CHARACTERIZATION OF GLYCOSYLATION OF PROLACTIN IN GALLIFORMES

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ABSTRACT

Prolactin (PRL) is a highly versatile hormone in terms of its biological functions. In Galliformes, high levels of PRL are associated with incubation behaviour and hatching. It has been shown that these high levels of PRL were associated with an increased ratio of glycosylated PRL (G-PRL) versus non-glycosylated PRL (NG-PRL) in turkey (Bédécarrats *et al.,* 1999a). This suggests that glycosylation of PRL is related to its proper function during those stages of life. However, the mechanism(s) controlling post-translational modification of PRL are unknown. In order to investigate genes associated with the glycosylation of PRL, an *in vitro* study was undertaken. The pituitary glands of day 24 turkey embryos (n=60) were collected and pooled into two groups which were incubated in medium 199 for 4 hours in the absence or presence of 10^{-7} M vasoactive intestinal peptide (VIP). Western blot analysis of PRL was used to assess the response of the pituitary glands to the VIP stimulation. As expected, the absolute level of PRL as well as the percentage of glycosylated PRL isoform increased following stimulation with VIP. The mRNA of both stimulated and non-stimulated samples were extracted and reverse transcribed into cDNA. Using suppression subtractive hybridization (SSH), a cDNA subtractive library which only contained differentially expressed cDNAs between VIP stimulated and non-VIP stimulated turkey pituitary glands was constructed. Seventeen percent of the genes from the library are related to cell proliferation and apoptosis. Ten genes were selected for real-time PCR analysis. The functions of these genes and their potential roles in response to the stimulation by VIP and glycosylation of PRL are discussed.

RÉSUMÉ

La prolactine (PRL) est une hormone très universelle au point de vue de ses fonctions biologiques. Dans les galliformes, les niveaux élevés de PRL sont associés au comportement d'incubation et à l'éclosion. Il a été reporté que ces niveaux élevés de PRL ont été associés à un plus grand rapport de PRL glycosylée (G-PRL) par comparaison à la PRL non-glycosylée (NG-PRL) chez la dinde (Bédécarrats *et al.,* 1999). Cela suggère que la glycosylation de la PRL soit requise pour qu'elle fonctionne correctement pendant ces étapes de la vie. Cependant, les mécanismes contrôlant la modification post-traductionelle de PRL sont inconnus. Afin d'étudier des gènes associés à la glycosylation de PRL, une étude *in vitro* a été conduite. Les glandes pituitaires des embryons de dinde du jour 24 (n=60) ont été rassemblées et mises en commun dans deux groupes qui ont été incubés dans le Milieu 199 pendant 4 heures en l'absence ou en la présence du peptide intestinal vasoactif de 10^{-7} M (VIP). L'analyse western blot de PRL a été employée pour évaluer la réaction des glandes pituitaires à la stimulation par le VIP. Comme prévu, le niveau absolu de PRL ainsi que le pourcentage de l'isoforme glycosylée de PRL ont augmenté suivant la stimulation par le VIP. Les ARNm des échantillons stimulés et des échantillons non-stimulés ont été extraits et réverse transcrits en ADNc. En utilisant l'hybridation Soustractive Suppressive (SSH), une banque soustractive d'ADNc a été construite comprenant seulement les ADNc qui étaient exprimés différemment dans les glandes pituitaires de dinde stimulées par le VIP. Dix-sept pour cent des gènes de la banque sont liés à la prolifération et à l'apoptose cellulaires. Dix gènes ont été choisis pour l'analyse PCR en temps réel. Les fonctions de ces gènes et leurs rôles potentiels en réponse à la stimulation par le VIP ainsi que la glycosylation de la PRL sont discutés.

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CHAPTER I. INTRODUCTION

Prolactin (PRL) is a highly versatile hormone that is responsible for more than 300 biological functions in vertebrates (Sinha, 1995). One of the causes for the diversity of biological functions of PRL may be due to the different isoforms of PRL, which either arise from post-translational modification or genetically determined factors. Different isoforms of PRL have different biological half-lives, receptor binding capacities and biological activities.

The earliest described functions of PRL were related to milk secretion in mammalian species, and crop sac development in Columbiformes (Riddle *et al.*, 1933). After years of studies, the most prominent function of PRL is its role in reproduction in mammal and avian species.

In many avian species, levels of PRL vary throughout the reproductive cycle. In turkey hens, many studies have shown that the levels of PRL, both in plasma and pituitary glands, rise at the onset of incubation behaviour. These high levels of PRL are required to maintain incubation behaviour. Studies have indicated that the expression of incubation behaviour was accompanied by other behaviours such as increased nesting frequency, care of young, anorexia, adipsia, substantial shifts in metabolism and termination of egg production (Porter *et al.,* 1991a,b; Sharp *et al.*, 1979; Zadworny *et al.,* 1985). Moreover, the hens undergo some physical changes such as the development of brood patches (Book *et al.,* 1991).

In the order Galliformes, only females exhibit parental behaviour. Although the cause and effect relation between incubation behaviour and PRL is still debatable, several studies have indicated that inhibition of PRL would alter incubation behaviour. For example, Crisostomo *et al.* (1997) successfully reduced the frequency of incubation behaviour by passively immunizing turkey hens with anti-tPRL serum. Furthermore, incubation behaviour was prevented by active immunization with a GST-tPRL fusion protein (Crisostomo *et al.,* 1998). Moreover, active immunization against VIP, a PRL releasing factor, prevented the increase in plasma prolactin, which in turn prevented

incubation behaviour (El Halawani *et al.,* 1995a). Thus, hyperprolactinemia appears to be a prerequisite for expression of incubation behaviour.

Although not many studies have been done on the function of prolactin during embryogenesis, fluctuations in the levels of PRL in chicken (Harvey *et al.,* 1979), and in turkey embryo (Bédécarrats *et al.,* 1999a) around the time of hatching suggests it plays a role in embryogenesis. The increased levels of PRL around the time of hatch may be associated with osmoregulation (Murphy *et al.,* 1986). Moreover, it has been shown that PRL played a major role in the maturation of embryonic thyroid metabolism by converting the T4-5-mono-deiodination to a T4-5'-mono-deiodination (Kuhn *et al.,* 1983).

In 1991, Corcoran and Proudman reported that the ratio of non-glycosylated PRL (NG-PRL) and glycosylated PRL (G-PRL) was approximately the same in adult turkey hens. This was further elaborated by Bédécarrats *et al.,* (1999b). They showed that the transition from egg laying to egg incubating behaviours was associated with a significant increase in the proportion of PRL which was glycosylated. Similarly, during embryogenesis, the transition from chorio-allantoic to pulmonary gas exchange around the time of hatching was associated with a 2-fold increase in G-PRL (Bédécarrats *et al.,* 1999a).

The association between higher levels of glycosylation around the time of hatch and during the reproductive cycle suggests that G-PRL may have specific physiological effects different from those of NG-PRL. Since G-PRL is likely to have a different affinity than NG-PRL for the PRL receptors, changes in the ratio of isoforms may act to modulate biological effects in different target tissues.

To date, the glycosylation process of PRL is poorly understood. In order to investigate the mechanism of glycosylation of PRL, this study is conducted to explore the genes that are potentially involved in glycosylation.

CHAPTER II. LITERATURE REVIEW

2.1 Prolactin

2.1.1 General information

Prolactin (PRL) was first identified about 70 years ago. In 1928, Stricker and Greuter reported that an extract from bovine pituitary gland promoted lactation in ovariectomized rabbits. Subsequently, this factor in the bovine extract was named PRL by Riddle *et al.* (1933) due to its ability to stimulate the synthesis of crop milk in pigeons and lactation in mammals.

Prolactin is a single-chain polypeptide hormone belonging to the GH-PRL family. The members in this family comprise growth hormone (GH), PRL, somatolactin and placental lactogen. All the hormones in this family exert their biological functions by interacting with and dimerizing specific single transmembrane-domain receptors (Forsyth and Wallis, 2002). Their molecular weight ranges from 20 to 26 kDa and their sequence contains about 200 amino acid residues. They are also very similar in terms of their structure; they all have five exons and four introns (Cooke *et al.*, 1981). The relatively high similarity amongst their amino acid sequences suggested that they originated from the same ancestral gene (Cooke and Baxter, 1982) by gene duplication about 400 million years ago.

Prolactin has more biological functions than the other hormones in the same family and more than 300 different biological functions have been reported (Bole-Feysot *et al.*, 1998). These functions have been grouped into six categories: 1) water and electrolyte balance; 2) growth and development; 3) endocrinology and metabolism; 4) brain and behaviour; 5) reproduction; and 6) immunoregulation and protection. However, most of the actions of PRL are not distinct and involve modulation of the effects of other hormones on biological processes.

2.1.2. Transcription of PRL

The transcription of PRL is regulated by many transcription factors (TFs) which affect the assembly and binding of the RNA polymerase complex to the TATA box. These TFs bind to specific response elements within the promoter to enhance transcription.

One of the most studied TFs associated with lactotrophes is Pit-1 that belongs to a gene family containing the POU domain. The POU domain contains helix-turn-helix motifs that directly associate with binding sites (consensus sequence ATGNATA$^{A}/_{T}$$^{A}/_{T}$AT) in the promoter of somatotroph, lactotroph, and thyrotroph (Augustijn *et al.*, 2002) to regulate the expression of GH, PRL and TSH (Ingraham *et al.*, 1988; Nelson *et al.*, 1988; Mangalam *et al.*, 1989; Fox *et al.*, 1990; Haugen *et al.*, 1994). It has been reported that Pit-1 was also present in human corticotrophs suggesting that the gene transcription induced by ACTH was also regulated by Pit-1 (Yamada *et al.*, 1996). Chowdhury and Yoshimura (2003) reported the expression of Pit-1 protein in six pituitary cell types *in vitro*, and suggested that Pit-1 may also regulate the gene expression of ACTH, LH and FSH in chicken. Pit-1, as other transcription factors in the same family, contains a POU homeodomain which is linked to another POU-specific domain binds to the motifs as a dimer (Andersen and Rosenfeld, 1994).

In mammals, the Pit-1 gene encodes a 291 amino acid protein of 33-kDa (Weatherly *et al.*, 2001). The transcription factor binds to the motifs 5'-$^{T}/_{A}$ $^{T}/_{A}$ TATNCAT-3' (Nelson *et al.*, 1988) in the promoter of the PRL gene. The binding of Pit-1 to its promoter causes helix-turn-helix configurational changes that facilitate the assembly of other factors or cofactors and RNA polymerase II, which in turn initiates the transcription of PRL (Jacobson *et al.*, 1997; Cohen *et al., 1996*). Numerous studies also indicated that Pit-1 was required for cell differentiation in pituitary glands (Mangalam *et al.*, 1989, Karin *et al.*, 1990) and cellular proliferation (Castrillo *et al.*, 1991).

The Pit-1 gene of chicken and turkey has been cloned (Kurima *et al.*, 1998, Wong *et al.*, 1992, Tanaka *et al.*, 1999) and a consensus octamer sequence (5'-($^{T}/_{A}$)NCTCAT-3') has been proposed (Ohkubo *et al.*, 1996) for the binding of teleost and avian Pit-1

transcription factors. Comparison of mammalian Pit-1 gene with avian and telestean Pit-1 gene indicated that an additional exon was located between exons 2 and 3 in both avian and telestean Pit-1 gene (Weatherly *et al.*, 2001). Moreover, this additional exon encodes an amino acid sequence of 38 and 33 residues in turkeys and in fish respectively (Weatherly *et al.*, 2001; Kurima *et al.*, 1998; Ono and Takayama, 1992). Although the function of these additional residues is not clear, it has been demonstrated that the presence of the additional 38 amino acids does not significantly affect Pit-1 activity *in vitro* (Weatherly *et al.*, 2001). Six Pit-1 binding sites were reported in the PRL promoter of the chicken: three are located at the proximal region (-128 to -67) and three at the distal region (-1313 to -1128). However, the significance of these binding sites is not known. In turkeys, the role of Pit-1 in stimulating transcription of the PRL gene *in vivo* is not known since Pit-1 is not detectable in lactotrophes (Weatherly *et al.*, 2001). However, a similar distribution of putative Pit-1 binding sites is observed in the promoter of the turkey PRL as in the chicken (Sotocinal, 2000).

Recently, a 35-bp activator element located within nt −74/−40 in the tPRL promoter was identified and named the VIP response element. The motif is a cis-acting element for PRL gene expression stimulated by VIP (Kang *et al.*, 2004). In addition, the VIP response element is essential for VIP-stimulated promoter transactivation and the assembly of nuclear proteins. Moreover, a short core sequence of the VIP response element is found to be highly conserved between mammalian and avian species. It has been shown that the tPRL gene failed to respond to VIP stimulation when the short core sequence was deleted (Kang *et al.*, 2004).

Genes from the activator protein 1 (AP1) family have been shown to modulate the transcription of PRL. The AP1 family is comprised of the Fos and Jun family. Genes from these 2 families form homo-dimer (Jun-Jun or Fos-Fos) or hetero-dimer complexes via leucine zippers and bind to the DNA motif 5'-TGCATCA-3' to regulate gene transcription (Yoneda *et al.*, 2001). Moreover, Jun and Fos can also modulate the transcription of PRL by forming dimers with TFs from other families such as CREB (Curran and Franza, 1988).

Cyclic adenosine 3',5'-monophosphate (cAMP) dependent PRL gene expression is believed to be mediated by cAMP response element binding protein (CREB) through binding to the specific motif CRE (TGACGTCA) (Bokar *et al.*, 1988; Deutsch *et al.*, 1988). The latter is supported by the presence of CRE-like element that responds to the cAMP stimulation in the proximal promoter region of the rat PRL gene (Yan *et al.*, 1994; Keech *et al.*, 1992; Iverson *et al.*, 1990; Liang *et al.*, 1992). However, sequence analysis of the cPRL promoter by Ohkubo *et al.*, (2000) revealed that the cPRL upstream region did not contain the CRE DNA motif. However, a possible alternative sequence TGACGTGC, similar to CRE DNA motif has been reported 2 bp downstream of the TATA box. In addition, Ohkubo *et al.*, (2000) also suggested that Pit-1 may involve the regulation of PRL gene expression by associating with cAMP-induced factor such as CREB binding protein (CBP) in the chicken.

After transcription of PRL is activated, pre-mRNA is synthesized and this pre-mRNA will lead to different isoforms of PRL by genetically determined factors or post-translational modification.

2.2 PRL isoforms

One of the possible reasons to explain the range of the biological effects of PRL in different target tissues is the different molecular variants of the PRL molecule. All these variants arise either from genetically determined factors or post-translational modifications (Sinha, 1995). Prolactin is transcribed from a single copy gene (Miller and Eberhardt, 1983) and it is synthesized as a prohormone that contains 229 amino acids. After cleaving the leader peptide, mature PRL consists of 199 amino acids and is the main biologically active form (Miller and Eberhardt, 1983).

To date, nine different variants of PRL have been described. These are the result of genetic variants, alternative splicing, proteolytic cleavage, deamidation, dimerization and polymerization, sulfation, phosphorylation and glycosylation (Freeman *et al.*, 2000). All of the post-translational modifications of PRL are either carried out in the pituitary gland or the blood. The presence and the levels of the isoforms are species specific and dependent on the pathological and physiological stage of the animal. Different isoforms

may have different molecular weights which can be observed by western blot assay. For example, post-translational modifications result in different size variants such as G-tPRL (27 kDa) verses NG-tPRL (24 kDa) (Bédécarrats *et al.*, 1999a,b). However, some other variants may have a very similar molecular weight which can only be detected by other assay systems. For example, it has been reported that chicken PRL, tPRL and turkey G-PRL could be phosphorylated by protein kinase A *in vitro* (Aramburo *et al.,* 1992).

2.2.1 Phosphorylated isoforms of PRL

In Galliformes, phosphorylated PRL has been identified (Aramburo *et al.*, 1992). However, little is known about the effects of phosphorylation on PRL in avian species.

Phosphorylation is one of the most studied post-translational modifications of proteins. PRL can be phosphorylated by one or two phosphates which link to serine or threonine residues (Greenan *et al.*, 1989). Phosphorylated PRL has been reported in many species. For example, in the bovine, PRL is phosphorylated at serine-26, -34, and -90 (Kim and Brooks, 1993). Serine-90 was the major phosphorylation site. Since serine-90 is conserved in PRL, GH and PL, they suggested that it was the phosphorylation site for all three hormones.

It has been suggested that phosphorylated PRL has reduced biological activity. However, it may act as an autocrine regulator of PRL secretion in rat pituitary since phosphorylated PRL suppresses the release of non-phosphorylated PRL. In addition, relative levels of phosphorylated and non-phosphorylated PRL vary during the estrous cycle which suggests that the isoform may affect the biological activity. Recently, it was shown that phosphorylated PRL signalled primarily through the MAP kinase pathway, whereas non-phosphorylated PRL signalled primarily through Jak 2-Stat 5 pathways (Wu *et al.*, 2003). They suggested that changes in levels of phosphorylated PRL may result in different patterns of gene induction in target cells.

2.2.2. Glycosylated isoforms of PRL

Glycosylation is a common post-translational modification of PRL and G-PRL has been reported in many different species. The proportion of G-PRL to NG-PRL varies tremendously in different species according to their physiological and pathological state. Among species studied, the degree of glycosylation in bovine pituitary gland was the smallest, which account for only 1 to 2 % of the total PRL (Lewis *et al.*, 1984), whereas, the degree of glycosylation was the highest in the pituitary of turkey during incubation, representing 68% of the total PRL (Bédécarrats *et al.,* 1999b). The levels of G-PRL in the turkey pituitary vary according to the stage of the reproductive cycle. The levels of G-PRL are lowest during the out-of-lay and moulting stages (38 and 33% respectively), and highest during incubation (68%). In porcine, pituitary G-PRL was reported to be the dominant isoform during embryogenesis (Sinha *et al.,* 1990) but its levels decreased during the first year of life and NG-PRL became the dominant form after sexual maturation and in adulthood. Similarly, in turkeys, G-PRL has been shown to be the major form of PRL in the late stages of embryogenesis and NG-PRL is dominant throughout subsequent development. However, G-PRL is again the main isoform during the transition to and the maintenance of incubation behaviour in adults.

The type of linkage between the oligosaccharide and PRL differs among species. For example, O-link glycosylation has only been reported in rats (Bollengier *et al.*, 2001) and in turkey (Corcoran and Proudman, 1991). In the latter study, the conclusion was based solely on carbohydrate composition; whereas, a study using specific deglycosylase suggested that glycosylation in turkey was exclusively N-linked (Bédécarrats *et al.*, 1999b). The N-link glycosylation of PRL starts in the rough ER, where an oligosaccharide transferase adds an oligosaccharide sequence to the Asn residue on consensus sequence (Asn-X-Ser/Thr) of the PRL. This consensus sequence is found at position 31 in most species (Sinha, 1995). Subsequently, the glycosylated protein will be transported to the Golgi apparatus for further processing. In the Golgi apparatus, the N-linked oligosaccharide is modified and/or O-linked oligosaccharide is added.

Although glycosylation is not always associated with the presence of a consensus sequence (Sinha, 1995), the observation that bovine has an Asp instead of an Asn in the consensus sequence may explain its low levels of G-PRL in pituitary (Lewis *et al.*, 1984, Strickland and Pierce, 1985). In ovine, an alternative site (Asn-X-Cys) for N-linked glycosylation was proposed by Strickland and Pierce (1985). To date, in avian species, it has been shown that no consensus sequence for N-link glycosylation is found at position 31. However, analysis of chickens, ducks and turkeys has revealed substantial levels of glycosylation. As a result, an alternative sequence (Asn-X-Cys) has been proposed (Karatzas *et al.,* 1990; Corcoran and Proudman, 1991). In the turkey, these sites are located at position 56 and 197. Since the amino acids at those positions are involved in disulphide bond formation, glycosylation at these sites may alter the secondary structure, receptor binding kinetics and biological activity by affecting the tertiary structure.

The composition of oligosaccharide varies among species and within species. It has been shown that the carbohydrate unit of porcine containing glucosamine, galactosamine, mannose, fucose, and galactose was linked to Asn at position 31 (Pankov and Butnev, 1986). In primates, the carbohydrate composition of G-PRL mainly consists of fucose and sialic acid (Cole *et al.*, 1991). In turkey, two different oligosaccharides were identified (Corcoran and Proudman, 1991). One of the oligosaccharides was composed of mannose, galatose, fucose, glucosamine, galactosamine and sialic acid, while the other contained exclusively galactose, glucosamine and sialic acid.

Different compositions of oligosaccharide affect the biological activity of PRL differently. For instance, sialic acid may extend the biological half life of PRL. However, the carbohydrate chain could also interfere with the binding of PRL to its receptor. Glycosylation may act in a different way depending on the target system. In most mammals, N-linked glycosylation occurs within helix I (position 31) and in general, receptor binding and activation are greatly reduced by glycosylation (Lewis *et al.*, 1984, Atkinson *et al.*, 1988, Kacsoh *et al.*, 1991). This may not be surprising since 3 of the 14 amino acids required for receptor binding are located in the proximity of this site and hence glycosylation may cause steric hindrance. In support of the latter, both human and ovine G-PRL have about 50% binding affinity of NG PRL in the mammary gland

receptor assay (Pellegrini *et al.*, 1988; Lewis *et al.*, 1989). G-tPRL has also been observed to bind to chicken kidney membranes with lower affinity than the NG-PRL (Corcoran and Proudman, 1991). Similarly, recombinant tPRL (NG) has greater bioactivity than G-PRL in the Nb2 assay (Karatzas *et al.*, 1993). Conversely, the bioactivity of the porcine G-PRL has been shown to be 40% more bioactive than NG-PRL in the mammary gland explant system (Pankov and Butnev, 1986). The effects of different PRL isoforms on the target tissue could be due to the binding capacity of different PRL isoforms to its specific receptor.

2.3. Sites of action of PRL and its receptor

Prolactin is a ligand that requires binding to its specific receptor in order to exert its biological function. The receptor has been identified in many different tissues supporting the versatility of PRL in the body (Ouhtit *et al.,* 1994; Bole-Feysot *et al.,* 1998).

The prolactin receptor (PRLR) belongs to the class 1 cytokine receptor family (Bazan, 1990). It is a specific, high affinity, transmembrane protein (Kelly *et al.*, 1974; Posner, 1975). The PRLR contains an extracellular domain and an intracellular domain (Boutin *et al.*, 1988; Kelly *et al.*, 1991; Murakami *et al.*, 1988). In mammals, several PRLR isoforms resulting from alternative splicing have been identified (Ali *et al.*, 1991; Boutin *et al.*, 1988; Boutin *et al.*, 1989; Davis and Linzer, 1989). Several studies suggested that the PRLR isoforms could also arise from proteolytic cleavage (Amit *et al.*, 1997; Fuh and Wells, 1995). The intracellular domain of the PRLR isoforms differs in length in many mammals and is referred to as long, intermediate and short (Goffin *et al.*, 1998; Bole-Feysot *et al.,* 1998). Before 1999, only one form of PRLR was identified in birds (Tanaka *et al.*, 1992; Chen and Horseman, 1994; Zhou *et al.*, 1996). In 1999, Mao *et al.* identified at least 5 types of testis-specific unique truncated PRLR transcripts in sexually immature male chicken. Two of these unique truncated PRLR transcripts lack the extracellular and transmembrane domains.

The extracellular domain of mammalian PRLR contains four paired disulfide-linked cysteines in the N-terminal subdomain D1 and a WSXWS motif in the membrane

proximal region (Boutin *et al.*, 1988; Kelly *et al.*, 1991). These two features are a characteristic feature of the extracellular domain of cytokine receptors. In birds, the extracellular domain of PRLR has a double antenna structure that contains two copies of the WSXWS region (Tanaka *et al.*, 1992; Chen and Horseman, 1994; Zhou *et al.*, 1996). However, the significance of this double antenna structure is still not clear. Studies in pigeons have indicated that the deletion of one WSXWS copy did not affect the ligand specificity (Chen and Horseman, 1994) or the signal transduction mechanism (Gao *et al.*, 1996).

Similar to other members in the cytokine receptor family, the intracellular domain of PRLR contains two consensus sequences named box 1 and box 2 (Kelly *et al.*, 1991; Murakami *et al.*, 1988). Box 1 is a proline rich region that is present in all forms of PRLRs whereas, box 2 is a hydrophobic region that is absent in the short isoform of PRLR (Kelly *et al.*, 1991). It has been reported that box 1 was required for signal transduction (Goffin and Kelly, 1996; Ihle and Kerr, 1995) while box 2 was essential for internalization of medium and long PRLR isoforms (Vincent *et al.*, 1997). However, despite of the absence of the hydrophobic region, the short isoform of PRLR is observed to internalize to a greater extent compared to the long isoform of PRLR. The reason is still unknown but the short isoform of PRLR possesses a proposed internalization motif (the tetrapeptide $L^{276}PGG^{279}$) which is not conserved in the long PRLR isoform (Vincent *et al.*, 1997).

