Appendix A: Production of Biologically Active Porcine Prorelaxin in the Milk of Transgenic Mice

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A.1 Abstract

Relaxin, a peptide hormone and member of the insulin family, regulates vasodilation, angiogenesis, and remodeling of extracellular matrix components. The natural scarcity of relaxin and the low productivities of conventional bioreactors have limited investigations into its potential clinical uses. Transgenic mice were generated for expression of recombinant porcine relaxin in milk. Expression levels up to 1 mg relaxin per ml of milk were achieved, though the murine mammary epithelial cells do not efficiently process 20 kDa prorelaxin into its mature 6 kDa form. Prorelaxin differs from other prohormones, however, in that it is biologically active. Prorelaxin purified from the milk of transgenic mice stimulated cAMP production in monocyte THP-1 cells, though at a lower specific activity than mature porcine relaxin. Importantly, the presence of prorelaxin in the mouse milk did not have adverse effects on either the mother or pups. The ability to produce relaxin at high levels in transgenic animals could lead to greater availability of the hormone for future research and therapeutic use.

Keywords: **Relaxin, transgenic animal, bioreactor**

A.2 Introduction

Relaxin is a 6 kDa peptide hormone structurally similar to insulin and other members of the relaxin/insulin family of hormones; the prohormone, prorelaxin, consists of B-C-A chains (20 kDa), with the C chain proteolytically excised to form the mature relaxin molecule consisting of the disulfide-linked A and B chain.[1] Although relaxin shares structural homology with insulin, there is little primary amino acid sequence homology, and relaxin has a unique receptor.[2] Unlike many other prohormones, prorelaxin retains its biological activity.[3,4,5,6] The primary site of synthesis and storage of relaxin in humans is within the corpora luteal cells of ovaries in females where it is secreted into the bloodstream at different levels throughout pregnancy, and within the prostate gland and testes in males.[7,8]

Historically, relaxin has been classified as a pregnancy hormone that acts on reproductive tissues only during pregnancy, preparing the female for parturition by "relaxing" the pelvic ligaments and tendons.[9,10] The most recognized effects of relaxin on target cells are induction of matrix metalloproteinase (MMP) expression and inhibition of collagen synthesis.[11,12,13] Recent evidence suggests that relaxin may be classified as a "master hormone" that induces biochemical changes in a number of non-reproductive tissues.[14,15,16,17] In addition to up-regulating MMP expression in reproductive tissues such as the cervix and placenta.,[18,12,19] relaxin up-regulates expression of MMP-1 and MMP-3 in lung fibroblasts, skin fibroblasts, and fibrocartilaginous cells.[20,11,21] Relaxin receptors are also found in the brain, heart, skin, nipples, small intestine, mammary gland, blood vessels, and testes.[22,23,24,25,26,27,28] Besides its role in extra-cellular matrix (ECM) remodeling, human relaxin promotes angiogenesis[29] and has been associated with vasodilation in the heart and other organs.[17] Schwabe and Bullesbach[30] have speculated that *porcine* relaxin is a more potent MMP stimulator in humans than *human* relaxin. This is not a unique occurrence for peptide hormones, as salmon calcitonin is more bioactive in humans than human calcitonin.[31]

Recent studies have demonstrated the potential of relaxin as a therapeutic agent outside the realm of reproduction. The effect of relaxin on collagen turnover motivated clinical trials investigating the use of recombinant human relaxin as a treatment for

scleroderma,[32] a life-threatening illness characterized by the overproduction of collagen in the skin and other organs. In rodents, relaxin has been shown to be capable of regulating the proliferation and differentiation of cardiac fibroblasts, as well as suppressing the expression of collagen in both *in vitro* and *in vivo* systems.[33] This data suggests a therapeutic role for relaxin in the treatment of heart diseases characterized by cardiac fibrosis. From their study on the effects of relaxin on arterial resistance and compliance, Conrad *et al*.[34] proposed the use of relaxin to prevent or reverse arterial stiffness as a consequence of aging or in reducing cardiac afterload during heart failure. Dschietzig *et al*.[35] have also speculated on the applicability of relaxin as a vasodilator for cardiac arrest treatments. Due to its angiogenic properties, relaxin has been studied for use in wound-healing applications. Schwabe and Bullesbach[30] reviewed anecdotal evidence from limited trials with porcine relaxin in the 1960's that supported wound-healing claims. More recently, two groups reported on the ability of recombinant human relaxin to increase blood vessel content at wound sites in rodents well as to stimulate the expression of the angiogenic stimulators vascular endothelial cell growth factor (vEGF) and basic fibroblast growth factor in multiple cell types.[29,36]

