EXPRESSION OF THYROID STIMULATING HORMONE IN CERVICAL EPITHELIAL CELLS

A Thesis

by

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ABSTRACT

Thyroid stimulating hormone (TSH) is an important endocrine hormone of the pituitary affecting metabolism, development, and immunity. However, TSH has lesser known roles in other bodily processes. Cells of the peripheral immune system are known to produce TSH, and epithelial tissues in the intestine and dermis have also been found to express TSH. We hypothesized that TSH may play a role in the mechanism of action of human papilloma virus infection in cervical epithelia, and subsequent tumorigenesis. As a first step in testing that hypothesis, the objective of this project was to evaluate human cervical-derived epithelial cell lines for endogenous TSH production. We examined TSH expression in four cell lines that included cells derived from normal epithelium (Ect1) and three cervical cancer cell lines (C33A, HeLa, SW756). Three of the cell lines (Ect1, HeLa, SW756) were also positive for human papilloma virus (HPV) infection. The expression of TSH mRNA and protein in human cervical epithelial cells was demonstrated for the first time in this study. Physical data generated show strong evidence that TSH is produced in several cervical epithelial cell lines. The IL-7 protein was also present in all cell lines, consistent with findings in other tissue types. The data in this experiment also suggested a possible relationship between TSH expression and the presence of HPV infection. This research establishes a foundation for further research to address the functional role of TSH in cervical epithelium.

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1. INTRODUCTION

Thyroid stimulating hormone (TSH) is an important endocrine hormone of the pituitary that plays a role in metabolism, development, and immunity. It has been well studied in its classical role of stimulating the thyroid gland, and much is known about its mechanisms of action. TSH is a pituitary hormone produced in the thyrotrope cells of the anterior pituitary and released into systemic circulation via the hypothalamus-pituitary-thyroid (HPT) axis. It acts directly on the thyroid gland to induce production and secretion of thyroxine (T_4) and triiodothyroxine (T_3). More recently there have been new findings that suggest TSH may have many lesser known roles in other bodily processes. Cells of the peripheral immune system are known to produce TSH, but the significance of this is not known. Epithelial tissues in the intestine and dermis have also been found to express TSH. Rotaviral infection of intestinal cells leads to an increase in production of TSH suggesting that this may also play a role in viral immune responses. TSH may play a role in the mechanism of action of human papilloma virus (HPV) infection in cervical epithelia, and subsequent tumorigenesis. The objective of this project was to evaluate human cervical-derived epithelial cell lines for endogenous TSH production. Four cell lines were chosen for evaluation that differed in cancerous phenotype and HPV infection status.

2. LITERATURE REVIEW

2.1 Traditional TSH Structure and Function

Gene Structure

The gene that encodes the alpha subunit of TSH (*TSHA*) is located on the q-arm of chromosome 6, 21 cM from the centromere (Szkudlinski et al. 2002). It is 9.4 kb long, and consists of four exons and three introns (Szkudlinski et al. 2002). The gene that encodes the beta subunit of TSH (*TSHB*) is located on p-arm of chromosome 1, 13 cM from the centromere (Grossmann 1997). It is 4.9 kb long, and consists of three exons and two introns (Szkudlinski et al. 2002). *TSHA* is produced broadly in both thyrotrope and gonadotrope cell types of the anterior pituitary (Wood et al. 1999), while *TSHB* production is restricted to the thyrotrope cells (Szkudlinski et al. 2002). Only about 5% of the cells in the anterior pituitary are TSH-producing thyrotrope cells (Szkudlinski et al. 2002).

An alternatively spliced variant of *TSHB* also exists (Miranda et al. 2009). This alternative protein retains the ability to dimerize with the alpha subunit, and bind to the TSH receptor (TSHR) to induce cAMP activity (Miranda et al. 2009). This variant transcript is expressed in many TSH expressing tissues, but is most common in the cells of the immune system (Miranda et al. 2009). Alternative variant splicing increases in expression following a systemic virus infection, and

may induce the formation of anti-TSH antibodies and autoimmune thyroiditis (Miranda et al. 2009).

Protein Structure

Thyroid stimulating hormone is a dimer that consists of an alpha and beta subunit (Rose 2004). It belongs to the glycoprotein family and shares a common alpha subunit with follicle stimulating hormone, luteinizing hormone, and chorionic gonadotropin (Grossmann 1997; Rose 2004). As with all members of the glycoprotein family, TSH carries a beta subunit that is unique to its particular function.

Thyroid stimulating hormone is a 28-30 kDa protein that varies in size based on the number of carbohydrate chains attached (Grossmann 1997). The alpha subunit of TSH consists of 92 amino acids, while the functionally-specific beta unit consists of 118 amino acids (Grossmann 1997). The subunits are linked together non-covalently, and the long C-terminal tail of the beta subunit acts as a "seat belt" to stabilize dimerization and aid in subunit cohesion (Szkudlinski et al. 2002). The glycoprotein hormone family is structurally classified in the cysteine knot group super-family (Szkudlinski et al. 2002). This means that during tertiary folding the subunits fold around a central cysteine knot formation. The cysteine knot is made of three central disulfide bridges where one bridge threads a ring produced by the other two bridges (Szkudlinski et al. 2002). The remaining loops

and sheets of the final tertiary structure of the protein then form around the cysteine knot.

As a member of the glycoprotein family, the TSH proteins undergo post-transcriptional modification to accommodate the addition of oligosaccharide side chains. Glycosylation of the protein is important in determining its bioactivity, and both subunits become glycosylated (Rose 2004). Once attached the oligosaccharide chains account for about 30% of the molecular weight of the active hormone (Szkudlinski et al. 2002). The alpha unit acquires two oligosaccharide chains which make up 40% of the subunits weight (Rapoport 1998), while the beta unit receives one side chain(Rose 2004). The oligosaccharides are linked to the subunits by binding to specific asparagine molecules in their amino acid chains (Szkudlinski et al. 2002).