Prolactin exerts its biological functions on the target tissue through interaction with PRLR to induce the signal transduction pathway. The main signal transduction pathway associated with the binding of PRL and PRLR is the JAK/STAT pathway (Ihle and Kerr, 1995). JAK (Janus Kinase) is a family of protein tyrosine kinases which includes JAK1, JAK2, JAK3, and Tyk2 (Ihle, 1994; Bole-Feysot *et al.*, 1998). Stat (Signal Transduction and Activator of Transcription) is a family of latent cytoplasmic proteins consisting of Stat1 (α and ß), Stat2, Stat3, Stat4, Stat5a, Stat5b, Stat6 (or IL-4 Stat), and dStat, (Bole-Feysot *et al.*, 1998). The signal transduction pathway induced by the binding of PRL and PRLR mainly involves JAK2 and Stat5. In mammals, only JAK2 has been identified; however, Gao *et al.*, (1996) suggested that JAK1 and JAK3 might

also play a role in the signal transduction pathway of PRL in avian species. Other than Stat5, Stat1 and Stat3 also have been identified as transducer molecules for PRLR (Bole-Feysot *et al.*, 1998). The activated JAKs associated with the intracellular domain of PRLR at box 1 (Clevenger *et al.*, 1998; Boyle-Feysot *et al.*, 1998; Hennighausen *et al.*, 1997) then recruit the Stat proteins into the PRL:PRLR:JAKs complex by interacting with the Stat protein SH2 domain. The activated JAKs in turn phosphorylate the Stat proteins. Subsequently, the activated Stats dimerize and migrate to the nucleus to initiate gene transcription by binding to specific motifs on genes. (Hennighausen *et al.*, 1997; Clevenger *et al.*, 1998; Boyle-Feysot *et al.*, 1998)

Numerous studies reported that the JAK/STAT transduction pathway was presumed to be the main pathway used by PRL and PRLR; nevertheless, other signal transduction pathways through MAP kinase involving the Shc/SOS/Grb2/Ras/Raf/MAPK cascade have also been reported (Avruch *et al.*, 1994; Bole-Feysot *et al.*, 1998). A recent study has shown that PRL could possibly signal through RUSH/SMARCA3 instead of Stat5; however, the ability of JAK2 to phosphorylate RUSH still needs to be confirmed (Hewetson *et al.*, 2004).

Dimerization of PRLR occurs upon the binding of PRL which brings two JAK molecules together to a sufficient proximity for transphosphorylation of JAKs (Finidori and Kelly, 1995). The binding of PRL and PRLR forms a 1 PRL : 2 PRLR complex which initiates the PRL signal transduction pathway (Gertler *et al.*, 1996). In mammals, it has been shown that the 1 PRL: 1 PRLR complex does not activate the subsequent steps of the JAK/STAT pathway, and the 1 PRL: 2 PRLR complex is essential for this activity (Goffin and Kelly, 1996). However, it is possible that a 2 PRL : 2 PRLR complex exists in birds because the extracellular domain of avian PRLR possesses a double antenna structure that provides an additional binding site for PRL. In avian species, the presence of 2 potential ligand binding sites in the extracellular domain allows for the possibility of more complex interactions. These complexes could be agonistic or antagonistic and could provide an additional mechanism for partitioning the biological actions of PRL to different tissues. Moreover, since the relative proportion of glycosylation varies with the physiological status of the hen (Bédécarracts *et al.*, 1999b),

additional interactions between isoforms with different binding affinities and the 2 putative binding domains are possible (Zhou *et al.,* 1996). Nevertheless, the significance of the 2 PRL : 2 PRLR complex remains unknown.

To date, the singular argument against this hypothesis is the observation that the membrane distal repeat has no functional role since deletion of this unit has no apparent affect on the binding affinity or ligand specificity (Chen and Horseman, 1994). However, the latter study used a heterologous ligand (rat PRL to pigeon PRLR), thus it is possible that different effects may be observed in a homologous system.

2.4. The regulation of secretion of pituitary PRL

The secretion of PRL is primarily under inhibitory control in mammals (Lamberts and Macleod, 1990), whereas, the secretion of PRL is under stimulatory control in birds (Hall *et al.*, 1986). In addition, in contrast to mammals, the hypothalamus of birds secretes one or more PRL releasing factors (Hall *et al.*, 1986). Many endogenous and exogenous factors can affect the secretion of PRL at different physiological stages. The endogenous factors are often mediated by exogenous factors such as photostimulation (Burke and Dennison, 1980; Etches and Cheng, 1982; Bédécarrats *et al.*, 1997) suckling (Frawley *et al.,* 1983; Terkel *et al.*, 1972) and stress (Neill, 1970; Neill and Smith, 1974). For example, stimulating the pituitary glands of incubating hens with dopamine induced the secretion of PRL; however, the same stimulation to the pituitary glands of laying hens suppressed the secretion of PRL (Youngren *et al.*, 1995).

2.4.1 Exogenous factors

2.4.1.1 Photoperiod

One of the best known exogenous factors that control the secretion of PRL is photoperiod. In mammals, the reproductive cycle of seasonal breeders is controlled by the day length and hence their offspring are born at times of the year when their chance of survival is maximized. For example, in sheep raised in temperate latitudes, blood levels of PRL and dopamine decrease with exposure to shorter day lengths (Kennaway *et al.*, 1982; Viguie *et al.*, 1996). Associated with the decrease in circulating levels of PRL,

the reproductive axis is activated and animals enter the breeding season such that births are timed to coincide with increased availability of food. It is suggested that melatonin was secreted from the pineal gland to exert the effect of photoperiod (Daveau *et al.*, 1994; Poulton *et al.*, 1989; Thiery *et al.*, 1989) acting through the suprachiasmatic nucleus (Scott *et al.*, 1995), or by acting directly on the pituitary gland (Lincoln and Clarke, 1994). Although studies have shown that the levels of dopamine decreased with the levels of PRL in sheep as a result of short photoperiod, the effect of photoperiod on prolactin secretion seemed to be independent of dopaminergic activity (Lincoln and Clarke, 1995; Lincoln and Tortonese, 1995).

In turkeys, increased levels of PRL in mature turkey hens were observed after photostimulation (Burke and Dennison, 1980; Etches and Cheng 1982; Wong *et al.*, 1991; Karatzas *et al.*, 1997; Tong *et al.*, 1997). However, the levels of PRL were shown to decrease in laying or photorefractory turkey hens (Siopes and El Halawani, 1986). These studies indicated that the effect of photoperiod on the release of PRL was dependent on the physiological stage of the birds. Siopes and El Halawani (1989) demonstrated that the release of PRL in turkeys was influenced by photostimulation through light perception by the retina in both male and female or by the pineal gland in female.

2.4.1.2 Physical stimulation

Physical stimuli including audible, olfactory and tactile cues have been proven to affect the secretion of PRL in numerous studies. It has been shown that the release of PRL was induced by ultrasonic cries from infant rats in lactating female rats and in virgin female rats (Grosvenor *et al.*, 1977; Terkel *et al.*, 1979; Voloschin and Tramezzani, 1984). Moreover, loss of pregnancy due to a dopamine-induced suppression of prolactin secretion was observed in female rats when they were exposed to the pheromone of an unfamiliar male (Freeman *et al.*, 2000; Dominic, 1967; Rosser *et al.*, 1989; Li *et al.*, 1989). This phenomenon is referred to as the Bruce effect (Bruce, 1965). In addition, Grosvenor *et al.*, (1977) reported that the odour of pups stimulated the secretion of milk and PRL in female rats.

In Galliformes, cessation of incubation behaviour and decreased levels of circulating PRL were observed in incubating turkey hens when their nest was removed (El Halawani *et al.*, 1980; Zadworny *et al.*, 1985) or when they were confined in sand and wire floored pens (Guémené and Etches, 1990). The incubation behaviour of the turkey hens was resumed if the nest was reintroduced within 3 days; even though the levels of circulating PRL were low (El Halawani *et al.*, 1980). These results corresponded to the observation in incubating broiler breeders (Richard-Yris *et al.*, 1998) which indicated that high levels of circulating PRL were not required for the resumption of incubation (El Halawani *et al.*, 1980).

2.4.1.3 Stress

The effect of stress on the secretion of PRL depends on the type of stress and the physiological state of the animal (Freeman *et al.*, 2000; Kehoe *et al.*, 1992).

In rats, it has been demonstrated that stress due to restraint increased PRL secretion and reduced the anterior pituitary dopamine content (Demarest *et al.,* 1984). Fujikawa *et al.,* (1995) reported that the gene expression for PRLR in the choroid plexus of rats was induced in concert with a rapid increase of serum PRL. Nevertheless, it was reported that the responsiveness of PRL to restraint stress was lower as a result of prenatal stress (Kinsley *et al.*, 1989).

In women, it has been shown that heat stress increased the levels of PRL by 82% in non-pregnant women while only a 12% increase was observed during late pregnancy (Vaha-Eskeli *et al.*, 1991). In heifers, increased levels of plasma PRL in response to heat stress was observed and this was suggested to be a direct effect to hyperthermia (Schams *et al.*, 1980). This suggestion was confirmed by Ronchi *et al.*, (2001). The increased levels of PRL were thought to be responsible for modifying the resumption of ovarian function in heat-stressed cattle (Weiss *et al.,* 1981).

In avian species, reduced reproductive performance as a result of heat stress has been associated with increased levels of prolactin (PRL) in response to heat stress (Rozenboim *et al.*, 2004) and vasodilatation (Marsh and Dawson, 1982). The decline of

egg production as a result of heat stress could be reversed by oral treatment with PCPA; however, active immunization against VIP did not maintain the egg-laying activity of turkey hens subjected to high temperature stress even though the levels of PRL were lowered (Rozenboim *et al.*, 2004).

Other than heat stress, feed and water deprivation have also been studied in avian species. It was shown that water deprivation in cockerel induced secretion of PRL both *in vivo* and *in vitro* (Harvey *et al.*, 1984). Neither feed nor water deprivation nor force feeding significantly affected the concentration of PRL or corticosterone in non-laying, and non-incubating turkey hens (Zadworny *et al.*, 1985). However, turkey breeder hens fed with standard diet had higher levels of PRL compared to hens fed with low energy high protein or high energy low protein diet (Emmerson *et al.*, 1991). Therefore, the feed composition may only affect the levels of PRL at a certain physiological state in turkey hens. During the incubation period, nest removal with and without feed and water deprivation significantly reduced the levels of plasma PRL in turkey hens (Zadworny *et al.*, 1986). Moreover, turkey hens resumed incubation behaviour in minutes after returning to their pen even though levels of circulating PRL remained low. Hence, the maintenance of high levels of PRL during incubation was associated with a stimulus induced by the nest itself or other environmental stimuli (Zadworny *et al.*, 1985).

2.4.2 Endogenous Factors

The endogenous effectors of the release of PRL are partially controlled by the exogenous factors; however, the release of PRL can also be controlled by inherent factors. For example, during avian embryogenesis, the embryo is isolated from maternal influences and has minimal external influences such as ambient temperature. Therefore, the hormonal changes in avian embryo are possibly controlled by some inherent factors which are activated at different stages of embryogenesis. A number of PRL releasing factors (PRF) such as angiotensin II, thyrotropin releasing hormone, vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), beta-adrenergic catecholamines, galanin, and estrogens (Lamberts and MacLeod, 1990) or PRL inhibitory factors (PIF) such as dopamine (Huhman *et al.*, 1995) have been identified. In mammals, the secretion

of PRL is under inhibitory control and the primary PIF is believed to be dopamine; whereas, in avian species, the release of PRL is under stimulatory control and the most potent PRF is VIP.

2.4.2.1 Dopamine (DA)

Dopamine has been shown to have a dual effect on the secretion of PRL both in mammalian and avian species. Freeman *et al.*, (2000) has reviewed the evidence that DA was the major PIF in mammals. Supporting evidence includes the high concentration of DA in median eminence (Fuxe *et al.,* 1977) as well as the hypophysial stalk blood (Ben-Jonathan *et al.,* 1978; Gibbs and Neill, 1978; Plotsky *et al.*, 1978), and the presence of DA receptor (D_2 receptor subclass) on lactotroph membrane (Meador-Woodruff *et al.*, 1989). In addition, knockout of the receptor results in anterior lobe lactotroph hyperplasia and hyperprolactinemia in mice (Kelly *et al.*, 1997; Saiardi *et al.*, 1997). However, other studies indicated that DA stimulated the secretion of PRL. For example, a stimulatory effect of DA was observed in pituitary culture from suckled lactating rats (Hill *et al.*, 1991). Moreover, it was shown that DA stimulated the secretion of PRL in ovariectomized female rats in the presence of estradiol and progesterone (Close and Freeman, 1997).

In avian species, early studies indicated that dopamine also had a dual action on the secretion of PRL. During the egg laying period, DA was shown to inhibit the secretion of PRL while DA had a stimulatory effect on PRL secretion during incubation period (Youngren *et al.*, 1995). Evidence indicated that the inhibitory effect of DA was exerted through the DA receptor on pituitary glands (Youngren *et al.*, 1998a) at the transcriptional level of PRL (Maurer, 1980). Similarly, DA exerted an inhibitory response on the VIP-stimulated PRL mRNA at the transcriptional and post-transcriptional levels via pituitary DA receptors belonging to the D_2 subclass (Al Kahtane *et al.*, 2003). Conversely, DA had a stimulatory effect on the release of PRL in the presence of VIP in turkey hypothalamic explants (Chaiseha *et al.*, 1997).

2.4.2.2 Vasoactive intestinal peptide (VIP)

Vasoactive intestinal peptide is a 28-amino acid peptide that is widely distributed in the peripheral and central nervous systems (Besson *et al.*, 1979; Dalcik and Phelps, 1993; Nylander *et al.*, 1993; Mezey and Kiss, 1985; Pelletier *et al.*, 1981; Sims *et al.*, 1980). It belongs to the secretin-glucagon-VIP superfamily and was first identified in the porcine small intestine (Said and Mutt, 1970). The stimulatory effect of VIP on the secretion of PRL has been reported in mammals and birds both *in vitro* and *in vivo* (Falsetti *et al.*, 1988; Kato *et al.*, 1978; Ruberg *et al.*, 1978; Shaar *et al.*, 1979; Vijayan *et al.*, 1979; El Halawani *et al.*, 1988; Opel and Proudman, 1988). It has been shown that VIP first bound to its receptor in anterior pituitary gland (Bataille *et al.*, 1979) and exerted its effects on the secretion of PRL through the cAMP/PKA pathway (Fernandez *et al.*, 2003; Le Péchon-Vallée *et al.*, 2000) In rats, Kato *et al.*, (1978) demonstrated that VIP stimulated the secretion of PRL by blocking the inhibitory effect of DA. In addition, it has been shown that the stimulatory effects of VIP acted in paracrine (Hagen *et al.*, 1986) and autocrine fashion (Nagy *et al.*, 1988) in anterior pituitary of rats. Passive immunization against VIP in rats completely suppressed the secretion of PRL under ether stress (Shimatsu *et al.*, 1984). However, passive immunization only partially suppressed the release of PRL induced by suckling stimulation (Abe *et al.*, 1985). These findings suggested that the release of PRL induced by different exogenous factors might be mediated through different pathways. Moreover, the variations in the levels of DA with different stimulation could also affect the stimulatory effect of VIP on the secretion of PRL.

In avian species, the control of secretion of PRL is mainly regulated by VIP (Lea and Vowles, 1986; Maney *et al.*, 1999; El Halawani *et al.*, 1990; Sharp *et al.*, 1998). In Galliformes, numerous studies have demonstrated the stimulatory effects of VIP on the secretion of PRL (Tong *et al.*, 1998; Bédécarrats 1999a; Macnamee *et al.*, 1986; Proudman and Opel, 1988). The abundance of PRL mRNA (Mauro *et al.*, 1989; Talbot *et al.*, 1991; Tong *et al.*, 1998; Chaiseha *et al.*, 1998) and plasma levels of PRL (Youngren *et al.*, 1996) vary in concert with the levels of VIP in the hypothalamus during the various stages in turkey reproductive cycle. Moreover, the pulsatile release patterns of VIP into

hypophysial portal blood (Chaiseha *et al.,* 1998) and the number of VIP immunoreactive cells in the hypothalamus (Mauro *et al.*, 1989) were correlated with the levels of plasma PRL (Kang *et al.*, 2004). Furthermore, it has been shown that photostimulation increased the levels of VIP in the hypothalamus of turkey hens. In contrast, levels of VIP were decreased by 44% after exposure to short photoperiod (Mauro *et al.*, 1992). Tong *et al.* (1998) reported that VIP was mainly regulated at the transcription level, and it induced PRL gene expression by increasing transcription rate and enhancing mRNA stability. Therefore, it is possible that VIP is the neural link between photoperiodic changes and PRL secretion (Mauro *et al.*, 1992). In agreement with the proposed role of VIP as the major releasing factor for PRL in avian species, the concentration of VIP specific receptors in pituitary cell membrane was shown to be associated with the levels of PRL both in pituitary and plasma during the various stages of the turkey reproductive cycle (El Halawani *et al.*, 1990).

It has also been shown that incubation behaviour in turkeys could be prevented by either passive (Crisostomo *et al.*, 1997) or active immunization (Crisostomo *et al.*, 1998) against PRL. Similarly, the high levels of plasma PRL associated with incubation are shown to be inhibited by immunization against VIP in both chickens and turkeys (El Halawani *et al.*, 1995a,b; Sharp *et al.*, 1989).

Recently, a 35-bp activator element in the tPRL promoter was identified and named the VIP response element. This motif was suggested to be an important cis-acting element for VIP-stimulated gene expression of tPRL (Kang *et al.*, 2004).

2.4.2.3 Serotonin (5-HT)

Serotonin (5-HT) is the precursor of melatonin which is involved in various physiological changes in response to photoperiod in vertebrates. It has been shown that inhibition of 5-HT synthesis reduced the secretion of PRL induced by estrogen in rats (Caligaris and Taleisnik, 1974; Chen and Meites, 1975). Moreover, inhibition of 5-HT synthesis completely prevented the release of prolactin induced by nursing and the effect was reversed by administrating 5-HT (Kordon *et al.*, 1973). However, it was suggested that 5-HT acted as a neurotransmitter rather than a neurohormone since 5-HT did not

stimulate the secretion of PRL *in vitro* (Lamberts and MacLeod, 1978; Freeman *et al.*, 2000). Pilotte and Porter (1981) showed that the release of PRL induced by 5-HT was independent of the concentration of DA; therefore, 5-HT might exert its function via system other than dopaminergic system.

In avian species, Fehrer *et al.* (1983) demonstrated that 5-HT induced the release of PRL in young turkeys. It has been shown that 5-HT played an important role in the release of PRL in response to photoperiod in turkey (Youngren *et al.*, 1996; Pitts *et al.*, 1996). On the other hand, it was demonstrated that 5-HT required both the dopaminergic system (Youngren *et al.*, 1998b) and VlPnergic systems (El Halawani *et al.*, 1995b; Pitts *et al.*, 1996) to promote a stimulatory PRL response.

2.4.2.4 Thyrotropin releasing hormone (TRH)

TRH has been known to be a PRF in mammals. It has been shown that TRH stimulated prolactin release from pituitary gland both *in vitro* and *in vivo* (Blake, 1974; Bowers *et al.*, 1971; Tashjian *et al.*, 1971). However, the stimulatory effect of TRH may depend on the physiological state of the animal. It has been reported that secretion of PRL was stimulated by TRH in estrogen-primed male rats (Piercy and Shin, 1980) but TRH failed to induce a significant response in normal male or lactating female rats (Riskind *et al.*, 1984; Grosvenor and Mena, 1980). Studies indicated that the stimulatory effect of TRH on PRL was enhanced by transient dopamine antagonism (Haisenleder *et al.*, 1986) or transient dopamine withdrawal (Martinez De La Escalera *et al.*, 1992)

In birds, TRH does not seem to be a potent PRF. In 1978, Harvey *et al.*, reported a dose-dependent stimulatory effect of TRH on the release of PRL *in vitro* in chicken pituitary cell culture. In 1985, Hall *et al.* suggested that TRH could stimulate PRL release from the pituitary gland through the cAMP pathway. Studies indicated that TRH only induced a transient increase in the levels of plasma PRL (Proudman, 1984; Fehrer *et al.*, 1985) but TRH failed to induce a response in adult birds and *in vitro* pituitary cell cultures (Proudman, 1984; Fehrer *et al.*, 1985; Saeed and El Halawani, 1986).

2.5 Roles of PRL in avian reproduction

The role of PRL in avian reproduction has mainly focused on the expression of reproductive behaviour. Although the causal relationship between hyperprolactinemia and incubation behaviour is still debatable, numerous studies have indicated high levels of PRL were required to initiate and maintain incubation behaviour. Studies indicated that the expression of incubation behaviour accompanied other behaviours such as increased nesting frequency (Zadworny *et al.,* 1985), care of poults, anorexia, adipsia (Zadworny *et al.*, 1985) and termination of egg production (Sharp *et al.*, 1989). Moreover, the hens undergo physical changes such as the appearance of brood patches (Riddle *et al.*, 1933) and ovarian regression (Sharp *et al.*, 1989; Porter *et al.*, 1991a,b). The latter may be associated with an inhibition of estradiol biosynthesis by PRL in the small follicles of the follicular hierarchy (Zadworny *et al.*, 1989).

Several studies indicated that the inhibition of PRL would alter incubation behaviour. In 1997, Crisostomo *et al.* successfully reduced incubation behaviour by passively immunizing turkey hens with anti-tPRL serum. The incubation behaviour was fully prevented by active immunization with GST-tPRL fusion protein (Crisostomo *et al.*, 1998). Furthermore, a study done by El Halawani *et al.*, (1995a) indicated that active immunization against VIP prevented the increase in plasma PRL, which in turn blocked the expression of incubation behaviour.

The effect of PRL on parental behaviour varies among avian species. In red-cockaded woodpeckers, a species in which both male and female care for juvenile birds, Khan *et al.*, (2001) reported that high levels of PRL were observed in female, male and helper male birds even though the male helper bird is sexually inactive. It was shown that the levels of PRL decreased dramatically within 1 or 2 days after the removal of nest or egg (Etches, 1979; El Halawani *et al.*, 1980; Goldsmith *et al.,* 1984; Ramsey *et al.,* 1985; Sharp *et al.*, 1988; Lea and Sharp, 1982). When exogenous PRL was administrated to ring doves, incubation behaviour was resumed even after 10 days of nest deprivation (Lehrman and Brody, 1964; Janik and Buntin, 1985). Moreover, tactile stimulation from the poults was shown to reduce the release of PRL in turkey (Opel and Proudman, 1988)

whereas, the release of PRL was independent of tactile stimulation from poults in Adélie penguins (Vleck *et al.*, 2000).

2.6. Roles of PRL in embryogenesis

Sources of PRL in mammalian embryos are of foetal and maternal origin. The mother transfers PRL to the embryo either by blood exchange (Josimovich *et al.*, 1974) or colostrum (Grosvenor and Whitorth, 1983). The presence of foetal PRL in mammalian embryo has been reported in mice (Komoto and Bern, 1971), rats (Hoeffler *et al.*, 1985; Khorram *et al.*, 1984) and humans (Asa *et al.*, 1986). Moreover, the numerous functions of PRL during embryogenesis were suggested based on the wide distribution of PRLR (Royster *et al.*, 1995; Tzeng and Linzer, 1997). These functions include growth and development, metabolism, osmoregulation and immunoregulation (Bole-Feysot *et al.*, 1998). Recently, PRL is suggested to be involved in non-shivering thermogenesis in newborns due to the observation that the high levels of PRLR positively correlate with the levels of uncoupling proteins during development in brown adipose tissue, a main source for non-shivering thermogenesis (Pearce *et al.*, 2003). Furthermore, an elevated level of uncoupling proteins and improved thermoregulation were observed in neonatal lamb and pregnant rats treated with PRL.

The avian embryo provides a unique system for studies of endocrine systems during embryogenesis because of its independency of maternal influence. Although a limited number of studies have been done on the function of embryonic PRL, the fluctuation of the levels of PRL in chicken embryo (Harvey *et al.*, 1979), and in turkey embryo (Bédécarrats *et al.*, 1999a) around the time of hatching indicated that it played a role in embryogenesis.

In Galliformes, it has been shown that the levels of PRL changed dramatically around the time of hatching. In turkey embryos, the levels of pituitary PRL remained low until 5 days before hatching, whereas, the levels of plasma PRL remained low until 2 days before hatching and increased to peak 1 day before hatching (Bédécarrats *et al.*, 1999a). These changes were paralleled by coincident changes in pituitary levels of PRL

mRNA. Similar changes about the time of hatch have been observed in chicken (Kansaku *et al.*, 1994).