For practical therapeutic applications, large-scale production of relaxin must be achieved. Historically, porcine relaxin has been isolated from ovaries of pregnant sows, yielding approximately 30 mg relaxin/kg of ovaries.[37] This source is unregulated, and extraction from tissue is an inefficient and expensive process that cannot be easily scaled up. To date, recombinant porcine relaxin has been produced in *E. coli,*[38] *S. cerevisiae,*[39] and Chinese hamster ovary (CHO) cells.[4] Each system presents certain advantages, yet a limitation shared by all is low expression levels; none of the systems published were capable of producing relaxin at levels above 1.2 μg/ml.

The use of transgenic animals to produce therapeutic proteins in milk is an established technique, and often yields gram/liter levels of protein due to efficient mammary-specific promoters.[40] We have investigated the potential of producing recombinant porcine relaxin in the milk of transgenic mice as the first step towards large-scale production of recombinant relaxin in transgenic livestock. In this work, we focus on addressing the following issues: 1) can porcine prorelaxin be produced in transgenic mouse milk; 2) is the expression stable; 3) is prorelaxin processed to the mature form in

the mouse mammary epithelial cells; 4) is the recombinant relaxin active; and 5) does the presence of the porcine relaxin affect the mouse physiologically on a qualitative level. From our studies, we have found that biologically active porcine prorelaxin can be stably produced in transgenic mouse milk at levels of 1 mg/ml.

A.3 Experimental

A.3.1 Materials

Purified porcine relaxin (B29) standard was obtained from Professor Chris Schwabe (Medical University of South Carolina). Rabbit anti-relaxin IgG was obtained via custom polyclonal antibody synthesis from Research Genetics (formerly of Huntsville, AL, now part of Invitrogen, Carlsbad, CA). The porcine relaxin A chain peptide was chemically synthesized at Research Genetics and conjugated to keyhole limpet haemocyanin (KLH). The KLH-peptide was mixed with an equal volume of Freund's Adjuvant and injected into rabbits. Serum was collected and the antibodies were purified via affinity chromatography by immobilizing the synthetic A chain onto the resin. The following buffers and solutions were used throughout this work: PBS-Tween: 20 mM Sodium Phosphate, 50 mM NaCl, 0.1% Tween-20, pH 7.4; PBS-Tween-BSA: PBS-Tween + 0.1% BSA; TBS-Tween: 20mM Tris, 500mM NaCl, 0.1% Tween-20, pH 7.2; TBS-Tween-Casein: TBS-Tween + 0.5% Casein.

A.3.2 Transgenic Mouse Generation

Porcine relaxin mRNA was isolated from an ovarian tissue cDNA library.[41] The cDNA, including the relaxin signal sequence, but no 3' untranslated region, was cloned into the pUCWAP plasmid. The pUCWAP plasmid includes a 4.2 kb mouse whey acid protein (WAP) promoter and 0.4 kb of 3' WAP sequences.[42] Sequencing confirmed the construct orientation. The mWAP-relaxin (Rlx) cDNA construct was excised with *Not* I and purified sequentially by agarose gel electrophoresis and $CsCl_2$ gradient centrifugation. Transgenic mice were produced by microinjecting purified, linearized

mWAP-Rlx cDNA into 1-cell embryos from B6C3F1 donors, and then transferring the embryos into pseudopregnant ICR recipient female mice using standard protocols.[43]