TSH Receptor

The TSH receptor is a member of the G-protein coupled seven trans-membrane domain family (Kleinau and Krause 2009). The *TSHR* gene is located on the q arm of chromosome 14, 31cM from the centromere (Davis and Camarillo 1996; Szkudlinski et al. 2002), and encodes a 84.5 kDa, 764 amino acid (Rapoport 1998) sequence that undergoes folding and glycosylation to produce the active TSHR (Davies et al. 2010). The active *TSHR* contains several functional domains: a leucine rich domain (LRD), a cleavage domain (CD), a trans membrane domain (TMD), and an intracytoplasmic tail (ICT) (Davies et al. 2010). TSHR is primarily

found in the cell membrane of thyroid cells, but is also found in lymphocytes, adipocytes, retroocular fibroblasts, neuronal cells, and astrocytes (Szkudlinski et al. 2002). When TSH binds to its receptor, it initiates second messenger pathways involving cAMP. When very high concentrations of TSH are present the inositol 1,4,5-triphosphate and diacylglycerol pathways are also activated (Kleinau and Krause 2009).

The ectodomain of the receptor is a large domain of 45.2 kDa, 397 amino acids, and includes the CD and LRD regions (Rapoport 1998). The LRD region of the TSHR is the binding site for TSH and makes up 2/3 of the extracellular region of the receptor (Kleinau and Krause 2009). The LRD is a "scythe-blade-like" structure whose shape is supported by the interaction of aromatic side chains with the hydrophobic core (Kleinau and Krause 2009). Thyroid stimulating hormone binds to the concave inner surface of the LRD via exposed side chains that provide selective interaction for recognition of TSH (Kleinau and Krause 2009). The CD or hinge region is attach to the c-terminal region of the LRD and resides in the extracellular region of the receptor (Kleinau and Krause 2009). It is a cystine rich region that is made up of approximately 130 amino acids (Kleinau and Krause 2009). The CD is constructed of two cystine box regions linked together by a linker region (Kleinau and Krause 2009). This region plays a significant role in TSHR conformation stabilization, and with interaction of TSH to elicit TSHR activation (Kleinau and Krause 2009). The extracellular loops of the TMD helices also play a fundamental role in TSHR signaling processes (Kleinau and Krause 2009).

Origin of TSH Producing Cells

The development of the pituitary is a synchronized effort of two separate cell types. The posterior lobe of the pituitary will develop from the ventral diencephalon while the anterior lobe of the pituitary develops as an outgrowth of the primitive oral ectoderm. These tissues will form through a complex and synchronized signaling induction by both tissues (Kelberman et al. 2009). During embryonic development of the mouse, the first signs of ectodermal thickening occur at 7.5 dpc. The thickening then invagenates and gives rise to a structure called Rathke's pouch, which continues to grow inward toward the infundibulum to create the functional anterior lobe (Sheng and Westphal 1999). The anterior pituitary will give rise to the thyrotrope cells and at 11.5 dpc prospective thyrotrope cells in the ventral region of Rathke's pouch can be identified by the expression of glycoprotein hormones, alpha peptide (CGA) and ISL LIM homeobox 1 (ISL1) (Kelberman et al. 2009). At 12.5dpc the thyrotropes begin to differentiate in the anterior pituitary as TSHB expression is initiated. The thyrotropes continue to mature up until about 17.5dpc and at birth the thyrotropes are the least abundant cell type in the anterior pituitary (Japon et al. 1994).

Traditional Function

The primary target of TSH is the thyroid gland. It is an organ of the endocrine system that controls body weight, metabolism, body temperature, muscle growth and reproduction (Reinehr 2010). Thyroid stimulating hormone

acts via its receptor to stimulate several activities in the thyroid gland, and is the key regulator in thyroid control. The primary activity of the thyroid is synthesis and secretion of T_3, T_4 and calcitonin (Grossmann 1997; Kleinau and Krause 2009), critical molecules for development and metabolism (Kress et al. 2009). Thyroid stimulating hormone initiates the process of thyroid hormone production by increasing the production of ATP in order to facilitate the active transport of iodine ions from the blood to the thyroid tissue via the apical iodine channels (Cavalieri 1997). Thyroid stimulating hormone then activates the peroxidases in the thyroid cells to oxidize iodine so that it can be used in the synthesis of thyroid hormones (Mansourian 2011). When TSH binds to its receptor it acts via the cAMP pathway to upregulate the production of thyroglobulin, the precursor for the functional thyroid hormones (Cavalieri 1997). The main product of the thyroid is T4, a pro form of the active thyroid hormone T3 which is responsible for binding to the receptor in the target cells and initiating a cellular response (Jansen et al. 2005). Thyroid stimulating hormone also acts as a survival factor to protect thyrotrope cells in the thyroid from undergoing apoptosis (Grossmann 1997; Kleinau and Krause 2009).

Regulation

Thyroid stimulating hormone synthesis is regulated by the hypothalamus-pituitary-thyroid (HPT) axis feedback mechanism (Figure 1). Thyrotropin releasing hormone (TRH) secreted from the para-ventricular nucleus of the hypothalamus is the primary stimulator of TSHB production and secretion

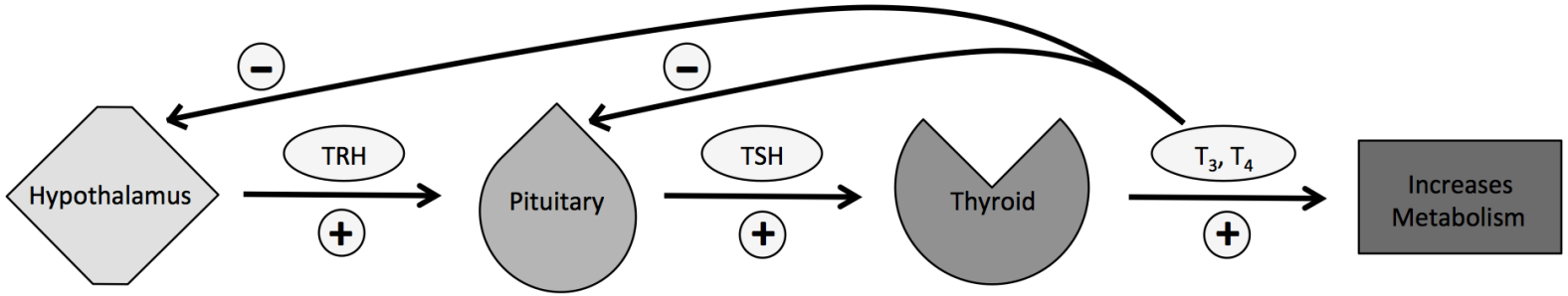

Figure 1- Hypothalamus-pituitary-thyroid axis feedback mechanism.