The significantly increased levels of PRL around time of hatching may play a role in assisting the poult to cope with the stress associated with post hatching life such as changes in ambient temperature, exposure to pathogens and initiation of breathing. Evidence indicated that uncoupling proteins might play a role in thermoregulation through skeletal muscle in birds; however, unlike mammal, birds do not have distinct brown adipose tissue (BAT) or a related thermogenic tissue; thus, the possible role of PRL in non-shivering thermoregulation requires further investigation (Toyomizu *et al.*, 2002).

Numerous studies indicated the importance of PRL on the mammalian immune system such as maturation of T cells (Carreno *et al.*, 2004) and the modulation of B cell function (Peeva *et al.*, 2004). In chicken embryo, it was suggested that PRL assisted the maturation of T cells through its capacity for inducing IL-2 receptor expression on the thymocytes (Moreno *et al.*, 1998).

Surfactant is a protein found in all vertebrate lungs which aids in the initiation of breathing. It has also been shown that PRL was involved in lung maturation of bull toad as metamorphosis progressed (Oguchi *et al.*, 1994). Hauth *et al.* (1978) reported a correlation between PRL and the production of surfactant in human. However, Hylka and Doneen (1982) reported that PRL had no significant effect on the prehatching stimulation of surfactant phospholipids synthesis in chicken embryos. Studies in human foetal lung culture have shown that PRL alone did not stimulate production of phosphatidylcholine (PC), a major constituent of surfactant. However, when PRL was used in combination with glucocorticoids and insulin, the rate of lamellar body PC synthesis increased to a value similar in human foetal lungs at term (Mendelson *et al.*, 1991). Therefore, PRL may exert an indirect effect on the production of surfactant or the initiation of breathing in combination with other hormones in avian species.

In summary, the association between higher levels of glycosylation around the time of hatch and during the reproductive cycle suggests that G-PRL may have specific

physiological effects different from those of NG-PRL. Since G-PRL is likely to have a different affinity than NG-PRL for the PRL receptors, changes in ratio of isoforms may act to modulate biological effects in different target tissues.

To date, the glycosylation process of PRL is poorly understood. In order to investigate the mechanism of glycosylation of PRL, this study was conducted to explore the genes that are potentially involved in glycosylation.

CHAPTER III. MATERIALS AND METHODS

3.1 Tissue source

Pituitary glands were collected from 60 day-24 turkey embryos that were purchased from Couvoir Unik Inc. in Quebec. Embryos were decapitated and pituitary glands were excised and stored in medium M199 (M199; GIBCO, Grand Island, NY) buffered with HEPES(25 mM), 0.1% BSA (w/v), L-glutamine (100 mg/L), penicillin (100 U/ml), streptomycin (100 µg /ml), and sodium bicarbonate (2200 mg/L) at 39 °C until the collection of all 60 pituitary glands were completed.

3.2 Tissue culture

Subsequently, pituitary glands were cultured in (1 ml / pituitary gland) medium M199 containing HEPES(25 mM), 0.1% BSA (w/v), L-glutamine (100 mg/L), penicillin (100 U/ml), Streptomycin (100 µg /ml), and sodium bicarbonate (2200 mg/L) at 39 °C and gas with 95% O_2 / 5 % CO_2 for 1 hour. After 1 hour of equilibration, the medium was removed and replaced with fresh medium with/without 10^{-7} M of synthetic VIP (Sigma, Inc).

The two groups of pituitary glands (30 pituitary glands in each group) were further incubated at 39 °C with 95% O_2 / 5 % CO_2 for 4 hours. All 60 pituitary glands were then snap frozen in liquid nitrogen and stored at – 70 °C.

3.3 Preparation of pituitary extracts

The protocol for preparation of pituitary extracts was adapted from Bédécarrats *et al.* (1999a). A pool of six pituitaries from each group was homogenized in 45 µl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5% Tween 20 by sonication 5 times for 10 sec on ice. The Bradford method was used to measure the protein concentration of each pool of protein extract. Two aliquots of each pool was mixed with an equal volume of 10 mM Tris-HCl (pH 8.0) containing PMSF (0.1 mM) and used for western blotting and radioimmunoassy (RIA).

3.4 Western blotting

3.4.1 Electrophoresis

The pituitary homogenate was adjusted to 1 μg protein / μl with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Tween 20. An aliquot of 2.5 μg protein from each pool was separated on 12% SDS polyacrylamide gel (0.75 mm, 8 cm). Prestained molecular weight markers (Bio-Rad Laboratories, Inc.) were included in each gel. The gel was electrophoresed at a constant voltage of 200 V for 30 min.

3.4.2 Electrotransfer

After electrophoresis, the polyacrylamide gel with separated proteins was equilibrated in electrotransfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 15 to 30 minutes on an orbital shaker. The proteins were electrotransfered to a PVFD membrane (Immobilon-P, 0.45 μm, Millipore) prewetted with 100% methanol at constant voltage of 100 V for 1 h at 4 °C with constant stirring in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3.

3.4.3 Labelling and Detection

Following electrotransfer, the membrane was removed from the transfer apparatus and blocked with Tris buffered saline pH 7.5 (TBS: Tris base 50 mM, NaCl 150 mM) with 5% non fat dry milk for 1 hour with shaking. The membrane was then incubated with rabbit anti recombinant turkey prolactin antibody (Guémené *et al.*, 1994) at a 1/2000 dilution in TBS with 0.5% non fat dry milk over night with shaking at 2 °C to 8 °C.

The membrane was washed 6 times for 10 minutes with TBS 0.05% with Tween 20 with shaking. Anti-rabbit IgG peroxidase (secondary antibody) at 1:20000 dilution in TBS with 0.5% non fat dry milk was added to the membrane, which was further incubated for one hour with shaking at room temperature. Subsequently, the membrane was washed 4 times for 5 minutes with TBS containing 0.05 % Tween 20.

The immunoreactive bands were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc.) according to the manufacturer's protocol. In brief, SuperSignal® West Pico Substrate Working Solution was prepared by mixing 5 ml of the luminol/enhancer solution and 5 ml of the stable peroxide solution. The membrane was incubated in working solution for 5 minutes, and then exposed to Kodak X-ray film for autoradiography. After the film was developed with Kodak Film Developer, the image was digitalized and the proportion of each band was measured by Quantity One Software (Bio-Rad Laboratories, Inc.).

3.5 Radioimmunoassay

The concentration of PRL from M199 was measured by radioimmunoassay (RIA) in order to investigate the amount of PRL released from the pituitary glands. Aliquots of 500 µl of M199 were collected from 6 ml of M199 with a pool of 6 day-24 embryonic pituitary glands after one hour of equilibration (time=0) and at the end of tissue culture with or without VIP stimulation (time=4). The levels of PRL were measured by RIA as described by Guémené *et al.* (1994). The recombinant turkey prolactin (rctPRL) used for iodination and as standard was obtained from Dr. A.F. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). The levels of PRL released by tissues in culture were measured in 100 µl aliquots of M199, and the levels of PRL pituitary content were assayed with 200 µl of a 1/50 dilution pituitary extracts. Briefly, samples were incubated with rabbit anti rctPRL antibody at a final dilution of 1/1000 (Guémené *et al.*, 1994) for 24 h at 4°C. On the second day, 15000 cpm of [^{125}I] rctPRL were added and tubes were incubated for an additional 24 h at 4°C. On day 3, goat anti rabbit second antibody (1/100 final dilution) was added, and tubes were incubated for 48 h at 4°C. After addition of 1 ml phosphate buffer saline, immuno-complexes were precipitated by centrifugation at 3000 x g for 30 min. Pellets were rinsed once with 1 ml phosphate buffer saline and radioactivity was counted using a gamma counter. The concentration of PRL in the samples was calculated using a variable slope sigmoidal dose-response equation (GraphPad Prism version 4.03, GraphPad Software, San Diego, CA). Intra- and inter-assay coefficients of variation were 5.8 % and 0.5 %, respectively.

3.6 Total RNA preparation

Total RNA was extracted using a NucleoSpin® RNA II Kit (BD Biosciences Clontech, Inc.) according to the manufacturer's protocol. In brief, a pool of 10 pituitary glands from each group of samples was lysed in RA1 buffer (lysis buffer provided from the kit) and 3.5 µl of β-mercaptoethanol by vortexing and RNA was precipitated by adding 350 µl of 70% ethanol. After treating the RNA with 350 µl Buffer MDB (membrane desalting buffer provided from the kit), DNA was digested by DNase I at room temperature for 15 min. Following two steps of washing with 200 µl of Buffer RA2 and 600 µl of Buffer RA3, total RNA was eluted by adding 50 µl of nuclease-free water twice. The concentration of total RNA was estimated by UV spectroscopy at a wavelength of 260 nanometers.

3.7 cDNA preparation for suppressive subtractive hybridization

3.7.1 First Strand cDNA synthesis

The Super Smart™ PCR cDNA Synthesis Kit (BD Biosciences Clontech, Inc.) was used to synthesize cDNA for suppressive subtractive hybridization. Five hundred ng of total RNA was annealed with 12 µM of 3' SMART CDS Primer II A and 12 µM of SMART A oligonucleotide (provided by Super Smart™ PCR cDNA Synthesis Kit) for 2 minutes at 65 °C, followed by 90 minutes of incubation at 42 °C for first strand cDNA synthesis. The single stranded cDNA was subsequently purified with NucleoSpin® Extraction Kit (BD Biosciences Clontech, Inc) using the protocol recommended by the manufacturer and eluted with 85 µl of Milli-Q H_2O.

3.7.2 Long Distance (LD) PCR amplification

For each LD PCR reaction, a reaction mix contained 80 µl of cDNA, 172 µl of deionized H_2O, 30 µl of 10X Advantage 2 PCR Buffer, 6 µl 50X dNTP (10 mM), 6 µl 5' PCR Primer II A (12 µM) and 6 µl 50X Advantage 2 Polymerase Mix. The mixture was aliquoted in 3 tubes and placed into a MJ Research, Inc. Model PTC-100 Programmable Thermal Cycler. Samples were initially denatured at 94 °C for 1 minute. Subsequently, each cycle consisted of 95°C for 15 seconds, 65°C for 30 seconds and 68°C for 6 minutes

for 36 cycles. After 21 PCR cycles, 2 tubes were removed and refrigerated at 4°C for later use. Aliquots of 5 µl were set aside every 3 cycles until the program was completed. These aliquots were electrophoresed in a 1% agarose gel at 70 V for 1 hour to optimize the number of cycles. Once the number of cycles was optimized, the 2 tubes stored at 4°C were placed back into the thermal controller for additional cycles.

PCR products were first purified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Afterward, the PCR products were concentrated to 70 µl with n-butanol extraction. PCR products were subsequently purified by centrifugation through CHROMA SPIN™-1000 DEPC-H_2O Columns (BD Biosciences Clontech, Inc.) using the protocol recommended by the manufacturer.

3.7.3 Rsa I digestion

The purified PCR products were digested with 10 units of Rsa I restriction endonuclease at 37 °C for 3 hours. Completion of restriction digestion was assessed on a 1% agarose gel. The digested PCR products were purified with NucleoSpin® Extraction Kit (BD Biosciences Clontech, Inc) using the protocol recommended by the manufacturer, and eluted with 50 µl of Milli-Q H_2O. The purified PCR products were precipitated with 50 µl of 4 M ammonium acetate and 375 µl of 95% ethanol, and finally dissolved in 6.7 µl of TNE buffer.

3.8 Suppressive subtractive hybridization

Suppressive subtractive hybridization was performed with the Clontech PCR-Select™ cDNA Subtraction Kit (BD Biosciences Clontech, Inc). Figure 3.1 represents the experimental plan for the suppressive subtractive hybridization. Figure 3.2 shows the experimental plan for the adaptor ligation. In the forward library, the sample without VIP stimulation is the driver cDNA while the sample with VIP stimulation is the driver cDNA in the reverse library.

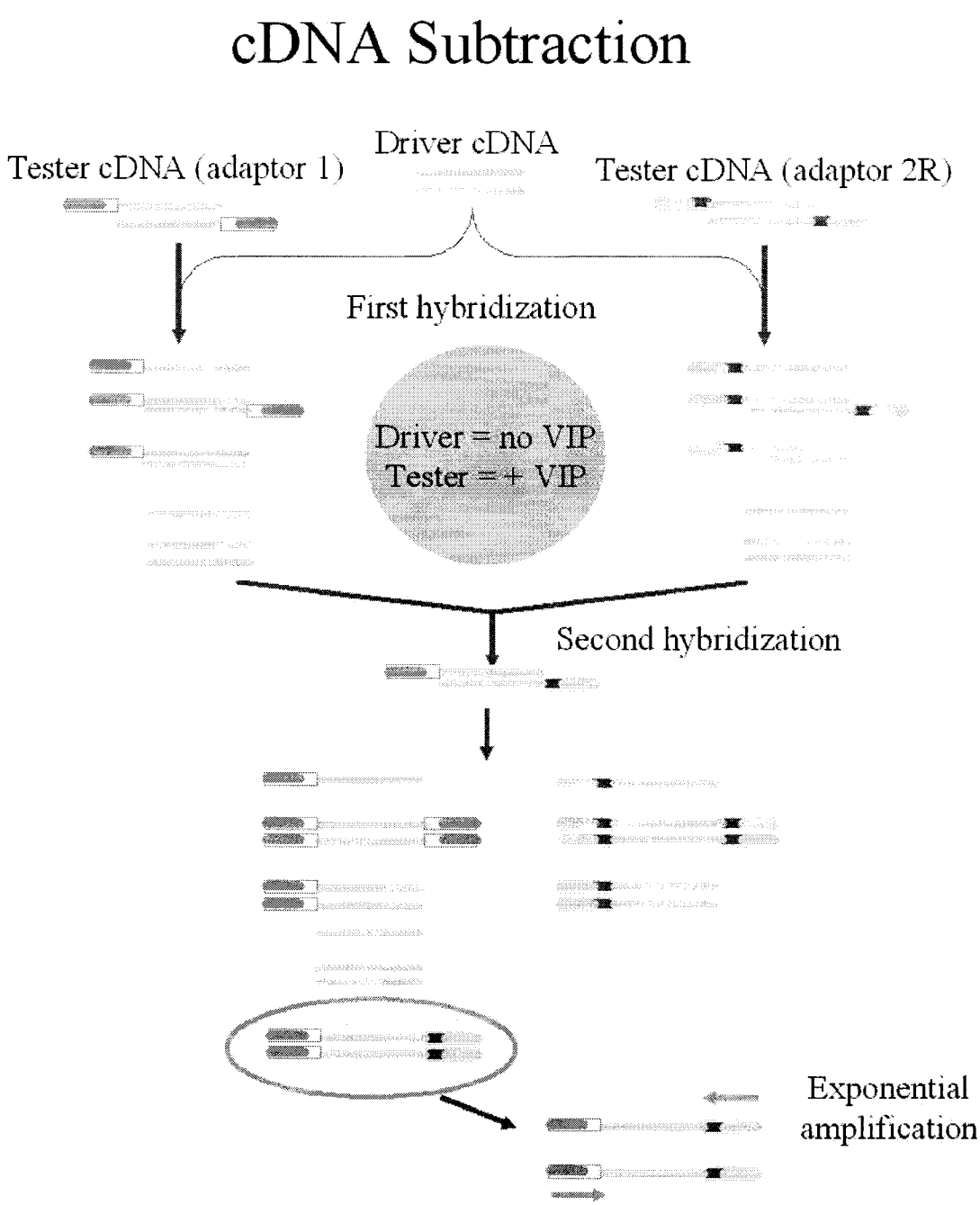


Figure 3.8a Overview of the experimental design of suppressive subtractive library of VIP treated and non-VIP treated day 24 turkey embryonic pituitary glands (Adapted from the Clontech PCR-Select^{TM} cDNA Subtraction Kit User Manual)

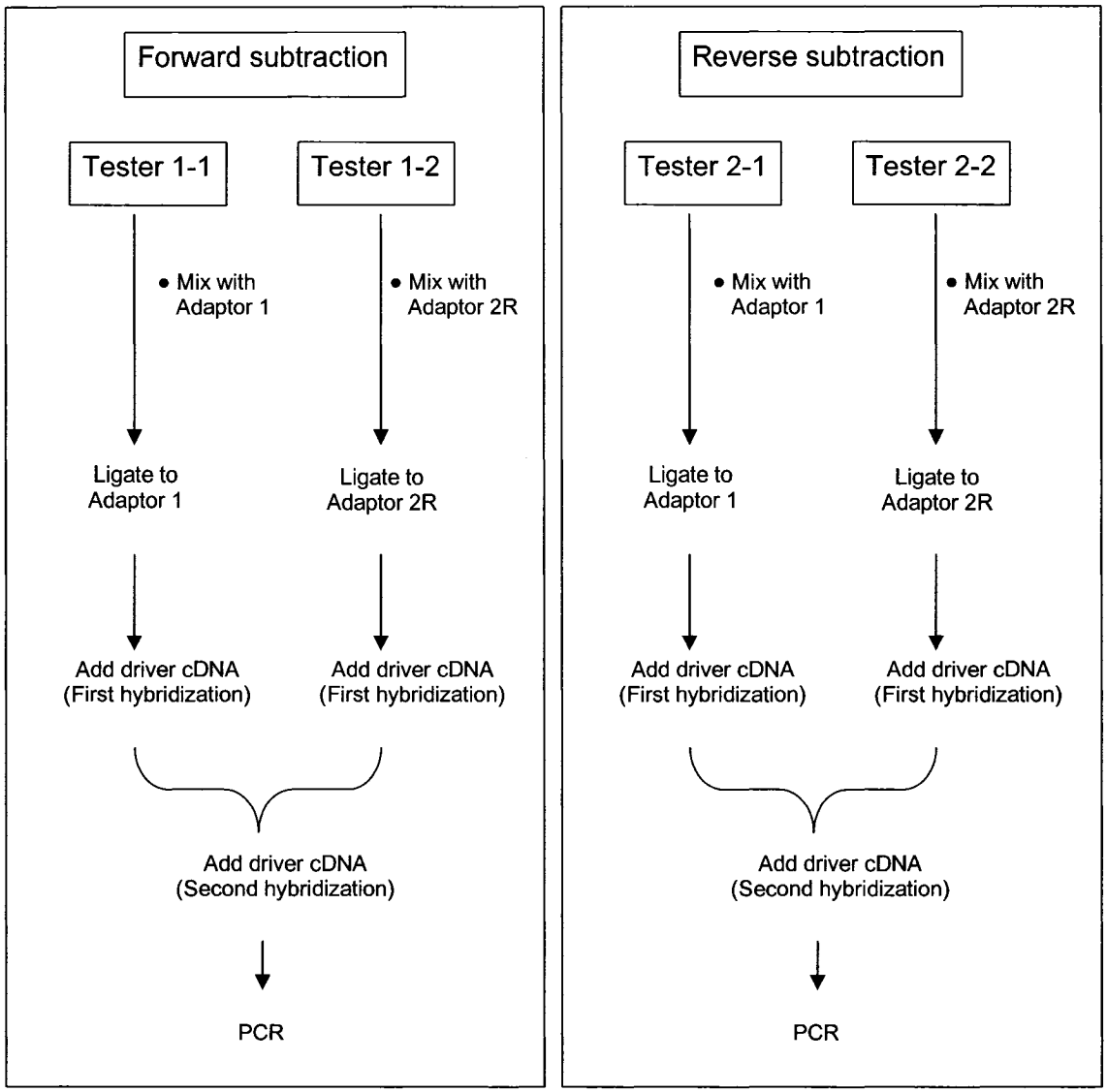


Figure 3.8b Graphical representation of the experimental plan for the adaptor ligation and hybridization (Adapted from the Clontech PCR-Select™ cDNA Subtraction Kit User Manual). Tester 1-1 and Tester 1-2 are the sample stimulated with VIP and Tester 2-1 and Tester 2-2 are the sample without VIP stimulation.

3.8.1 Adaptor ligation

An aliquot of 1 µl of purified Rsa I digested cDNA was diluted with 5 µl of sterile H_2O. Tester 1-1 and Tester 2-1 were ligated with Adaptor 1, while Tester 1-2 and Tester 2-2 were ligated with Adaptor 2R. The ligation procedure was carried out using 400 units of T4 DNA ligase at 16 °C overnight.

3.8.2 Hybridization

Hybridization was divided into 2 parts. In the first hybridization, a reaction mix containing 1.5 µl of Tester cDNA with one type of adaptors, 1.5 µl of driver cDNA and 1 µl of 4X hybridization buffer (provided from the kit) was incubated at 98 °C for 1.5 min followed by incubation at 68 °C for 8 hours. In the second hybridization, 1 µl of driver cDNA was overlaid with one drop of mineral oil and was denatured at 98 °C for 1.5 min. The two samples with different adaptors from the first hybridization were mixed together simultaneously in the presence of the freshly denatured driver cDNA. The reaction was incubated at 68 °C overnight. Finally, the hybridized cDNA was diluted with 200 µl of dilution buffer pH 8.3 (20 mM HEPES pH 6.6, 20 mM NaCl, 0.2 mM EDTA pH 8.0).

3.8.3 PCR Amplification

There are 2 PCR amplification reactions. In the primary PCR, a reaction mix contained 1 µl of diluted cDNA, 19.5 µl of deionized H_2O, 2.5 µl of 10 X Advantage 2 PCR Buffer, 0.5 µl of dNTP (10 mM), 1 µl of PCR Primer 1 (10 µM) and 0.5 µl of 50X Advantage 2 Polymerase Mix. The thermal cycler was programmed for an initial denaturation of 94 °C for 1 minute followed by 27 cycles consisting of 94°C for 10 seconds denaturation, 66°C for 30 seconds and 72°C for 1.5 minutes. Upon the completion of the primary PCR, 3 µl of the PCR mixture was diluted with 27 µl of sterile H_2O.

In the secondary PCR, a reaction mix contained 1 µl of diluted cDNA from primary PCR, 18.5 µl of deionized H_2O, 2.5 µl of 10 X PCR Buffer, 0.5 µl of dNTP (10 mM), 1 µl of Nested PCR Primer 1 (10 µM), 1 µl of Nested PCR Primer 2R (10 µM) and 0.5 µl of rTaq DNA polymerase (5000 units/ml) (Amersham Biosciences, Inc). The

thermal cycler was programmed for an initial denaturation of 94 °C for 1 minute followed by 15 cycles. Each cycle consisted of 94°C for 10 second, 66°C for 30 second and 72°C for 1.5 minutes. An extension step of 72°C for 10 minutes was added to the end of the PCR program.

3.8.4 Cloning of genes

Genes were cloned using Qiagen PCR Cloning^{plus} Kit (Qiagen, Inc.). PCR products were purified with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Inc) using the protocol recommended by manufacturer. An aliquot of 2 µl (70ng/µl) of PCR products with an average size of about 1000 bp were mixed with 50 ng of pDrive Cloning Vector, 2 µl of distilled water and 5 µl of 2 X ligation master mix, and ligated at 4-16°C overnight.

An aliquot of 2 µl of the ligated mixture was added to 50 µl of Qiagen EZ Competent Cells and incubated for 5 minutes followed by a heat shock procedure at 42°C for 30 seconds. Subsequent to 2 minutes of incubation on ice, 250 µl of SOC medium was added to the mixture. The transformation mixture, 50 µl each, was plated onto 6 LB agar plates containing ampicillin (100 µg/ml), IPTG (5 mM) and X-gal (80 µg/ml). The plates were incubated at 37°C overnight.

After blue/white screening, white colonies were individually inoculated in 3 ml of liquid LB medium containing ampicillin (100 µg/ml) and grown at 37°C overnight with vigorous shaking. Subsequently, an aliquot of 750 µl of each culture was mixed with 750 µl of LB medium containing 30% sterile glycerol, and stored as bacterial stock at -70 °C.

3.9 Minipreparation of plasmid DNA

This protocol of minipreparation of plasmid was modified from the methods of Birnboim and Doly (1979). In brief, cells were pelleted by centrifugation at 15,000 rpm for 20 seconds. The bacteria were resuspended in 100 µl of Ice-cold Solution A containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA (pH 8.0), followed by the addition of 200 µl Solution B containing 0.2M NaOH and 1%SDS. The samples were gently mixed and incubated on ice for 15 minutes. An aliquot of 150 µl

ice-cold Solution C containing 3 M potassium acetate and 1.8 M glacial acetic acid was added to the samples to precipitate the protein and bacterial debris. Supernatant was collected after 6 minutes of centrifugation at 15,000 rpm. Subsequently, the supernatant was extracted with 450 µl of phenol:chloroform:isoamyl-alcohol (25:24:1), then extracted with 450 µl of chloroform:isoamyl-alcohol (24:1). RNA in the sample was digested with 0.5 µl of RNase (10mg/ml) at 37°C for 20 minutes. DNA was precipitated by the addition of 900 µl of ice-cold 100% ethanol and incubated at -20°C for 20 minutes. After 10 minutes centrifugation at 15,000 rpm, 500 µl of 70% ethanol was used to wash the DNA precipitate. The air dried DNA pellet was dissolved in 50 µl of deionized H_2O.

