REGULATORY BALANCE BETWEEN THE PEPTIDE TRASPORTER, PEPT1, AND AMINO ACID TRANSPORTER GENE EXPRESSION IN THE ENTEROCYTE

by

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(ABSTRACT)

Amino acids are assimilated by membrane-associated transporters into and out of enterocytes either in their free form or in the form of peptides. The peptide transporter, PepT1, is thought to be the major facilitator of peptide transport in the enterocyte. It is unknown if the peptide transporters and free amino acid transporters operate in a compensatory fashion to regulate the amino acid balance within the enterocyte. Therefore, the objective was to examine the regulatory balance between PepT1 and other peptide and free amino acid transporters in enterocytes.

The Mouse Small Intestinal Epithelial (MSIE) cells are conditionally immortalized. It was found that MSIE cells express $B^{o}AT1$, CAT1, CAT2, LAT1, $y^{+}LAT1$, and $y^{+}LAT2$, but not PepT1, EAAT3, $B^{o,+}AT$, or LAT2, making this model similar to the basolateral membrane of enterocytes. Growing MSIE cells at high temperatures did not affect the nutrient transporter gene expression profile of these cells. Thus, the human colon carcinoma (Caco-2) cell line was used as a small intestinal *in vitro* model for this study. These cells express PepT1, HPT1, PTR3 EAAT1, EAAT3, rBAT, $B^{o,+}AT$ CAT1, LAT1, $y^{+}LAT1$, $y^{+}LAT2$, ABCC3, ABCC4, which increased from D0 to D21 post confluency, indicating cell maturation. In Caco-2 cells, PepT1 gene silencing was induced in Caco-2 cells. Despite an reduction of PepT1 gene (82%, $P < 0.05$) protein

(96%), no significant difference in any peptide (HPT1, PTR3, ABCC3, ABCC4) or free amino acid transporters (EAAT1, EAAT3, rBAT, $B^{o,+}AT$, $B^{o}AT1$, CAT1, CAT2, LAT1, LAT2, $y^{+}LAT1$, $y^{+}LAT2$) between Caco-2 cells treated with PepT1 siRNA and Caco-2 cells treated with Control siRNA was observed. These results suggest no compensation at the gene expression level of these transporters in response to a reduction of PepT1.

To account for the limitations of an *in vitro* and PepT1 kockout mouse model, transgenic chicken models were pursued. Potential cPepT1 overexpressing, cPepT1 shRNA or control shRNA expressing G0 chickens were generated by embryo injection of pseudolentiviral particles followed by *ex ovo* egg culture. Overall, 9 potential G0 cPepT1 overexpressing chickens, 15 potential G0 cPepT1 shRNA expressing chickens, and 4 potential G0 control shRNA expressing chickens were generated.

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The work enclosed in this dissertation is a product of, and is dedicated to, all of those who support me. You are from all walks of my life and are both human and animal. It is because of all of you and your actions, large and small, that I succeed. No words exist that can encompass what you mean to me and communicate to you my gratitude for your efforts. Thank you all from the depth of my being. I am forever grateful.

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CHAPTER I. INTRODUCTION

Generally, the end products of upper-gastrointestinal digestion enter the small intestine where enzymatic digestion generates absorbable end products. These end products are transported from the lumen of the small intestine into the blood stream by transport systems located in the enterocyte, the intestinal absorptive epithelial cell. These transport systems are comprised of proteins that recognize, bind, and relocate substrate(s) across the enterocyte membranes. Amino acids among the end products of digestion are transported by these transport systems located in the enterocyte either in their free form by several different amino acid transporters or in the form of peptides by peptide transporters. The broad-specificity peptide transporter, PepT1, is believed to be responsible for the majority of peptide transport in the small intestine despite the presence of other peptide transporters (Hu et al., 2008).

Substrate specificity among the free amino acid transporters is quite limited, when compared to the specificity of PepT1. Theoretically, PepT1 could transport all 400 di- and 8,000 tri-peptide combinations derived from the 20 different dietary amino acids (Daniel, 2004). From an energetic viewpoint, assimilation of amino acids in the from of peptides is more efficient than assimilation of the same amount of amino acids in their free form. In other words, for the same expenditure of energy required to transport a single free amino acid, two or three amino acids can be transported in the form of peptides by PepT1 (Daniel, 2004). In addition to being more energetically efficient, transport of amino acids in the form of peptides is also a faster route of assimilation than their component amino acids in the free form (Adibi and Phillips, 1968; Craft et al., 1968; Adibi, 1971, 1986; Cheng et al., 1971; Burston et al., 1972).

CHAPTER I. INTRODUCTION

The discovery of peptide trnasporters stemmed from the knowledge that with disorders imparing transport of certain amino acids in their free form could nonetheless absorb the amino acid when it was provided in peptide form (Hellier et al., 1972; Silk et al., 1975; Leonard et al., 1976). Not only were these studies integral for establishing support for transport of peptide transport in the enterocyte, they also suggest peptide transport compensates for decreased amno acid transport. More recent evidence also suggests this possible compensatory relationship. Knockout PepT1 mice show no gross adverse phenotype, including no observable changes in growth rate or body weight (Hu et al., 2008). The gene expression of two other peptide transporters, PHT1 (PTR4, SCL15A4) and PHT2 (PTR3, SCL15A3) was not altered in PepT1 null mice, despite an 80% reduction in dipeptide uptake, as measured by Gly-Sar uptake. Taken on its face, the initial characterization of PepT1 null mice suggests that PepT1 is a, if not the, major source of dipeptide transport in the small intestine. Further, evaluation of dipeptide uptake in PepT1 null mice suggests that a compensatory dipeptide mechanism exists, as dipeptide uptake was not completely abolished.

This initial physiological characterization of PepT1 null mice leaves the door open for several more questions. First, do the free amino acid actually compensate for a lack or reduction of PepT1? Second, as several peptide transporters besides PTR3 and PTR4, are found in the enterocyte, how do these various peptide transporters respond to a lack or reduction of PepT1?

Therefore, the objective of the work presented in this dissertation was to examine the regulatory interaction between PepT1, and other peptide and free amino acid transporters in the enterocyte. To achieve this objective, human colon carcinoma cells

(Caco-2) were used to examine the effect of PepT1 knockdown on the effect of a panel of brushborder and basolateral free amino acid and peptide transporters. In addition, efforts to generate transgenic chicken models that over- or under-express PepT1 were undertaken.

The chicken is an economically important species, with the combined value of the U.S. poultry industry in 2010 being $34.7 billion with 557.4 million pounds of ready to cook chicken consumed in the U.S. (U.S. Poultry and Egg Association, 2012). The greatest expense in chicken production is feed costs, with $17.1 billion spent on feed in 2007 (2007 USDA Agriculture Census). Therefore, any incremental improvement in feed utilization of chickens, be it through improved feed formulation or genetic selection, would result in large-scale economic savings. Given this, it was said that a"turkey is NOT a feathered rat" (Weatherly K., 1998), and similarly, a chicken is NOT a feathered mouse. Therefore, although many genetic approaches utilize mouse models, investigating protein utilization in chickens is necessary to shed light into poultry nutrient utilization and feed efficiency that mouse systems cannot. Hopefully, this research may lead to economic savings within the poultry industry through improved feed formulation and/or genetic selection. Further, the chicken embryo is widely used as a developmental model and the mechanisms underlying nutrition during development are not fully understood. Lastly, the knowledge generated from this research will add beneficial new model systems to further characterize embryonic nutritional physiology.

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CHAPTER II. LITERATURE REVIEW

CHICKEN DIGESTIVE ANATOMY AND PHYSIOLOGY

Chicken Gastrointestinal Anatomy

Upper Gastrointestinal Anatomy

The information provided in the following section is referenced to "Scott's Nutrition of the Chicken" (Leeson and Summers, 2001). The process of feed digestion begins with the beak, which is used to gather and mechanically break down feed. The efficiency of feed breakdown by the beak is low. Feed then enters the esophagus and moves into the crop. The crop, a structure unique to avians, functions to store and moisten feed. In the domestic chicken the crop serves little purpose except for young broiler breeders or where there are severe limitations in food availability. Feed then enters the proventriculus, which is analogous to the stomach of other monogastrics. The normal pH within the proventriculus is about 2.5 due to HCl secretion. From the proventriculus, feed and secretions are moved into the gizzard, another structure unique to avians. The gizzard is a strong, muscular organ that functions to mechanically break down feed in to particles fit for enzymatic digestion and to mix the feed with secretions from the proventriculus. In the domesticated chicken the gizzard is rudimentary as compared with the gizzard of wild birds. This difference is a consequence of the feed milling processes used to increase the digestibility of feed fed to domesticated chickens. See Figure 2.1.

Lower Gastrointestinal Anatomy

The information provided in the following section is referenced to "Scott's Nutrition of the Chicken" (Leeson and Summers, 2001). Contents of the gizzard are then passed into the small intestine, which is divided into three defined sections: duodenum, jejunum, and ileum. The duodenum starts at the gizzard, forms a loop surrounding the pancreas and ends at the top of the loop. The jejunum begins at the end of the duodenal loop and is separated from the ileum by Meckel's diverticulum. Meckel's diverticulum is the remnant of the yolk stalk. The ileum starts at Meckel's diverticulum and extends to the ileal-cecal junction. The last part of the gastrointestinal tract is a pair of ceca followed by the large intestine. Fecal material is then excreted through the cloaca. See Figure 2.1.

