the 3' and 5' probes were successfully integrated into the pBluescript plasmid. Isolated DNA from 14 DH5 α colonies (2 control and 12 experimental) were digested and run on the agarose gel with a 1kB DNA ladder. Samples c1 and c2 were colonies transformed with intact pBluescript. Samples 1-5 were transformed with 5' probe ligated into pBluescript, and samples 6-12 were transformed with 3' probe ligated into pBluescript

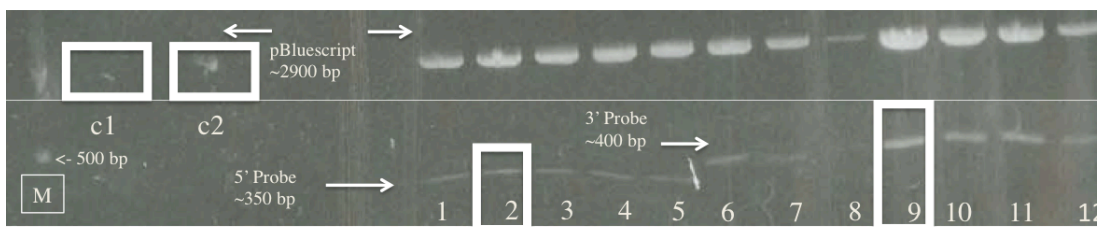


Figure 5: Condensed and magnified depiction of the gel shown in figure 1. Controls 1 and 2 each show a faint high molecular weight (MW) linearized pBluescript band. Samples 1-5 have 2 bands each – a high MW linearized pBluescript and a low MW 5' probe band. Samples 6-12 also have 2 bands as well – a high MW pBluescript band and low MW 3' band that is slightly higher in MW weight than the 5' band. Samples 2 had the strongest signal for the 5' probe and sample 9 had the strongest signal for the 3' probe.

Figure 4 shows the gel in its entirety and figure 5 is a more detailed and enlarged image of the bands in the verification gel electrophoresis. Samples control 1 (c1) and control 2 (c2) each showed one, albeit faint, band in their lanes. These bands had a relatively high molecular weight (MW) that corresponded to around 2800-3000 base pairs (bp) in length. This was most assuredly the linearized pBluescript sequence. Samples 1-12 showed two bands each. The band located towards at the top of the gel was the high MW pBluescript sequence, as it aligned at the same location as the control

pBluescript bands. The other, relatively lower MW band was clearly absent in both of the controls. These bands were our probes.

There were two distinct versions of the small band. One group, in lanes 1-5, was a slightly smaller MW sequence than the sequence in lanes 6-12. The slightly smaller MW bands corresponded with the 5' probe, and the bands in lanes 6-12 corresponded with the 3' probe. This is consistent with the fact that the 5' probe is approximately 50 nucleotides shorter than the 3' probe. Both the 3' probe and 5' probe bands fell in the range of the 500 bp band in the marker. Since the 5' probe is approximately 350 bp and the 3' probe is about 400 bp, it is likely that the identity of these sequences were our desired probes.

Theoretically, these bands could have been any 350-400 nucleotide sequence; so, further sequencing is necessary to unquestionably confirm the presence of the probes in the plasmid. For sequencing (and the eventual use of the probe), greater quantities of the recombinant plasmids were required. The strongest band from the 5' probe samples found in lane 2 and the strongest from the 3' probe samples from lane 9 will be chosen to be sequenced and used as the probes in the Southern blot if the sequencing results came back positive. The cell cultures from which these recombinant DNA samples were harvested were used in the QIAGEN Plasmid Maxi Kit in order to obtain massive quantities of purified DNA. This DNA will then be sent to a nucleotide-sequencing lab for ultimate verification of the successful amplification of both probes.

Nucleotide Sequencing

The purified and concentrated 5' and 3' probe DNA sequences were sent to a lab for nucleotide sequencing verification. The sequences of the 5' and 3' probes were confirmed:

3' Probe (5' → 3')

**CTCGAGGGGTGCACAGGGCTGGCAGGTTGGGACCCTTTCCTCCCCATGTGGA
TGCCGATGTGTTCCATGGTTTTACGAACATCTGCTCCACAGGCCTCTCACCCA
CCTGTTTGTGGCCGTGTGACAGAGCAAGGTGATGTCTCGGTACCCACCTTG
GAGCAACTGGAGAAGCTAAAGAAGATGGGGGACCTCCCCAGAGACCGTGCA
GGCAATGAAGGCATGGCTCTGCTTGCCTTCTCTACCTGTACCTGGCAATCTG
CCGGAAGCAGAGGTGTGTGCATGGTCTATAGGAGCCCGTCCCTGGGAAGCAT
TCCGGAGAATCCCTGTGCCTCCACAGAGCAGGGAGTCCTGTAGTTTACAGTG
ATCCACTTGAGGTCTGGTGCCCATTTCTCTGGTTCGAAGCTT**