The presence and size of the DNA insert were confirmed by EcoRI enzyme digestion. An aliquot of 2 µl of the plasmid DNA (approximately 3 µg of plasmid DNA) was mixed with 6 µl of the digestion master mix containing 4.5 µl of deionized H_2O, 1 µl of 10 X H buffer (500 mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, 10 mM Dithiothreitol, and 100 mM NaCl), and 0.5 µl of EcoRI (15 units/ µl) (Amersham Biosciences Inc.). The mixture was incubated at 37 °C for 3 hours. The digested samples were analyzed on a 1.5% agarose gel after electrophoresis at 70 volts for 2 hours.

3.10 DNA sequencing and blast search

The plasmid DNA samples with DNA insert were sequenced at the Genome Québec Innovation Centre. The primer SP6 and M13 reverse were used for DNA sequencing in both directions. The DNA sequences obtained were analyzed using the search engines provided by National Center for Biotechnology Information (NCBI).

3.11 Dot blot assay

3.11.1 Preparation of the plasmid DNA

For blotting, 400 ng of plasmid DNA containing the insert was denatured in 800 µl of 6 X SSC containing 0.1 vol of 1 M NaOH at 37°C for 5 minutes. Aliquots of 200 µl of the samples containing 100 ng of plasmid DNA were spotted onto a positively charged nylon membrane, Hybond N+ (Amersham Biosciences, Inc) prewetted with 10 x SSC with a Minifold® System I Dot Blot apparatus (Schleicher and Schuell BioScience, Inc.).

Subsequently, the membrane was soaked in denaturing solution containing 1.5M NaCl and 0.5M NaOH for 5 minutes followed by a neutralization step in solution containing 1.5M NaCl, 0.5M Tris-HCl (pH 7.2) and 0.001M EDTA for 1 minute. The plasmid DNA was immobilized on the membrane by baking at 80°C for 2 hours. The detection of nucleic acid was done using The ECL Direct Nucleic Acid Labelling and Detection Systems (Amersham Biosciences, Inc).

3.11.2 Probe preparation

The cDNA reversed transcribed from the mRNA of the samples with or without stimulation by VIP was used for probe preparation. The concentration of the cDNA was adjusted to 10 ng/µl. Two aliquots of 10 µl of the DNA sample from the stimulated and non-stimulated sample were denatured in boiling water bath for 5 minutes and cooled on ice immediately for 5 minutes. After adding 10 µl of labelling reagent (provided from the kit) and 10 µl of glutaraldehyde solution, the probes were incubated at 37°C for 10 minutes and held on ice.

3.11.3 Hybridization

Blocking reagent was added to ECL Gold Hybridization Buffer (Amersham Biosciences Inc.) with 0.5M NaCl to a final concentration of 5% (w/v). The blot was prewetted with 5 X SSC before being prehybridized with 10 ml of hybridization buffer at 42°C for 15 minutes in a rotisserie hybridization incubator. An aliquot of 10 µl of the labelled probe per blot was added to the hybridization buffer and hybridized overnight in a rotisserie oven at 42 °C.

3.11.4. Washing and Detection

Following the hybridization, the hybridization buffer was replaced by 50 ml 5 X SSC. The blots were incubated at 42°C for 5 minutes then washed with 25 ml of primary washing buffer (Urea 6M, 0.4 % SDS, and 0.5 X SSC) at 42°C for 20 minutes. This washing step was repeated twice for 10 minutes each time. The membrane was then washed twice with 10 ml of 2X SSC at room temperature for 5 minutes with gentle agitation.

The blot was placed on a sheet of Saran Wrap with the DNA side uppermost. The working solution for detection was prepared by mixing 5 ml of detection reagent I with 5 ml of detection reagent II and added directly on top of the blot. After one minute of incubation at room temperature, the excess working solution was drained off. The blot was then wrapped in Saran wrap and exposed to Kodak X-ray film for autography. After the film was developed with Kodak Film Developer, the image was digitalized using Quantity One Software (Bio-Rad Laboratories, Inc.).

3.12 Quantitative PCR (Q-PCR)

3.12.1 ssDNA synthesis

The SuperScript™ II Reverse Transcriptase kit (BD Biosciences Clontech, Inc.) was used to synthesize cDNA for Q-PCR according to the manufacturer's protocol. In brief, a mixture containing1 µl of random hexamer (132 ng/µl) (Amersham Biosciences, Inc.), 2 µl of total RNA (250 ng/µl) obtained from step 3.5, 1 µl of dNTP mix (10mM) and 8 µl of deionized H_2O was heated for 5 minutes at 65 °C, and quickly chilled on ice. After a brief centrifugation, 4 µl of 5 X First-Strand buffer (provided from the kit) and 2 µl of DTT (0.1 M) were added to the mixture and incubated for 2 minutes at 25 °C. Subsequently, 1 µl (200 units) of SuperScript™ II reverse transcriptase was added to the mixture and further incubated for 10 minutes at 25 °C, and then for 50 minutes at 42 °C. Finally, the reaction was inactivated by heating for 15 minutes at 70 °C.

3.12.2 Gene selection for real-time PCR assay

Genes from Table 4.6 were categorized into 3 groups, up-regulated (group A), down-regulated or no change (group B) and genes of interest (group C). Three genes from group A and group B, and four genes from group C were randomly selected for Q-PCR assay. Primers shown in Table 3.12 were designed using DNAMAN sequence analysis software program (Lynnon Corporation), based on the DNA sequencing results.

3.12.3 Real-time (Q-)PCR of transcripts

The Q-PCR assay was performed using Brilliant® SYBR® Green QPCR Master Mix kit (Stratagene Inc.) according to the manufacturer's protocol. In brief, a reaction mix containing 12.5 µl of 2X master mix (provided from the kit), 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 0.375 µl of reference dye (final =30 nM) (provided from the kit), 11.125 µl of deionized H_2O and 2 µl of diluted ssDNA (different dilutions were used depending on the abundance of the particular gene) was placed into the MX3000P™ real-time PCR system (Stratagene, Inc.). PCR conditions were: denaturation at 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C for 30 seconds and annealing at 60 °C for 1 minute. Following completion of the cycles, purity of the amplicons was assessed by melting curve analysis. Fluorescence data were collected twice at the end point of the annealing step and continuously on the 60-95 °C ramp of the dissociation curve. In addition to the genes shown in Table 3.12, PRL and a reference gene,18s rRNA (Ambion, Inc.) were also analyzed by Q-PCR.

The relative changes of the gene expression were analyzed by using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$) described by Livak and Schmittgen (2001). In brief, the change in fold is calculated based on the delta Ct of the sample, which is normalized to the Ct of the reference gene (18s rRNA) with the assumption that the amplification efficiency of the sample and the reference gene are approximately equal.

Table 3.12 List of primers used in the Q-PCR assay

Gene	Fragment	Primer	Size (bp)
FKBP12	Forward	5'-CGAAGATTAGAGTGGCGTTTGG-3'	22
	Reverse	5'-TCAGAGGGCGAAGATGACCA-3'	20
Hsp70	Forward	5'-CTGCTTGTCCTGGTCGCTTA-3'	20
	Reverse	5'-AACAGAGATAGGGTGGGAGC-3'	20
NAPOR	Forward	5'-CGCAGATGGTCTTACAGTTT-3'	20
	Reverse	5'-GGGTTACAGTTCCTTGGCT-3'	19
NPM	Forward	5'-GCTGGTCTCTTTGTTGAAGCA-3'	21
	Reverse	5'-GCCTGTTTATGTCAGTGGTCA-3'	21
PFN	Forward	5'-TGGCTGTCTTGTGATGTGG-3'	19
	Reverse	5'-GCAGGGAAAGAAATGGACAA-3'	20
CYP17	Forward	5'-TCTAAAGGTCACCGCATTGA-3'	20
	Reverse	5'-GCGATTCCTCATCTTACCCT-3'	20
sFRP1	Forward	5'-ATTGTTCTCTGGCTCGGGA-3'	19
	Reverse	5'-CATCCTAAATCCATCTCTCTGC-3'	22
TiPARP	Forward	5'-TCAGGGTTGCAGTAGAGTC-3'	19
	Reverse	5'-CAGGGCGCTTGTGTTTATG-3'	19
TRX	Forward	5'-GGCTGGAGATTAGACAAGACT-3'	21
	Reverse	5'-TGGTGATGTGGTGTTCATTG-3'	20
UBI	Forward	5'-GTCAAGCAAGATGCACA-3'	17
	Reverse	5'-CACTTAAACCACCGTGGAA-3'	19

Abbreviation: FK506 bing protein 12 (FKBP12), Hsp70 heat shock protein (Hsp70), Neuroblastoma apoptosis-related RNA-binding protein (NAPOR), Nucleophosmin (NPM), Profilin (PFN), Steroidogenic enzyme cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17), Secreted frizzed related protein 1 (sFRP1), TCDD inducible polyADP-ribose polymerase (TiPARP), Thioredoxin (TRX), Ubiquitin I (UBI).

CHAPTER IV. RESULTS

4.1 Tissue collection

Collection of pituitary tissue from day 24 embryos was done twice due to difficulty encountered in the SSH analysis and seasonal differences in the stage of development. Embryos collected in summer seemed to be further developed than those collected in winter. The vocal organs of the embryos collected in summer were developed and the chicks chirped before decapitation. Moreover, the yolk sac was obviously smaller in embryos collected in the summer than those collected in winter. The producer (Couvoir Unik, Inc. QC) confirmed that the embryos were on day 24 of development; however, they stated that turkey eggs incubated in winter hatched on day 28 or 29 instead of day 27. The reason for this discrepancy is presumably related to the higher temperature of barns in the summer which would promote increased development prior to be being collected and placed into the incubator. However, assessment of the pituitary content of PRL revealed no differences in either total levels or ratio of isoforms between the two collections. In addition, pituitaries from either preparation responded to VIP stimulation to the same extent both quantitatively and qualitatively.

4.2 Detection of PRL

Total protein was extracted from a pool of 6 pituitary glands from VIP stimulated or non-stimulated samples and aliquots of 2.5 μg (Figure 4.2a). Two immunoreactive bands were detected in pituitary extracts of 24 kDa and 27 kDa which represented NG-PRL and G-PRL respectively. There was about 2- fold increase in the expression of PRL following stimulation with VIP. The relative proportion of NG-PRL and G-PRL in the sample without stimulation by VIP (Figure 4.2a: Lane 1) was 87.2% and 12.8% respectively. The relative proportion of the G-PRL increased from 12.8% to 43.6 % upon the stimulation by VIP.

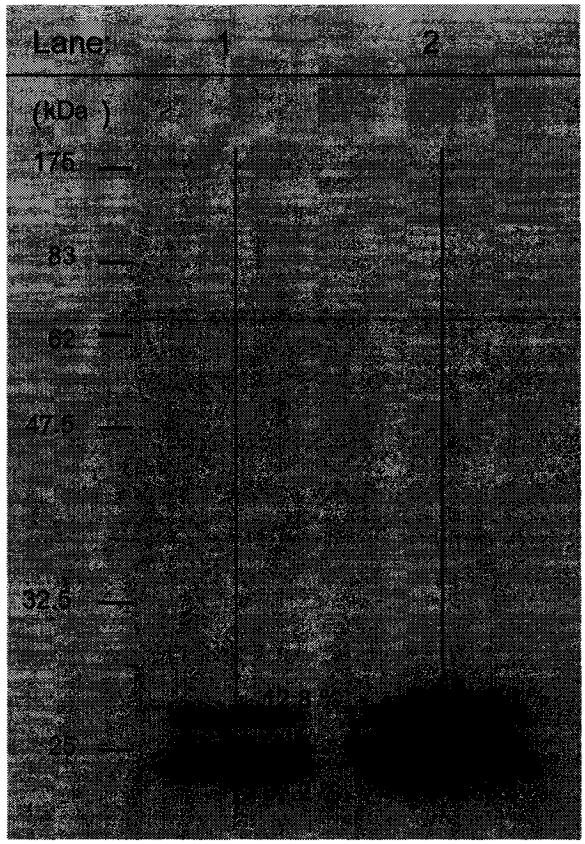


Figure 4.2a Western Blotting. SDS PAGE followed by western blot of 2 pools of 10 pituitary extract (1mg of total protein/ml) of day 24 turkey embryos with or without VIP stimlulation. Lane 1 contains total protein (2.5 μg) extracted from the sample without VIP stimulation, whereas, lane 2 contains the total protein extracted from the sample with VIP stimulation.

The results from RIA are summarized in Figure 4.2b. After 4 hours of incubation, the concentration of PRL in pituitary extracts and in medium increased 2.8 and 5.7 fold respectively, when pituitary glands were incubated with VIP. In the absence of VIP, the medium content of PRL did not change.

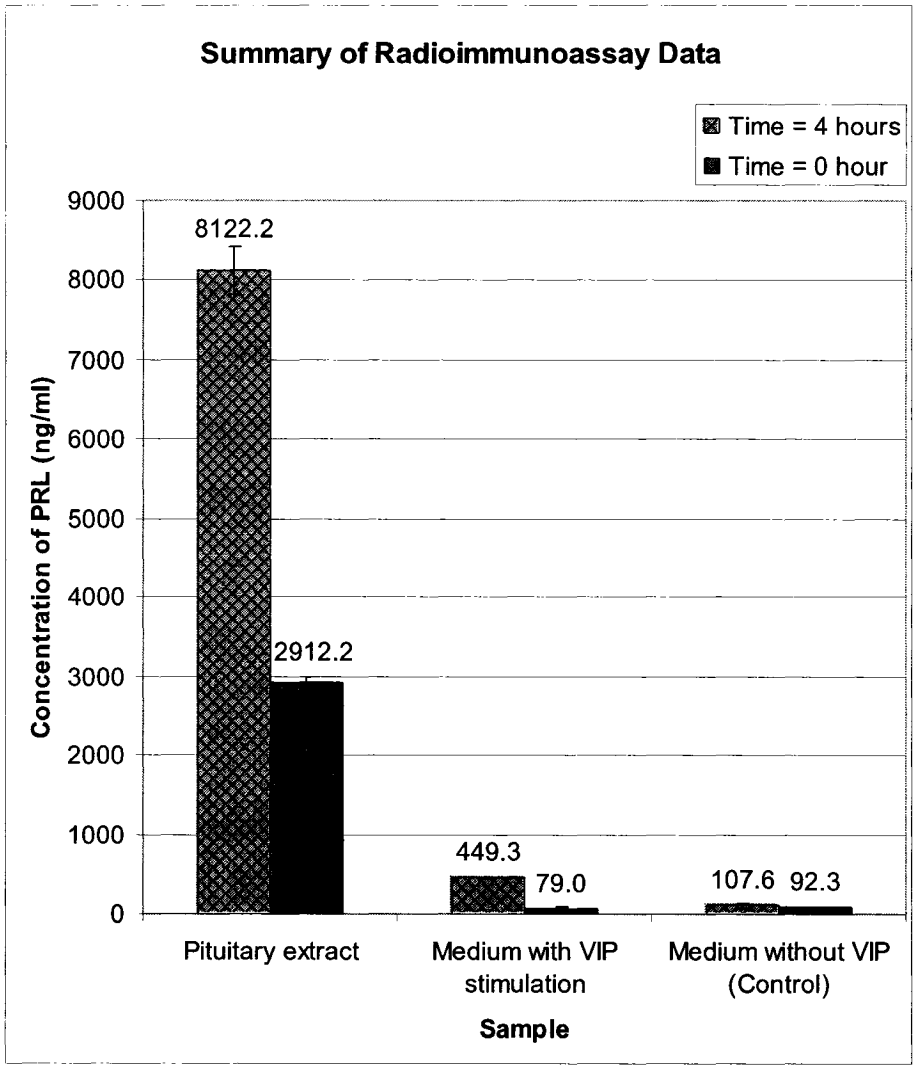


Figure 4.2b Changes in content of PRL measured by RIA in the pituitary gland and medium when glands (n=10) were incubated with 10^{-7} M VIP for 4 hours. The concentrations of PRL (ng/ml) in 2 pools of 10 pituitary extract (1mg of total protein/ml) of day 24 turkey embryos with VIP stimulation (1mg total protein/ml) and the concentrations (ng/ml) of PRL in medium 199 collected before VIP stimulation and 4 hours after VIP stimulation were accessed by RIA.

4.3 Suppressive subtractive hybridization

Suppressive subtractive hybridization was carried out after confirming that pituitary glands responded to stimulation by VIP and levels of PRL were significantly up-regulated (Fig. 4.2). Initially, Oligotex® mRNA Direct Mini Kit (Qiagen, Inc.) was used to isolate the mRNA from a pool of 54 pituitary glands to make a subtractive library. About 10 μg of mRNA was isolated from the pool. The SSH was performed according to the protocol provided by the manufacturer. However, no cDNA was detected after hybridization and PCR amplification. The SSH was repeated several times and in one of the attempts, cDNA was produced. However, sequence analysis of 6 clones indicated no homology to any known DNA sequence. This suggested that the clones were likely to be PCR artefacts. As a result, additional pituitary glands (n=60) were collected from day 24 embryos and total RNA was collected.

Due to the failure of previous attempts, the method of RNA isolation was modified and followed the recommendation from the manufacturer of the Super Smart™ PCR cDNA Synthesis Kit (BD Biosciences Clontech, Inc.). Total RNA was extracted with NucleoSpin® RNA II Kit (BD Biosciences Clontech, Inc.) in combination with Super Smart™ PCR cDNA Synthesis Kit (BD Biosciences Clontech, Inc.) to synthesize cDNA for SSH. About 17.9 μg of total RNA was isolated from a pool of 10 VIP-stimulated pituitary glands and 16.8 μg of total RNA was isolated from a pool of 10 non VIP-stimulated pituitary glands. Five hundred ng of total RNA from each pool was used to synthesize cDNA which was subsequently amplified by long distance PCR. After purification and Rsa I digestion of the PCR products, 12.73 μg and 15.73 μg of cDNA were obtained from the VIP-stimulated and non VIP-stimulated samples respectively. These cDNAs were used to produce both forward (-VIP stimulation as the driver cDNA) and reverse (+VIP stimulation as the driver cDNA) libraries. Figure 4.3 is a gel electrophoresis analysis of the SSH. After the primary PCR amplification, the amount of cDNA in the unsubtracted sample was noticeably more concentrated than the subtracted sample, which indicated that the majority of the cDNA was subtracted after SSH. In the secondary PCR, differentially expressed sequences were enriched with nested primers.

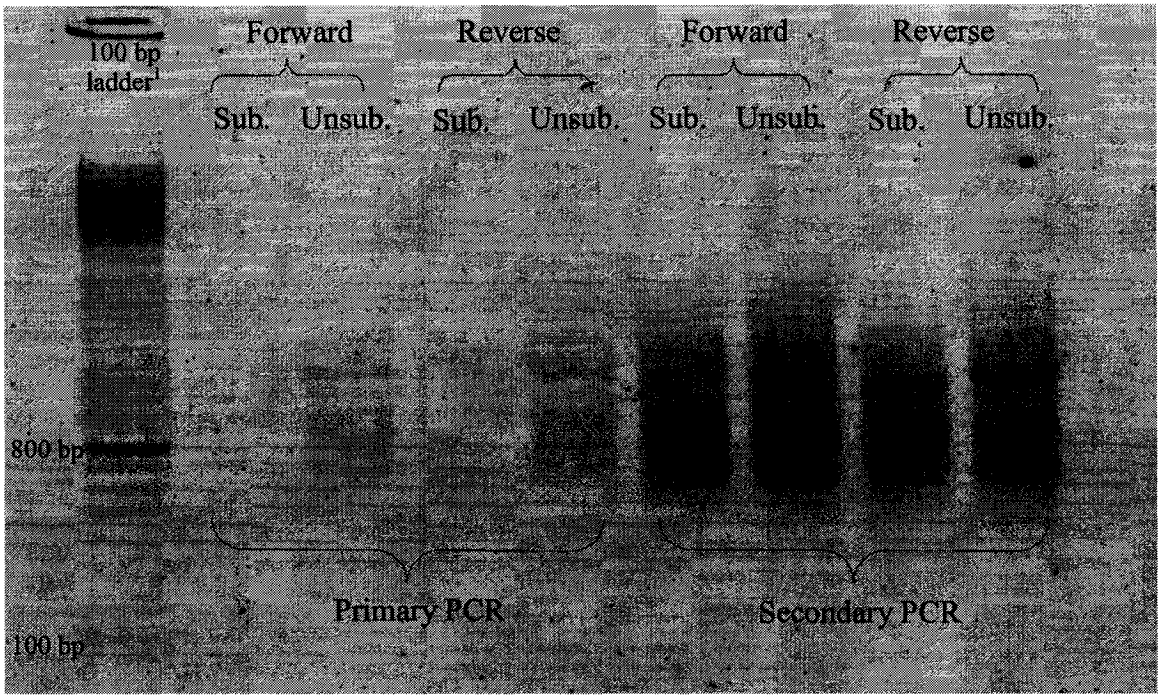


Figure 4.3 Gel electrophoresis analysis of SSH. The concentration of PCR products between the subtracted and unsubtracted library as well as the forward and the reverse library was analysed on a 2% agarose/ethidium bromide (EtBr) gel after electrophoresis at 70 volts for 1 hour. Forward: forward library; Reverse: reverse library; Sub: subtracted cDNA; Unsub: unsubtracted cDNA.
[1] 100 base-pair ladder (Amersham Biosciences, Inc).

4.4 Cloning of Genes

An aliquot of the subtracted cDNA pool was ligated into the plasmid cloning vector transformed into competent cells and plated onto LB agar. Approximately 200 colonies formed on 6 agar plates from each library. After β-galactosidase assay, about 80% of the colonies were white, which indicated that E.coli colonies contained plasmid with cDNA insert. Ninety-six clones from each library were randomly selected and inoculated into 3 ml of LB medium. Subsequent to minipreparartion of plasmid DNA, the presence and size of DNA insert were confirmed by EcoRI enzyme digestion. An

example of EcoRI enzyme digestion of plasmid DNA is shown in Figure 4.4. Different sizes of DNA insert (ranging from 200 bp to 2300 bp) were detected indicating various DNA sequences were picked up from the library. Moreover, two distinct insert bands were detected in some samples. DNA sequence analysis indicated that an internal EcoRI digestion site existed in the clones showing two bands except for clones FS89 and FS91 (not shown). Since DNA sequencing was not successful in sample FS89 and FS91 and two distinct insert bands were detected in both samples; therefore, it is likely that 2 different E.coli colonies were inoculated together and a mixture of two plasmids with different insert was loaded in the well.

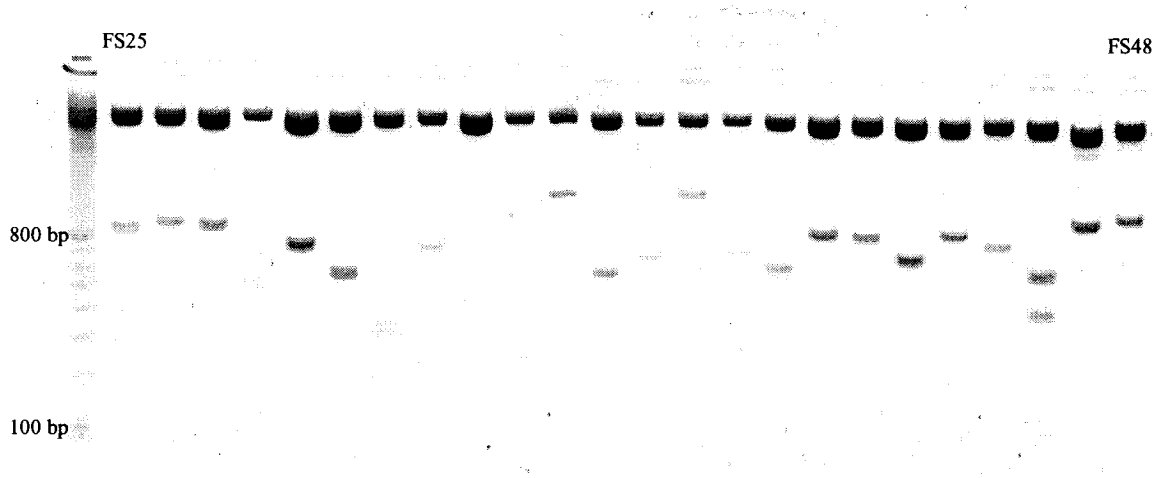


Figure 4.4 EcoRI digestion of plasmid DNA. The presence and size of the DNA insert were confirmed by EcoRI enzyme digestion. Approximately 3 μg of EcoRI-digested plasmid DNA from each sample were analysed on a 1.5% agarose gel after electrophoresis at 70 volts for 2 hours. The first lane is a 100 base-pair ladder (Amersham Biosciences, Inc). Twenty-four plasmids (FS25 to FS48) were shown here. The bands with higher molecular weight represent the plasmid, whereas, the bands with lower molecular weight represent the DNA insert.