(Grossmann 1997; Gogakos et al. 2010). Thyroid releasing hormone is primarily mediated by protein kinase C (PKC) in the TSH secreting cells of the pituitary (Steinfelder and Wondisfor 1997). TRH works through PKC to phosphorylate the transcription factor PIT-1 (Steinfelder and Wondisfor 1997). Once phosphorylated, PIT-1 has an increased binding affinity for the THSB gene where it binds to the promoter to stimulate gene expression (Steinfelder and Wondisfor 1997). Pit-1 has also been shown to be important in the development of the thyrotrope cells (Steinfelder and Wondisfor 1997).

The primary means of TSH inhibition is a classical negative feedback loop created by the secretion of the stimulated thyroid hormones T_3 and T_4 (Grossmann 1997). The thyroid hormones then act to inhibit both TRH and TSH (Gogakos et al. 2010). In the pituitary receptor bound T_3 targets a binding site located in the first exon of the THSB gene and blocks transcription of the gene (Steinfelder and Wondisfor 1997). The transcription factors c-Jun and c-Fos that form the heterodimer AP-1 also seem to play a role in this process by antagonizing the inhibitory action of the bound T_3, and allowing for fine control of TSHB gene expression (Steinfelder and Wondisfor 1997). Thyroid stimulating hormone circulation also occurs in a circadian pattern (Rose 2004). Concentration surges nocturnally, and diminishes as the day goes on (Rose 2004). Surges of TRH from the hypothalamus, or altered sensitivity to TRH nocturnally, are the likely causes (Rose 2004).

2.2 Non-Traditional TSH Function

In addition, to the functions TSH has in regulating metabolism, it also plays a role in the immune-endocrine network. Thyroid stimulating hormone is found in several extra pituitary sources in the body. It has been long known that TSHR is found on hematopoietic cells, and TSH binding has been shown in the peripheral blood leukocytes (PBL) (Klein 2003). High levels of TSHR have been detected in monocytes and macrophages, but only low expression in the lymphocytes and the natural killer cells (Klein 2003). Leukocytes preferentially express a splice variant of TSHB that can also be found in the thyroid, but it is unknown whether it is produced locally in the thyroid or by hematopoetic cells migrating to the thyroid (Schaefer and Klein 2009). A splice variant of TSHB derived from the immune system may micro-regulate thyroid hormone output by blocking or augmenting pituitary TSHB function to preserve energy during an active infection (Schaefer and Klein 2009).

Thyroid stimulating hormone has been found to be expressed in $CD11b^+$ bone marrow derived cells (Klein 2003). These TSH producing cells found in the bone marrow often migrate to the thyroid (Klein 2003), and are thought to be a paracrine source of TSH in thyroid (Klein 2006). $CD11b^+$ cells may act as a secondary source of TSH to mediate immune driven control of metabolic activity during an acute infection (Klein 2006). Inflammatory agents released during an acute infection would cause T4 to be converted to T3, and as a result, would drive down TSH production in the pituitary, and lower overall metabolic activity to

encourage energy conservation in the body (Klein 2006). CD11b+ cells would then migrate to the thyroid after recovery to restore metabolic activity (Klein 2006). The importance of bone marrow derived cells in thyroid function can be demonstrated by the disruption of thyroid function that occurs following a bone marrow transplantation (Harvey et al. 2011). TSH expression in these cells also stimulate the production of TNF-α through an autocrine pathway (Wang 2002). The effects of this action is undetermined, but could play a role in the formation of dendric cells from precursor cells in the bone marrow (Wang 2002). Dendric cells in the spleen are also a source of extrapituitary TSH expression (Klein 2006).

Thyroid stimulating hormone also plays a role in the development of T cells in the intestinal epithelium (Klein 2003). T cell-like intestinal epithelial lymphocytes (IELs) are highly abundant in the intestinal epithelium, and display a much more complex phenotype than traditional thymus-derived T cells (Wang 1996). Thyroid releasing hormone produced by IELs induces expression of TSH in the intestinal epithelium (Klein 2003). In both intestinal and peripheral immune systems, the TSH may be acting as a cytokine through autocrine or paracrine action to regulate further cytokine release (Klein 2003). Another possible function of TSH in these cells is to stimulate lymphocyte development and differentiation in the regions (Klein 2003). In the intestinal villi, TSH is localized to cryptopatches that coexpress IL-7 and c-kit (Scofield et al. 2005). These sites are thought to be the primary development sites of IELs (Scofield et al. 2005).

Viral infection increases TSH expression in the cells of the small intestine, and may play a role in activating IELs in response to an antigen being present (Scofield et al. 2005; Varghese et al. 2008). In rotavirus infected mice, new hotblocks of TSH expression in enterocytes were activated outside of the cryopatch region (Scofield et al. 2005). This suggests that TSH is being re-activated in the infected cells as a response to signaling within the cells, or from cytokines produced by nearby IELs.

Thyroid stimulating hormone transcript and protein has also been confirmed to be produced in normal human scalp epidermis, and is subject to the same mechanism of regulation that is found in the HPT-axis (Bodo et al. 2010). In the epidermal cells, TSH did not appear to have much effect on the proliferation rate, but did promote terminal differentiation in epidermal keratinocytes (Bodo et al. 2010). Thyroid stimulating hormone receptor is not found in the epidermis, but in the dermis indicating a paracrine action of the TSH as well as autocrine (Bodo et al. 2010). Most of the secondary functions of TSH are still not well understood and even more are likely still unknown.

2.3 TSH and Cancer

Thyroid stimulating hormone plays an active role in stimulating cell proliferation in the thyroid and may also have a similar affect in cancerous cell types (Schlumberger and Sherman 2011). Thyrotropin binding to its receptor initiates proliferation and differentiation of cells in the thyroid. García-Jiménez and

Santisteban described the mechanism by which this occurs in a 2007 paper: after binding, TSHR is stimulated by TSH to disassociate and activate the $G_{\alpha s}$ subunit. The $G_{\alpha s}$ subunit then activates adenylyl cyclase, which increases cAMP production. Next, PKA is then activated by cAMP to phosphorylate and activate the signaling molecules and transcription factors that promote growth and differentiation (García-Jiménez and Santisteban 2007).