A.3.3 Genomic DNA Isolation

Mouse genomic DNA was isolated from 1 cm tail sections excised from anesthetized mice in accordance with standards approved by the Virginia Tech Animal Care Committee. The tail sections were digested using a lysis buffer containing 100 µg/ml Proteinase K (Sigma, St. Louis, MO), 1% SDS, 10 mM EDTA, 150 mM NaCl, 50 mM Tris, and 1M $NaClO_4$ pH 8 at 55°C. The DNA-containing solution was then aspirated from the digested tissue and added to an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol and mixed by vortexing. The mixture was then centrifuged for 3 min at room temperature at 12,000 *x* g in a microfuge. The phenol:chloroform extraction was repeated. DNA was then precipitated from the second extraction aqueous phase by bringing solution up to 250 mM NaCl and then adding 1.2 volumes of 100% isopropanol followed by centrifugation for 10 min at 12,000 *x* g in a microfuge. The resulting DNA pellet was washed twice with 70% ethanol to remove salts, and the final pellet was then dried and reconstituted in deionized water. DNA concentrations were estimated by optical density measurements at 260 nm.

A.3.4 PCR Analysis

The following primers were used for PCR analysis of mice: forward (pig rlx CS1 PO4, anneals in B chain region) 5'-AAACCATGCCATCCTCCATCACCAAAG-3'; reverse (WAP 3'A2, anneals in 3' mWAP region) 5'-AGGAGTGAAGGGTCTTGCTGTA-3'. Final concentrations per 50 µl PCR reaction were as follows: 100 µM of both forward and reverse primers, 200 µM each dNTP, 1.5 mM $MgCl_2$, 1 Unit Taq DNA polymerase (Qiagen, Valencia, CA), and 150 ng genomic DNA. The reactions were topped with mineral oil and cycled as follows in a thermocyler (Onigene, Hybaid, Ashford, Middlesex, UK): 10 minutes at 96°C followed by 35 cycles

of 30 seconds 96°C, 20 seconds 60°C, 30 seconds 72°C, and a final extension for 10 minutes at 72°C. The reaction products were then loaded onto a horizontal 0.9% agarose gel and stained with ethidium bromide (Gibco BRL, Gaithersburg, MD).

A.3.5 Mouse Milking

Female mice were milked in accordance with standards approved by the Virginia Tech Animal Care Committee. Lactating females were separated from their offspring for approximately one hour prior to milking. The mice were injected intra-peritoneal with 400 µl Avertin (2,2,2 tribromoethanol (Sigma, St. Louis, MO) in t-amyl alcohol) for sedation, as described in Hogan *et al.*[43] Oxytocin, 50 µl, (Vedco, St. Joseph, MO) was then injected intramuscularly in the hind leg to promote milk letdown. Milk was collected using a small vacuum pump-assisted milking machine.

A.3.6 Enzyme Linked Immunosorbent Assay – ELISA

Relaxin concentrations were estimated by ELISA. Samples and standards diluted in 0.1 M sodium carbonate pH 9.2 were applied in triplicate to Immulon 2 microtiter plates (Dynex Technologies, Chantilly, VA), 100 µl/well. After overnight incubation at 4°C, the wells were washed three times with PBS-Tween and then blocked for 1 hr at room temperature with 100 µl PBS-Tween-BSA. Rabbit anti-relaxin IgG diluted 1:1000 in PBS-Tween-BSA was then added to the wells and the microplate was incubated at 4°C overnight**.** The wells were again washed with 200 µl PBS-Tween, followed by incubation with 100 µl horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG, 1:2000 in PBS-Tween-BSA, (Sigma, St. Louis, MO) at 37°C for 1 hour. After washing with PBS-Tween, the plates were developed by the addition of 100 µl o-phenyl-diamine (OPD Sigma Fast, Sigma, St. Louis, MO), then the reaction was stopped with 100 µl 3N sulphuric acid. The plates were read at 492 nm using a microplate reader (Multiscan RC, Labsystems, Vantaa, Finland).