4.5 DNA sequencing and blast search

After the confirmation of the presence and size of DNA insert, the plasmids were submitted to the Genome Québec Innovation Centre for DNA sequencing. Since VIP stimulates the production of PRL, the presence of PRL in the blast search results was expected. Moreover, genes related to the glycosylation of PRL were expected to be found in the library. In addition, PRL is a highly versatile hormone; therefore, genes that are regulated by the PRL were also expected to be found in the library. Table 4.6 is a summary of the blast search results for the forward library only. A more detailed table of the blast search results is presented in appendix A.

Of the 96 clones, which were sequenced, 59 were represented once, 7 were represented by multiple clones (2 to 9) and from 4 clones, readable sequence was not obtained. Most of the sequences represented by multiple clones were house-keeping genes with high abundance transcripts such as myelin basic protein (5 clones) and cytochrome I (7 clones). However, ariadne 1 was represented by 9 clones and would normally be expressed at low levels. In total, 66 different putative genes were identified of which 34 (52%) have, as yet, not been assigned a function.

Table 4.5 A summary of blast search results from NCBI		
Identity of Gene	**Brief description of gene product**	**Sample ID**
Glycoprotein hormone alpha-subunit precursor	It is the common alpha subunit of LH, FSH and TSH.	FS01
Heat shock protein	It associates with environmental stress. Anti-apoptotic property of Hsp has also been reported.	FS06, FS55, FS79, FS84
Myelin basic protein	It binds to multiple target proteins such as calmodulin, actin, and tubulinit also appears to function as a signalling molecule in attempts at remyelination.	FS07, FS43, FS60, FS74, FS93
Gap junction protein	It is responsible for cell-cell communication.	FS09
Hemoglobin beta chain	It assembles with the hemoglobin alpha chain to form hemoglobin.	FS28
Ribosomal protein	It is essential for protein translation.	r15S: FS32 r60S: FS03, FS81 r18S: FS52, FS64, FS80
Ubiquitin family (Ubiquitin I)	It has been reported that members of the ubiquitin family affected signal transduction or protein half-life in various disorder. Moreover, it has been reported that these proteins played a role in post-translational modifications and other signal transduction phenomenon by acting as portable recognition tags (Finley, 2001; Larsen and Wang, 2002).	FS35, FS50, FS94
FKBP12	It mediates immunosuppression in mammals by acting as the intracellular receptor for FK506 (Arévalo-Rodríguez *et al.*, 2004).	FS37
Cytochrome P450 17α-hydroxylase/17,20 lyase	It is an steroidogenic enzyme	FS90

Cytochrome family	Members of this family act as catalysts of oxidation-reduction reactions.	Cytochrome I: FS12, FS18, FS38, FS62, FS72, FS82, FS87 Cytochrome III: FS13 Cytochrome VII: FS58
Thioredoxin	It plays a role in oxidant buffering system that controls the cellular redox state (Holmgren, 1985).	FS39
Nucleophosmin	It is a nucleolar phosphoprotein that is responsible for the maturation of rRNA (Okuwaki *et al.*, 2002).	FS48
Vimentin	It is a structural protein found in many cell types.	FS69
Secreted frizzled related protein 1	Secreted frizzled-related protein (sFRP) is an extracellular antagonist of the Wnt signaling pathway (Jones and Jomary, 2002).	FS83
Mitochondrial ATPase 8 and ATPase 6	It involves in the synthesis of ATP.	FS92
Neural cell-adhesion molecule	It is a structural protein found in neural cells.	FS04
FUSE binding protein 3	It is a transcription factors that binds to the far upstream element (c-myc) of single-strand DNA (Davis-Smyth *et al.*,1996).	FS40
Oxidative stress responsive protein	Several oxidative stress responsive proteins have been identified. It has been reported that its expression level increased in human pancreatic cancer cell line (Katoh, 2002).	FS70
TCDD-inducible poly(ADP-ribose) polymerase (TiPARP)	Its function is not clear, but it has been suggested to serve as an adaptive response to TCDD; hence reduce the effects of TCDD on the cell in the future encounter.	FS96
Profilin 2	It mainly regulates the actin polymerization in brain cells.	FS14
Clathrin light chain a gene	It plays a role in cellular transport	FS11

Mitogen-activated protein kinase 6 (MAPK6)	It involves in signal transduction pathway.	FS26
Neuroplastoma apoptosis-related RNA-binding protein (NAPOR)	It has been suggested that NAPOR involved in the regulation of RNA processing events such as alternative splicing and RNA editing (Ladd and Cooper, 2004).	FS57
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	It is a house keeping gene that has a multiple functions such as glycolysis and apoptosis.	FS76
Gallus gallus similar to Rnps1 protein, mRNA	It is a pre-mRNA splicing activator. It has been suggested to be involved in the post-mRNA splicing process (Sakashita *et al.*, 2004).	FS29
Gallus gallus similar to Extracellular superoxide dismutase [Cu-Zn] precursor (EC-SOD) (LOC422810), mRNA	It removes superoxide radicals from the extracellular space by catalyzing the dismutation of superoxide radical to hydrogen peroxide and oxygen (Skrzycki and Czeczot, 2004).	FS53
Gallus gallus similar to calnexin precursor - dog (LOC416288), mRNA	Calnexin is an intergral protein found on the membrane of endoplasmic reticulum, which plays an important role in the folding of glycoprotein (Ellgaard and Frickel, 2003)	FS88
Gallus gallus similar to ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1; ariadne 1 mRNA	Its possible role is to transfer ubiquitin from specific E2 ubiquitin-conjugating enzymes to substrates by acting as an E3 ubiquitin-protein ligase	FS23, FS25, FS41, FS42, FS47, FS56, FS59, FS73, FS75
Unknown genes	N/A	FS02, FS05, FS08, FS10, FS15, FS17, FS19, FS21, FS22, FS24, FS27, FS30, FS31, FS33, FS34, FS36, FS44, FS45, FS46, FS49, FS51, FS54, FS61, FS63, FS65, FS66, FS67, FS71, FS77, FS78, FS85, FS86, FS91, FS95
Error	N/A	FS16, FS20, FS68, FS89

4.6 Dot blot assay

The 96 clones were spotted in duplicate (400 ng of plasmid) onto Hybond N+ nylon membrane (Amersham Biosciences, Inc) using a Minifold® System I Dot Blot apparatus (Schleicher and Schuell BioScience, Inc.). Therefore, there were 4 membranes, 2 for the forward library and 2 for the reverse library. The appropriate cDNA reversed transcribed from the mRNA of the samples with or without stimulation by VIP was used as probe for hybridization and the resultant images were digitalized.

The subtraction efficiency was assessed by calculating the ratio of intensity of the clones hybridized to the VIP-stimulated and non VIP-stimulated probes. A ratio greater than one indicated up-regulation of the particular gene, whereas, a ratio smaller than one indicated down-regulation. A ratio equal to one indicated no changes in the expression level. Theoretically, all 96 clones produced in the forward library should be up-regulated as a result of stimulation by VIP. The ratio of intensity between the VIP-stimulated and non VIP-stimulated samples at different exposure time are shown in Table 4.6. This dot blot assay was intended to be used as a differential screening of the subtraction library to verify the subtraction efficiency and to decide genes that would be analysed with Q-PCR. The subtraction efficiency is represented by the percentage of samples showing differential expression in the VIP stimulated samples, which are 39%, 57% and 46% at 2 minutes, 15 minutes and 2 hours of exposure time, respectively. Results are not consistent at different exposure times. Sixteen percent of the samples showed differential expression at all three different exposure times. Thirty one percent of the samples showed differential expression at two or more exposure time. Finally, 72% of the samples showed differential expression at one or more exposure time. The intensity of the 96 clones on the blot did not indicate substantial differences. The ratio of intensity differences among the samples ranged from 0.95 to 1.07. The clones were categorized into 3 groups, up-regulated (group A), down-regulated or unchanged (group B) and genes of interest (group C) based on the ratio of intensity between the VIP-stimulated and non VIP-stimulated and the sequence analysis. Three genes from group A and group B, and four genes from group C were randomly selected for Q-PCR assay.

Table 4.6 Ratio of intensity of clones hybridized to probes derived from VIP stimulated and non VIP stimulated samples

Sample ID	Exposure Time = 2 minutes	Exposure Time = 15 minutes	Exposure Time = 2 hours	Sample ID	Exposure Time = 2 minutes	Exposure Time = 15 minutes	Exposure Time = 2 hours
FS01	1.03	1.03	1.04	FS49	0.99	1.00	0.96
FS02	0.99	0.99	0.96	FS50	1.10	1.05	1.02
FS03	1.01	1.01	0.95	FS51	0.97	1.01	0.99
FS04	0.98	0.99	0.94	FS52	0.99	1.03	1.06
FS05	0.99	0.98	0.93	FS53	0.98	0.99	0.98
FS06	0.98	0.98	0.91	FS54	1.00	1.00	0.99
FS07	0.97	0.98	0.90	FS55	0.99	1.02	1.03
FS08	1.00	0.97	0.91	FS56	1.05	1.06	1.04
FS09	0.98	0.97	0.89	FS57	1.00	1.02	1.04
FS10	0.99	0.98	0.95	FS58	0.98	1.00	1.05
FS11	1.01	0.98	0.98	FS59	1.06	1.05	1.06
FS12	1.10	1.01	1.01	FS60	1.00	1.01	1.09
FS13	1.02	1.04	1.08	FS61	0.98	1.01	0.99
FS14	1.03	1.02	1.04	FS62	1.08	1.02	1.01
FS15	0.99	1.03	1.06	FS63	0.97	1.00	0.95
FS16	0.97	1.01	1.03	FS64	0.97	1.01	0.97
FS17	0.95	1.01	1.02	FS65	0.98	0.99	0.95
FS18	0.98	1.00	0.97	FS66	0.97	0.99	0.96
FS19	0.98	0.97	0.88	FS67	0.97	1.00	0.99
FS20	0.98	0.98	0.93	FS68	1.08	0.99	1.00
FS21	0.99	0.99	0.94	FS69	0.98	0.99	1.03
FS22	1.01	0.98	0.94	FS70	0.99	0.98	1.03
FS23	1.07	1.01	1.01	FS71	0.99	1.01	1.08
FS24	1.04	1.00	1.03	FS72	1.09	1.04	1.04
FS25	1.08	1.07	1.03	FS73	1.06	1.03	1.02
FS26	0.99	1.01	1.05	FS74	0.96	1.01	0.99
FS27	1.01	1.04	1.13	FS75	1.06	1.02	1.01

FS28	0.99	1.02	1.06	FS76	0.99	1.00	0.98
FS29	0.96	1.03	1.07	FS77	0.99	1.01	0.99
FS30	0.95	1.00	0.99	FS78	0.95	0.99	0.96
FS31	0.97	1.00	0.97	FS79	0.96	0.99	0.95
FS32	1.00	1.01	1.00	FS80	0.95	0.99	0.96
FS33	1.00	1.00	0.97	FS81	1.04	0.98	0.97
FS34	1.01	1.02	0.99	FS82	1.08	0.99	0.99
FS35	1.04	1.04	1.05	FS83	0.98	1.00	0.99
FS36	1.04	1.02	1.09	FS84	1.00	1.01	1.02
FS37	0.99	1.01	0.95	FS85	0.98	1.00	0.93
FS38	1.11	1.00	1.00	FS86	0.98	1.01	0.97
FS39	0.98	1.01	1.01	FS87	1.11	0.99	0.98
FS40	0.99	1.02	1.02	FS88	0.99	0.99	0.97
FS41	1.03	1.04	1.03	FS89	1.07	1.00	1.00
FS42	0.98	1.04	1.02	FS90	0.97	1.00	0.97
FS43	1.00	1.02	1.04	FS91	0.98	0.98	0.94
FS44	0.99	1.00	0.99	FS92	1.02	0.98	0.95
FS45	0.99	1.01	1.00	FS93	1.00	1.00	0.97
FS46	0.99	1.00	1.00	FS94	1.06	1.00	1.00
FS47	1.07	1.05	1.04	FS95	1.01	1.00	1.01
FS48	1.04	1.00	1.01	FS96	0.98	0.99	1.01

Note: The number represents the ratio of the intensity of the VIP-stimulated sample: the intensity of the non VIP-stimulated sample. A value greater than 1 represents up-regulation of the particular gene; in contrast, a value less than 1 represents down-regulation, and zero represents no changes after the stimulation by VIP.

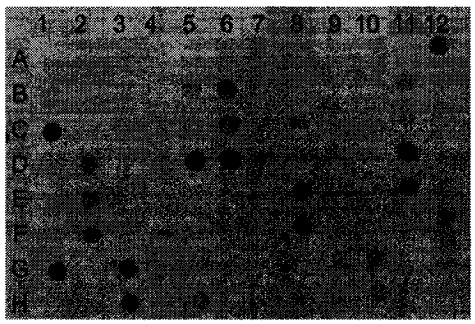
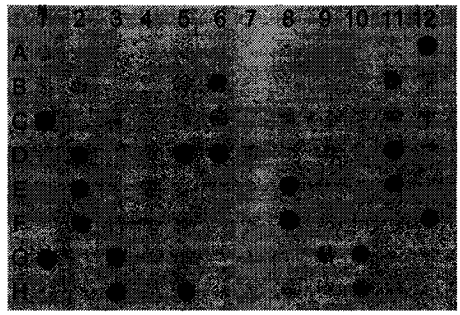
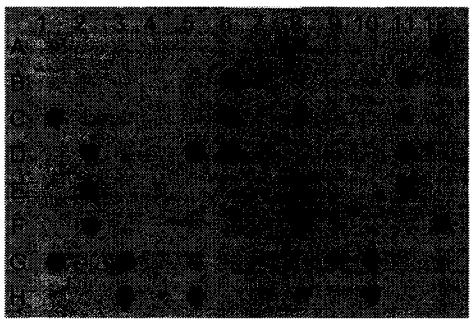
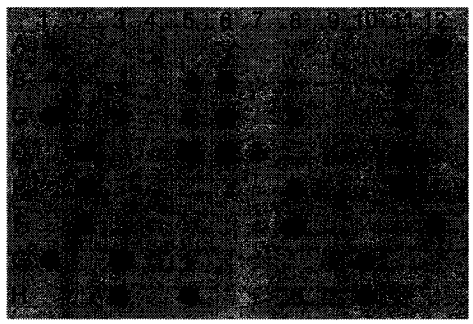
Exposure time	Membrane with VIP simulation	Membrane without VIP simulation (Control)
2 minutes		
15 minutes		
2 hours		

Figure 4.6 Dot blot image at different exposure time. Dot blot assay was performed with 100 ng of plasmid of each sample loaded onto a Hybond N+ (Amersham Biosciences, Inc) in order to screen for differentially expressed genes. Samples were loaded onto the membrane in increasing order from left to right and top to the bottom i.e. Well A1 is sample FS01, well H12 is sample FS96. Different exposure times, 2 minutes, 15 minutes and 2 hours were used in order to visualize the less abundant genes.

4.7 Real-time (Q-) PCR of transcripts

The relative changes of the gene expression were analyzed by using the comparative threshold cycle (Ct) method described by Livak and Schmittgen (2001). The normalized value in fold relative to the control equals to $2^{-\Delta\Delta Ct}$. In order to have a valid relative quantification of the genes, the Ct of the reference gene, 18s rRNA was adjusted to be within a range of 5 Ct difference of the target gene by different dilution of cDNA. Figure 4.7 is a graphic representation of the results from real-time PCR.

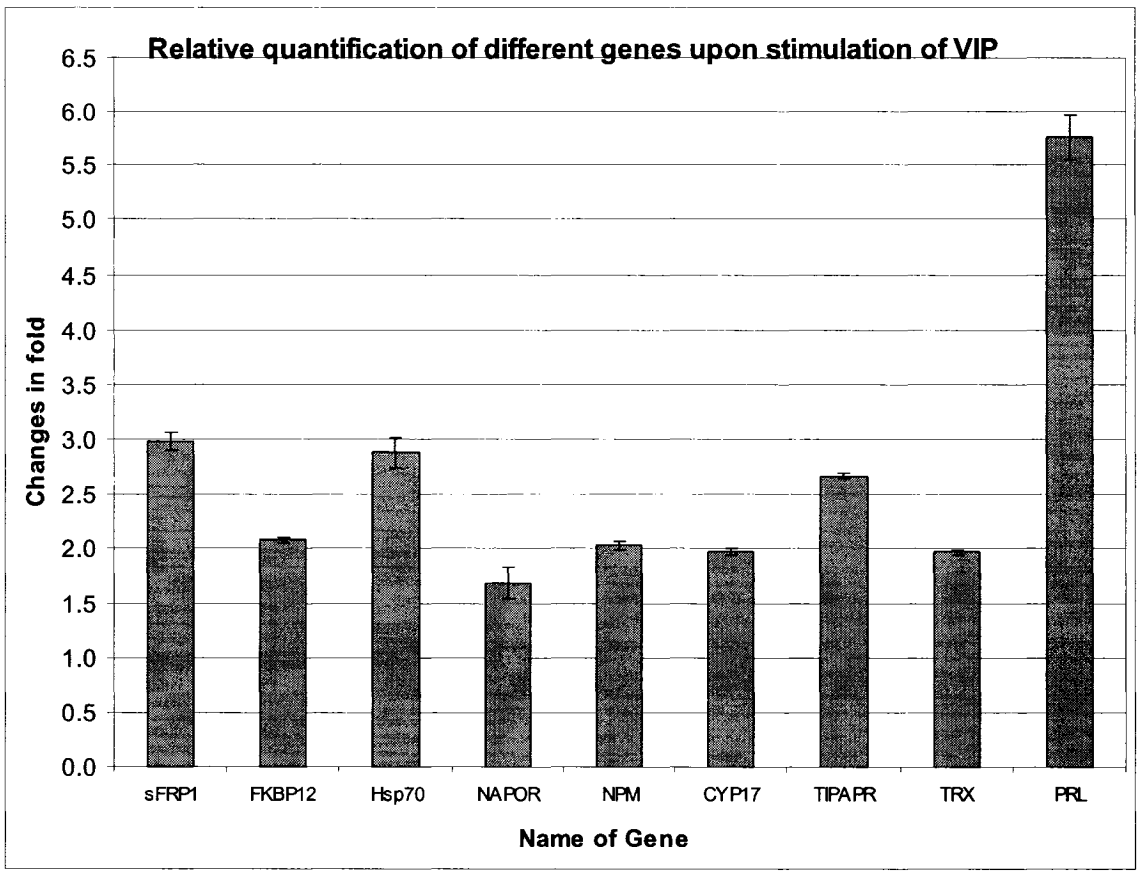


Figure 4.7 Graphical representation of the relative quantity of the target genes
The relative quantity of different genes presented in this graph was normalized with 18s rRNA and calculated according to the comparative threshold cycle (Ct) method described by Livak and Schmittgen (2001).

Table 4.7 Relative changes of gene expression upon stimulation by VIP

Turkey embryonic pituitary glands	Target genes	Mean Ct (n=3)	△Ct	△△Ct	Normalized amount relative to the control
Control (Without VIP)	sFRP1[a]	25.94	5.08	0.00	1.00
	FKBP12[a]	23.32	2.47	0.00	1.00
	Hsp70[a]	22.38	1.53	0.00	1.00
	NAPOR[b]	31.88[c]	11.02	0.00	1.00
	NPM[a]	21.88	1.02	0.00	1.00
	CYP17[a]	22.11	1.25	0.00	1.00
	TiPAPR[a]	24.01	3.15	0.00	1.00
	TRX[a]	22.34	1.49	0.00	1.00
	PRL[b]	33.29[c]	4.16	0.00	1.00
	PFN	No amplification			
	UBI	No amplification			
Sample (With VIP)	sFRP1[a]	25.31	3.50	-1.58	2.99
	FKBP12[a]	23.22	1.41	-1.06	2.08
	Hsp70[a]	21.81	0.00	-1.53	2.88
	NAPOR[b]	32.08[c]	10.27	-0.76	1.69
	NPM[a]	21.81	0.00	-1.02	2.03
	CYP17[a]	22.08	0.27	-0.98	1.97
	TiPAPR[a]	23.54	1.74	-1.42	2.67
	TRX[a]	22.32	0.51	-0.98	1.97
	PRL[b]	31.22[c]	1.59	-2.53	5.76
	PFN	No amplification			
	UBI	No amplification			

[a] The expression of these genes were normalized with 18s rRNA that had a mean Ct of 20.86 and 21.81 in the control and sample respectively.
[b] The expression of these genes were normalized with 18s rRNA that had a mean Ct of 29.13 and 29.63 in the control and sample respectively.
[c] n=2 instead of n=3

CHAPTER V. DISCUSSION

5.1. General discussion

Two immunoreactive bands were detected on the western blot assay. These two bands represent G- and NG-PRL isoforms, which have a molecular weight of 27 kDa and 24 kDa respectively as described by Corcoran and Proudman (1991). Moreover, analysis of western blot data indicated that stimulating turkey embryonic pituitary glands with VIP not only induced the secretion of PRL, but also the glycosylation of PRL since the relative levels of G-PRL increased from 12.8 % to 43.6 %. In addition, the RIA data revealed that the concentration of PRL in the pituitary extracts (n=6) had increased 2.8 fold, and the concentration in the medium containing pituitary stimulated by VIP has increased 5.7 fold. It has been reported that the levels of PRL started to increase on day 22 of the embryonic stage in turkey (Bédécarrats *et al.,* 1999a). Therefore, the slight increase of concentration of PRL in M199 containing the control pituitary glands (1.2 fold) was probably due to the effect of endogenous release of PRL by hypothalamic releasing factors prior to sample collection. The observations from western blot and RIA data were supported by Bédécarrats *et al.* (1999b) where adult turkey pituitary glands were stimulated by VIP using a perfusion system. The PRL released upon the stimulation by VIP was newly synthesized and possibly originated from the stored PRL in the cell. Results from real-time PCR analysis of PRL gene indicated that there was a 5.8-fold increase in the levels of PRL mRNA after the stimulation by VIP. Therefore, de novo synthesis of PRL was responsible for the increased level of PRL. Since an increased level of glycosylation of PRL was observed, other genes must have been also expressed and involved in the glycosylation process. Glycosylation occurs in the rough ER (N-link glycosylation) and Golgi apparatus (O-link glycosylation or modification of the N-link glycosylation) where carbohydrate chains are added to the protein. Thus genes specifically involved in glycosylation of PRL are possibly responsible for signalling and anchoring PRL to the proper organelle for processing.

In this thesis, only the forward library, which represents genes differentially expressed upon stimulation by VIP, is reported. The results of blast search from NCBI

(table 4.3) indicated a high homology between the genes from the forward library and the chicken genome project. Although half of the genes (52 %) have not been assigned a function, a match in the chicken genome indicated that these genes were not PCR artefacts. It is interesting to note that PRL gene was not picked up from the subtracted cDNA pool even though the mRNA levels of PRL has increased 5.8 fold. It is possible that the abundance of the repeated genes in Table 4.5 is much higher than PRL. For example, multiple independent clones encoding house keeping genes such as cytochrome I (7 clones), myelin (5 clones) and rRNA 18s (3 clones) were randomly sequenced. The detection of house keeping genes in the subtracted cDNA pool indicated that not all the commonly expressed genes in the stimulated and non-stimulated samples were indeed subtracted. Conversely, 9 independent clones were sequenced which encoded the ariadne 1 gene. The product of this gene is involved in protein ubiquitination and is normally expressed at low levels. The high levels of ariadne 1 in the library (9 out of 92 clones) suggested that it was greatly enriched during the subtraction process.

Theoretically, all 96 clones produced in the forward library should be upregulated as a result of stimulation by VIP and this was assessed using dot blotting. The subtraction efficiency is the percentage of genes that indicates upregulation in the dot blot assay. The subtraction efficiency was accessed at 2 minutes, 15 minutes and 2 hours of exposure time, which were 39%, 57% and 46% respectively. Multiple exposure times were used since the required exposure time was dependent on the abundance of a particular gene. Genes that had low abundance required a longer exposure time, whereas, highly abundant genes required a shorter exposure time. When the membrane was exposed for 2 minutes, some of the genes were not detected. On the other hand, when the exposure time was increased to 15 minutes or 2 hours, signal saturation occurred in some of the samples. For example, in Figure 4.6, the signal emission in well D5 and D6 had already been saturated at 2 minutes exposure. Conversely, the signal in well H12 was not detected. The cause was possibly due to non-specific binding of the probes to the membrane. The probes and the cDNA used for the SSH were both reversed transcribed using Super Smart^{TM} PCR cDNA Synthesis Kit (BD Biosciences Clontech, Inc.), in which an oligonucleotide (Smart^{TM} II A oligonucleotide, provided from the kit) was added to the cDNA. Since both the probe and the cDNA insert of the plasmid possessed

this oligonucleotide, non-specific binding occurred to a certain degree. Therefore, candidate genes for real-time PCR were randomly selected from 3 categories, up-regulated, down-regulated or unchanged, and genes of interest.