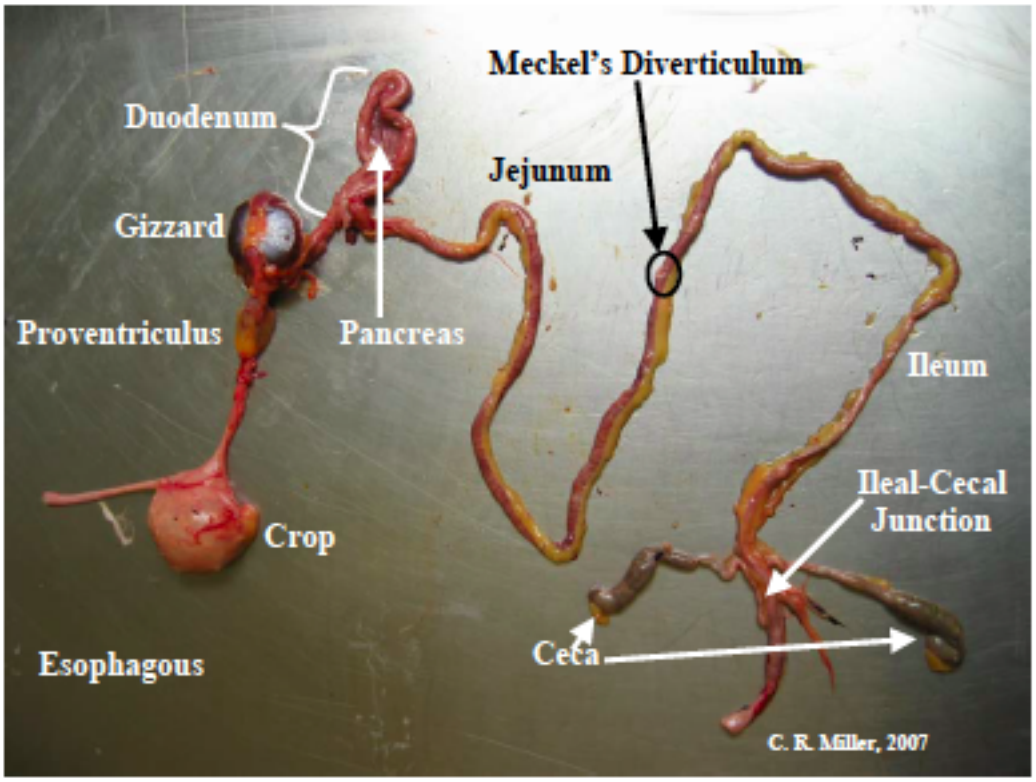

Figure 2.1 Chicken Gastrointestinal Tract.

Small Intestine Microanatomy

The information in the following section is referenced to "Molecular and Cellular Basis of Digestion" (Desmulle et al., 1986). The microanatomy of the small intestine is paramount to the overall function of this absorptive organ. The primary objective of the microanatomical features of the small intestine is to maximize absorptive area. Three structures accomplish this goal: mucosal folds, villi, and microvilli that constitute the brush border. Goblet cells are also present in the intestinal epithelium and play an indirect role in nutrient absorption.

The mucosal folds are the circular folds on the luminal side of the intestine, which give the luminal side the rough appearance. Not only do these folds increase absorptive surface area, they also aid in mixing of luminal contents. On the surface of these mucosal folds are many finger-like projections called villi. The villous is divided into two anatomical parts: the villous and the crypt. The crypt is an invagination of the epithelial surface and the villous is the part that projects in to the lumen of the small intestine.

On the surface of the villous are the enterocytes, which are the absorptive epithelial cells of the small intestine. Enterocytes originate from stem cells in the crypts. As the enterocyte migrates towards the villous tip it gains its absorptive function. After reaching the tip it is sloughed off into the lumen of the small intestine. The life span of an enterocyte is short, lasting only about 2-5 days.

Enterocytes are polarized cells that have a basolateral membrane that faces the blood stream and an apical membrane that faces the lumen of the small intestine. The apical membrane is comprised of more microscopic finger-like projections called

microvilli. These microvilli form the structure known as the brush border membrane. The brush border membrane is the absorptive interface of the small intestine. The transport systems and enzymes that facilitate nutrient assimilation reside in the enterocyte on the brush border and basolateral membrane.

Arising from stem cells in the villi crypts, goblet cells are part of the villous epithelium along with enterocytes. Goblet cells secrete mucus, which forms the glycocaylx of the small intestine. This mucus layer of the small intestine plays an important role in nutrient digestion and absorption by creating a microenvironment, sometimes called the unstirred water layer. The unstirred water layer surrounds the brush border membrane and serves to protect the fragile brush border from the movement of gut contents in the mainstream of the lumen, which may cause premature sloughing of the enterocytes or damage to the microvilli. This microenvironment also functions as a pathogen barrier and maintains an environment that has a consistent pH and levels of ions and other molecules to optimize digestive enzyme and nutrient transporter function. See Figure 2.2

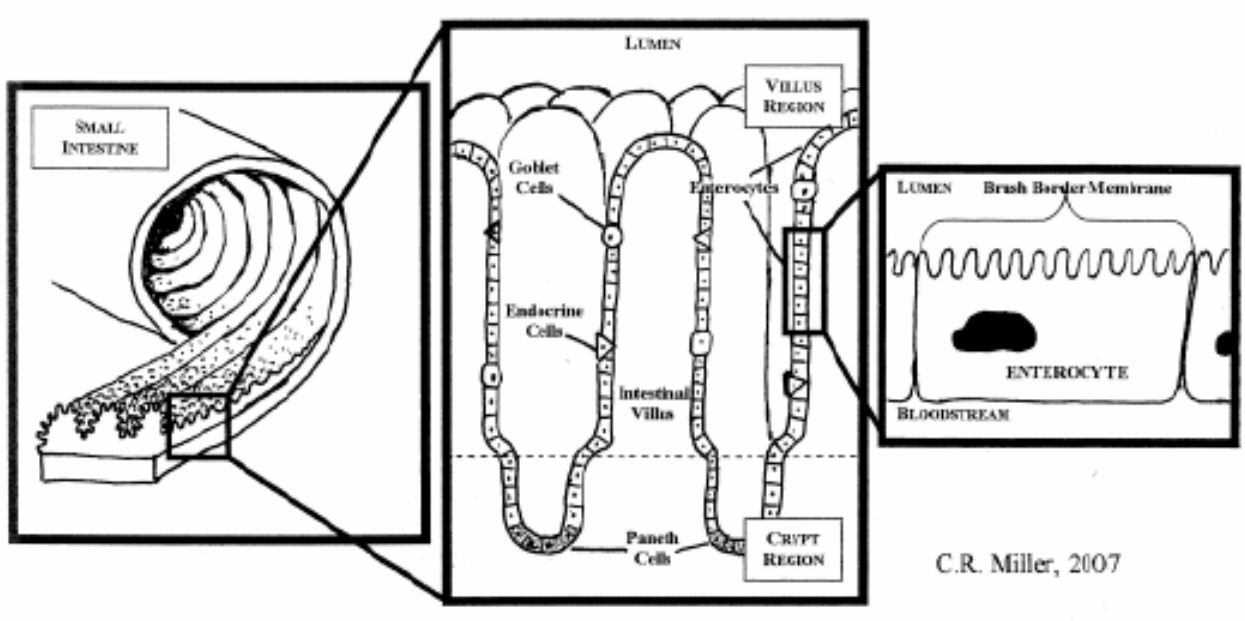

Figure 2.2 Small Intestine Microanatomy.

Chicken Protein Digestion Physiology

The information provided in the following section is referenced to "Scotts Nutrition of the Chicken" (Leeson and Summers, 2001) and "Molecular and Cellular Basis of Digestion" (Desmulle et al., 1986). Even though there is no meaningful digestion of dietary protein in the mouth or crop, the physical process of ingesting feed stimulates the vagus nerve, which initiates the secretion of gastric juices into the proventriculus in preparation for feed arrival. The main components of this gastric juice are HCl and the zymogen pepsinogen, which is transformed into its active form, pepsin, by acid hydrolysis. Pepsin cleaves proteins between amino acids Leu-Val, Leu-Tyr, or Leu-Leu, as well as between aromatic amino acids Phe-Phe, or Phe-Tyr. Thus, the proventriculus is the first site of protein digestion in the chicken. The mix of feed and secretions, now called chyme, passes into the gizzard where it is mixed and undergoes further proteolysis by pepsin. Other proteolytic enzymes produced by the small intestine further break down the resulting peptides.

As the acidic chyme enters the duodenum, it causes the luminal pH to decrease. The decrease in pH and other mechanisms stimulate gastrin secretion, which stimulates increased HCl secretion in the proventriculus, and thus promotes further conversion of pepsinogen to pepsin. The pancreas produces and releases elastase and they zymogens trypsinogen and chymotrypsinogen into the duodenum. Elastase hydrolyzes collagen proteins. Enterokinases convert trypsinogen into its active form called trypsin, which is an important proteolytic enzyme as it activates many other zymogens including chymotrypsinogen. Trypsin and chymotrypsin further hydrolyze polypeptides produced from protein breakdown by pepsin. The result of hydrolysis by these enzymes is the

release of many terminal peptide bonds. The newly formed terminal peptide bonds are further broken down by aminopeptidases and carboxypeptidases A and B, as well as numerous other specific peptidases produced by the mucosa of the duodenum. Peptides are also produced from the breakdown of collagen by collagenase in the duodenum. In the jejunum, the proteolytic enzyme erepsin is produced, which breaks down polypeptides into amino acids.

The end result of hydrolysis by these proteolytic enzymes in the duodenum and jejunum are small oligopeptides and free amino acids. Interestingly, the majority of the hydrolysis of peptides by the proteolytic enzymes occurs in the unstirred water layer rather than in the intestinal lumen. There are more digestive and other enzymes near the tip of the microvilli, which is also where the majority of the nutrient transport systems are located. The free amino acids and oligopeptides produced by hydrolysis in the unstirred water layer are assimilated by the enterocytes by specific transport systems located in the brush border membrane. Brush border membrane transporters are discussed in greater detail in the subsequent section. Amino acids and oligopeptides located in the lumen of the enterocyte are transported into the bloodstream via transporters located in the basolateral membrane. Basolateral membrane transporters are discussed in greater detail in the subsequent section.

Summary

The chicken is a monogastric with the addition of several unique structures including the crop, gizzard, proventriculus and Meckel's diverticulum. The small intestine of the chicken is the absorptive organ of the digestive tract and the enterocyte is the absorptive cell of the intestinal epithelium. The apical surface of the enterocyte is the

brush border membrane, where nutrient transporters and enzymes are located to facilitate nutrient assimilation. Transporters on the basolateral membrane of the enterocyte serve to move nutrients from the enterocyte into the bloodstream.