A.3.7 SDS PAGE and Western Blots

Samples were reduced and denatured in LDS sample buffer pH 8.5 (Invitrogen, Carlsbad, CA) containing 50 mM DTT and incubated for 10 minutes at 95°C. Proteins were separated on a NuPage pre-cast gradient 4-12% Bis-Tris SDS-PAGE gel using MES running buffer pH 7.3 (Invitrogen, Carlsbad, CA). Gels were silver stained according to the protocol described in Walker.[44]

For Western blots, after protein separation on the SDS PAGE gel, proteins were transferred onto PVDF membrane (Sequi-Blot, Bio-Rad, Hercules, CA) using the Invitrogen MiniCell transfer apparatus and a 25 mM Tris, 190 mM glycine, 20% methanol, pH 8.3 transfer buffer. The membrane was then blocked using TBS-Tween-Casein for 30 minutes. The blot was incubated with rabbit anti-porcine relaxin IgG for 30 minutes, washed, and then incubated with goat anti-rabbit IgG-HRP for 30 minutes (Sigma, St. Louis, MO), both antibodies diluted 1:1000 in TBS-Tween-Casein. The blot was developed by addition of metal enhanced diaminobenzidine (DAB) substrate (Pierce, Rockford, IL).

A.3.8 Purification of Recombinant Porcine Relaxin

Frozen milk samples were thawed and the casein micelles in milk were solubilized by the addition of an equal volume of 100 mM Tris 200 mM EDTA, pH 8 to the milk samples. The milk/EDTA mixture, in 1.5 ml microcentrifuge tubes, was centrifuged at 12,000 *x g* at 4°C for 10 minutes. The skim milk/EDTA layer was aspirated from the bottom of the tube using a syringe and needle. For purification, 250 µl of milk/EDTA was diluted in 3.75 ml of 50 mM ammonium acetate, pH 5 (loading buffer) prior to loading onto a SP-Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ) ion-exchange column previously equilibrated in loading buffer. Column dimensions were 1.0 cm internal diameter by 9 cm length. The samples were loaded at a flow rate of 1ml/min and then washed with at least three column volumes of loading buffer. The relaxin-containing fraction was eluted in 50 mM ammonium acetate, 1M NaCl, pH 5 buffer at 1ml/min. The relaxin-containing fractions were then dialyzed against deionized

water and lyophilized. The protein pellet was then reconstituted in 2 ml of 7M Urea, 50 mM Tris, pH 7.8. The sample was reloaded onto the SP-Sepharose column equilibrated with 50 mM Tris, 7M Urea, pH 7.8, and two isocratic step elutions were performed. Impurities were eluted in 50mM Tris, 0.06 M NaCl, 7M Urea pH, 7.8; relaxin was eluted with 50 mM Tris, 4M NaCl, pH 7.8, both at 1ml/min. The relaxin-containing fraction was then dialyzed against deionized water for activity analysis.

A.3.9 Activity Assay Using THP-1 Cells

Relaxin activity was measured utilizing cAMP stimulation in the human monocyte THP-1 cell line.[45] Briefly, THP-1 cells were grown in RPMI media to a cell concentration of 8 x 10^4 cells/ml, and then incubated with varying concentrations of porcine relaxin standard and the enriched porcine relaxin product from transgenic mice for 30 minutes at 27°C. All experiments were performed in duplicate. cAMP levels were measured using a BioTrak cAMP enzyme immunoassay kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) per the manufacturer's instructions. After incubation with relaxin, the THP-1 cells were pelleted by centrifugation, then resuspended in lysis buffer (supplied in the assay kit) and agitated for 10 minutes at room temperature to ensure efficient lysis. 100 µl each of cell lysate or cAMP standard (in duplicate) followed by 100 µl of antiserum were added to the wells of the pre-coated anti-cAMP plate. The plates were incubated for 2 hours at 4°C. Subsequently 50 µl of anti-cAMP IgG-HRP conjugate was added to each well and incubated for 1 hour at 4°C. After washing, 150 µl of the 3,3',5,5' trimethybenzidine substrate was added and the plate was developed for 1 hour at room temperature. Development was stopped by addition of 100 µl 1 M sulfuric acid and the plate was read at 450nm using a Multiscan RC microplate reader.