TSH has also been found to play a role in stimulating adhesion, migration, and invasion of differentiated thyroid cancer cells. Zielke et al. (1999) used transwell chambers with polycarbonate cell permeable membranes to demonstrate the effect of TSH to stimulate migration in a well-differentiated human thyroid cancer line. Titrating the concentration of TSH between 1-10mU/mL in the serum free media caused significant increases in the number of migrated cells in the chambers. In less differentiated thyroid cancer cells the effect of the presence of TSH is not as apparent (Zielke et al. 1999).

TSH may drive tumorigenesis in other ways too. In thyroid carcinoma cell lines TSH also has a moderate effect in inducing angiogenesis required for tumor growth by stimulating vascular endothelial growth factor (VEGF) production through the PKC pathway (Hoffmann 2004). The anti-apoptotic actions of TSH may also play a role in tumor development. In the thyroid, TSH stimulates iodine uptake and acts as a survival factor to protect thyrotrope cells from undergoing apoptosis (Grossmann 1997; Kleinau and Krause 2009). More work will be needed to fully understand the effects of TSH presence in cancer cells.

2.4 Human Papilloma Virus

Human papilloma viruses (HPV) are small DNA viruses that infect the mucosal squamous epithelium of the oral and anogenital regions. After infection, HPV acts on the cellular mechanisms to promote transformation of the infected tissue and can lead to the formation of lesions and carcinomas (Hausen 1996). In the cervix, abrasions allow the virus to invade the actively dividing cells of basal layer of the epidermis (Doorbar 2006). In a normal infection the virus reproduces extrachromosomally in the nucleus of the basal layer at low levels until it reaches the upper epithelium. Once it reaches this layer, a massive round of amplification is undergone and the virus is packaged before being released when the cells slough off (Chow and Broker 1994). This process can go awry though if the viral DNA inserts itself into the host chromosome and can result in neoplastic transformation (Southern and Herrington 2000).

Human Papilloma Virus Genome

The HPV genome is 7.9Kb long and is separated into early (E), late (L), and long control (LCR) regions (Figure 2). The early region contains six separate genes that are responsible for replication, regulation and transformation. The late region contains two genes that control the formation of the viral capsid (Hausen 1996). Viral genes E1 and E2 are the primary genes responsible for genome replication and maintenance. The E2 protein recruits E1 to act as a helicase and unwind the

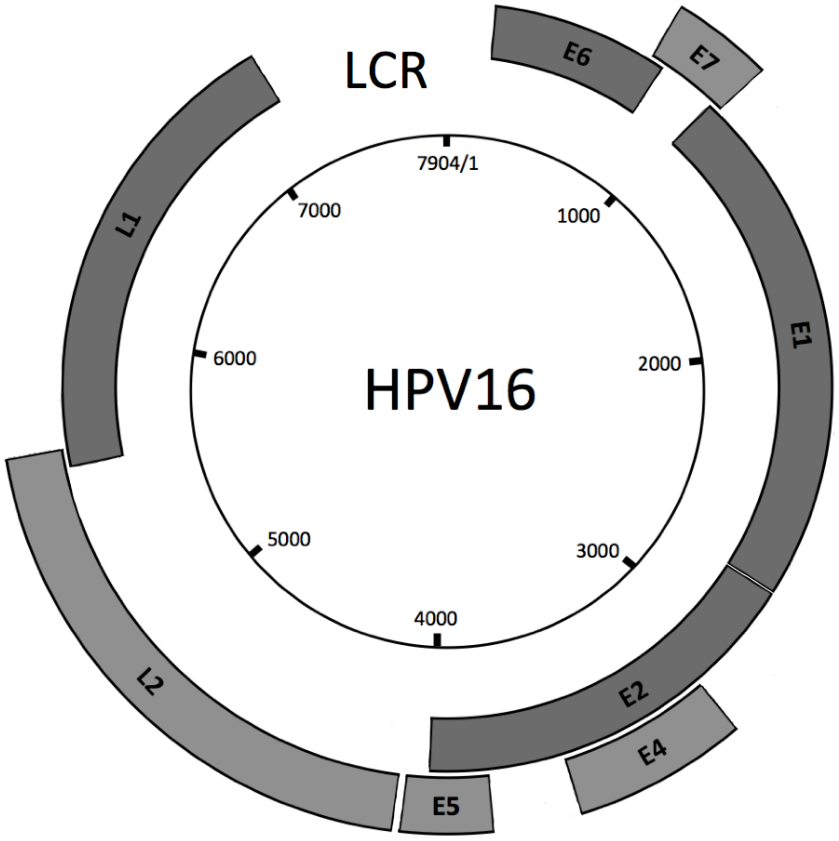

Figure 2- Human Papilloma Virus genome structure.

DNA before replication (Conger et al. 1999). The E2 protein also acts to repress the activity of E6 and E7, the primary genes responsible for transformation (Demeret et al. 1994). The E4 protein acts later in replication to disrupt the cells' keratin network and facilitate release of the virons (Doorbar et al. 1991). The E5 protein interacts with cell membrane growth factor receptors and may play a role in transformation of the cell (DiMaio and Mattoon 2001). The E6 and E7 proteins are the main proteins responsible for transforming the cell. The E6 product degrades p53, while the E7 product degrades pRb (Scheffner et al. 1990; Jones and Wells 2006). In low risk HPV types E6 and E7 have separate promoters, but in High risk HPV types the E6 and E7 genes are combined into one gene that transcribes a full length E6/E7 transcript plus several spliced products (Smotkin et al. 1989). These gene products are only found in high risk HPV types. The L1 and L2 genes encode structural proteins and are activated in the late stages of viral production to facilitate production of the viral capsid (Finnen et al. 2003). The Long Control Region directs and regulates gene expression across the entire HPV genome (Thierry and Yaniv 1987).

Human Papilloma Virus Infection

Human papilloma virus is the most prevalent viral sexually transmitted disease in the world. Infection most often occurs in young adults and decreases with age (Sheet 2008). Most infections are cleared by the body in a short amount of time, and some immunity seems to be developed after infection. Infections that are

not cleared by the body may go on to develop into lesions or growths on the cervix (Kirwan and Herrington 2001). During an infection of high risk HPV types the cell cycle of the infected cells is disrupted in many ways in order to facilitate replication of the HPV DNA.