PEPTIDE TRANSPORT IN THE ENTEROCYTE

Introduction

Until the 1970's it was generally thought that all amino acids derived from dietary protein were absorbed by the small intestine in the form of free amino acids and that oligopeptides did not cross the brush border membrane. The physiological importance of oligopeptides became apparent in studies that documented their hydrolysis and disappearance from the lumen of the small intestine as well as their appearance in the blood after a meal (Adibi, 1971; Adibi and Mercer, 1973; Adibi et al., 1975). Although these findings were met with skepticism, studies using patients with impaired transport of certain single amino acids supported the new hypothesis. These studies showed normal assimilation of these amino acids when they were supplied to these patients in peptide form (Hellier et al., 1972; Silk et al., 1975; Leonard et al., 1976). Peptide transport systems, first functionally characterized in humans (Adibi, 1971; Adibi and Soleimanpour, 1974; Adibi et al., 1975), exist in every animal species studied. Today, assimilation of amino acids in the form of peptides by an intestinal peptide transport system is recognized as an energy efficient pathway for the uptake of amino acids.

Peptide flux in the enterocyte is complex and involves enzymes that break down substrates in the unstirred water layer, brush border peptide transporter, cytosolic peptidases, basolateral peptide transporters, and free amino acid transporters (Figure 2.3). Generally, enzymes located in the unstirred water layer break down proteins and

oligopeptides into smaller peptides that serve as substrates for brush border peptide transporters. Once inside the enterocyte the peptides can be transported out of the enterocyte into the bloodstream via basolateral membrane peptide transporters or further broken down by cytosolic peptidases into free amino acids. These free amino acids can then be transported out of the cell via free amino acid transporters located on the basolateral membrane. This section discusses in greater detail the border and basolateral transporters that play a significant role in peptide assimilation.

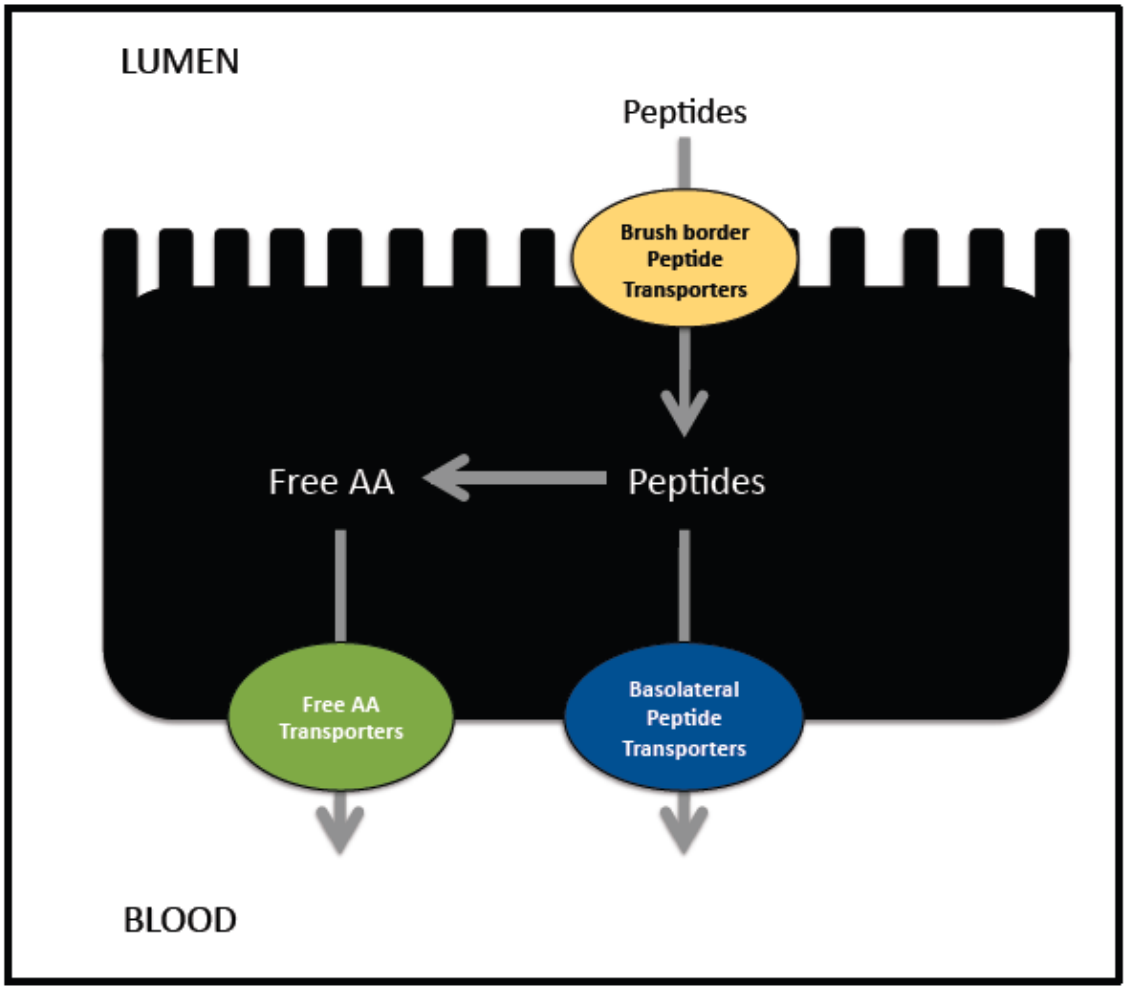

Figure 2.3. General peptide transport in the enterocyte. Brushborder enzymatic digestion of proteins in the lumen of the small intestine results in oligopeptides and free amino acids. The peptides are transported across the brushborder membrane from the lumen of the small intestine into the enterocyte by peptide transporters located on the brushborder membrane. Intracellular enzymes break down the peptides into free amino acids, which are transported across the basolateral membrane into the blood stream by free amino acid transporters. Alternatively, peptides can be transported from the cytosol into the bloodstream via peptide transporters located in the basolateral membrane of enterocytes.

The Oligopeptide Transporter, PepT1

Introduction

The peptide transporter (PepT1) facilitates the assimilation of di- and tripeptides in the small intestine and is generally regarded as being responsible for the majority of peptide transport across the brush border membrane. The PepT1 was first cloned in rabbit (Fei et al., 1994). Since this time, PepT1 has been cloned and characterized from several other animal species including human (Liang et al., 1995), mouse (Fei et al., 2000), rat (Saito et al., 1995), chicken (Chen et al., 2002), turkey (Van et al., 2005), cattle (Chen et al., 1999), sheep (Chen et al., 1999), pig (Klang et al., 2005) and salmon (Ronnestead et al., 2010).

Tissue and Cellular Distribution of PepT1

The PepT1 protein is expressed primarily in the small intestine with lesser expression in the kidney (Meredith and Boyd, 2000), although there are exceptions. For example, PepT1 is expressed in the omasum and rumen of dairy cattle and sheep (Chen et al., 1999). Expression of PepT1 is also found in the nasal epithelium (Agu et al., 2011) and the yolk sac membrane of developing chicken embryos (Yadgary et al., 2011). Abundance of PepT1 mRNA and protein in the small intestine in animals under normal physiologic and environmental conditions is varied. In rabbits (Fei et al., 1994) and pigs (Chen et al., 1999; Klang et al., 2005), maximal PepT1 mRNA abundance and/or protein are in the proximal intestine. In sheep (Chen et al., 1999), dairy cattle (Chen et al., 1999), and black bear (Gilbert et al., 2007a), maximal PepT1 mRNA abundance is in the distal small intestine. Tissue mRNA expression of chicken PepT1 (cPepT1) is greatest in the small intestine with lesser expression in the kidney and minimal expression in the ceca

(Chen et al., 1999, 2002). In the small intestine, greatest cPepT1 mRNA levels are seen in the duodenum followed by jejunum and ileum (Chen et al., 1999, 2002; Gilbert et al., 2007b). The physiological importance governing these species differences is not fully understood.

The PepT1 has a specific cellular distribution that is independent of intestinal region (Groneberg et al., 2001; Hussain et al., 2002). In the small intestine of mice (Groneberg et al., 2001) and rats (Ogihara et al., 1999; Groneberg et al., 2001; Hussain et al., 2002), PepT1 protein expression is limited to the brush border membrane of mature enterocytes. Protein expression level of PepT1 is likely related to the maturity of the enterocytes as the density of PepT1 t decreases from the apical tip to the base of the villous in rats (Ogihara et al., 1999). The observation that there is no PepT1 expression in the crypts of the villous in mice and rats (Ogihara et al., 1999; Hussain et al., 2002) further supports this rationale. The cellular location of PepT1 is also influenced by developmental stage. Immediately after birth in rats, PepT1 is localized in the subapical cytoplasm and basolateral membrane of the enterocyte (Hussain et al., 2002). This basolateral localization of PepT1 in newborn rats may serve an important physiological function as the newborn rat switches from assimilation of nutrients from the bloodstream to the lumen of the small intestine.

Substrates of PepT1

The PepT1 has a broad substrate range that includes di- and tri- peptides, amino acid derivatives (Brandsch et al., 2004; Vig et al., 2006) and pharmacologically important compounds such as the β-lactam antibiotics and several prodrugs (Brodin et al., 2002). Substrates for PepT1 do not posses a common structural feature. However, there are

structural characteristics of PepT1 substrates that dictate the binding affinity as well as transport efficiency (Amasheh et al., 1997; Brandsch et al., 2004; Vig et al., 2006). Substrate size, charge, amino and carboxy termini modifications, side chain modifications, presence of proline, stereospecificity all affect the ability of a substrate to bind to and be transported by PepT1.

Although a compound may bind the PepT1 protein, this does not necessarily mean that PepT1 transports the compound. Vig et al. (2006) showed that transport by PepT1 is influenced by substrate charge, hydrophobicity, size, and side chain flexibility. Di-peptides with two positively charged amino acids or with extreme physical bulk are not substrates of PepT1 (Vig et al., 2006). In studies with cPepT1, transport of di-and tri-peptides is saturable and requires an acidic environment to facilitate optimal peptide transport (Chen et al., 2002). Of the 16 di- and tri- peptides tested (all contained the essential amino acids Met, Lys, or Trp) most had substrate affinities in the 20 to 100 micromolar range and were transported by cPepT1 in Chinese hamster ovary cells. However, the dipeptide Lys-Lys, the tripeptide Lys-Trp-Lys, and tetrapeptides tested had substrate affinities in the 4 to 27 millimolar range. Further, free amino acids were not transported by cPepT1 in Chinese hamster ovary cells.