A.4 Results

PCR analysis of pups from the first round of microinjections resulted in identification of one male founder, designated #9. This founder male mouse was bred

with nontransgenic B6C3F1 females, and transgenic female offspring were identified using PCR analysis. Relaxin transgene transmission stability was confirmed by PCR analysis of four generations, shown in Figure A-1. The expected DNA product of 400 bp DNA is detected in the transgenic offspring.

Porcine relaxin expression was demonstrated by Western blot of milk samples from three generations of transgenic mice (Figure A-2). The major form of secreted relaxin is the 20 kDa prorelaxin, indicating significant rate limitations in proteolytic excision of the C chain. Trace amounts of fully processed 6 kDa relaxin can be seen in overloaded blots (data not shown). The nontransgenic negative control milk samples had no signals in the 20 kDa or 4-6 kDa ranges, confirming the specificity of the anti-relaxin antibody. The expression level was measured by ELISA using a 6 kDa porcine relaxin standard. The expression levels and the proteolytic processing of porcine prorelaxin in transgenic mice were consistent within a single lactation, and from lactation to lactation for each mouse. However, there were two phenotypes noted: mice with consistent expression levels of approximately 200 μg/ml, and mice with consistently higher expression levels of approximately 1 mg/ml.

Endogenous mouse milk proteins are at a concentration of approximately 80 mg/ml. The major protein contaminants are the caseins and whey acid protein. Recombinant relaxin was enriched by solubilizing the casein micelles with EDTA and then performing two cation-exchange chromatographic steps, based on a process similar to that used for purification of relaxin from ovarian tissue extracts.[37] Methods based on initial casein precipitation steps (acid precipitation and ethanol precipitation) resulted in significant losses of relaxin to the pellets. The final product from the SP-Sepharose column, 4 M NaCl fraction, was significantly enriched for recombinant porcine prorelaxin (Figure A-3).

The activity of purified prorelaxin was determined by measuring cAMP stimulation in a human monocyte THP-1 cell line. As shown in Figure A-4, recombinant porcine prorelaxin does stimulate cAMP production in THP-1 cells, although the specific activity of prorelaxin is lower than mature relaxin. For comparison, to induce an intracellular cAMP level of approximately 1600 fmol requires 75 nM recombinant porcine prorelaxin, while the same response was induced by only 25 nM mature porcine

relaxin. This was similar to observations of recombinant porcine prorelaxin produced in CHO cells[4] and *E. coli.*[38]

A.5 Discussion

Transgenic animal bioreactors have been utilized to produce a number of recombinant proteins in their milk. Three factors contribute to making the transgenic animal a productive bioreactor: a high density of mammary epithelial cells, a high protein synthesis and secretion capacity, and the utilization of efficient transgene promoters. Protein expression levels of grams/liter are now routine, and commercial entities have many proteins in the product pipeline. All transgenic bioreactor development begins with the analysis of protein production in mice. This is especially important for the production of low molecular weight peptide hormones and growth factors. Even though the alveoli in the mammary gland are separated from the rest of the animal's body by the tight junctions of the mammary epithelial cells, endogenous milk proteins do leak into the bloodstream at trace levels[46] and could potentially affect the physiology of the lactating animal. Additionally, intact milk proteins or significant fragments of the milk proteins may be absorbed into the circulations of the nursing young, and could affect their growth and development.[47] The dramatic example of the effects of recombinant human growth hormone production in transgenic animals emphasizes the seriousness of this aspect.[48,49] Due diligence must be exerted to ensure that animal health is not negatively affected by the presence of the recombinant protein.