The ten genes analysed with real-time PCR have shown at least 2 fold increase in their expression after stimulation by VIP. No amplification was observed on PFN and UBI. In order to investigate the cause of the failure in amplification, standard PCR with different PCR conditions such as optimization of annealing temperature and variable concentration of $MgCl_2$ were tested. It is likely that the primers for these two genes were not properly designed. As for NAPOR, amplification was successful; however, the Ct of the sample (31.88 in control sample, 32.28 in the stimulated sample) is within a range of five Ct of the no template control (36.80). Therefore, analysis of the NAPOR would not be as accurate as the analysis of the other genes. Nonetheless, the relative quantity of NAPOR was calculated and presented in Figure 4.7 and Table 4.7.

5.2 Discussion on selected genes analyzed with Q-PCR

It is interesting to note that 17% of the known genes isolated from the subtracted cDNA pool were related to the control of cell proliferation and apoptosis. One of the possible causes is the changes of cell type in the pituitary gland during hyperprolactinemia. It has been reported that the replacement of somatotrophes by lactotrophes in the caudal lobe of pituitary gland was associated with hyperprolactinemia (Ramesh *et al.*, 1996). In mammals, studies have indicated that lactotrophes differentiated from somatotrophes through an intermediate cell type called mammosomatotrophes which secreted both GH and PRL (Voss and Rosenfeld, 1992; Rhodes *et al.*, 1994; Dasen and Rosenfeld, 2001; Burgess *et al.*, 2002; Scully and Rosenfeld, 2002). However, it has been reported that embryonic chicken pituitary glands had a low abundance of mammosomatotrophes; thus it is unlikely that lactotrophes arised from somatotrophes in chicken embryo as reported in mammals (Fu *et al.*, 2004). Moreover, it has been reported when the incubation behaviour of turkey hen was interrupted, lactotrophes underwent apoptosis and somatotrophes differentiated from non-somatotrophic cell, which in turn decreased the levels of PRL (Ramesh *et al.*, 2001). A reciprocal cellular

response could also occur at the onset of incubation. Therefore, the expression of genes associated with cell proliferation and apoptosis observed in this study may be an early indicator of the genes expressed associated with the differentiation of somatotrophes to lactotrophes. Moreover, both the presence of PRLR and the positive correlation between the levels of PRL and the levels of PRLR in the pituitary glands of turkey indicated that PRL could act as autocrine/paracine hormone (Zhou *et al.,* 1996). In other words, the changes in the pituitary gland including recruitment, hypertrophy and increased cellular population of lactotrophes (Proudman and Wentworth., 1996) may be increased by PRL through an autocrine/paracrine system.

Nevertheless, the mitogenic activity of PRL has been reported in many tumour cells such as mammary tumours (Nandi *et al.,* 1995), prostatic hyperplasia and neoplasia (Lane *et al.,* 1997), tumours of the female reproductive tract (Nowak *et al.,* 1999), and T-leukemic cell line (Matera *et al.,* 1997). It has been suggested that PRL had a mitogenic effect on the pituitary glands of juvenile cockerels (Maiti and Chakraborty, 1981). Although the mitogenic effects of PRL in pituitary gland have not been well documented, its potential in promoting cell proliferation cannot be excluded.

Studies have indicated that VIP was both a neurotrophic factor releasing agent and an astroglial mitogen on cultures derived from murine spinal cord (Brenneman *et al.,* 1990). In addition, VIP has been shown to be a mitogen in human pituitary adenoma cell cultures (Fazekas *et al.,* 2000). Therefore, the induction of cell proliferation related genes could be a result of the combined mitogenic property of VIP and PRL.

5.2.1 FKBP12 for FK506 bing protein 12

FKBP12 belongs to a subclass of immunophilin proteins, FK506-binding proteins (FKBPs) which are under the prolyl-isomerase enzyme family (Davies and Sánchez, 2005). It is reported that FKBP mediated immunosuppression in mammals by acting as the intracellular receptor for FK506 (Arévalo-Rodríguez *et al.,* 2004). It has been demonstrated that immunosuppressant ascomycin, which bound to FKBP12 and inhibited calcineurin activity by forming a complex with calcineurin (Liu *et al.*, 1991; Kay, 1996), abolished both PACAP-evoked VIP biosynthesis and mRNA up-regulation,

at 100 nM (Lee *et al.*, 1999). Therefore, it is possible that the 2 fold increased level of FKBP12 4 hours after VIP stimulation may be a mechanism to compensate for the high level of VIP in the culture medium.

5.2.2 Secreted frizzled related protein 1

Secreted frizzled-related protein (sFRP) is an extracellular antagonist of the Wnt signalling pathway. Five different sFRP have been identified in human, and 4 sFRP have been identified in chicken (Jones and Jomary, 2002). The variants differ in their location of expression, and molecular weight. In terms of function, they are very similar. All members in this group contain a frizzled-like cysteine-rich domain and function as modulators of Wnt-Frizzled signals (Hsieh *et al.*, 2003). The antagonist effects of sFRP are exerted through interaction with Wnt or competing for the Wnt receptors (Bafico *et al.*, 1999). It has been reported that Wnt played key roles in differentiation, embryonic development and cell proliferation (Cadigan and Nusse, 1997). It has been shown that Wnt induced the expression of the transcription factor Pitx2, which in turn activated other specific growth-regulating genes to promote cell proliferation during organogenesis of the anterior pituitary in mice (Kioussi *et al.,* 2002). It has been suggested that up-regulation of Wnt signalling pathway led to tumourigenesis and development of pituitary adenoma in human (Semba *et al.,* 2001). Hence sFRP alters cell differentiation and cell proliferation by down-regulating the Wnt activity. Furthermore, it has been reported that PRL up-regulated the expression of sFRP-4 in hypophysectomized rat ovaries, and stimulated the secretion of sFRP-4 protein from luteinized rat granulosa cells in culture. Hence, regulation of sFRP by PRL might be important for modulating Wnt-Frizzled signals (Hsieh *et al.*, 2003). Therefore, the 3 fold up-regulation of sFRP1 observed in our experiment might be a mechanism for the cell to control cell proliferation in response to the increased levels of PRL.

5.2.3 Heat shock protein 70

Heat shock proteins (Hsp) are a group of proteins that are present in all prokaryotes and eukaryotes. They are not only expressed when the cells undergo environmental stress (heat shock and UV radiation), pathological (viral, bacterial,

inflammation and autoimmunity), or physiological stimuli (growth factors, cell differentiation, hormonal stimulation, or tissue development), but also in normal cells and function as chaperones (De Maio, 1999; Lindquist and Craig, 1988; Asea *et al.,* 2002). It has been reported that administration of PRL to the rat Nb2 T lymphoma cell line induced the expression of heat shock protein 70 (Hsp70) gene (Yu-Lee, 1990). An anti-apoptotic property of Hsp has also been reported. It has been shown that Hsp70 suppressed apoptosis by forming a complex with p53-inducible cell-survival factor (Park and Nakamura, 2005) which in turns bound to the apoptosis protease activating factor-1 to interfere with the apoptosome complex formation (Saleh *et al.,* 2000; Beere *et al.,* 2000). Therefore, the expression of Hsp70 is to maintain the cellular homeostasis by counterbalancing the effect of apoptotic genes.

In our experiment, many different factors could directly or indirectly contribute to the increased levels of Hsp70 (3 fold) including physical damage of pituitary glands during sample collection, administration of VIP, increased levels of PRL and other apoptosis related genes. The increased levels of Hsp70 were probably a cellular response to maintain cellular homeostasis.

5.2.4 Neuroblastoma apoptosis-related RNA-binding protein

It has been reported that neuroblastoma apoptosis-related RNA-binding protein (NAPOR) was an alternative RNA slicing regulator that repressed the alternative splicing at exon 5 and promoted alternative splicing at exon 21 on the N-methyl-D-aspartate (NMDA) receptor R1 transcript in rat forebrain (Zhang *et al.,* 2002). NMDA R1 receptor has been reported to be crucial for neuronal survival and maturation, and stabilization of synaptic connections in mammals (Zukin and Bennett, 1995). The physiological function of NAPOR is still unclear. Studies indicated that the expression of NAPOR was associated with programmed neuronal cell death, which suggested its role in the development of central nervous system (Choi *et al.,* 1999). Since the cellular population of the pituitary was changing during hyperprolactinemia upon the stimulation by VIP, it is conceivable that NAPOR (2 fold) played a role in the apoptosis of somatotroph in the pituitary gland.

5.2.5 Nucleophosmin

Nucleophosmin (NPM) is a nucleolar phosphoprotein that is responsible for the maturation of rRNA (Okuwaki *et al.,* 2002). Over expression of NPM has been reported in actively proliferating cells and cancer cells (Li *et al.*, 2004). It has been suggested that the function of NPM in hypoxia-driven cancer progression was to suppress p53 activation and to maintain cell survival by preventing the phosphorylation of p53 at serine-15 (Li *et al.*, 2004). As mentioned in section 5.1.1, apoptosis of somatotrophes and cell proliferation of lactotrophes were occurring at the same time. The expression of NPM (2 fold) might be responsible to control apoptosis in order to maintain cellular homeostasis.

5.2.6 Steroidogenic enzyme cytochrome p450 17α-hydroxylase/17,20 lyase

Steroidogenic enzyme cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17) belongs to the steroidogenic enzyme cytochrome p450 (p450) family encoding six distinct steroid hydroxylases (Nelson *et al.,* 1993) that are involved in the conversion of cholesterol to pregnenolone in steroidogenesis (Miller, 1988). It has been reported that p450 enzymes were expressed in the adrenals as well as gonads and pituitary peptide hormones such as VIP in a hypothalamus–pituitary–adrenal regulatory axis controlled the expression of p450 (Chung and Bresnick, 1997). De novo steroidogenesis from cholesterol in the brain has been reported in many vertebrates including mammals (Beaulieu *et al.*, 1997), birds (Tsutsui *et al.,* 1995), amphibians (Beaujean *et al.,* 1999) and fish (Sakamoto *et al.,* 2001). It has been reported that dehydroepiandrosterone (DHEA), one of the most abundant neurosteroids in the avian brain was converted from pregnenolone by CYP17 (Matsunaga *et al.,* 2001). High levels of gene transcription of CYP17 have been reported in the quail diencephalon and mesncephalon (Matsunaga *et al.,* 2001). Although no data concerning the expression of CYP17 in the avian pituitary glands have been reported, it has been suggested that CYP17 was expressed in the pituitary gland of male zebra finch due to the observation of high aromatization activity in pituitary gland which was believed to be a result from local aromatization rather than peripheral aromatization (Schlinger and Arnold, 1991). This speculation supported our finding of CYP17 (2 fold) in the pituitary gland where aromatization took place.

5.2.7 3,7,8 Tertrachlorodibenzo-p-dioxin inducible polyADP-ribose polymerase

3,7,8 Tertrachlorodibenzo-p-dioxin (TCDD) is an environmental pollutant that is proven to be a carcinogen. It has been reported that TCDD inducible polyADP-ribose polymerase (TiPARP) was induced by TCDD in mouse cells (Ma *et al.,* 2001). TiPARP belongs to the PARP family consisting of six polymerases that might function in genome protection, telomere replication, cellular transport and other cellular functions (Smith, 2001). Moreover, PARPs are reported to alter the function of nuclear protein by using NAD+ as a substrate to transfer ADP-ribose onto glutamic acid residues of a protein acceptor; repeated rounds of ADP-ribosylation leads to the formation of poly(ADPribose) chains on the protein (Homburg *et al.,* 2000; Ma *et al.,* 2001; Smith, 2001). It is known that the activation of PARP-1 is a response to transient and localized DNA strand breaks (Nguewa *et al.,* 2005); however, the activation of other PARPs is still not clear. The function of TiPARP is not clear, but it has been suggested that TiPARP served as an adaptive response to TCDD; hence reducing the effects of TCDD on the cell in the future encounter (Nguewa *et al.,* 2005).

As mentioned above, TiPARP can be induced by TCDD. One of the sources of TCDD is from PVC plastic. It is possible that TCDD from the plastic ware diffused into the medium and triggered the expression of TiPARP. It is interesting to note that TCDD also induced the expression of cytochrome p450 in human cell culture (Li *et al.,* 1998) and chicken embryo (Walker *et al.,* 2000). Although whether or not there was enough amount of TCDD, if any from the plastic ware that diffused into the medium is uncertain, the possible effect from TCDD cannot be excluded. However, it is unlikely to be the case because the samples without VIP stimulation were also incubated in the same type of plastic ware; thus the expression of TiPARP in both VIP stimulated samples and non VIP stimulated sample should be the same. The TiPARP found in turkey embryonic pituitary glands shared only 78% of identity with human TiPARP (Sample FS96 in Table 4.3). It is possible the turkey gene has other cellular functions such as cellular transport and chromosome maintenance and stability. For example, PARP-1 has been reported to played an important role in programmed cell death due to the fact that high levels of poly(ADP-ribose) was observed during the early stages of apoptosis (Simbulan-

Rosenthal *et al.,* 1998; Diefenbach and Bürkle, 2005). Moreover, it has been demonstrated that PARP-1 induced a caspase-independent pathway of programmed cell death by signalling the translocation of apoptosis-inducing factor from the mitochondria (Cregan *et al.,* 2004). The turkey TiPARP may have similar functions.

5.2.8 Thioredoxin

It has been shown that reduction/oxidation (redox) reactions are involved in the control of biological processes including the functional modulation of transcription factors (Nakamura *et al.*, 1997). In the case of the endocrine system, the cellular redox state appears to regulate the secretion and action of hormones (Stamler *et al.*, 1992). An important constituent of the oxidant buffering system that controls the cellular redox state is thioredoxin (TRX), a 12-kDa protein with a redox-active disulfide/dithiol in the conserved active site sequence Cys-Gly-Pro-Cys (Holmgren, 1985). This molecule has a variety of activities including serving as a hydrogen donor for various intracellular molecules (Matthews *et al.*, 1992). Studies suggested the presence of a control mechanism by the TRX system in certain endocrine systems (Demarquoy *et al.*, 1991). For example, it has been reported that in the hypothalamic-pituitary-adrenal axis, TRX modulated cellular glucocorticoid responsiveness (Makino *et al.*, 1996). Recent immunohistological studies have demonstrated an intense level of staining for TRX in the pig anterior pituitary gland (Padilla *et al.*, 1993), supporting the idea that the TRX system might play a role in the regulation of PRL secretion. However, to date, no data are available regarding the role of TRX in the PRL secretion.

A relation has been established between a glycoprotein, selenoprotein P and TXR in rats (Ma *et al.*, 2003). It is suggested that the function of selenoprotein P depended on the redox reaction at the glycosylation site. In turkey, the glycosylation sites of prolactin are found at positions 56 and 197, located in a consensus sequence (Asn-X-Cys). These amino acids are involved in disulfide bond formation that is also a candidate to be a redox center. Therefore, the glycosylation of PRL may depend on the redox function via TXR.

CHAPTER VI. CONCLUSION

It has been reported that the quantity of non-glycosylated PRL (NG-PRL) and glycosylated PRL (G-PRL) was approximately the same in adult turkey hens (Corcoran and Proudman, 1991). This was further elaborated by Bédécarrats *et al.,* (1999b). They showed that the transition from egg laying to egg incubating behaviour was associated with a significant increase in the proportion of PRL which was glycosylated. A similar pattern of PRL isoform secretion was also observed during embryogenesis, and a two fold increase in G-PRL (Bédécarrats *et al.,* 1999a) was reported. The association between higher levels of glycosylation around the time of hatch and during the reproductive cycle suggested that G-PRL might have specific physiological effects different from those of NG-PRL. Since G-PRL and NG-PRL are likely to have a different affinity for the PRL receptors, changes in ratio of isoforms may act to modulate biological effects in different target tissues.

In order to study the glycosylation process of PRL, the pituitary glands of day 24 turkey embryos were collected and cultured with VIP, a potent PRF in avian species to stimulate the production and glycosylation of PRL. In this study, we reported several genes that were differentially expressed when the turkey embryonic pituitary glands were challenged with VIP. The relative quantities of the 8 genes analyzed with real-time PCR have shown at least a 2 fold increase. Five of these eight genes were related to cellular homeostasis. FKBP12 and sFRP1 served to control cell proliferation caused by high levels of VIP and PRL, NPM and Hsp70 assisted cell proliferation by interfering with the apoptosis process, and NAPOR promoted apoptosis of somatotrophes. All these genes support the mitogenic ability of both VIP and PRL, and may be involved in the cell type changes in the pituitary gland during hyperprolactinemia. Since TiPARP in turkey may carry out other cellular functions than the other members in the PARP family, it is possible that TiPARP also assists in cell proliferation through interaction with the genomic DNA such as preventing cell death by interacting with telomerase (a function of Tankyrase, member of the PARP family) or conversely induces apoptosis by signalling the translocation of AIF from mitochondria. The increased levels of TRX indicated that it played a role in the cellular response to the VIP stimulation. Although the target of

TRX is uncertain, it is conceivable that the glycosylation of PRL may depend on TRX. Further investigations have to be done in order to confirm the role of TRX in the glycosylation of PRL.

The expression pattern of other genes in Table 4.6 has not been confirmed with real-time PCR. Those genes are potentially involved in the process of glycosylation or the function of G-PRL. Moreover, results of the blast search indicated that some of the unidentified genes from the subtracted cDNA library had a high identity match with some unknown chicken genes, which suggested that they could possibly be novel proteins yet to be characterized.

In order to minimize the expression of genes that are associated with PRL induced cell proliferation and not directly involved in glycosylation of PRL, a system such as perfusion system which can remove the secreted PRL around the cultured tissue could be utilized. In addition, it also provides an environment to be created for the cells that is much closer to that seen within biological tissue.

Moreover, the blast search results only represent a portion of genes that might have been differentially expressed upon the stimulation by VIP. A complete library requires more work and the collection of rare genes depends on random chance. Microarray analysis could be an alternative to SSH to compensate for this limitation. The main advantage of microarray is that the differential expression of thousands of genes can be detected at the same time. However, the preparation of the cDNA microarray chip for turkey embryonic pituitary glands could be costly, labour intensive and highly variable. Due to the high homology between chicken and turkey, commercially prepared chicken microarray chip may be a good alternative.

To conclude, this study demonstrated the mitogenic effects of VIP and PRL in the pituitary gland of turkey and opened new research areas for the study of genes associated with high levels of G-PRL during the prehatching time and during incubation in turkey hens. For example, a study of the effects of TRX could be done by verifying the levels of PRL, G-PRL and PRLR after the administration of TRX, which would indicate whether TRX exerts its functions on the process of glycosylation or the function of G-PRL.

Moreover, our study also supported Dr. Porter's hypothesis that somatotrophes might not transform to lactotrophes through an intermediate cell type, mammosomatotrophes, in avian embryogenesis; instead, the cell type changes could be a combined result of lactotrophe proliferation and apoptosis of somatotrophes.

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CHAPTER VIII. APPENDICES

Appendix A: A detailed table of blast search results from NCBI

Table 8a A detailed table blast search results from NCBI			
Sample number	**Name of the gene**	**Accession number**	**Score / Expect value / Identities**
FS01	Meleagris gallopavo pituitary glycoprotein hormone alpha-subunit precursor, mRNA, complete cds	M33698	Score = 1461 bits (760), Expect = 0.0 Identities = 760/760 (100%)
FS02	Gallus gallus finished cDNA, clone ChEST383i22	CR354143.1	Score = 825 bits (429), Expect = 0.0 Identities = 706/817 (86%), Gaps = 35/817 (4%)
FS03	Gallus gallus finished cDNA, clone ChEST376p22	BX930684.1	Score = 842 bits (425) Expect = 0.0 Identities = 487/508 (95%)
FS04	Chicken neural cell-adhesion molecule (N-CAM) gene, exon 19	M15861.1	Score = 675 bits (351), Expect = 0.0 Identities = 488/539 (90%)
	Gallus gallus finished cDNA, clone ChEST134p17	CR353195.1	Score = 462 bits (233) Expect = e-127 Identities = 302/321 (94%)
FS05	tgy34f10.b1	262815773	Score = 1052 bits (547) Expect = 0.0 Identities = 704/779 (90%)
FS06	meleagris Hsp70 mRNA for heat shock protein, complete cds	AB096696.1	Score = 615 bits (310) Expect = e-173 Identities = 343/354 (96%)
FS07	Gallus gallus myelin basic protein (MBP), mRNA	NM_205280.1	Score = 305 bits (154) Expect = 2e-80 Identities = 169/174 (97%)
FS08	tlg40f07.g1	281737674	Score = 685 bits (356) Expect = 0.0 Identities = 368/373 (98%)
FS09	Gallus gallus gap junction protein, beta 1, 32kDa (connexin 32, Charcot-Marie-Tooth neuropathy, X-linked) (GJB1), mRNA	NM_204371.1	Score = 971 bits (490) Expect = 0.0 Identities = 641/691 (92%),
FS10	tqp13f04.b1	285660119	Score = 969 bits (504) Expect = 0.0 Identities = 680/763 (89%)
FS11	svg81f06.b1	249763767	Score = 783 bits (407) Expect = 0.0 Identities = 428/438 (97%)
	Gallus gallus mRNA for hypothetical protein, clone 33p3	AJ721067.1	Score = 1087 bits (565), Expect = 0.0 Identities = 585/595 (98%)

FS12	japonica mitochondrial DNA, complete genome	AP003195.2	Score = 264 bits (133) Expect = 9e-68 Identities = 211/237 (89%)
FS13	gallus mitochondrial DNA, complete genome, sub_species:domesticus, strain:White Plymouth Rock	AP003318.1|	Score = 490 bits (247) Expect = e-135 Identities = 502/587 (85%)
FS14	Gallus gallus finished cDNA, clone ChEST251p16	CR353461.1	Score = 515 bits (260) Expect = e-143 Identities = 272/276 (98%)
	Homo sapiens profilin 2 (PFN2), transcript variant 1, mRNA	NM_053024.1	Score = 406 bits (205) Expect = e-110 Identities = 269/284 (94%)
FS15	tam61a06.b1	260150973	Score = 1221 bits (635) Expect = 0.0 Identities = 755/811 (93%)
FS16	NO SIGNIFICANT RESULT		
FS17	tls13h12.g1	282393440	Score = 316 bits (164) Expect = 3e-83 Identities = 181/189 (95%)
FS18	Coturnix chinensis mitochondrial DNA, complete genome	AB073301.1	Score = 706 bits (356) Expect = 0.0 Identities = 641/736 (87%)
	Gallus gallus mitochondrial DNA, complete genome, strain:silky chicken	AB086102.1	Score = 690 bits (348) Expect = 0.0 Identities = 633/728 (86%)
FS19	Gallus gallus finished cDNA, clone ChEST370c15	CR354045.1	Score = 305 bits (154) Expect = 4e-80 Identities = 242/267 (90%)
FS20	NO SIGNIFICANT RESULT		
FS21	rxl14g06.b1	224530216	Score = 810 bits (421) Expect = 0.0 Identities = 535/589 (90%)
FS22	tcb96f04.b1	261445327	Score = 358 bits (186) Expect = 1e-95 Identities = 257/289 (88%)
FS23	Gallus gallus finished cDNA, clone ChEST260i9	CR354213.1	Score = 99.6 bits (50) Expect = 3e-18 Identities = 128/151 (84%)
	tcd55f11.b1	260234877	Score = 200 bits (104) Expect = 1e-48 Identities = 174/206 (84%)
FS24	Gallus gallus finished cDNA, clone ChEST968n22	BX930752.2	Score = 988 bits (514), Expect = 0.0 Identities = 630/683 (92%)
FS25	Gallus gallus finished cDNA, clone ChEST260i9	CR354213.1	Score = 91.7 bits (46) Expect = 8e-16 Identities = 127/151 (84%)