The ablation of PepT1 is not lethal and leads to no gross differences in phenotype in mice. (Hu et al., 2008). The non-metabolizable di-peptide Glycylsarcosine (GlySar) is transported primarily by PepT1 (Jappar et al., 2011). In a study evaluating the in vivo absorption and disposition of GlySar, after escalating oral doses, in wild-type and PepT1 null mice, PepT1 ablation resulted in significant reductions, *in vivo*, in the rate and extent of GlySar absorption. Furthermore, the absorption of GlySar, as measured by the area

under the plasma concentration-time curve, indicates that the transport system becomes saturated at doses over 100nmol/g. In both genotypes, the area under the curve (AUC) plasma concentration was proportional to dose up to the saturation dose, albeit the absolute AUC was reduced in PepT1 null mice. As uptake was not completely abolished in PepT1 null mice, other mechanisms of GlySar assimilation exist. In agreement with these results, Ma et al. (2012) found a 78% reduction in GlySar uptake in everted intestinal rings from PepT1 null mice as compared to wild type controls.

Peptide substrates for proteins involved in the innate immune response are also transported by PepT1. Many of these are derived from pathogenic bacteria, thus allowing PepT1 to play a role in innate immunity. However, not all bacterial or yeast derived peptides are transported by PepT1. For example, Lactoferricin derived cationic antimicrobial di- and tripeptides displayed a moderate affinity for interaction with PepT1, but were not transported by PepT1 (Flaten et al., 2011).

Structure of PepT1

The PepT1 protein is an integral membrane protein with 12 trans-membrane domains with a large extracellular loop between domains 9 and 10 (Fei et al., 1994; Meredith and Boyd, 2000; Chen et al., 2002). Evidence from studies using PepT1 and PepT2 chimeras indicate that the putative peptide-binding site in PepT1 consists of transmembrane domains 7, 8, 9 and the loops in between (Chen et al., 2000). The PepT1 protein has several potential N-linked glycosylation sites (Fei et al., 1994; Saito et al., 1995; Chen et al., 2002; Van et al., 2005). The number and location of these putative N-linked glycosylation sites are species dependent. The PepT1 protein also has been shown in some species to contain potential phosphokinase C (PKC) and cyclic AMP (PKA)

dependent phosphorylation sites (Fei et al., 1994; Saito et al., 1995; Chen et al., 2002; Meredith and Boyd, 2000; Van et al., 2005). While rabbit PepT1 has both the putative PKA and PKC dependent phosphorylation sites (Fei et al., 1994), most other species have only the putative PKC dependent phosphorylation site (Saito et al., 1995; Meredith and Boyd, 2000; Chen et al., 2002; Van et al., 2005). The implications of the modifications by glycosylation or phosphorylation have yet to be fully characterized.

Chicken PepT1 has 714 amino acids with a molecular weight of 79.3 kDa and an isoelectric point of 7.48. The predicted cPepT1 is 62.4, 62.5, 63.8, 64.8, 65.1 percent homologous to rabbit, human, mouse, rat, and sheep PepT1 respectively, but has no sequence identity in the first 15 amino acids (Chen et al., 2002). Two cPepT1 cDNAs were cloned which encode two naturally occurring cPepT1 protein variants (Chen et al., 2002). The point mutation in the cDNA results in a Leu 703 Ser change. The model of cPepT1 indicates that it has several features that are similar to mammalian PepT1 as well as many signatures of the POT superfamily, including 12 transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10 and cytoplasmic amino- and carboxy-termini (Chen et al., 2002). Chicken PepT1 contains a putative PKC dependent phosphorylation site.

Transport Mechanism of PepT1

The PepT1 is a proton-coupled transport protein (Fei et al., 1994) that is dependent on a pH gradient as well as a negative intracellular membrane potential (Adibi, 1997) to drive the transport of its substrates. The pH of the unstirred water layer that surrounds the tip of the villi is approximately 6.0 (Shimada, 1987). Transport of neutral and cationic substrates by PepT1 is most efficient at this slightly acidic physiologic pH

(Steel et al., 1997). Di- and tri peptides as well as other substrates of PepT1 are co-transported along with one or two protons by the PepT1 transporter across the brush border membrane of enterocytes (Steel et al., 1997). Neutral and cationic peptides are transported with a proton:peptide stoichiometry of 1:1, while anionic peptides are transported with a proton:peptide stoichiometry of 2:1 (Steel et al., 1997). It is thought that one proton transported with the acidic peptides neutralizes the peptide, while the second proton binds the PepT1 transporter (Steel et al., 1997). The proton first binds to the binding site on PepT1 followed by substrate binding and translocation (Mackenzie et al., 1996).

Intracellular pH is maintained by a Na+ / H+ exchanger protein (NHE) located on the brush border membrane so that the intracellular proton concentration is less than the extracellular concentration (Adibi, 1997). The NHE transports a proton from the cytosol of the enterocyte to the lumen of the small intestine, while it transports one Na+ from the lumen of the small intestine into the enterocyte (Adibi, 1997). A basolateral Na+ / K+ ATPase pumps out the cytosolic Na+ in exchange for a K+, thus maintaining the negative intracellular membrane potential (Adibi, 1997).

The intracellular peptide concentration is regulated by two mechanisms. Peptides can be broken down by intracellular peptidases into free amino acids (Adibi, 1997). These free amino acids can be used by the enterocyte or they can be transported out of the enterocyte and into the blood by free amino acid transporters located on the basolateral membrane (Adibi, 1997). Peptides not broken down by intracellular peptidases are transported across the basolateral membrane as peptides by basolateral peptide transporters, which are distinct from PepT1 (Adibi, 1977; Adibi and Krzysik,

1977; Saito and Inui, 1993; Thwaites et al., 1993; de Waart et al., 2011). The general transport mechanism of PepT1 is shown in Figure 2.4. Basolateral peptide transporters are discussed in greater detail in the following section.

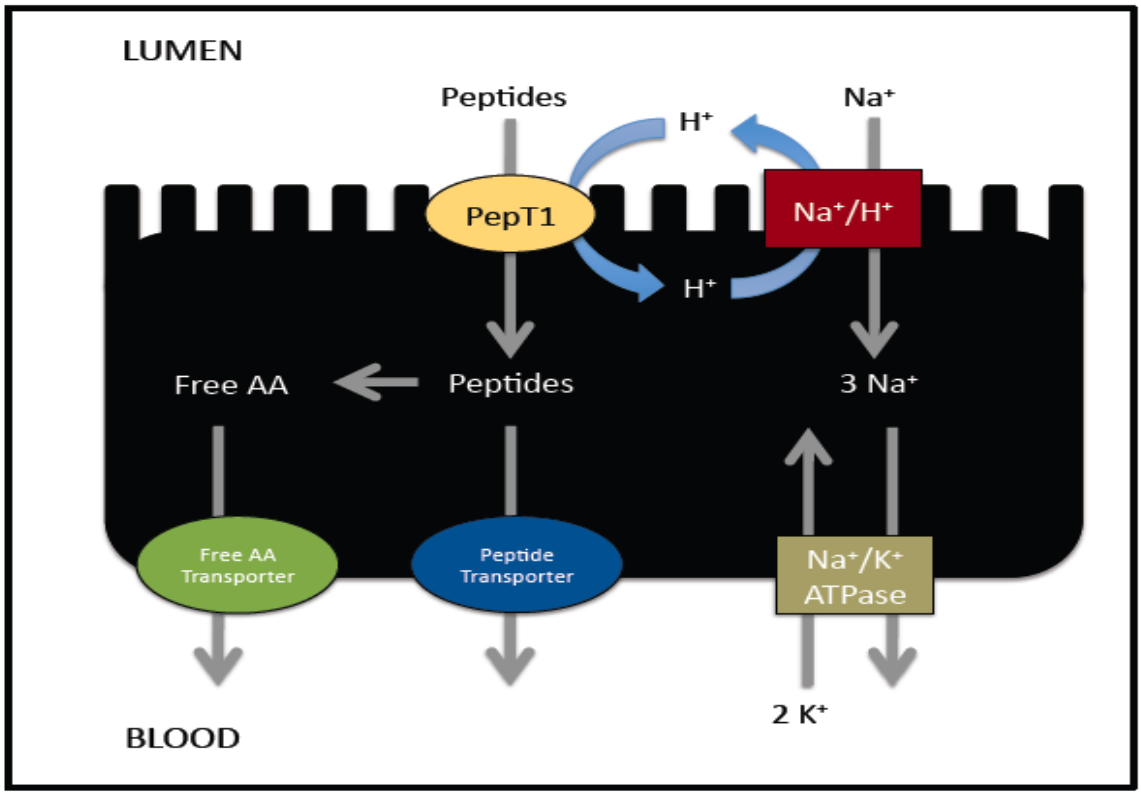

Figure 2.4. Transport mechanism of PepT1. Brushborder enzymatic digestion of proteins in the lumen of the small intestine results in oligo peptides and free amino acids. PepT1 transports di and tri peptides across the brushborder membrane from the lumen of the small intestine into the enterocyte, which is located on the brushborder membrane. Intracellular enzymes break down the peptides into free amino acids, which are transported across the basolateral membrane into the blood stream by free amino acid transporters. Alternatively, peptides can be transported from the cytosol into the bloodstream via peptide transporters located in the basolateral membranes of enterocytes. The proton gradient driving this transport is maintained by a brushborder Na+/H+ exchanger and a basolateral Na+/K+ ATPase.