The mouse WAP promoter used here for porcine relaxin production has successfully driven production of gram/liter levels of other recombinant proteins in the milk of mice and pigs.[42,50,51] The relaxin cDNA sequence was that of Accession A17335 (NCBI Nucleotide database), which has an amino terminal B-chain sequence of QSNERFIKAC. We note that another version of porcine relaxin having an amino terminal B-chain sequence of QSTNDFIKAC (Accession A06852) has also been reported. Both cDNA and genomic DNA transgene constructs can be used to express proteins at these high levels with the WAP promoter. Using a cDNA construct for porcine relaxin, one male founder was used to generate all the transgenic females

included in this study. The relaxin expression levels of 0.2 to 1 mg/ml were typical for the mWAP promoter. As seen with studies of recombinant protein production in transgenic pigs and sheep,[52,50] the relaxin transgene was transferred to the offspring in a manner indicating that multiple copies of the transgene were inserted into two or more loci in the founder. Of the PCR-positive mice, one subset had consistent expression levels of approximately 200 µg/ml, and another subset had consistent expression levels of approximately 1 mg/ml. This behavior is typical of outbreeding an animal with multiple loci, where each locus is responsible for a portion of the total protein expression.

A major objective was to determine if murine mammary epithelial cells could perform the post-translational excision of the C chain. Western blots of milk samples showed that only trace amounts of the prorelaxin were being proteolytically processed to the 6 kDa mature relaxin. Porcine prorelaxin, unlike human prorelaxin, does not have paired basic amino acid residues at the termini of the C chain (Figure A-5). Thus porcine prorelaxin is not likely to be proteolytically processed to its mature form by the common proprotein convertases (PCs) such as furin, PC1, PC3, or Kex2.[53] The enzyme responsible for excising the C-chain from prorelaxin in the porcine ovary has yet to be identified, but the enzyme SKI-1 cleaves at RGLT↓SL or RSVL↓SF sequences, and has been postulated to be a candidate.[54] Whatever the protease involved, these data indicate that the murine mammary epithelial cells are similar to CHO cells in the very low capacity for proteolytic post-translational processing of porcine prorelaxin.

Unlike other polypeptide prohormones, however, prorelaxin is biologically active. The activity of the recombinant prorelaxin was measured by cAMP stimulation in a human monocyte cell line. Similar to the observations of prorelaxin produced in *E. coli* and CHO cells, the data showed that prorelaxin produced in transgenic mouse milk has a lower specific activity than mature relaxin. The reasons for the difference between prorelaxin and relaxin activity in this cell line are not known, and beyond the scope of this work. Additional engineering of transgenic animals to improve post-translational proteolytic processing has been successful with other recombinant proteins.[55] Discovery of the protease responsible for prorelaxin processing could thus lead to transgenic animal bioreactors that are further engineered for enhanced proteolytic processing and provide new productive sources for both recombinant relaxin and prorelaxin.

Importantly, neither the presence of the porcine relaxin gene nor the prorelaxin itself had any noticeable effect on the overall health of the mice, even though porcine relaxin is active in mouse-based assays such as the pubic symphisis assay.[30] Studies on the effect of porcine relaxin infusion in rats indicated that the presence of relaxin in plasma prolonged both the gestation period and length of labor, but had no other apparent effect on the health of either the mother or pups.[56] Transgenic females producing recombinant porcine pro-relaxin showed no outwardly visible health problems with respect to reproductive behavior, litter sizes, and lifespan. Lactation performance was also normal, as pups feeding on the milk had the same rate of weight gain as pups nursing from non-transgenic females in the same colony.

The implication of these results for scale-up to transgenic livestock animals is promising. The data in mice indicate that production of recombinant relaxin should be feasible in transgenic livestock animals, and the animals' health will not likely be adversely affected. Transgenic pigs may be a natural first choice for large-scale expression of porcine relaxin; pigs have advantages of shorter generation times, of producing more offspring/year than other more traditional livestock dairy animals, and producing milk at the rate of up to 0.5-1 liter/milking. Likewise, the mouse WAP promoter works just as well, if not better, at driving high expression levels in pigs.[50,51] Thus the potential productivity of a transgenic pig bioreactor would be at least three orders of magnitude higher than *E. coli* or CHO cell bioreactors. One key question will be whether the putative porcine ovarian protease that excises the C chain of prorelaxin is also expressed in the porcine mammary epithelial cells during lactation. Secondly, it must be determined what the rate limitations of this proteolytic processing would be in the mammary gland. If the protease is not expressed in the mammary gland, the termini of the C-chain could be re-engineered to dibasic peptide sequences, and furin could be co-expressed to achieve successful secretion of the mature 6 kDa porcine relaxin.[55]