FS26	H.sapiens ERK3 mRNA	HSERK3	Score = 264 bits (133) Expect = 3e-67 Identities = 240/268 (89%)
	sks60g08.g1	254393723	Score = 1236 bits (643) Expect = 0.0 Identities = 738/781 (94%)
FS27	Gallus gallus finished cDNA, clone ChEST496a15	CR387093.1	Score = 1010 bits (525), Expect = 0.0 Identities = 615/655 (93%)
FS28	chicken hemoglobin beta chain mrna	CHKHBBM	Score = 464 bits (234) Expect = e-127 Identities = 330/362 (91%)
FS29	PREDICTED: Gallus gallus similar to Rnps1 protein (LOC416756), mRNA	XM_415051.1	Score = 825 bits (429), Expect = 0.0 Identities = 590/658 (89%)
FS30	tci05a02.g1	261336420	Score = 592 bits (308) Expect = e-166 Identities = 409/458 (89%)
FS31	Cloning vector pHS4, luciferase reporter vector containing HS4 insulator sequence	AJ277959.1	Score = 694 bits (361), Expect = 0.0 Identities = 363/364 (99%)
	tjm57d12.g1	289840002	Score = 217 bits (113) Expect = 2e-53 Identities = 121/125 (96%)
	svf89g04.b1	250970886	Score = 410 bits (213) Expect = e-111 Identities = 264/288 (91%)
FS32	Lapemis hardwickii ribosomal protein S15 isoform mRNA, complete cds	AF159546	Score = 400 bits (202) Expect = e-108 Identities = 359/410 (87%),
	Mus musculus ribosomal protein S15a (Rps15a), mRNA	|NM_170669.1	Score = 367 bits (185) Expect = 2e-98 Identities = 362/421 (85%)
	Homo sapiens ribosomal protein S15a, mRNA (cDNA clone MGC:57552 IMAGE:5736078), complete cds	BC046113.1	Score = 351 bits (177) Expect = 1e-93 Identities = 348/405 (85%)
	Gallus gallus 18S ribosomal RNA gene, complete sequence	AF173612	Score = 252 bits (127) Expect = 8e-64 Identities = 133/135 (98%)
FS33	Cloning vector pHS4, luciferase reporter vector containing HS4 insulator sequence	AJ277959.1	Score = 917 bits (477), Expect = 0.0 Identities = 477/477 (100%)
	svf89g04.b1	250970886	Score = 508 bits (264) Expect = e-141 Identities = 406/472 (86%)

FS34	svf89g04.b1	250970886	Score = 515 bits (268) Expect = e-143 Identities = 408/473 (86%)
FS35	Chicken ubiquitin I (UbI) gene, partial cds	CHKUB1A	Score = 1078 bits (544) Expect = 0.0 Identities = 691/740 (93%)
FS36	txg48a10.g1	291791917	Score = 812 bits (422) Expect = 0.0 Identities = 496/530 (93%)
FS37	gallus FKBP12 mRNA for FK506 bing protein 12, complete cds	AB055761.1	Score = 222 bits (112) Expect = 8e-55 Identities = 127/132 (96%)
	sis03c12.g1	253753965	Score = 446 bits (232) Expect = e-122 Identities = 359/415 (86%)
FS38	Gallus gallus mitochondrial DNA, complete genome, sub_species:domesticus, strain:White Plymouth Rock	AP003318.1	Score = 779 bits (393) Expect = 0.0 Identities = 696/797 (87%)
	Oreophasis derbianus cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	AF165495.1	Score = 577 bits (291) Expect = e-161 Identities = 657/779 (84%)
FS39	Gallus gallus thioredoxin (TRX), mRNA	NM_205453.1	Score = 829 bits (431), Expect = 0.0 Identities = 551/601 (91%)
FS40	Gallus gallus finished cDNA, clone ChEST948b2	BX933677.1	Score = 704 bits (355) Expect = 0.0 Identities = 373/379 (98%)
	Gallus gallus mRNA for hypothetical protein, clone 2o21	AJ719508.1	Score = 717 bits (373), Expect = 0.0 Identities = 389/397 (97%)
FS41	Gallus gallus finished cDNA, clone ChEST260i9	CR354213.1	Score = 200 bits (101) Expect = 2e-48 Identities = 149/163 (91%)
FS42	Gallus gallus finished cDNA, clone ChEST260i9	CR354213.1|	Score = 99.6 bits (50) Expect = 3e-18 Identities = 128/151 (84%)
	tcd55f11.b1	260234877	Score = 200 bits (104) Expect = 1e-48 Identities = 174/206 (84%)
FS43	Gallus gallus myelin basic protein (MBP), mRNA	NM_205280.1	Score = 1013 bits (511) Expect = 0.0 Identities = 573/595 (96%)
FS44	Gallus gallus finished cDNA, clone ChEST963b22	CR338819.1	Score = 408 bits (206) Expect = e-111 Identities = 259/282 (91%)
FS45	Gallus gallus finished cDNA, clone ChEST130h23	CR389266.1	Score = 398 bits (207), Expect = e-108 Identities = 254/270 (94%)

FS46	tch11h04.b1	261454331	Score = 1042 bits (542), Expect = 0.0 Identities = 740/827 (89%)
FS47	tji89e10.b1	281892895	Score = 139 bits (72) Expect = 9e-30 Identities = 248/330 (75%)
FS48	Gallus gallus nucleophosmin (NPM1), mRNA	NM_205267.1	Score = 418 bits (211) Expect = e-114 Identities = 252/267 (94%)
FS49	ttm86a08.b1	283986990	Score = 1023 bits (532), Expect = 0.0 Identities = 580/603 (96%)
FS50	PREDICTED: Gallus gallus similar to polyubiquitin (LOC417602), mRNA	XM_415847.1	Score = 875 bits (455), Expect = 0.0 Identities = 583/637 (91%)
FS51	Cloning vector pHS4, luciferase reporter vector containing HS4 insulator sequence	AJ277959.1	Score = 823 bits (428), Expect = 0.0 Identities = 428/428 (100%)
	svf89g04.b1	250970886	Score = 433 bits (225) Expect = e-118 Identities = 283/310 (91%)
FS52	Meleagris gallopavo 18S rRNA gene	MGA419877	Score = 367 bits (185) Expect = 1e-98 Identities = 191/193 (98%)
FS53	PREDICTED: Gallus gallus similar to Extracellular superoxide dismutase [Cu-Zn] precursor (EC-SOD) (LOC422810), mRNA	XM_420760.1	Score = 1088 bits (566), Expect = 0.0 Identities = 679/733 (92%)
FS54	Cloning vector pHS4, luciferase reporter vector containing HS4 insulator sequence	AJ277959.1	Score = 492 bits (256), Expect = e-136 Identities = 256/256 (100%)
	svf89g04.b1	250970886	Score = 425 bits (221) Expect = e-116 Identities = 272/296 (91%)
FS55	Gallus gallus heat shock protein Hsp70 (Hsp70) gene, Hsp70-1 allele, complete cds	AY143691.1	Score = 950 bits (479) Expect = 0.0 Identities = 533/551 (96%)
FS56	Gallus gallus finished cDNA, clone ChEST260i9	CR354213.1	Score = 216 bits (109) Expect = 4e-53 Identities = 151/163 (92%)
FS57	Mus musculus apoptosis-related RNA binding protein (Napor-1) mRNA, complete cds	AF090696	Score = 278 bits (140) Expect = 2e-71 Identities = 178/190 (93%)
	thg28b06.b1	262835649	Score = 1108 bits (576), Expect = 0.0 Identities = 618/638 (96%)

FS58	Meleagris gallopavo cytochrome c oxidase subunit VIIb precursor, mRNA, complete cds; nuclear gene for mitochondrial product	AF255353.1	Score = 658 bits (332) Expect = 0.0 Identities = 358/368 (97%)
FS59	tcd55f11.b1	260234877	Score = 185 bits (96) Expect = 5e-44 Identities = 163/194 (84%)
FS60	Gallus gallus myelin basic protein (MBP), mRNA	NM_205280.1	Score = 1023 bits (516), Expect = 0.0 Identities = 575/596 (96%)
FS61	Gallus gallus mRNA for hypothetical protein, clone 7d18	emb|AJ719849.1	Score = 860 bits (447), Expect = 0.0 Identities = 605/679 (89%)
FS62	Gallus gallus mitochondrial DNA, complete genome, strain:silky chicken	AB086102.1	Score = 973 bits (506), Expect = 0.0 Identities = 706/806 (87%)
	Dinornis novaezealandiae clone Dino699 cytochrome oxidase subunit I gene, partial cdsmitochondrial genes for mitochondrial products	AY326193.1	Score = 200 bits (101), Expect = 1e-48 Identities = 155/173 (89%)
FS63	ttg96c12.g1	282544688	Score = 794 bits (413), Expect = 0.0 Identities = 479/510 (93%)
	Cloning vector pHS4, luciferase reporter vector containing HS4 insulator sequence	AJ277959.1	Score = 371 bits (193), Expect = 1e-99 Identities = 207/209 (99%)
FS64	ust18f03.g1	324760517	Score = 469 bits (244) Expect = e-129 Identities = 248/250 (99%)
FS65	Gallus gallus finished cDNA, clone ChEST671n9	CR524460.1	Score = 442 bits (230), Expect = e-121 Identities = 409/481 (85%)
	tsw37d08.b1	288079393	Score = 196 bits (102) Expect = 2e-47 Identities = 193/235 (82%)
FS66	tro93h06.b1	284239888	Score = 454 bits (236) Expect = e-124 Identities = 288/311 (92%)
	svf89g04.b1	250970886	Score = 446 bits (232) Expect = e-122 Identities = 294/323 (91%)
FS67	ryj63a08.b1	230650153	Score = 675 bits (351) Expect = 0.0 Identities = 405/429 (94%)
FS68	NO SIGNIFICANT RESULT		

FS69	PREDICTED: Gallus gallus similar to vimentin - chicken (LOC420519), mRNA	XM_418622.1	Score = 821 bits (427), Expect = 0.0 Identities = 527/577 (91%)
	Gallus gallus vimentin gene 3'-nontranslated region	GGVIMEZ	Score = 591 bits (298) Expect = e-166 Identities = 310/314 (98%)
FS70	PREDICTED: Gallus gallus similar to chromosome 20 open reading frame 111 (LOC419203), mRNA	XM_417380.1	Score = 1131 bits (588), Expect = 0.0 Identities = 654/687 (95%)
FS71	tqy49b01.b1	288007608	Score = 212 bits (110) Expect = 3e-52 Identities = 170/197 (86%)
FS72	Gallus gallus mitochondrial DNA, complete genome, strain:silky chicken	AB086102.1	Score = 817 bits (412), Expect = 0.0 Identities = 721/824 (87%)
	Pipile jacutinga cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	AF165500.1	Score = 609 bits (307) Expect = e-171 Identities = 679/803 (84%)
FS73	Gallus gallus finished cDNA, clone ChEST177l14	CR389011.1	Score = 306 bits (159), Expect = 6e-80 Identities = 217/241 (90%)
FS74	Gallus gallus myelin basic protein (MBP), mRNA	NM_205280.1	Score = 813 bits (410) Expect = 0.0 Identities = 478/502 (95%)
FS75	Gallus gallus finished cDNA, clone ChEST260i9	CR354213.1	Score = 99.6 bits (50) Expect = 3e-18 Identities = 128/151
FS76	Chicken gapdh (glyceraldehyde-3-phosphate dehydrogenase) mrna, 3' end	J00850.1	Score = 1377 bits (716), Expect = 0.0 Identities = 751/766 (98%)
FS77	Gallus gallus finished cDNA, clone ChEST873k15	CR406775.1	Score = 483 bits (251), Expect = e-133 Identities = 291/306 (95%)
FS78	tcm05h11.g1	261452670	Score = 644 bits (335) Expect = 0.0 Identities = 438/485 (90%)
FS79	Gallus gallus mRNA for heat shock cognate 70	GGAJ4940	Score = 285 bits (144) Expect = 2e-74 Identities = 156/160 (97%)
FS80	svf89g04.b1	250970886	Score = 496 bits (258) Expect = e-137 Identities = 368/419 (87%)

FS81	Homo sapiens ribosomal protein L3, mRNA (cDNA clone IMAGE:2905497), complete cds	BC006483.1	Score = 529 bits (267), Expect = e-147 Identities = 564/661 (85%)
FS82	Gallus gallus mitochondrion, complete genome	AY235570.1	Score = 129 bits (65) Expect = 2e-27 Identities = 168/201 (83%)
	Pipile jacutinga cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	AF165500.1	Score = 117 bits (59) Expect = 8e-24 Identities = 104/119 (87%)
FS83	Gallus gallus secreted frizzled related protein 1 (sFRP1), mRNA	NM_204553.1	Score = 698 bits (352) Expect = 0.0 Identities = 466/504 (92%)
FS84	tyu94a10.g1	298764284	Score = 887 bits (461) Expect = 0.0 Identities = 497/514 (96%)
FS85	uat71d12.b1	298838143	Score = 715 bits (372) Expect = 0.0 Identities = 440/470 (93%)
FS86	Gallus gallus finished cDNA, clone ChEST651l3	CR405924.1	Score = 348 bits (181), Expect = 7e-93 Identities = 352/425 (82%)
	svf89g04.b1	250970886	Score = 254 bits (132), Expect = 2e-64 Identities = 151/160 (94%)
FS87	Gallus gallus mitochondrial DNA, complete genome, sub_species:domesticus, strain:White Plymouth Rock	AP003318.1	Score = 887 bits (461), Expect = 0.0 Identities = 651/746 (87%)
FS88	Gallus gallus mRNA for hypothetical protein, clone 2d15	AJ719429.1	Score = 1019 bits (530), Expect = 0.0 Identities = 560/575 (97%)
FS89	NO SIGNIFICANT RESULT		
FS90	Taenopygia guttata steroidogenic enzyme cytochrome P450 17α-hydroxylase/17,20 lyase mRNA, partial cds	AY313844.1	Score = 605 bits (305) Expect = e-170 Identities = 492/553 (88%)
FS91	NO SIGNIFICANT RESULT		
FS92	Gallus gallus mitochondrial DNA, complete genome	AP003580.1	Score = 337 bits (170) Expect = 2e-89 Identities = 404/482 (83%)
	Gallus gallus mitochondrial ATPase 8 and ATPase 6 genes	MIGGATP8	Score = 329 bits (166) Expect = 4e-87 Identities = 403/482 (83%)

FS93	Gallus gallus myelin basic protein (MBP), mRNA	NM_205280.1	Score = 151 bits (76) Expect = 9e-34 Identities = 139/160 (86%)
FS94	Chicken ubiquitin I (UbI) gene, partial cds	CHKUB1A	Score = 391 bits (197) Expect = e-106 Identities = 257/277 (92%)
FS95	tte45c05.g1	291815351	Score = 1000 bits (520), Expect = 0.0 Identities = 566/588 (96%)
FS96	Gallus gallus finished cDNA, clone ChEST597c14	BX932479.1	Score = 1122 bits (566), Expect = 0.0 Identities = 644/670 (96%)
	Homo sapiens TCDD-inducible poly(ADP-ribose) polymerase (TIPARP), mRNA	NM_015508.2	Score = 97.6 bits (49), Expect = 4e-17 Identities = 289/369 (78%)

Appendix B: Differential gene expression between day 8 old male and female chicken embryonic genital ridge (gonad, mesonephros, Müllerian duct)

8.1 FORWARD

This experiment is conducted in collaboration with Yonju Ha (Ph. D candidate) and Dr. Kiyoshi Shimada from Laboratory of Animal Physiology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan. The work presented in this thesis is still in progress. Due to the length limitation of thesis, only part of the results is presented.

8.2 INTRODUCTION

During avian embryogenesis, the gonads develop from two sources during embryonic development. When the nephric ridges are well established, genital ridges appear on their surface. This tissue forms sex cords, which will develop into testes if the bird becomes a male or into the ovary if the bird becomes a female. The cells near the base of the yolk sac become very large, migrate individually into the gut mesoderm, up the mesenteries and invade the genital ridges. These are the primitive sex cells that will form ova and spermatozoa. Each embryo starts out with two sets of ducts: the Wolffian and the Müllerian ducts. However, during the course of differentiation of female avian embryos (except for Order Falconiforms), only the left ovary and Müllerian duct develop; whereas, the right ovary and Müllerian duct regress. Since bilateral ovaries and oviducts develop in other nonmammalian vertebrates including reptiles and amphibians, this is a unique characteristic in birds.

In the chicken, both female and male embryos initially have a pair of undifferentiated gonads and Müllerian ducts that develop in the coelomic cavity from day 5 of incubation and grow to the region of the cloaca (Romanoff, 1960). Each Müllerian duct consists of epithelial cells, basement membrane, and mesenchymal cells (Allard *et al.*, 2000). In the male embryo, the Müllerian ducts undergo regression and disappear by day 12 of incubation. In the female embryo, both the left and right Müllerian duct maintain a linear rate of growth until day 12 of incubation. Subsequently, the right

Müllerian duct undergoes a relatively slow involution from anterior to posterior. Eventually the right Müllerian duct completely disappears at the time of hatching (Romanoff, 1960). Conversely, the left Müllerian duct continues to grow and develops into oviduct; i.e., infundibulum, magnum, isthmus, shell gland and vagina.

The regression of the Müllerian duct is caused by anti Müllerian hormone (AMH), a member of the transforming growth factor-β family (Hutson *et al.*, 1981). One of the receptors for AMH, a type II AMH receptor, was cloned in mammals (di Clemente *et al.*, 1994), and expression of this receptor was found only in the mesenchymal cells of the duct (Baarends *et al.*, 1994; Josso *et al.*, 2001). This suggests that AMH binds to AMH-II-R in mesenchymal cells and induces regression of the Müllerian duct. This hypothesis is supported by Roberts *et al.*, (1999) who demonstrated that AMH initiates the Müllerian duct regression through mesenchymal cells in mammals.

Several reports have suggested that estrogen protected the left Müllerian duct from regression. It has been shown that the exposure to estrogen during embryogenesis prevented the right Müllerian duct regression in female chicken embryos (Hutson *et al.*, 1982; Doi and Hutson, 1988). IT has been reported that exogenous estrogen induced ovarian like cortical development of the left testis in male chicken embryos, although a sufficient amount of AMH was still secreted to cause regression of the Müllerian duct (Hutson *et al.*, 1982). These data indicated that the inhibitory effect of estrogen on the Müllerian duct regression might not occur by inhibition of AMH secretion as was proposed by Stoll *et al.*, (1990). Furthermore, treatment with aromatase inhibitor, which causes reduction of estrogen synthesis, was shown to induce regression of left and right Müllerian ducts in female chicken embryo (Elbrecht and Smith., 1992).

The Müllerian duct is derived from the urogenital ridge at coleomic body. The development of Müllerian duct begins from day 4-5 of incubation. Both Müllerian ducts of male embryo and the right duct of female embryo stop development on day 8 of embryogenesis and start to regress. Surgical removal of the Wolffian duct was reported to prevent the development of Müllerian duct (Romanoff, 1960). The exact mechanism of the development of Müllerian duct is unknown.

Since the development of Müllerian duct depends on Wolffian duct, and Wolffian duct is part of the mesonephros, we hypothesized that some signals related to regression of Müllerian duct come from mesonephros and gonad. In order to investigate the initiation of the regression of Müllerian duct, two subtractive libraries were created from the embryonic genital ridge (including Müllerian duct) from day 8 male and female chicken embryos.

8.3 MATERIALS AND METHODS

8.3.1 Tissue source

The embryonic genital ridge including Müllerian duct was collected from forty-eight day 8 chicken embryos that were purchased from Couvoir Simentin-Les Entreprises Simentin Inc. in Quebec. The collected tissues were immediately immersed in liquid nitrogen and kept at -70°C until use.

8.3.2 Sexing of the chicken embryos

The sex of the embryo was determined by the PCR amplification of the CHD gene (Griffiths *et al.*, 1998). The liver of the embryo was collected and DNA was extracted by boiling method from about 50 mg of liver tissue that was taken from each embryo. Genomic DNA was then extracted with an equal volume of phenol:chloroform. An aliquot of 1 µl of genomic DNA was used for PCR of the CHD gene. The primers for CHD genes (Griffiths *et al.*, 1996) were 5'- CAA GCA TGA GAA ACT GTG CAA AAC AG -3' (sense) and 5'- CTA TCA GAT CCA GAA TAT CTT CTG C -3' (antisense). PCR reactions were carried out in 25µl of reaction mixture (20mM Tris-HCl, 100mM KCl, 2mM $MgCl_2$, pH 8.0) containing 1mM dNTPs, 25mM $MgCl_2$, 10mM primers and 3 units of rTaq DNA polymerase (Amersham Biosciences, Inc). The thermal cycler was programmed for an initial denaturation of 94°C for 1 minute followed by 40 cycles, each cycle consist of a denaturation at 94°C for 20 seconds, an of annealing at 58°C for 30 seconds annealing and 72°C for 30 seconds extension time. PCR products were run on a 3% agarose gel in 1×TBE buffer at 70 volts for 2 hours.

8.3.3 Total RNA extraction

The left genital ridge including Müllerian duct of male or female embryos were homogenized in 1 ml of Trizol™ Reagent (GibcoBRL, Gainthersburg, MD). After incubation of the homogenized samples with 200µl of chloroform for 5 minutes on the ice, the samples were centrifuged at 13,000 rpm for 15 minutes. The supernatant of each sample was transferred to new tube and mixed with 500µl of isopropyl alcohol. Following incubation for 10 minutes at room temperature, samples were centrifuged at

13,000 rpm for 10 minutes. The supernatant was removed and the RNA pellet was washed once with 1 ml of 75% ethanol. The pellet was air dried and dissolved in 10µl of diethyl pyrocarbonate (DEPC)-treated water. The RNA quantity was determined by spectrophotometry at 260nm. An aliquot of 10 µg of the total RNA with 40 µl of assay buffer (40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$) and 2 units of DNase I (Amersham Biosciences, Inc.) were incubated at 37 °C for 15 minutes. Samples were then centrifuged at 15,000 rpm for 5 min after mixing with 100 µl of phenol-chloroform. The supernatant of each sample was collected and mixed with 125 µl of 100% ethanol. After incubation at -70 °C for 1 hour, the supernatant was discarded and the RNA pellet was washed once with 150 µl of 75% ethanol. The pellet was air dried and dissolved in 10 µl of diethyl pyrocarbonate (DEPC)-treated water. The RNA quantity was determined by spectrophotometry at 260nm. Samples were stored at -70 °C until use.

8.3.4 cDNA preparation for suppressive subtractive hybridization

8.3.4.1 First Strand cDNA synthesis

The Super Smart™ PCR cDNA Synthesis Kit (BD Biosciences Clontech, Inc.) was used to synthesize cDNA for suppressive subtractive hybridization. Five hundred ng of total RNA was annealed with 12 µM of 3' SMART CDS Primer II A and 12 µM of SMART A oligonucleotide (provided by Super Smart™ PCR cDNA Synthesis Kit) for 2 minutes at 65 °C, followed by 90 minutes of incubation at 42 °C for first strand cDNA synthesis. The single stranded cDNA was subsequently purified with NucleoSpin® Extraction Kit (BD Biosciences Clontech, Inc) using the protocol recommended by the manufacturer and eluted with 85 µl of Milli-Q H_2O.

8.3.4.2 Long Distance (LD) PCR amplification

For each LD PCR reaction, a reaction mix contained 80 µl of cDNA, 172 µl of deionized H_2O, 30 µl of 10X Advantage 2 PCR Buffer, 6 µl 50X dNTP (10 mM), 6 µl 5' PCR Primer II A (12 µM) and 6 µl 50X Advantage 2 Polymerase Mix. The mixture was aliquoted in 3 tubes and placed into a MJ Research, Inc. Model PTC-100 Programmable Thermal Cycler. Samples were initially denatured at 94 °C for 1 minute. Subsequently,

each cycle consisted of 95°C for 15 seconds, 65°C for 30 seconds and 68°C for 6 minutes for 36 cycles. After 21 PCR cycles, 2 tubes were removed and refrigerated at 4°C for later use. Aliquots of 5 µl were set aside every 3 cycles until the program was completed. These aliquots were electrophoresed in a 1% agarose gel at 70 V for 1 hour to optimize the number of cycles. Once the number of cycles was optimized, the 2 tubes stored at 4°C were placed back into the thermal controller for additional cycles.

PCR products were first purified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Afterward, the PCR products were concentrated to 70 µl with n-butanol extraction. PCR products were subsequently purified by centrifugation through CHROMA SPIN™-1000 DEPC-H_2O Columns (BD Biosciences Clontech, Inc.) using the protocol recommended by the manufacturer.

8.3.4.3 Rsa I digestion

The purified PCR products were digested with 10 units of Rsa I restriction endonuclease at 37 °C for 3 hours. Completion of restriction digestion was assessed on a 1% agarose gel. The digested PCR products were purified with NucleoSpin® Extraction Kit (BD Biosciences Clontech, Inc) using the protocol recommended by the manufacturer, and eluted with 50 µl of Milli-Q H_2O. The purified PCR products were precipitated with 50 µl of 4 M ammonium acetate and 375 µl of 95% ethanol, and finally dissolved in 6.7 µl of TNE buffer.

8.3.4.4 Suppressive subtractive hybridization

Suppressive subtractive hybridization was performed with the Clontech PCR-Select™ cDNA Subtraction Kit (BD Biosciences Clontech, Inc). Figure 8.3 shows the experimental plan for the adaptor ligation. In the forward library, the male cDNA is the driver cDNA while the female is the driver cDNA in the reverse library.