Several of the viral protein products play crucial roles in the disruption of the host cell cycle. The most important of these proteins are E6 and E7. The E6 protein disrupts cell cycle checkpoints by blocking the apoptotic and tumor suppressing pathways of p53 (Scheffner et al. 1990), BAK (Thomas and Banks 1998), BAX (Li and Dou 2000), and c-Myc (Gross-Mesilaty et al. 1998). In Hela cells, E6 inhibits p53 function by blocking phosphorylation (Ajay et al. 2012). The E6 protein also stimulates endothelial growth by increasing the rate of transcription of vascular endothelial growth factor (VEGF) (Chakrabarti and Krishna 2003). The E7 protein interacts with proteins that regulate cell growth and causes an increase in cell proliferation. The E7 protein is also responsible for immortalization and transformation of a cell (Chakrabarti and Krishna 2003). The E2 protein is important in the regulation of the transformative E6/E7 protein as increased expression can repress E6/E7 expression (Jeon et al. 1995). The E2 protein can induce apoptosis in a host cell (Demeret et al. 2003) and is responsible for tethering the viral DNA to the host cell DNA during mitosis (You et al. 2004). When E2 is overexpressed it can also stimulate multiple rounds of host genome replication without cellular division resulting in increased genome copy number within a cell (Frattini et al. 1997).

High risk HPV infection is a primary risk factor for cervical cancer and over 93% of cancerous tissues from the cervix contain HPV DNA (Walboomers et al. 1999). Human papilloma virus infection is transmitted through mucosa and infects only epithelial cells. Since there is no bloodstream phase to infection, the virus often goes undetected by the host immune system. Only 50-60% of host women go on to produce antibodies to infection despite large numbers of surface markers on HPV infected cells (Carter et al. 2000). In recent years, vaccines have been developed to guard against the more dangerous HPV 16 and 18 infections. The vaccines are based on virus like particles and when taken prophylactically are very effective at preventing HPV infection (Koutsky and Harper 2006).

3. OBJECTIVE

We hypothesized that TSH may play a role in the mechanism of action of human papilloma virus infection in cervical epithelia, and subsequent tumorigenesis. As a first step in testing that hypothesis, the objective of this project was to evaluate human cervical-derived epithelial cell lines for endogenous TSH production. Four cell lines were chosen for evaluation with varying cancer states that differed in cancerous phenotype and HPV infection status. Thus, this project was designed to evaluate cervical epithelia as another possible source of TSH production and its expression in virally infected and tumorigenic cervical epithelia.

4. MATERIALS AND METHODS

4.1 Cell Culture

Human cervical epithelial carcinoma cell lines ECT1/E6E7, C-33A, SW756 and HeLa (Table 1) were obtained from the American Type Culture Collection (Manassas, VA) and expanded two passages in T75 tissue culture flasks (Corning, Inc., Corning, NY) at 37°C in the presence of 5% CO2. The SW756, ECT1/E6E7, and C-33A cell lines were maintained in Basal Medium Eagle (BME) (Invitrogen, Carlsbad, CA) and supplemented with 10% FBS, The HeLa cell line was maintained in BME and supplemented with 2.5% FBS. All media were supplemented with gentamycin (50ng/mL) and 5mL glutamine (Invitrogen, Carlsbad, CA). Culture of human cell lines was performed in a class II type A2 biological safety cabinet (NuAire, Inc., Plymouth, MN).

4.2 RNA Extraction

RNA was extracted with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) and phase separation was done with 1-bromo-3-chloro-propane (BCP) (Molecular Research Center, Inc., Cincinnati, OH). After cell lines were expanded 4-6 T-75 flasks were serially lysed and washed with 6ml Tri-Rreagent. Tri-Reagent was then transferred to six 1.7 ml low retention microcentrifuge tubes (Phenix Research Products, Candler, NC) and cooled to 4°C. The tubes were then

Cell Line	Tissue Morphology	HPV
C33A	Epithelial Carcinoma	Negative
Ect1/E6E7	Normal Epithelia	Type 16+
HeLa	Adenocarcinoma	Type 18+
SW756	Squamous cell carcinoma	Type 18+

Table 1- Cell lines evaluated for TSH and IL-7 activity.

centrifuged at 14,000xg and 4°C for 10 min to remove cell debris. The supernatant was transferred to a new microcentrifuge tube containing 200 mL BCP, vortexed until a milky pink color appeared, and centrifuged at 14,000xg and 4°C for 10 min. The aqueous phase was then transferred to a new microcentrifuge tube containing 200 mL BCP, and vortexed until a milky color appeared, and centrifuged at 14,000xg and 4°C for 10 min. The aqueous phase was again transferred to a new microcentrifuge tube containing 800 mL isopropanol to precipitate the RNA, vortexed 10 s., and centrifuged at 14,000xg and 4°C for 10 min. Supernatant was discarded, and samples were washed in 1 mL 70% ethanol, vortexed 10 s., and centrifuged at 14,000xg and 4°C for 10 min. Supernatant was discarded, and samples were washed in 1 mL 95% ethanol, vortexed 10 s., and centrifuged at 14,000xg and 4°C for 10 min. Supernatant was discarded, and samples were washed in 1 mL 100% ethanol, vortexed 10 s., and centrifuged at 14,000xg and 4°C for 10 min. The supernatant was removed, and the samples were dried down at room temperature. They were then re-suspended in nuclease free water, and stored at -80°C. Samples were DNased (Qiagen, Valencia, CA) and total RNA was quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA quality assessed by capillary electrophoresis on an RNA 6000 NanoChip with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California).

4.3 Reverse Transcriptase Polymerase Chain Reaction

Reverse transcription (RT) was performed using the SuperScript™ II Reverse Trancriptase Kit with either random hexamer primers (Invitrogen, Carlsbad, CA) or direct priming of the target gene. DNase treated RNA samples were used as the template for the reaction.

4.4 Quantitative Reverse Transcriptase Polymerase Chain Reaction

For quantitative assessment of mRNA, cDNA from RT treaded RNA samples were anylized with quantitative RT-PCR (Applied Biosystems, Inc., Foster City, CA). SYBR GreenER qPCR SuperMix, or AmpliTaq Gold™ Master Mix (Invitrogen, Carlsbad, CA) were used to amplify the cDNA samples. Primers used for *18S* gene analysis were F TGCCGGAGTCTCGTTCGT, R GGTGCATGGCCGTTCTTAGT, TaqMan® gene expression arrays were used for *ACTB, TSH, B2M* and *IL7* (Applied Biosystems, Foster City, CA). Samples were loaded into a 384-well optical PCR plate (catalog # MPS-3898, Phenix, Hayward, CA) using Eppendorf Research® Plus pipetters (Eppendorf, Westbury, NY), and sealed with optical PCR sealing tape (catalog # LMT-OPCT, Phenix Research Products, Candler, NC). Samples were loaded onto a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). for thermal cycling (60°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min) utilizing SDS 2.3 software (Applied Biosystems, Foster City, CA). Quantitative RT-PCR data were analyzed by relative quantification (Livak and Schmittgen 2001).