Regulation of PepT1

Substrates of PepT1 can influence expression and activity of this transporter. In human intestinal cells (Caco-2), dipeptides were shown to increase the transport of substrate by PepT1, which correlated with an increased amount of PepT1 protein in the membrane, resulting from an increase in PepT1 mRNA stability coupled with an increase in gene expression (Walker et al., 1998; Shiraga et al., 1999). In Caco-2 cells, certain amino acids could up-regulate PepT1 transport activity and amount of PepT1 protein in the membrane (Shiraga et al., 1999). In transient expression studies using luciferase reporter vectors and Caco-2 cells, selected peptides as well as the amino acids Phe, Arg, and Lys stimulated the rat PepT1 promoter (Shiraga et al., 1999). Analysis of the rat PepT1 promoter revealed the presence of an AP-1 binding site as well as an amino acid response element, which may mediate the effect of the amino of the positive and negative acting elements in the chicken PepT1 promoter region (approximately 1000 bp upstream of the transcription start) revealed that no amino acids or peptides, in contrast to the rat, had an effect on chicken PepT1 promoter activity (Frazier et al., 2008). Sequence analysis showed that the amino acid response element found in rat PepT1 promoter was not present in the chicken PepT1 promoter.

In addition to dietary substrates, immunoactive substrates can also influence PepT1 expression and function. In vitro and in vivo studies reveal that PepT1 transports the antimicrobial peptide JBP485 (Liu et al., 2011), which reduces the decrease in PepT1 expression and function caused by indomethacin-induced intestinal injury in rats and damage in Caco-2 cells (Wang et al., 2011).

Hormones also play a role in the regulation of PepT1. In Caco-2 cells, insulin increased the abundance of PepT1 in the membrane by increasing PepT1 trafficking from the cytoplasmic pool to the membrane (Thamotharan et al., 1999). However, PepT1 expression is influenced by sex, as insulin decreases PepT1 gene expression in male mice and increases PepT1 gene expression in female mice. Leptin can also increase the amount of PepT1 protein present in the membrane (Buyse et al., 2001). Like insulin, leptin increases PepT1 trafficking from the cytoplasmic pool to the membrane (Buyse et al., 2001). Epidermal growth factor (Nielsen et al., 2001) and thyroid hormone (Ashida et al., 2004) decreased the expression of PepT1 protein in the membrane in Caco-2 cells. Thyroid hormone also decreased PepT1 expression in vivo in rats (Ashida et al., 2004). The decrease in PepT1 protein was caused by a decrease in PepT1 gene expression and/or PepT1 mRNA stability (Ashida et al., 2002). Corticosterone administration increased duodenal PepT1 relative mRNA abundance in 7 day-old broiler chickens (Hu et al., 2010) . Subsequent injection of Glucagon-like peptide-2 further increased duodenal and jejunal PepT1 relative mRNA abundance in the broiler chickens (Hu et al., 2010).

The diurnal rhythm in rats influences both PepT1 mRNA and protein expression and transport (Pan et al., 2003). Transport of Gly-Sar and levels of PepT1 mRNA and protein were low during the light phase and high during the dark phase (Pan et al., 2003). The diurnal rhythm in PepT1 expression and function may be controlled by the vagus nerve in response to anticipation of food intake (Mourad and Saade., 2011). Vagal capsaicin sensitive primary afferent fibers exert tonic inhibitory effects on amino acid absorption and a role in the mediation of the inhibitory effect of intestinal amino acids on their own absorption at the level of the proximal or distal intestinal segment. Chronic

extrinsic denervation leads to a decrease in intestinal amino acid absorption. Conversely, adrenergic agonists as well as activation of SPA fibers enhance peptide uptake through PepT1. Furthermore, complete abdominal vagotomy abolished diurnal variation of PepT1 mRNA only in the ileum (Quandeel et al., 2009). However, diurnal variations in expression of PepT1 protein and GlySar uptake were absent post vagotomy (Quandeel et al., 2009). Therefore, vagal innervation appears to mediate in part diurnal variations in protein expression and transport function of PepT1, but not diurnal variation in mRNA expression of PepT1.

PepT1 gene expression changes during development. The PepT1 gene expression was greatest in 4-day old rats and decreased to adult levels by day 28 of age (Miyamoto et al., 1996). Similarly, Shen et al. (2001) found that PepT1 gene and protein expression was greatest in rats at 3-5 days of age and then decreased rapidly thereafter. They also observed an increase of PepT1 expression at day 24 of age, which was attributed to the stresses of weaning. Adult PepT1 protein levels in rats were 70 percent that of day 3-5 levels. In chickens and turkeys, PepT1 is developmentally regulated. In turkeys there is a 3.2-fold increase in PepT1 mRNA abundance from embryonic day 25 to day of hatch (Van et al., 2005). In chickens there is a 14- to 50-fold increase in PepT1 mRNA abundance from embryonic day 16 to day of hatch (Chen et al., 2005). In chickens selected for high or low juvenile body weight, PepT1 gene expression was greater in chicks selected for low body weight as compared to high or their reciprocal crosses. (Mott et al., 2008). PepT1 is expressed in the yolk sac membrane and increased from embryonic day 11 to 15 and decreased from embryonic day 15 to 20 (Yadgary et al., 2011).

Diet also influences PepT1 expression. When rats were fed a high protein diet of 50% gelatin, PepT1 mRNA abundance increased (Erickson et al., 1995). The greatest increase in PepT1 mRNA abundance occurred in the distal regions of the small intestine and therefore, Erickson et al. (1995) concluded that these regions were more responsive to dietary induced changes in peptide transport. There was a decrease in PepT1 mRNA abundance in intestinal tissue as well as transport of Gly-Sar in brush border membrane vesicles (BBMV) isolated from rats that were on a protein free diet (Shiraga et al., 1999). This was in contrast to the increase in Gly-Sar transport in BBMV isolated from rats that were on a high protein, 50% casein, diet (Shiraga et al., 1999). The increase in functionality of the PepT1 protein in the presence of a high protein diet was due to enhanced PepT1 protein expression as a result of increased gene transcription (Shiraga et al., 1999).

Chicken PepT1 is regulated by dietary protein level. Chicks that were fed a 12% crude protein diet had a decrease in PepT1 mRNA abundance from day of hatch to 35 days of age (Chen et al., 2005). This was in contrast to an increase in PepT1 mRNA abundance in chicks that had been fed an 18% or 24% crude protein diet (Chen et al., 2005). In this study, the feed intake of chicks fed the higher protein diets were restricted to the amount of feed consumed by the 12% group and therefore the results may have been confounded because of effects of restricted food intake. To separate out the effects of food restriction and protein level on PepT1 gene expression, Chen et al. (2005) measured PepT1 mRNA abundance in chicks that were fed a 24% crude protein *ad libitum* diet and observed a decrease of PepT1 mRNA abundance through day 14 of age followed by an increase in PepT1 mRNA abundance so that by day 35 of age PepT1

mRNA abundance was greater than chicks on the 12% crude protein diet but lower than chicks on the 18% crude protein diet and the chicks on restricted 24% crude protein diet. These results indicate that dietary protein levels affect gene expression of PepT1, however amount of food intake can influence these effects.

Metabolic conditions affect PepT1 expression. The major metabolic regulator, AMP activated protein kinase, regulates PepT1 in Caco-2 cells (Peri et al., 2010). Vazquez et al. (1985) noted that starvation altered peptide transport in the human jejunum. Thamotharan et al. (1999) observed that transport by PepT1 increased 2-fold in rats that had undergone a 24 hour fast. This increase in transport was coupled with a 3-fold increase in PepT1 protein and mRNA abundance. These results indicate that acute food withdrawal induces an up-regulation in PepT1 gene expression leading to an increase in membrane PepT1 protein levels and an overall increased capacity to transport substrates. Naruhashi et al. (2002) observed that in fed rats, PepT1 mRNA abundance as well as transport activity was greatest in the distal small intestine. In starved rats, PepT1 mRNA abundance and transport activity increased in all segments of the small intestine, with the greatest increase seen in the proximal small intestine. The effect of increased PepT1 gene expression in response to feed restriction was also observed in chickens (Masden and Wong, 2011).

The increase in PepT1 gene and protein expression during feed restriction also corresponds to an increase in PepT1 substrate uptake as shown by increased Gly-Sar uptake in mice undergoing feed restriction (Ma et al., 2012). This increase in Gly-Sar uptake during feed restriction was due to PepT1, and not other peptide transporters, as the effect was not observed in PepT1 null mice undergoing feed restriction (Ma et al., 2012).

This response in PepT1 expression is different in fish. Gene expression of PepT1 decreases during fasting and increases during refeeding, opposite of what is observed in mammals and birds (Verri et al., 2011). This suggests that in telostat fish, PepT1 is involved in compensatory growth.

Shimakura et al. (2006) focused their investigations on the transcription factor PPAR**α**, which plays a role in the adaptive response to fasting in the liver and other tissues. In rats that had been fasted for 48 hours, PPAR**α** mRNA in the small intestine increased and was accompanied by an elevation of serum levels of free fatty acids, which are endogenous PPAR**α** ligands. Oral administration of a PPAR**α** agonist to rats also increased intestinal PepT1 mRNA levels. Further, treatment of Caco-2 cells with the same PPAR**α** agonist increased PepT1 mRNA and transport of Gly-Sar. This group concluded that PPAR**α** plays a role in mediating the increase in fasting levels of PepT1 mRNA in the small intestine. In chickens undergoing feed restriction, a correlated response in PPARα gene expression to PepT1 gene expression was observed (Masden and Wong, 2011). The PepT1 may also be regulated by the PPARγ receptor. *In vitro* experiments using Caco-2 cell and *in vivo* experiments in mice fed a high-fat diet indicate that rosiglitazone, a PPARy agonist, increased PepT1 gene expression after 7 days of treatment with the drug (Hindlet et al., 2012).

Micro RNAs play key roles in modulating the expression of proteins by reducing translation efficiency to varying degrees. These molecules involved in post-transcriptional gene silencing work through targeting and binding transcripts within the 3' untranslated region (UTR), thereby limiting access of the transcript by translational machinery. The expression of PepT1 is regulated by microRNA-92b (miR-92b)

(Dalmasso et al., 2011). During differentiation of Caco-2 cells there was an inverse correlation between expression levels of PepT1 and miRNA-92b. The miR-92b suppressed PepT1 mRNA abundance and protein level, resulting in decreased PepT1 transport activity in Caco2 cells by targeting the PepT1 3'UTR.

Summary

Transport of di- and tri-peptides across the brush border membrane is facilitated by the proton-dependent oligopeptide transporter, which is localized to the brush border membrane of mature enterocytes. The intestinal expression and functionality of PepT1 is dependent on species, nutritional or metabolic status, diet, hormones, age, time of day, functionality of proteins that maintain chemical, electrical or pH gradients, as well as other factors such as second messengers, PepT1 substrates and amino acids. In the chicken, PepT1 is proximally expressed in the small intestine and varies with diet and developmental age.