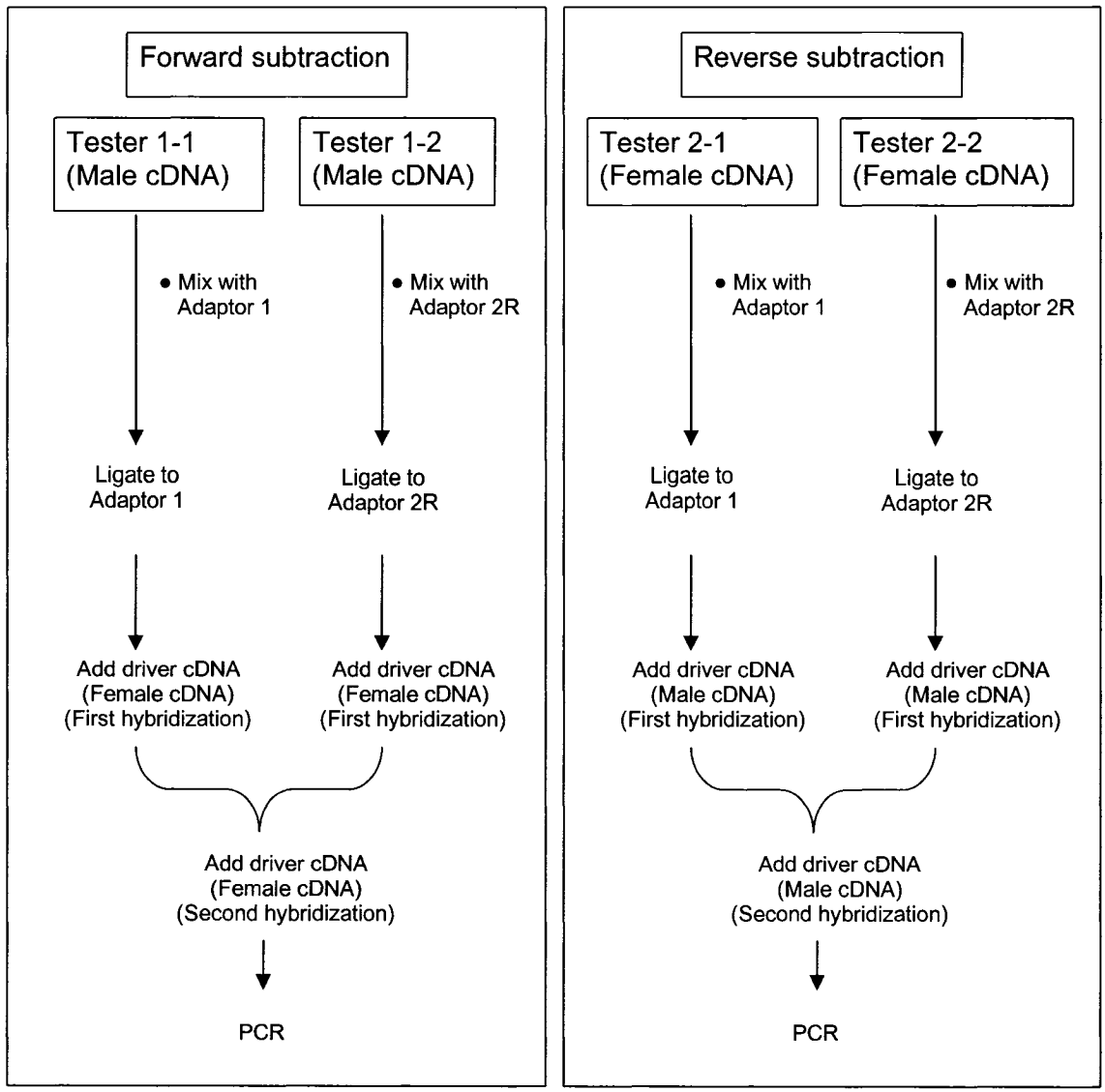

Figure 8.3 Graphical representation of the experimental plan for the adaptor ligation and hybridization of day 8 old male and female chicken embryonic genital ridge (Adapted from the Clontech PCR-Select™ cDNA Subtraction Kit User Manual). Tester 1-1 and Tester 1-2 are female cDNA and Tester 2-1 and Tester 2-2 are male cDNA.

8.3.4.5 Adaptor ligation

An aliquot of 1 µl of purified Rsa I digested cDNA was diluted with 5 µl of sterile H_2O. Tester 1-1 and Tester 2-1 were ligated with Adaptor 1, while Tester 1-2 and Tester 2-2 were ligated with Adaptor 2R. The ligation procedure was carried out using 400 units of T4 DNA ligase at 16 °C overnight.

8.3.4.6 Hybridization

Hybridization was divided into 2 parts. In the first hybridization, a reaction mix containing 1.5 µl of Tester cDNA with one type of adaptors, 1.5 µl of driver cDNA and 1 µl of 4X hybridization buffer (provided from the kit) was incubated at 98 °C for 1.5 min followed by incubation at 68 °C for 8 hours. In the second hybridization, 1 µl of driver cDNA was overlaid with one drop of mineral oil and was denatured at 98 °C for 1.5 min. The two samples with different adaptors from the first hybridization were mixed together simultaneously in the presence of the freshly denatured driver cDNA. The reaction was incubated at 68 °C overnight. Finally, the hybridized cDNA was diluted with 200 µl of dilution buffer pH 8.3 (20 mM HEPES pH 6.6, 20 mM NaCl, 0.2 mM EDTA pH 8.0).

8.3.4.7 PCR Amplification

There are 2 PCR amplification reactions. In the primary PCR, a reaction mix contained 1 µl of diluted cDNA, 19.5 µl of deionized H_2O, 2.5 µl of 10 X Advantage 2 PCR Buffer, 0.5 µl of dNTP (10 mM), 1 µl of PCR Primer 1 (10 µM) and 0.5 µl of 50X Advantage 2 Polymerase Mix. The thermal cycler was programmed for an initial denaturation of 94 °C for 1 minute followed by 27 cycles consisting of 94°C for 10 seconds denaturation, 66°C for 30 seconds and 72°C for 1.5 minutes. Upon the completion of the primary PCR, 3 µl of the PCR mixture was diluted with 27 µl of sterile H_2O.

In the secondary PCR, a reaction mix contained 1 µl of diluted cDNA from primary PCR, 18.5 µl of deionized H_2O, 2.5 µl of 10 X PCR Buffer, 0.5 µl of dNTP (10 mM), 1 µl of Nested PCR Primer 1 (10 µM), 1 µl of Nested PCR Primer 2R (10 µM) and 0.5 µl of rTaq DNA polymerase (5000 units/ml) (Amersham Biosciences Corp). The

thermal cycler was programmed for an initial denaturation of 94 °C for 1 minute followed by 15 cycles. Each cycle consisted of 94°C for 10 second, 66°C for 30 second and 72°C for 1.5 minutes. An extension step of 72°C for 10 minutes was added to the end of the PCR program.

8.4.8 Cloning of genes

Genes were cloned using Qiagen PCR Cloning^{plus} Kit (Qiagen, Inc.). PCR products were purified with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences Corp) using the protocol recommended by manufacturer. An aliquot of 2 µl (70ng/µl) of PCR products with an average size of about 1000 bp were mixed with 50 ng of pDrive Cloning Vector, 2 µl of distilled water and 5 µl of 2 X ligation master mix, and ligated at 4-16°C overnight.

An aliquot of 2 µl of the ligated mixture was added to 50 µl of Qiagen EZ Competent Cells and incubated for 5 minutes followed by a heat shock procedure at 42°C for 30 seconds. Subsequent to 2 minutes of incubation on ice, 250 µl of SOC medium was added to the mixture. The transformation mixture, 50 µl each, was plated onto 6 LB agar plates containing ampicillin (100 µg/ml), IPTG (5 mM) and X-gal (80 µg/ml). The plates were incubated at 37°C overnight.

After blue/white screening, white colonies were individually inoculated in 3 ml of liquid LB medium containing ampicillin (100 µg/ml) and grown at 37°C overnight with vigorous shaking. Subsequently, an aliquot of 750 µl of each culture was mixed with 750 µl of LB medium containing 30% sterile glycerol, and stored as bacterial stock at -70 °C.

8.3.4.9 Minipreparation of plasmid DNA

This protocol of minipreparation of plasmid was modified from the methods of Birnboim and Doly (1979). In brief, cells were pelleted by centrifugation at 15,000 rpm for 20 seconds. The bacteria were resuspended in 100 µl of Ice-cold Solution A containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA (pH 8.0), followed by the addition of 200 µl Solution B containing 0.2M NaOH and 1%SDS. The samples were gently mixed and incubated on ice for 15 minutes. An aliquot of 150 µl

ice-cold Solution C containing 3 M potassium acetate and 1.8 M glacial acetic acid was added to the samples to precipitate the protein and bacterial debris. Supernatant was collected after 6 minutes of centrifugation at 15,000 rpm. Subsequently, the supernatant was extracted with 450 µl of phenol:chloroform:isoamyl-alcohol (25:24:1), then extracted with 450 µl of chloroform:isoamyl-alcohol (24:1). RNA in the sample was digested with 0.5 µl of Rnase (10mg/ml) at 37°C for 20 minutes. DNA was precipitated by the addition of 900 µl of ice-cold 100% ethanol and incubated at -20°C for 20 minutes. After 10 minutes centrifugation at 15,000 rpm, 500 µl of 70% ethanol was used to wash the DNA precipitate. The air dried DNA pellet was dissolved in 50 µl of deionized H_2O.

The presence and size of the DNA insert were confirmed by EcoRI enzyme digestion. An aliquot of 2 µl of the plasmid DNA (approximately 3 µg of plasmid DNA) was mixed with 6 µl of the digestion master mix containing 4.5 µl of deionized H_2O, 1 µl of 10 X H buffer (500 mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, 10 mM Dithiothreitol, and 100 mM NaCl), and 0.5 µl of EcoRI (15 units/ µl) (Amersham Biosciences Inc.). The mixture was incubated at 37 °C for 3 hours. The digested samples were analyzed on a 1.5% agarose gel after electrophoresis at 70 volts for 2 hours.

8.3.5. DNA sequencing and blast search

The plasmid DNA samples with DNA insert were sequenced at the Genome Québec Innovation Centre. The primer SP6 and M13 reverse were used for DNA sequencing in both directions. The DNA sequences obtained were analyzed using the search engines provided by National Center for Biotechnology Information.

8.3.6 Dot blot assay

8.3.6.1 Preparation of the plasmid DNA

For blotting, 400 ng of plasmid DNA containing the insert was denatured in 800 µl of 6 X SSC containing 0.1 vol of 1 M NaOH at 37°C for 5 minutes. Aliquots of 200 µl of the samples containing 100 ng of plasmid DNA were spotted onto a positively charged nylon membrane, Hybond N+ (Amersham Biosciences) prewetted with 10 x SSC with a Minifold® System I Dot Blot apparatus (Schleicher and Schuell BioScience, Inc.).

Subsequently, the membrane was soaked in denaturing solution containing 1.5M NaCl and 0.5M NaOH for 5 minutes followed by a neutralization step in solution containing 1.5M NaCl, 0.5M Tris-HCl (pH 7.2) and 0.001M EDTA for 1 minute. The plasmid DNA was immobilized on the membrane by baking at 80°C for 2 hours. The detection of nucleic acid was done using The ECL Direct Nucleic Acid Labelling and Detection Systems (Amersham Biosciences).

8.3.6.2 Probe preparation

The cDNA reversed transcribed from the mRNA of the male or female samples from genital ridge including Müllerian duct was used for probe preparation. The concentration of the cDNA was adjusted to 10 ng/µl. Two aliquots of 10 µl of male cDNA and female cDNA were denatured in boiling water bath for 5 minutes and cooled on ice immediately for 5 minutes. After adding 10 µl of labeling reagent (provided from the kit) and 10 µl of glutaraldehyde solution, the probes were incubated at 37°C for 10 minutes and held on ice.

8.3.6.3 Hybridization

Blocking reagent was added to ECL Gold Hybridization Buffer (Amersham Biosciences Inc.) with 0.5M NaCl to a final concentration of 5% (w/v). The blot was prewetted with 5 X SSC before being prehybridized with 10 ml of hybridization buffer at 42°C for 15 minutes in a rotisserie hybridization incubator. An aliquot of 10 µl of the labeled probe per blot was added to the hybridization buffer and hybridized overnight in a rotisserie oven at 42°C.

8.3.6.4 Washing and Detection

Following the hybridization, the hybridization buffer was replaced by 50 ml 5 X SSC. The blots were incubated at 42°C for 5 minutes then washed with 25 ml of primary washing buffer (Urea 6M, 0.4 % SDS, and 0.5 X SSC) at 42°C for 20 minutes. This washing step was repeated twice for 10 minutes each time. The membrane was then washed twice with 10 ml of 2X SSC at room temperature for 5 minutes with gentle agitation.

The blot was placed on a sheet of Saran Wrap with the DNA side uppermost. The working solution for detection was prepared by mixing 5 ml of detection reagent I with 5 ml of detection reagent II and added directly on top of the blot. After one minute of incubation at room temperature, the excess working solution was drained off. The blot was then wrapped in Saran wrap and exposed to Kodak X-ray film for autography. After the film was developed with Kodak Film Developer, the image was digitalized using Quantity One Software (Bio-Rad Laboratories, Inc.).

8.4. RESULTS

The results from the sex determination PCR is shown in Figure 8.4a. Samples showing two-bands are expected to be female; whereas, the one-band pattern indicated that the individuals were male birds.

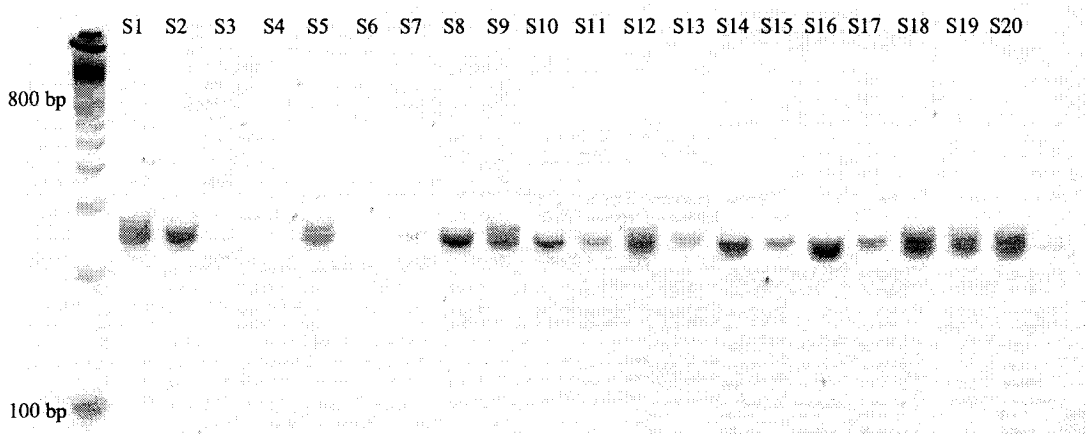

Figure 8.4a The sex determination of day 8 chicken embryo. Twenty amplicons of the CHD gene from the genomic DNA of day 8 chicken embryo were analysed on a 3% agarose/EtBr gel after electrophoresis at 70 volts for 2 hours. The first lane is a 100 base-pair ladder (Amersham Biosciences, Inc).

After the confirmation of the sex of the embryos, total RNA was extracted from the left genital ridge including Müllerian duct of male or female embryos, and was reverse transcribed into cDNA. Two SSH libraries were created by subtracting the cDNA from the male and the female. An aliquot from each library was ligated into vectors, transformed into E. coli and cultured on LB agar. Ninety-six genes from each library were randomly selected by blue/white screening. Subsequent to mini-preparation of plasmid DNA, a total of 192 genes were digested with EcoRI restrictions enzyme. The presence and size of the DNA insert were confirmed by EcoRI enzyme digestion. An example of EcoRI enzyme digestion of plasmid DNA is shown in Figure 8.4b. Different sizes of DNA insert were detected indicating various DNA sequences were picked up from the library. Furthermore, 2 or 3 distinct insert bands were detected in some samples suggesting that the insert contained an internal EcoRI digestion site, or a mixture of two plasmids with different insert was loaded in the well.

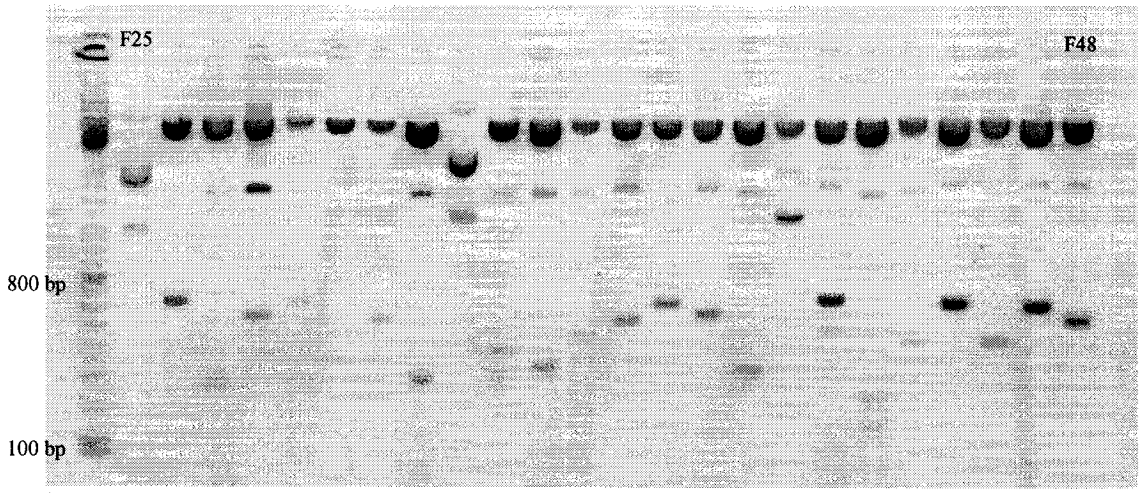

Figure 8.4b EcoRI digestion of plasmid DNA. The presence and size of the DNA insert were confirmed by EcoRI enzyme digestion. Approximately 3 μg of EcoRI-digested plasmid DNA from each sample were analysed on a 1.5% agarose gel after electrophoresis at 70 volts for 2 hours. The first lane is a 100 base-pair ladder (Amersham Biosciences, Inc). Twenty-four plasmids (FS25 to FS48) were shown here. The bands with higher molecular weight represent the plasmid, whereas, the bands with lower molecular weight represent the DNA insert.

The selection of plasmids subjected to DNA sequencing was based on the EcoRI enzyme digestion assay. Since all plasmids contained a maximum of three digested products, all 192 plasmids were submitted to the Genome Québec Innovation Centre for DNA sequencing. A selected list of the blast search results is presented in Table 8.4a and 8.4b.

Dot blot assay was performed (data not shown). However, no substantial differences were found among blots. Therefore, Q-PCR is required to confirm the differential gene expression.

Table 8.4a Selected list of genes from the female library	
Name of Genes	**Score / Expect value / Identities**
Gallus gallus frizzled-7 (FZ7) mRNA, partial cds	Score = 712 bits (370), Expect = 0.0 Identities = 419/439 (95%)
PREDICTED: Gallus gallus vacuolar H-ATPase B subunit osteoclast isozyme (VATB), mRNA	Score = 454 bits (236), Expect = e-124 Identities = 236/236 (100%)
PREDICTED: Gallus gallus similar to putative NADH dehydrogenase (ubiquinone) 1 beta subcomplex 1 (LOC423415), mRNA	Score = 456 bits (237), Expect = e-125 Identities = 259/270 (95%)
Gallus gallus collapsin response mediator protein-1B (CRMP1B) mRNA, complete cds	Score = 992 bits (516), Expect = 0.0 Identities = 522/525 (99%)
PREDICTED: Gallus gallus similar to cyclin I (LOC422634), mRNA	Score = 806 bits (419), Expect = 0.0 Identities = 425/428 (99%)
Gallus gallus lamin B2 (LMNB2), mRNA	Score = 1002 bits (521), Expect = 0.0 Identities = 521/521 (100%)
Chicken apolipoprotein AI (Apo-AI) mRNA, complete cds	Score = 813 bits (423), Expect = 0.0 Identities = 430/431 (99%)
PREDICTED: Gallus gallus similar to epsilon isoform of 14-3-3 protein	Score = 1152 bits (599), Expect = 0.0 Identities = 634/644 (98%)
PREDICTED: Gallus gallus similar to epsin 2 isoform a; Eps15 binding protein (LOC416524), mRNA	Score = 673 bits (350), Expect = 0.0 Identities = 352/353 (99%)
Gallus gallus OL-protocadherin isoform mRNA, complete cds; alternatively spliced	Score = 665 bits (346), Expect = 0.0 Identities = 350/352 (99%)
PREDICTED: Gallus gallus similar to peptidyl-prolyl isomerase G (cyclophilin G); Clk-associating RS-cyclophilin	Score = 1060 bits (551), Expect = 0.0 Identities = 559/563 (99%)
PREDICTED: Gallus gallus similar to Nucleostemin (LOC415897), mRNA	Score = 671 bits (349), Expect = 0.0 Identities = 357/361 (98%)
PREDICTED: Gallus gallus similar to arginine/serine-rich splicing factor 6 (LOC419116), mRNA	Score = 539 bits (280), Expect = e-150 Identities = 282/283 (99%)
Gallus gallus GCN5 general control of amino-acid synthesis 5-like 2 (yeast) (GCN5L2), mRNA	Score = 446 bits (232), Expect = e-122 Identities = 236/238 (99%)
PREDICTED: Gallus gallus similar to Tpr (LOC424457), mRNA	Score = 1017 bits (529), Expect = 0.0 Identities = 541/547 (98%)
PREDICTED: Gallus gallus similar to CG6340-PB (LOC416864), mRNA	Score = 1283 bits (667), Expect = 0.0 Identities = 675/679 (99%)
PREDICTED: Gallus gallus similar to 3110004L20Rik protein	Score = 479 bits (249), Expect = e-132 Identities = 251/252 (99%)
PREDICTED: Gallus gallus similar to protein tyrosine phosphatase, non-receptor type 6 isoform 2; protein-tyrosine phosphatase 1C; hematopoietic cell phophatase; hematopoietic cell protein-tyrosine phosphatase; 70 kda SHP-1L protein (LOC427947), mRNA	Score = 289 bits (150), Expect = 6e-75 Identities = 165/170 (97%)

Table 8.4b Selected list of genes from the male library	
Name of Genes	**Score / Expect value / Identities**
predicted: Gallus gallus similar to protein KIAA0103(LOC420273) mRNA	Score=1121bits(583) expect=0.0 Identities=572/592 (96%)
PREDICTED: Gallus gallus similar to Fraser syndrome 1 isoform 1;extracellular matrix protein (LOC418893), mRNA	Score=902bits(469) expect=0.0 Identities=477/481 (99%)
G.gallus PG-M mRNA for proteoglycan	Score=230bits(116) expect=3e-57 Identities=119/120 (99%)
Gallus gallus transcription factor Crx (LOC395160), mRNA	Score = 469 bits (244), Expect = e-129 Identities = 257/270 (95%)
predicted: Gallus gallus similar to UDP-N-acetylglucosamine-dolichyl-phosphate, (LOC419699) mRNA	Score=754bits(392) expect=0.0 Identities=448/479 (93%)
PREDICTED: Gallus gallus similar to NIF3L1 (LOC424076), mRNA	Score=858bits(446) expect=0.0 Identities=440/455 (96%)
Gallus gallus collapsin response mediator protein-1B (CRMP1B), mRNA	Score=1385bits(720) expect=0.0 Identities=708/738 (95%)
PREDICTED: Gallus gallus similar to oxysterol-binding protein-like 1A isoform B; oxysterol-binding protein-related protein 1; oxysterol-binding protein-like 1B; OSBP-related protein 1 (LOC421079), mRNA	Score = 960 bits (499), Expect = 0.0 Identities = 514/519 (99%)
PREDICTED: Gallus gallus similar to TUBULIN BETA-5 CHAIN (BETA-TUBULIN CLASS-V) (LOC421037), mRNA	Score = 667 bits (347), Expect = 0.0 Identities = 329/353 (93%)
predicted: Gallus gallus similar to transmembrane 9 superfamily protein member 4(LOC419281), mRNA	Score = 632 bits (319), Expect = e-178 Identities = 328/331 (99%)
Gallus gallus endothelin receptor type A (EDNRA), mRNA	Score = 1186 bits (617), Expect = 0.0 Identities = 623/626 (99%)
PREDICTED: Gallus gallus similar to beta prime cop (LOC424823), mRNA	Score = 937 bits (487), Expect = 0.0 Identities = 495/499 (99%)
prdicted:Gallus gallus similar to glutaminyl cyclase(LOC421479), mRNA	Score=1185bits(616) expect=0.0 Identities=632/640 (98%)

8.5. FURTHER INVESTIGATION

Several genes will be selected from the Table 8.4a and 8.4b for the Q-PCR analysis. Moreover, another two libraries from day 6 or day 7 embryos will be created because we suspected that the signal of the regression of the Müllerian duct might start earlier than day 8.