4.5 Western Blotting

Samples were boiled for 3 minutes at 100 ^{O}C under non-reducing conditions, then loaded into a 12% Mini-PROTEAN® TGX™ polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA). Samples were electrophoresed for 50 minutes at a constant 150V in a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, Inc., Hercules, CA) electrophoresis unit. The protein was then transferred to a PVDF membrane (EMD Millipore, Billerica, MA) in a Mini Trans-Blot® module (Bio-Rad Laboratories, Inc., Hercules, CA) for 90 minutes at a constant 400mA. Membranes were stained with 2.5μg/mL IL7 primary antibody (R&D Systems, Minneapolis, MN) and 0.4μg/mL TSHB primary antibody (Santa Cruz, Dallas, TX) overnight and ACTB (EMD Millipore, Billerica, MA) was used for a control. Membranes were washed and then incubated in secondary antibody (Dako, Carpenteria, CA) for 45 minutes. Membranes were then imaged with Immoblion Western Chemiluminescent Substrate (EMD Millipore, Damstadt, Germany) on a Kodak Image Station 4000MM digital developer (Carestream, Rochester, NY). HEK293 cells, human embryonic kidney tumor origin, were used as a negative control for both proteins.

5. RESULTS

5.1 Expression Analysis of TSH mRNA

Quantitative realtime RT-PCR was performed on lysates from each cell line to determine presence of TSH transcript (Figure 3). Transcript was amplified from all four cell lines at very low levels with detection of threshold cycles (Cts) in the 31-34 range. Three genes were evaluated as potential reference genes, *ACTB*, *18S*, and *B2M*, for normalization of *TSH* and *IL7* expression. The transcripts *ACTB* and *18S* were stably expressed across samples, but *B2M* had severely suppressed expression in the cancerous cell types. After a quick literature search, we found that cancerous phenotype suppresses expression of B2M and therefore depressed the Ct values of the cancerous cell lines when evaluated with real-time RT-PCR (Nihon-Yanagi et al. 2013). Because of this effect B2M was no longer considered a candidate for reference gene. The two remaining reference genes were then compared using the method outlined in Vandesompele *et al*. (2002), and a combined reference was calculated using geometric mean (Vandesompele et al. 2002). This combined reference proved to be the most stable and was therefore used to calculate the relative quantifications (RQ) for the two genes of interest.

When the RQ was calculated, the SW756 cell line had the highest expression of TSH when compared to the reference. The C33A and HeLa cell lines had similar TSH expression, but lower levels than Ect-1 and SW756 when normalized.

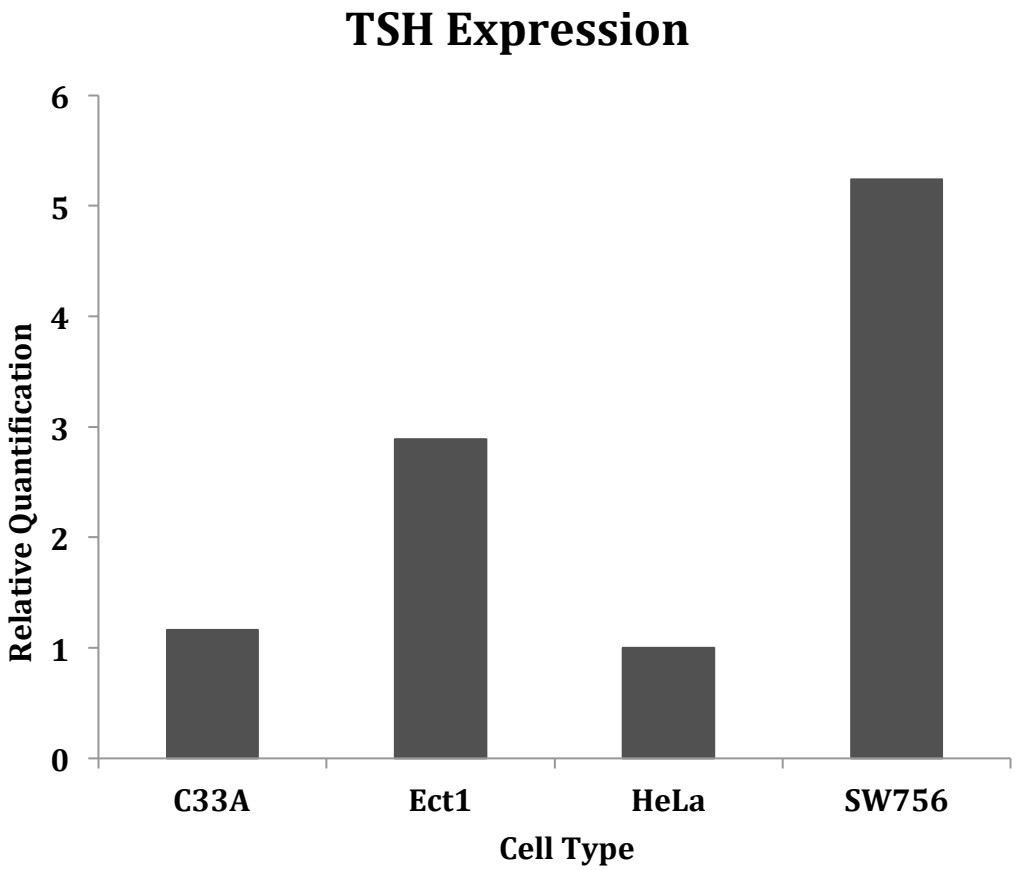

Figure 3- Quantitative PCR results for TSH activity normalized to two reference genes

5.2 Expression Analysis of IL-7 mRNA

Quantitative real-time RT-PCR was performed on RNA extracted from lysates from each cell line to determine presence of IL-7 transcript (Figure 4). When the RQ was calculated the ECT-1 cell line had the highest expression of IL-7 when compared to the reference. In the C33A and HeLa cell lines IL-7 was undetectable.