Other Peptide Transporters in the Enterocyte

Other Brushborder Peptide Transporters

Several other oligopeptide transporters have been identified in the brushborder of the small intestine in addition to PepT1. Human intestinal peptide transporter 1 (HPT1) (CDH17) belongs the cadherin family of proteins and was discovered almost simultaneously with PepT1 (Dantzig et al., 1994). Analysis of HPT1 revealed that it has 832 amino acid residues, an approximate mass of 120 kDa and only 16% identity and 41% similarity to PepT1 (Dantzig et al., 1994; Fei et al., 1994). The HPT1 is expressed in the apical membrane of Caco-2 cells and is predicted to have one to six membrane

spanning domains (Hoffman and Stoffel, 1993; Dantzig et al. 1994). Gene and protein expression of HPT1 is found throughout the gastrointestinal tract (Dantzig et al., 1994; Herrera-Ruiz et al., 2001). A correlation in expression of the heavy chain protein, 4F2hC, was observed (Landowski et al., 2003). The heavy chain 4F2hC is predicted to have a similar role in HPT1 expression as it does with some amino acid transporters (See below). Interestingly, microarray analysis, validated by qPCR) showed that in differentiated Caco2 cells, PepT1 expression was 45-fold lower than HPT1 expression (Sun et al., 2002). Furthermore, up to 90% of cephalexain (a peptidiomimentic substrate of PepT1) uptake in Caco-2 cells was attributed to HPT1 (Dantzig et al., 1994).

Formally named PTR3, the peptide-histidine transporter 2, PHT2 (SLC15A3), was first isolated from the placenta (Herrera-Ruiz, 2002) and is also expressed in the small and large intestine (Herrera-Ruiz et al., 2001). This protein shares homology with the PTR family of proteins (Steiner et al., 1995). Like the peptide-histidine transporter 1 (PHT1/PTR4), PHT2 transports free histidine and di- and tri peptides. Little is known about substrate preference and role in nutrient absorption the enterocyte and what is known is purely speculative.

Basolateral Peptide Transporters

Compared to peptide transport across the apical membrane of enterocytes, little is known about transport of peptides across the basolateral membrane of enterocytes. This may be due to the general holding that there is little transport of amino acids across the basolateral membrane in the form of peptides. Despite this belief, peptides are found in the portal blood stream after passage through the small intestine in a variety of species (Gardner et al., 1975, Koeln et al., 1993; Seal and Parker, 1996; Redmond et al., 2000,

Han et al., 2001 a, b; Tagari et al., 2004, 2008). Although evidence for the presence of a basolateral peptide transport exists, little is know about the proteins involved in basolateral peptide transport. Recently, transport across the basolateral membrane of a peptidiomimentic drug, also a substrate of PepT1, has been attributed to the ABCC3 and ABCC4 proteins. Therefore, this review discusses these two transporters and their relevance to intestinal peptide transport.

Closely related to ABCC2, which resides in the brush border membrane of enterocytes (Mottino et al., 2000), ABCC3 and ABCC4 are also found in enterocytes. Expression of ABCC3 is limited to the basolateral membrane of enterocytes (Scheffer et al., 2002). Localization of ABCC4 is cell type dependent and it remains unclear where ABCC4 resides in the enterocyte. In Caco-2 cells, ABCC4 preferentially localizes to the basolateral membrane (Ming and Thakker, 2010). However, in the colonic cell line HT29-CL19A expression was found in both the brush border and basolateral membrane.

Cefadroxil, a peptidiomimentic substrate of PepT1, was transported by membranes isolated from insect cells expressing the ABCC2 (positive control), ABCC3 or ABCC4 (de Waart et al., 2011). Transport was saturable with Km values of 2.5 ± 0.7 (ABCC3) and 0.25 ± 0.07 (ABCC4) mM. The involvement of ABCC3 and ABCC4 in the transport of cefadroxil from enterocytes was examined using jejunal explants in Ussing chambers from wild-type, and homozygous ABCC3, ABCC4 and ABCC3/ABCC4 null mice (de Waart et al., 2011). Transport of cefadroxil from the apical to the basolateral side of jejunal tissue explants was unchanged in ABCC3 null mice, but significantly reduced two-fold in jejunal explants from ABCC4 and ABCC3/ABCC4 null mice compared to explants from wild type mice. To support the *ex*

vivo transport experiment, the appearance of cefadroxil in the portal blood of wild-type, and homozygous ABCC3, ABCC4 and ABCC3/ABCC4 null mice was measured *in vivo* after administration of cefadroxil to the jejunum (de Waart et al., 2011). Portal and peripheral blood cefadroxil concentrations were similar in ABCC3 and ABCC4 null mice compared to wild type mice. However, portal and peripheral blood cefadroxil concentrations were reduced two-fold in ABCC3/ABCC4 null mice compared to wild-type mice. Taken together, these findings indicate that transport of the peptidiomimentic, cefadroxil, across the basolateral membrane depends partly on ABCC3 and ABCC4.

Summary

Amino acids are assimilated in the form of peptides. The peptide transporter PepT1 plays a major role in transport of peptides across the brush border membrane of the small intestine. Other transporters, such as HPT1 and PHT2/PTR3 also contribute to peptide flux across the brush border membrane. Transport of peptides across the basolateral membrane into the blood stream also occurs. Although the key players in basolateral membrane peptide transport are not fully characterized, the ABCC3 and ABCC4 may be responsible for some of this peptide transport. Generally speaking, the majority of expression of these transporters is localized to the villous tip, which corresponds with expression in the mature and functional enterocyte.

AMINO ACID TRANSPORT IN THE ENTEROCYTE

Introduction

Free amino acid transport systems exist in the small intestine and throughout the body. For example, free amino acid transport systems exist to traffic amino acids across

the blood brain barrier (Wagner et al., 2001) and yolk sac membrane (Yagarady et al., 2011). Free amino acid transporters may transport unidirectionaly or operate as exchangers. Together, in addition to the peptide transporters, the free amino acid transporters operate in a complex network to regulate cellular amino acid content. While free amino acid transporters are classically divided into families based on substrate, this review focuses on individual members of amino acid transporter families involved in amino acid transport in the small intestine and are presented here according to their location in the enterocyte. Table 2.1 shows an overview of the amino acid transporters discussed in this review.

Amino Acid Transporters in the Enterocyte			
Brush border		**Basolateral**	
Transporter	**Substrate**	**Transporter**	**Substrate**
EAAT3	Anionic Amino Acids	CAT1	Cationic amino acids
$b^{o,+}AT$	Hetrodimerizes with rBAT , cationic amino acids	CAT2	Cationic amino acids
rBAT	Hetrodimerizes with $b^{o,+}AT$, cationic amino acids	y^+LAT1	Heterodimerizes with 4F2hC, dibasic and neutral amino acids
B^oAT1	Neutral amino acids	y^+LAT2	Heterodimerizes with 4F2hC, neutral and cationic amino acids
		LAT1	Heterodimerizes with 4F2hC, large neutral amino acids
		LAT2	Heterodimerizes with 4F2hC small and large neutral amino acids

Table 2.1. **Amino Acid Transporters in the enterocyte.**

Brushborder Amino Acid Transporters

The Intestinal Excitatory Amino Acid Transporter, EAAT3

Introduction

The intestinal glutamate/aspartate transporter, EAAT3, is a high-affinity low-capacity transporter of free aspartate and glutamate in the small intestine. Cloning of EAAT3 first occurred in rabbit (Kanai and Hediger, 1992). In addition to contributing to the assimilation of aspartate and glutamate, EAAT3 is important to the enterocyte, as glutamate is the primary energy source for the enterocyte (Newsholme et al., 2003). The EAAT3 is a member of the amino acid transporter system X_{AG}, which includes the other aspartate/glutamate amino acid transporters.

Tissue and Cellular Distribution of EAAT3

Expression of EAAT3 occurs in the small intestine (Kanai and Hediger, 1992; Erickson et al., 1995; Howell et al., 2001; Fan et al., 2004) and a variety of other tissues including kidney (Kanai and Hediger, 1992; Howell et al., 2001), brain neurons (Rothstein et al., 1994; Lehre et al., 1995), liver (Kanai and Hediger, 1992; Howel et al., 2001), pancreas (Howell et al., 2001), rumen (Howell et al., 2001), omasum (Howell et al., 2001) and heart (Kanai and Hediger, 1992).

Messenger RNA of EAAT3 is differentially expressed along the longitudinal axis of the small intestine with greatest quantities in the distal small intestine (Erickson et al., 1995; Rome et al., 2002; Iwanaga et al., 2005, Gilbert et al., 2007a, Gilbert et al., 2007b, Mott et al., 2008). *In situ* hybridization revealed that EAAT3 mRNA is also differentially expressed along the crypt-villous axis with greatest quantities seen in the crypts and basal half of the intestinal villi (Iwanaga et al., 2005). The EAAT3 protein is

localized to the brush border membrane of enterocytes (Iwanaga et al., 2005) and only found in differentiating enterocytes in the crypt of the intestinal villous (Rome et al., 2002). This villous location is independent of intestinal region. The localization of the EAAT3 transcript and protein suggests that EAAT3 and its substrates play a role in the growth and differentiation of the premature enterocyte.

Substrates and Structure of EAAT3

Intestinal EAAT3 is highly and sterospecific for the anionic amino acids L-glutamate and D- or L-aspartate (Nicholson and McGivan, 1996; Castagna et al., 1997). Studies with neuronal and bran EAAT3 indicate that EAAT3 may also have a low affinity for cysteine (Zerangue and Kavanaugh, 1996; Chen and Swanson, 2003). Transport of cysteine by intestinal EAAT3 has not been investigated.