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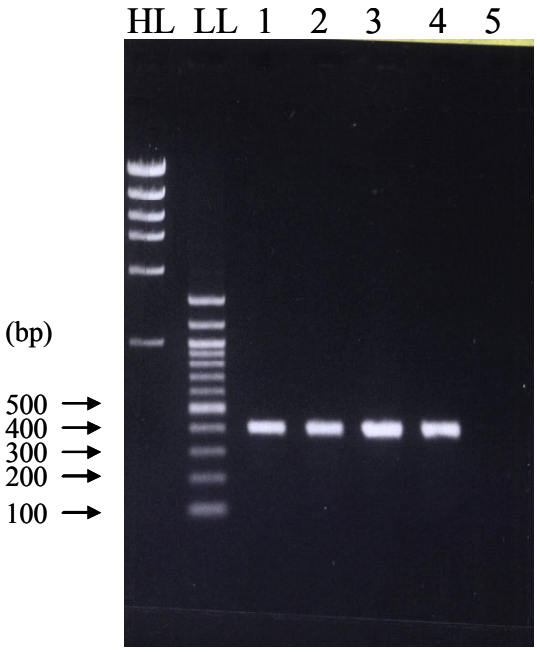

Figure A-1. Transgene Stability. PCR of mice genomic DNA across several generations. Mouse #9 - F0 (lane 1), #9.41 - F1 (lane 2), #9.41.6 - F2 (lane 3), #9.41.6.2 - F3 (lane 4), and negative control B6C3F1 mouse genomic DNA (lane 5). Lanes HL and LL represent high and low mass DNA ladders.

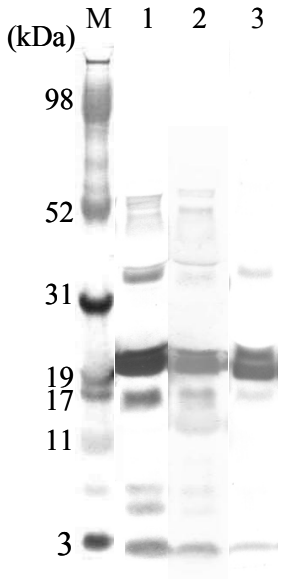

Figure A-2. Milk Western blots. Reduced gel loaded with whole milk from mouse # 9.41 - F1 (lane 1), # 9.41.6 - F2 (lane 2), and #9.41.6.2 - F3 (lane 3). M represents prestained molecular weight markers

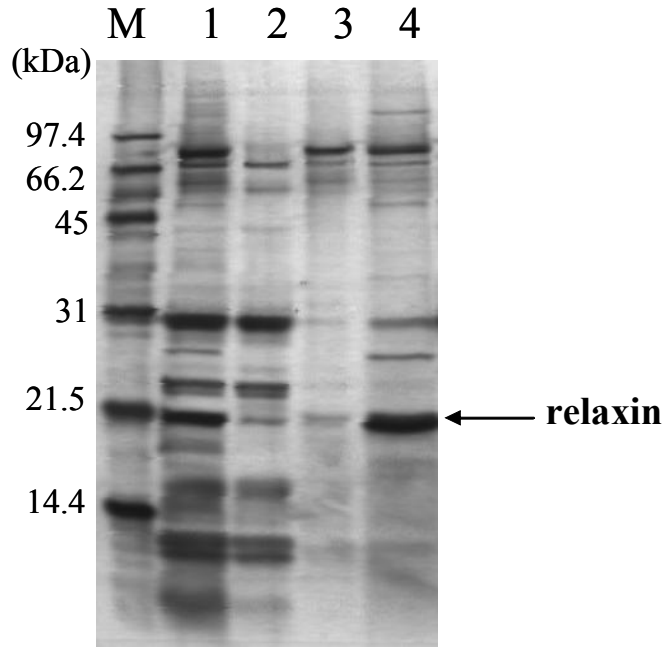

Figure A-3. Reduced SDS PAGE of relaxin purification. Second SP column fractions: feed (lane 1), flow-through (lane 2), 0.06 M NaCl elution (lane 3), and 4 M NaCl elution (lane4). M represents molecular weight standards.