5' Probe (5' → 3')

**CTCGAGTAGCAGCGCTACAACCTGTTTTGAACACTTGAACAGTGCACCTTTC
TTGTGGCTTCACACTTATGATGTATATTCAGACCCGTCACATGGCTACAGCGT
GGCCTACCTTTGTCTCCCGTCTTGGTGTAATCTTGGGGATCTTGGCTGCCGT
CGAGGACGGCTGCAGCCTGATGAGAAACAGACACAAAGGCGTGGTAAGTGG
GAACGGCTGCGCCTCGGGGAGTGACGCCAGCTTTCAGTCTGCAACCTCAGG
GGCAGCACTTCTCTGCCTCTGGGATGGCAATCTCCCGTCAGCCATTTCCAAGC
TT**

DISCUSSION

In this section, the design of the targeting vector, the breeding scheme to be conducted by the TAMC, and how the amplified 3' and 5' probes play a role in MvK cKO mice formation will all be discussed. The first step conducted in the creation of the MvK cKO mice was the creation of a BAC containing the mutated *Mevk1* targeting vector. The Cre-loxP approach was used as the targeting scheme to specifically delete *Mevk1* in the granulosa cells. In the scheme shown in figure 6, through homologous recombination in ES cells, a loxP recombination site was introduced in intron 1 of *Mevk1*, and another loxP site and an FRT-flanked PGK-Neo cassette were introduced in intron 3 of *Mevk1*, creating a “floxed” *Mevk1* gene.

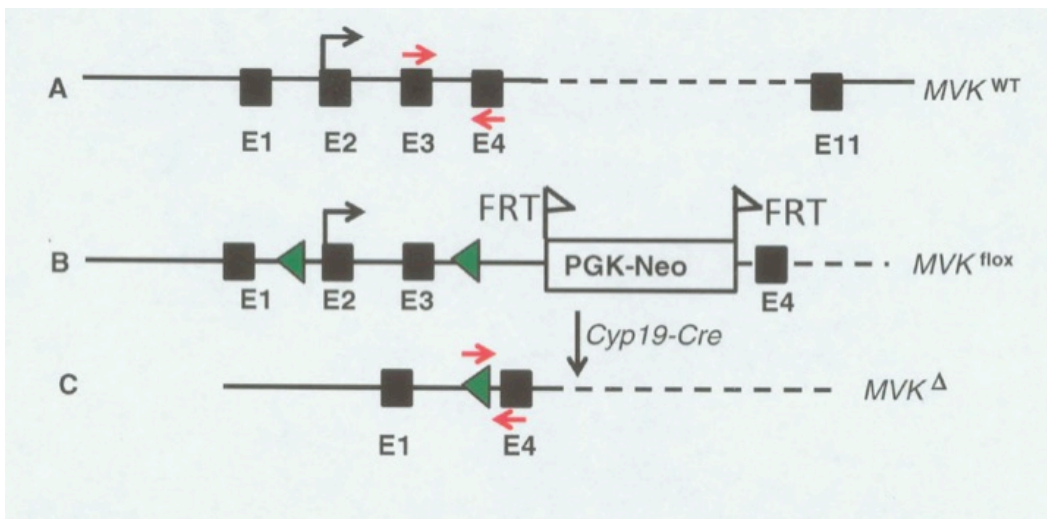


Figure 6: The experimental design of the creation of the targeting vector and the eventual knock-out of the MvK start codon in the ovaries. Construct A represents the wild-type gene which includes 11 exons with the ATG start codon located in exon 2. The ATG start codon is depicted in exon 2 by the black arrow. Construct B is the depiction of the the designed targeting vector. The loxP sites that are located in introns 1 and 3 are depicted by the green arrows, and the PGK-Neo selection cassette is shown in intron 3 flanked by the FRT restriction site. When the male *MVK*^{flox} heterozygotes are mated with female *Cyp19-Cre*⁺ mice, chimeras will be produced that selectively express Cre in the ovaries, resulting in the *MVK*^Δ mice cannot form functional MvK in the ovaries.

Lox P sites contain 34 bp – a 13 bp palindromic sequence followed by 8 random bp and another 13 bp palindromic sequence [13]. The two loxP sites introduced into

Mevk1 were oppositely oriented and are recombined by Cre recombinase, deleting the sequence between the two recombination sites when Cre recombinase is expressed. Since the ATG start codon for *Mevk1* is located in exon 2, the MvK cKO mice with deleted exon 2 are not able to form a functional MvK protein. The deletion of MvK throughout the entire body is fatal; so the expression of Cre recombinase must be limited to the mouse ovaries alone in this construct. This was achieved using the Cre genes controlled by a Cyp19 promoter. Cyp19-Cre⁺ mice show expression of Cre exclusively in the ovaries and have been successfully used in the past to create granulosa cell-specific knockout mice [14].