Sequence analysis indicates that EAAT3 has a primary structure of 524 amino acids in rabbit (Kanai and Hediger, 1992), human (GenBank Accession # P43005), and dog (Sato et al., 2001). Rat (Kanai et al., 1995a) and mouse (Tanaka, 1993) had primary EAAT3 sequences of 523 amino acids. Controversy exists regarding the membrane topology or EAAT3. The first model proposed that EAAT3 had 10 transmembrane domains with intracellular N- and C-termini (Kanai and Hediger, 1992). However, the large hydrophobic stretch of amino acids near the C-terminus makes other models, such as a12 transmembrane domain model, possible.

The tertiary and quaternary structures of EAAT3 are not well understood. However, freeze fracture electron microscopy did reveal a pentameric 3-D structure of neuronal EAAT3 expressed in *Xenopus* ooctyes (Eskandari et al., 2000). It remains

unclear if the pentameric structure is made of subunits of a single EAAT3 protein or individual EAAT3 proteins forming a homopentamer.

Transport Mechanism of EAAT3

Transport of glutamate and aspartate by EAAT3 is coupled to the transport of 2 Na^+ ions, either contransport of 1 K^+ ion (Kanai et al., 1995b). Kinetic studies indicate that transport of glutamate and aspartate by EAAT3 follows a 10 step ordered kinetic mechanism (Kanai et al., 1995b). First, one Na^+ ion binds to the extracellular face of EAAT3, which increases the affinity for glutamate. Glutamate (or aspartate) then binds followed by the binding of the second Na+ ion and translocation into the intracellular side of the membrane. The bound Na+ and glutamate (or aspartate) are released into the cytoplasm, then an OH- ion binds followed by binding of K^+. Relocation of the transporter to the extracellular side of the membrane occurs followed by release of K+ concentration is maintained by the basolateral Na^+/K^+ ATPase (Hyde et al., 2003).

Regulation of EAAT3

The expression of EAAT3 is modulated by levels of dietary protein. A change from a low (4% casein) to high protein (50% gelatin) diet fed to rats caused a 2- to 3- fold increase in EAAT3 mRNA in the mid small intestine, while there was little change in the proximal and distal small intestine (Erickson et al., 1995). Further, in the absence of luminal nutrients, induced by total parenteral nutrition feeding in rats, led to an increase in ileal EAAT3 mRNA (Howard et al., 2004). These results suggest that enterocytes have adaptation mechanisms to reduced luminal nutrients.

The expression of EAAT3 mRNA is influenced by growth stage of the animal. A comparison between growing and non-growing lambs that were fed diets that had similar

levels of metabolizable protein, found that the ileal epithelium of growing lambs contained approximately 313% more EAAT3 mRNA than non-growing lambs (Howell et al., 2003). The expression of EAAT3 mRNA was increased throughout the small intestine of rats from days 4 through 21 of age, indicating that gene expression of EAAT3 is developmentally regulated (Rome et al., 2002). The second messenger phosphokinase C (PKC) is also involved in the regulation of EAAT3). In neural cells, phosphorylation of EAAT3 at serine residues by PKC caused a 2-fold increase in L-glutamate transport *in vivo* (Casado et al., 1993).

Summary

The intestinal EAAT3 transporter facilitates the assimilation of glutamate and aspartate, thus providing the enterocyte with its primary fuel. Expression of this transporter is greatest in the rapidly growing and differentiating premature enterocytes located in the crypts of the intestinal villi, indicating the importance of EAAT3 to these cells during this time. Diet, growth, and second messengers also may influence EAAT3 gene expression.

The Cationic, Neutral and Cystine Amino Acid Transporter, System $b^{o,+}$

Introduction

The $b^{o,+}$ system is made up of $b^{o,+}AT$ and rBAT. These two proteins are members of the family of glycoprotein-associated amino acid transporters (Verrey et al., 2004). The $b^{o,+}AT$ and rBAT heterodimerize to facilitate the transport of neutral cationic amino acids (Verrey et al., 2004; Palacin et al., 2005). The heavy chain rBAT was discovered first by expression cloning (Tate et al., 1992; Wells and Hediger, 1992; Bertran et al., 1993) followed by discovery of the light chain, $b^{o,+}AT$, also by expression cloning

(Chairoungdua et al., 1999). Dysfunction of $b^{o,+}$AT and/or rBAT leads to cystinuria, which is characterized by cystine accumulation in the kidney and subsequent kidney failure.

Tissue and Cellular Distribution of System $b^{o,+}$

The $b^{o,+}$ system transporters are found in the small intestine, kidney, liver, lung, placenta, and pancreas (Verrey et al., 2000; Wagner et al., 2001; Palacin and Kanai, 2004). Protein expression of rBAT is limited to the brushborder membrane of enterocytes, kidney proximal tubule (Wagner et al., 2001), and enteroendocrine cells (Pickel et al., 1993). Despite the limited tissue distribution of rBAT protein, rBAT mRNA is found in a variety of tissues including kidney, intestine, pancreas, heart, adrenal gland, brain stem, and spinal cord (Wagner et al., 2001). The primary site of protein expression in the enterocyte of rBAT is in the brush border membrane (Pickel et al., 1993; Dave et al., 2004). In the rat small intestine, rBAT mRNA an expression gradient was observed, with expression decreasing from proximal to distal regions (Howard et al., 2004). A similar expression pattern of rBAT was observed in chickens, with expression being greatest in the ileum (Gilbert et al., 2007b). Protein expression of rBAT also showed an expression gradient along the crypt-villous axis, with expression increasing from the crypt-villous junction to the villous tip (Dave et al., 2004; Howard et al., 2004). No rBAT protein expression was observed in crypt cells (Howard et al., 2004.)

In the horse, b^{0+}AT gene expression was found in the distal regions of the small intestine and throughout the large intestine, with similar levels in all segments examined (Woodward et al., 2010). In bears, b^{0+}AT expression is greatest in the distal regions of the small intestine (Gilbert et al., 2007a). Expression of b^{0+}AT was found in all segments

of the chicken small intestine with greatest expression in the ileum (Gilbert et al., 2007b). Expression of b^{0+}AT protein within the enterocyte is confined to the brushborder membrane (Dave et al., 2004). An expression gradient exists along the crypt-villous axis, with greatest b^{0+}AT protein expression at the villous tip (Dave et al., 2004).

Substrates of System $b^{o,+}$

Generally, the $b^{o,+}$ system transports neutral and cationic amino acids in exchange for intracellular neutral amino acids (Palacin et al., 2001). The $b^{o,+}$ system shows a high affinity (K_m of 100 μM) for L-cystine and cationic amino acids and a lower affinity for neutral amino acids (Palacin, 1994). The $b^{o,+}$ system is the primary transporter for cystine in the kidney (Palacin and Kanai, 2004) and also transports ornithine (Wagner et al., 2001). The $B^{o,+}$ system is an obligatory antiporter or amino acid exchanger. As such, it functions to absorb cationic amino acids and cystine, while contributing to the efflux of neutral amino acids.

Structure of system $b^{o,+}$

The $b^{o,}$+AT contains 12 transmembrane domains, which associates with the rBAT through a disulfide bond between the third trnasmembrane domain of the $b^{o,}$+AT and the single transmembrane domain of rBAT (Verrey et a., 2004). Dimerization between $b^{o,}$+AT and rBAT is required for trafficking of the transporter to the brushborder membrane, and thus transporter function (Reig et al., 2002).Human and mouse $b^{o,}$+AT consists of 487 AA with 12 predicted transmembrane domains with an extracellular loop between transmembrane domains three and four (Chairoungdua et al., 1999; Wagner et al., 2001). Both C- and N- termini of $b^{o,+}$AT face the cytoplasm (Verrey et al., 2004). A single tyrosine kinase-dependent phyosphorylation site, five protein kinase C

phosphorylation sites, and a single camp-dependent phosphorylation site ere predicted to exist in $b^{o,}$+AT (Chairoungdua et al., 1999; Wagner et al., 2001).

The human rBAT protein consists of 685 amino acid residues and is highly glycoslylated (Bertran et al., 1993; Tate et al., 1992; Wells and Hediger, 1992). Four variant mouse rBAT transcripts were detected in the kidney corresponding to sizes of 2.4, 4.2, 5.4, and 8.0 kb (Segawa et al., 1997). Intestine expressed three of the four (Segawa et al., 1997), with the primary species being the 2.4 kb transcript. The 5.4 kb transcript was not expressed. The rBAT exists with an extracellular C terminus, intracellular N-terminus and a large extracellular domain (Palacin and Kanai, 2004). Mouse rBAT contains four potential N-glycosylation sites and a leucine zipper motif at the C-terminal end (Segawa et al., 1997).

Regulation of System $b^{o,+}$

Development effects rBAT gene expression in the kidney (Segawa et al., 1997). Microarry analysis in chickens found rBAT to upregulated between embryonic d 18 and d 14 post hatch (Li et al., 2008). Development affects rBAT expression in the chicken (Zeng et al., 2011). Nutritional signals influence rBAT expression. For example, rBAT was induced by the amino acid aspartate (Segawa et al., 1997). Diet supplementation with leucine resulted in increased $b^{o,+}$AT gene expression in jejunum of pigs. (Morales et al., 2011). In Caco-2 cells, dipeptides increased L-Arginine influx more efficiently than their corresponding free amino acids (Wenzel, et al., 2001). Increased levels of Na+ also led to increased transport activity of system $b^{o,+}$ (Bauch and Verrey, 2002; Pfeiffer et al., 1999). Genetic background can affect expression of system $b^{o,+}$ transporters. Chickens selected for performance on a soybean meal based diet have greater expression of $b^{o,+}$AT

then chicks selected for performance on a corn based diet when fed a diet where the majority of protein was supplied in the form of peptides. (Gilbert et al., 2010). This correlates to the effect of dipeptides on $b^{o,+}$AT expression observed in Caco-2s by Wenzel et al. (2001).

The Neutral Amino Acid Transporter, B^oAT1

Introduction

The transporter, B^oAT1 is a member of the transport system B^o and is the major neutral amino acid transporter in the brush border of the enterocyte (Broer et al., 2004). In addition to expression in enterocytes, the other major site of B^oAT1 expression is the kidney proximal tubule where it reabsorbs neutral amino acids from the urine (Romeo et al., 2006). Dysfunction of neutral amino acid absorption and the resulting Hartnups disorder correlates to mutations in B^oAT1 (Seow et al., 2004).