5.3 Western Blot analysis of TSH and IL-7 Protein

Translation of the expressed TSH and IL-7 RNA was confirmed by western blotting (Figure 5). Thyroid stimulating hormone protein was detected in all of the cell lines at approximately 37kD. The ECT-1 and SW756 cell lines had the largest staining protein bands and this correlates nicely with the observed mRNA expression data from the quantitative PCR assays. The secreted form of TSH was not present on the pictured western blots, but possibly could have been seen in culture media rather than cell lysate.

When blotting for IL-7, bands were present for all cell lines, even the cell lines that did not have detectable signal with quantitative PCR. Again, the ECT-1 and SW756 cell lines had the largest staining protein bands and this observation correlates with the observed mRNA expression data from the quantitative PCR assays. The presence of IL-7 bands for the cell lines that did not have detectable quantitative PCR signal could be due to a low level of non-specific binding of the

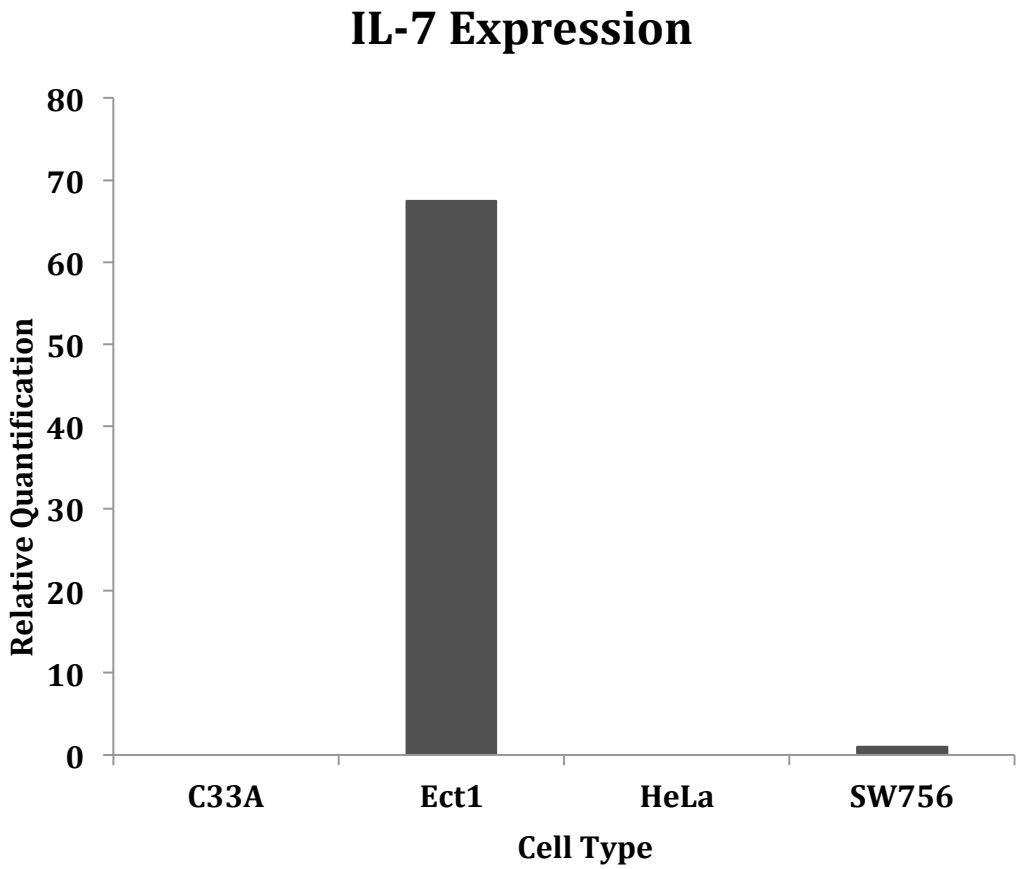

Figure 4- Quantitative PCR results for IL-7 expression normalized to two reference genes.

antibody used, or the mRNA was below the sensitivity threshold of the method used to detect it. The size location of the detected IL-7 protein was not where it was expected based on its predicted size. When formed, IL-7 folds into a 20kD protein that is then excreted from the cell. After excretion, IL-7 often forms a heterodimer with hepatocyte growth factor beta (HGF-β) and functions as a growth factor for pre-pro-B cell growth(Mackall 2006). The beta chain of HGF is 34kD and when dimerized with the 20kD IL-7 protein produces a 54kD heterodimer. When IL-7 was imaged on the western blot it was located between the 50 and 60kD protein markers, thus indicating the dimerized form was likely being detected. A follow up western blot to confirm the presence of HGF-β would have been ideal, but the reagents were not readily accessible.