The PGK-Neo cassette was introduced into the targeting vector to be used in the selection of ES cells that successfully obtained the targeting vector through homologous recombination. The cassette is a drug-selection cassette that renders cells resistant to the drug neomycin. When introduced into the targeting vector, the cassette was flanked by FRT recombination sites, as shown in figure 6. FRT recombination sites are recombined by FlpO recombinase [15]. Deletion of the drug-selection cassette in the final MvK cKO mice is conducted by breeding with FlpO transgenic mice.

The use of the amplified 3' and 5' probes comes into play after the homologous recombination has occurred in the ES cells and selection on neomycin plates due to the presence of the PGK-Neo cassette has transpired. The stem cells that are neomycin-resistant will be sent back to our lab for Southern blot analysis. The 5' and 3' probes are radioactively labeled for use in the assay. The sequences complimentary to the amplified 5' and 3' probes flank the targeting vector; therefore, if the ES cell DNA binds both the

3' and the 5' probes as displayed in the Southern blot assay, then the ES cell contains the desired genetically engineered *Mevk1* targeting vector.

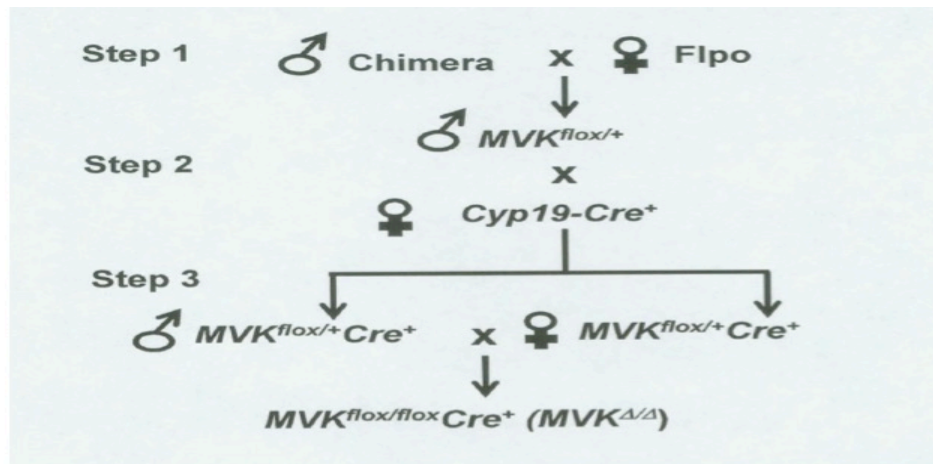


Figure 7: This is the generational breeding strategy that will be used by TAMC to engineer the proper transgenic knockout genotype once the targeting vector is successfully integrated into the male chimeras. The chimeras will be mated with Flpo females resulting in heterozygous $MVK^{lox/+}$ in which Flpo has removed the PGK-Neo cassette via the flanked FRT sites. These heterozygous males are then mated with Cyp19-Cre⁺ females whose progeny will have $MVK^{lox/+}Cre^+$ heterozygous genotypes. This generation is intercrossed in step 3 until mice homozygous for the knocked out *Mevk1* gene ($MVK^{\Delta/\Delta}$) are created.

Once the Southern blot analysis correctly identifies the ES cells containing the homologously recombined targeting vector, the results will be sent back to the TAMC, and the breeding scheme shown in figure 7 is conducted. The mutant ES cells carrying the floxed allele will be injected into blastocysts, which will be injected into pregnant female mice. Some of the progeny will be chimeric agouti mice carrying the floxed allele (F0 generation). Male chimeras will then be mated with FlpO transgenic females, excising the FRT-flanked PGK-Neo cassette in the F1 generation mice. The F1 $MVK^{lox/+}$ mice will be subsequently mated with Cyp19-Cre⁺ female mice to obtain heterozygous $MVK^{lox/+}Cre^+$ F2 generation mice. The heterozygous F2 mice will finally be intercrossed until homozygous $MVK^{lox/flox}Cre^+$ mice (MvK cKO mice) are produced that will have granulosa-specific deletion of LRBP/MvK.

The creation and amplification of the probes for use in the development of a Southern blot verification assay and the design of the targeting vector are two of the first steps required in the creation of MvK cKO mice. These are two of the primary responsibilities of our lab in the creation of the transgenic mice. Moving forward, before the TAMC will carry out the steps to create the MvK cKO mice, our lab must produce a mock Southern blot assay using our amplified probes to show the TAMC, which will prove that we can properly conduct an assay that will identify the correct ES cells. This is the final step in the path towards the creation of the MvK cKO mice as far as Menon Lab is concerned. Once the MvK cKO mice are created, we will be able to observe the hypothesized wide-ranging effects on mice that are unable to express LRBP in the ovaries. The MvK cKO mice will allow us to examine how important LRBP is in LHR mRNA down-regulation, and how the absence of LRBP affects key players in the post-LH surge LHR down-regulation.

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