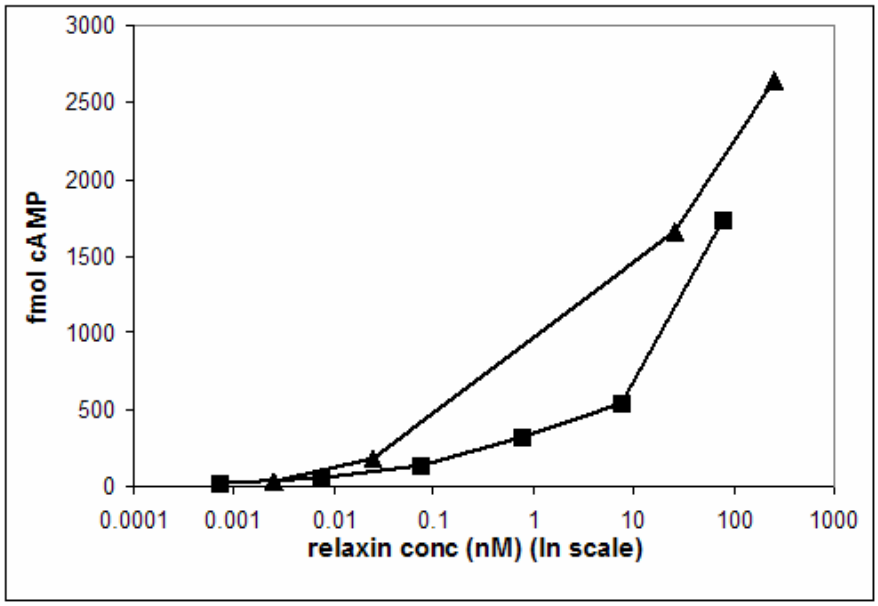

Figure A-4. Intracellular cAMP levels of THP-1 cells versus concentration of porcine relaxin exposure. Porcine relaxin (triangles) and recombinant porcine prorelaxin purified from transgenic mouse milk (squares) were incubated with THP-1 cells and the total intracelluar cAMP levels were measured by cAMP ELISA. Each point represents the mean values of duplicate determinations.

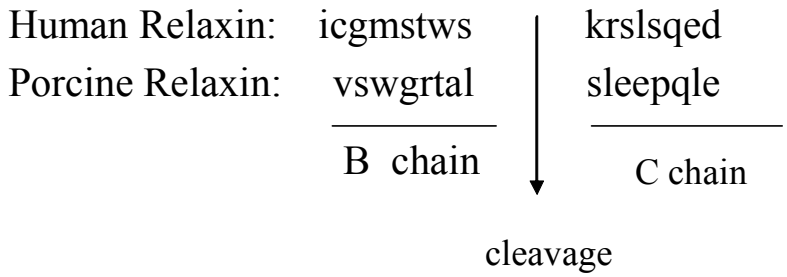

Figure A-5. C chain termini of human and porcine relaxin. Last 8 and first 8 amino acids (N terminus to C terminus) of the B and C chains of human and porcine prorelaxin. Human prorelaxin contains basic lysine and arginine at the N terminus of the C chain, whereas porcine prorelaxin does not.

Vita

Myles Lindsay was born in 1976 in Huntsville, Alabama. He was raised in Union Grove, Alabama until 1994 when he attended Auburn University. In the spring of 1999, he received his B.S. degree from Auburn University in chemical engineering. In the fall of that year, he began attending Virginia Polytechnic Institute and State University in Blacksburg to pursue his doctorate in chemical engineering under Dr. Kevin VanCott. In December of 2004, he successfully defended his dissertation and was awarded a Ph.D in chemical engineering.