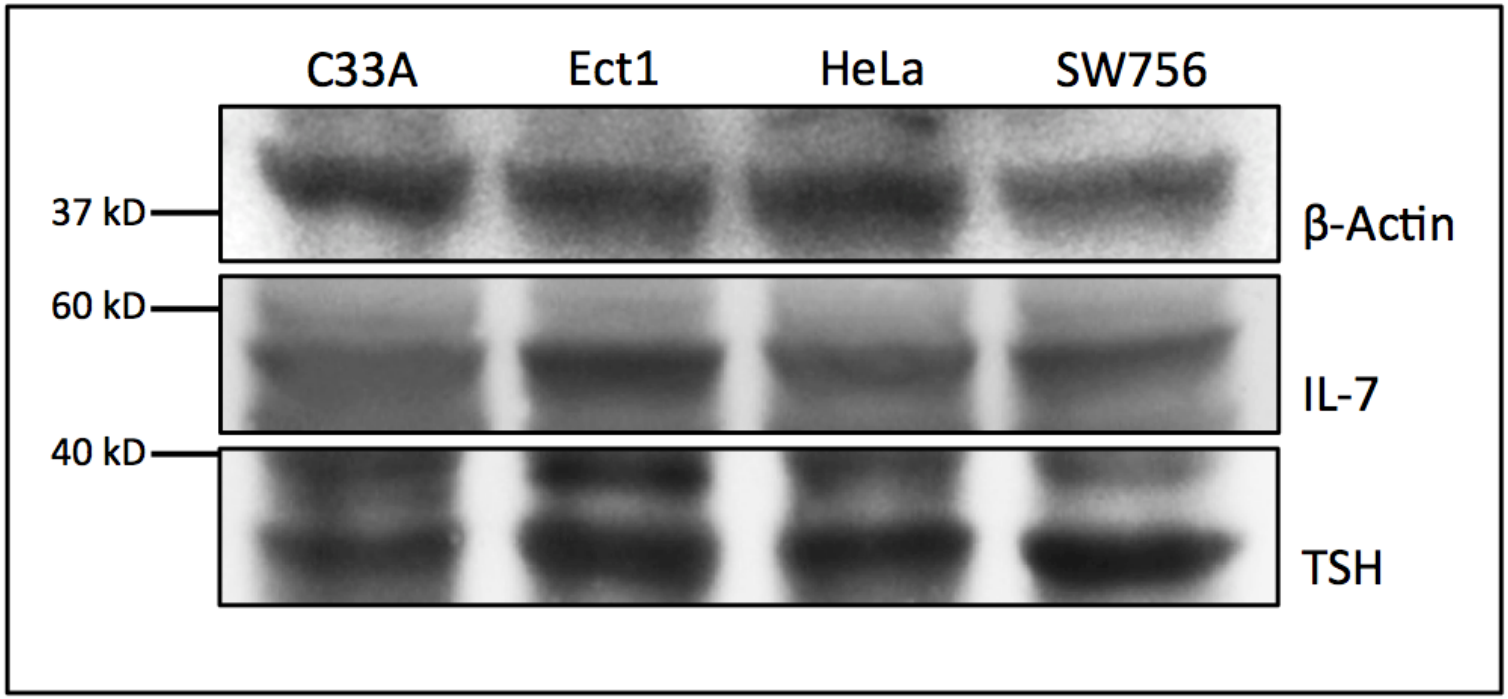

Figure 5- Western Blot of TSH, IL-7 and ACTB

6. DISCUSSION AND CONCLUSION

Thyroid stimulating hormone is found in several extra pituitary sources in the body. In addition to the functions TSH has in regulating metabolism, it also plays a role in the immune-endocrine network, and is expressed in lymphocytes, T-cells, intestinal epithelia, and keratinocytes. The objective of this project was to evaluate human cervical derived epithelial cell lines as an additional source of TSH production.

In the 2005 study by Scofield et al., TSH expression was investigated in the intestinal villi of the mouse small intestine. They found that in the intestinal villi TSH is localized to cryptopatches that co-express IL-7 and c-kit, locations are thought to be the primary development sites of intestinal epithelial lymphocytes (IELs). When these cells were exposed to rotavirus infection, TSH expression in the cells increased. They postulated that TSH is being re-activated in the infected cells, either as a response to signaling within the cells or from TRH produced by nearby IELs, and this increase in TSH levels may play a role in activating the IELs in response to a viral antigen being present.

In the cervical cell lines examined, this TSH-IL-7 relationship seemed to be maintained to a degree. The best example was in the non-cancerous ECT-1 cell line, which had a much higher expression of IL-7 than any of the other cell types, and also had the second highest expression of TSH. The other cell type that had

detectable levels of IL-7 expression was the SW756 cell line, and it also had the highest level of TSH expression. The C33A and HeLa cell lines did not have detectable IL-7 mRNA expression, but seemed to have low levels of detectable IL-7 protein, and were the lowest TSH producers.

The effect of viral infection in the cervical cell lines on TSH production cannot be determined from this small study, although it is notable that expression was greater in the HPV-infected normal epithelial line (Ect1) than the non-infected epithelial carcinoma line (C33A). The mouse study referenced above was performed on whole tissues so interactions between cell types could be observed. In the cervical cell lines analyzed, the two highest producers of TSH transcript were both HPV positive, but with only four cell types it is difficult to make any conclusions. Determining the effect of HPV infection on TSH production was not a goal of this particular paper, but the idea that TSH might have a functional role in HPV infection and subsequent carcinogenesis was a component of our initial hypothesis. In the mouse intestinal villi study viral infection up-regulated TSH expression. It will be interesting to expand this study into a much larger sample set that can appropriately evaluate the relationship between TSH expression and HPV infection.

This study was an important first step in determining whether TSH is produced by cervical epithelium and it's functions. The objective was to begin with a cell culture system for preliminary testing. It is acknowledged that this preliminary analysis has a few shortfalls. First and foremost is that no good

"normal" or control cell line was identified. Commercial non-transformed human cervical epithelial cell lines were simply unavailable for the study. Confirmation of TSH expression in tissue or a primary cell line would be optimal because it more closely mimics in vitro conditions. Now that our data has shown the presence of TSH in cell lines, primary cell or tissue experiments would be an appropriate follow-up. Another potential issue with the way this project was designed is that all the cell lines were morphologically different and no conclusive comparisons could be made to determine whether cancer state or viral infection had any effect on TSH production. To some extent, this reflects the nature of cell culture experiments versus primary tissue analysis. Conversely though, the inclusion of several types of cervical epithelial cell lines makes a better case that TSH is likely expressed in all lines and not just cell lines with a certain phenotype. These results would justify additional work to examine expression in tissue biopsies or tissues from other sources.

There are several future directions for this research to go into that are beyond the scope of this initial project. First would be to determine the role of TSH in cervical epithelial cells, and to answer the question of why this protein is being expressed in this tissue. In other tissues TSH plays a role in the immune-endocrine network, and it would be reasonable to hypothesize it has the same function in cervical cells. As with the intestinal villi studied in the Scofield paper, the cervical epithelia cells act as a barrier between internal and external spaces. Therefore it would be important in to have similar mechanisms in the cervical cells to protect

against a potential viral threat as those seen in the intestinal villi. This possible TSH action in response to a viral infection would be another future direction of study. A larger study would be needed to look at more cell types and determine if HPV infection or even the resulting cancerous state effect expression of TSH, or if it plays a role in the epidemiology. The cancerous phenotype is highly variable and different forms of cancer cells act very differently from each other and could have varying effects on, or interactions with, the regulatory pathways that are associated with TSH in cervical epithelial cells.

This project has provided physical data to show strong evidence that TSH is produced in several cervical epithelial cell lines. It has also shown that TSH is expressed in a relatively consistent manner despite differences in phenotype of the cell lines. This project has also shown that IL-7 mRNA is expressed at detectable levels in two cervical cell lines, and that western blot detected IL-7 protein is present in all cell lines. This project was simple in design and effective in showing that the genes of interest are being expressed in several cervical cell lines. This research provides data that establishes a foundation for further research to address the functional role of TSH in cervical epithelium.

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