Tissue and Cellular Distribution of B^oAT1

The primary sites of B^oAT1 expression are the kidney and the small intestine as demonstrated by *in situ* hybridization and immunocytochemical analysis (Broer et al., 2004; Romeo et al., 2006). Neither group observed B^oAT1 expression in the colon under normal pathophysiological conditions. Expression of B^oAT1 increased from the duodenum to the ileum (Terada et al., 2005; Romeo et al., 2006) in mammals. In chickens, B^oAT1 mRNA expression is greatest in the ileum. Expression of B^oAT1 increases from the crypt to the villous tip where the signal was most intense (Broer et al., 2004), which is consistent with enterocyte maturation and differentiation as it migrates from crypt to villous tip. Within the enterocyte, B^oAT1 expression is limited to the brush border membrane (Terada et al., 2005; Romeo et al., 2006).

Structure and Substrates of B^oAT1

Molecular cloning revealed that B^oAT1 is a protein with 634 residues and transcript of 1,904 bp (Broer et al., 2004) . There are no reports of B^oAT1 splice variants. There are 12 predicted transmembrane domains with intracellular amino and carboxyl termini. All neutral amino acids are transported by B^oAT1, as indicated by inhibition of radiolabeled leucine by all neutral amino acids (Broer et al., 2004). Functional studies indicate that V_{max} is the same for all neutral amino acids, but affinities are different for each amino acid. One Na+ ion is cotransported with each amino acid (Bohmer et al., 2005; Camargo et al., 2005). As cosubstrate concentration increases, the affinity for the substrate decreases (Bohmer et al., 2005). Thus, as the concentration of Na+ increases the affinity for neutral amino acids decreases and vice versa. This relationship is explained by the structure of B^oAT1, where the binding site for Na+ is partially formed by the carboxyl group on the amino acid substrate. (Broer, 2006).

Regulation of B^oAT1

Development affects gene expression of B^oAT1. In broiler chickens selected for performance on a corn-based or soy-based diet, gene expression of B^oAT1 increased linearly regardless of genetic line from embryonic day 18 to day 14 post-hatch (Gilbert et al., 2007b). Expression of auxiliary proteins, which may facilitate incorporation into the apical membrane of cells, may also influence expression and function of B^oAT1. Recently, collectrin was found to influence B^oAT1 expression in the kidney (Danilczyk et al., 2006) and is thought to influence B^oAT1 incorporation in the membrane. Coexpression of collectrin together with B^oAT1 increased surface expression of B^oAT1 in *Xenopus* oocytes and transfected cells (Kowalczuk et al., 2008).

Basolateral Amino Acid Transporters

The Cationic Amino Acid Transporters, CAT1 and CAT2

Introduction

While the $b^{o,+}$ system transports cationic amino acids across the brush border membrane, The y^+ system (system for cationic amino acid transport) members CAT1 and CAT2 facilitate cationic amino acid transport across the basolateral membrane in enterocytes (Verrey et al., 2004). The CAT family of transporters contains four members (CAT1-4) with CAT2 having two isoforms (CAT2A and CAT2B) (Verrey et al., 2004). These transporters play key roles in transporting arginine into cells, which can serve as substrate for constitutive and inducible nitric oxide syntheses (Verrey et al., 2004). Homozygous knockout of CAT1 is lethal (Kizhatil and Albritton, 2003), thereby demonstrating that CAT1 is the major transporter for system y^+ in most cells. Homozygous CAT2B appear phenotypically normal, but do exhibit decreased nitric oxide synthase production in macrophages.

Tissue, Cellular Distribution and Structure of CAT1 and CAT2

The CAT1 is ubiquitously expressed except for the liver, which has no expression (Verrey et al. 2004). Expression of CAT1 is greatest in the testis with lesser expression in other tissues including the small intestine (Deves and Boyd, 1998). In the chicken small intestine, expression of CAT1 mRNA is similar across all intestinal segments (Gilbert et al., 2007b). In epithelial cells, expression is localized to the basolateral membrane (Wolf et al., 2002). In kidney epithelial cells, CAT 1 formed clusters in the basolateral membrane (Kizhatail and Albrttion, 2003). The formation of CAT1 clusters in different microdomains may influence or be indicative of physiological functions or

states. There are two transcript products for CAT1 resulting from differential polyadenylation (Deves and Boyd, 1998). Mouse CAT1 protein has 622 amino acids and a molecular weight of approximately 67 kDa (Deves and Boyd, 1998).

Two isoforms, formed by alternative splicing, of CAT2 exist (CAT2A and CAT2B). Expression of the two CAT2 isoforms is quite different (Verrey et al., 2004). Expression of CAT2A is constitutive where as expression of CAT2B requires induction. When expressed, expression of both isoforms is greatest in the liver but is also found in skeletal muscle, heart muscle, vascular smooth muscle, and pancreas. In the chicken small intestine gene expression of CAT2A is greatest in the ileum (Gilbert et al., 2007b). Both isoforms are expressed on the basolateral membrane of cells (Verrey et al., 2004).

Transport Properties of Chicken CAT1 and CAT2

The CAT1 is a high-affinity, low-capacity transporter of cationic amino acids, where as CAT2 is a low affinity cationic amino acid transporter (Verrey et al., 2004). Transport by CAT1 is pH and Na^+ independent (Kizhatil and Albritton, 2003). In chickens, CAT1 and CAT2 (A/B or both? Need to look at primers) gene expression was downregulated with age, decreasing linearly from embryonic day 18 to day 14 post-hatch (Gilbert et al., 2007b; Li et al., 2008). Interestingly, in the yolk sac membrane of developing chickens, with gene expression of CAT1 decreasing from embryonic day 11 to day 13 and increasing from embryonic day 15 to day 17 (Yadgary et al., 2011).

The Heterodimeric Neutral Amino Acid Transporters, System L

Introduction

System L is responsible for the transport of large branched and aromatic neutral amino acids (Rajan et al., 2000). Transport is dependent on system L members heterodimerizing with the heavy chain protein 4F2bC. Most cells have System L transport. The light chain proteins LAT1 and LAT2 are members of system L located on the basolateral membrane (Verrey et al., 2004).

Tissue, Cellular Distribution, and Structure of LAT1 and LAT2

Gene expression of LAT1 is found throughout the body (Kanai et al., 1998, Wagner et al., 2001), including the small intestine (Dave et al., 2004; Gilbert et al., 2007b). Expression of LAT1 is also found in cancer tissues and cell lines including Caco2 (Kanai et al., 1998, Wagner et al., 2001), suggesting LAT1 is important in growing cells. Expression of LAT1 is limited to the basolateral membrane (Verrey et al. 2004). Expression cloning in *Xenopus* oocytes revealed that LAT1 rat cDNA encodes a 512 amino acid protein with 12 transmembrane domains, and no glycosylation sites (Kanai et al., 1998). Human LAT1 is a 507 amino acid protein (Prasad et al., 1999).

Expression of LAT2 is more limited than LAT1. Primary expression of LAT2 is in the epithelial cells of the proximal kidney tubule and small intestine (Bassi et al., 1999; Wagner et al. 2001; Dave et al., 2004). Expression is also found to a lesser extent in muscle, placenta, eye and tooth buds (Bassi et al., 1999). Expression is greatest in the jejunum of mouse (Dave et al., 2004) and horse (Woodward et al., 2010). No expression of LAT2 was found in the colon. Immunocytochemistry revealed an expression gradient of LAT2 along the crypt-villous axis of intestinal villi (Dave et al., 2004). Expression

increased towards the villous tip, with no LAT2 expression in the cells in the crypt. Expression of LAT2 was limited to the basolateral membrane within the enterocyte (Dave et al., 2004). Expression cloning of LAT2 cDNA isolated from a rabbit small intestinal cDNA library revealed a protein of 535 amino acids with 12 putative transmembrane domains and intracellular amino and carboxy termini (Rajan et al., 2000). The putative structure has no N-linked glycosylation sites, but does have a single phosphokinase C dependent phosphorylation site, three phosphokinase A dependent phosphorylation sites, and a single tyrosine kinase-dependent phosphorylation site (Rajan et al., 2000).

Transport Properties of LAT1 and LAT2

Similar to $b^{o,+}AT$, LAT1 and LAT2 are light chains that require heterodimerization to transport amino acids. Both LAT1 and LAT2 heterodimerize with the heavy chain protein 4F2hC to mediate transport of their respective substrates (Verrey et al., 2004). Similar to rBAT, the 4F2hC protein functions to traffic LAT1 and LAT2 to the basolateral membrane (Verrey et al., 2004). Transport by LAT1 and LAT2 is Na^+ independent and the complex of LAT1 or LAT2 with 4F2hC functions as an exchanger, transporting intracellular amino acids at a 1:1 ratio to extracellular amino acids (Verrey et al., 2004). The LAT1 has up to a 100-fold greater affinity for extracellular amino acids than for intracellular amino acids (Verrey et al., 2004). Therefore, it is hypothesized that these complexes function to equilibrate the concentrations of neutral amino acids across the cell membrane.

The substrates for LAT1 and LAT2 differ, with LAT2 having a broader specificity then LAT1 (Rajan et al., 2000). The LAT1 transporter complex has a high

affinity for large branched chain and aromatic amino acids (Verry et al., 2004). The LAT1 also transports D-isomers of leucine, methionine, and phenylalanine (Kanai et al., 1998). In addition to the large neutral amino acids transported by LAT1, LAT2 transports small neutral amino acids (L-isomers of alanine, glycine, cystine, serine, and glutamine) (Rajan et al., 2000). Although the breadth of substrates for LAT2 is greater then LAT1, LAT2 is a relatively low-affinity transporter compared to LAT1 with K_t values up to seven fold greater than LAT1 (Rajan et al., 2000). Transport by LAT2 is stimulated by an acidic pH, unlike LAT1 (Rajan et al., 2000).

The Heterotdimeric Cationic and Neutral Amino Acid Transporters, System y^+L

Introduction

Cationic and neutral amino acids are transported across the basolateral membrane of polarized epithelial cells by system y^+L (Wagner et al., 2001; Verrey et al., 2004). As with system L, System y^+L members y^+LAT1 and y^+LAT2 require dimerization with heavy chain 4F2hC to transport amino acids. Dysfunction of y^+LAT1 can lead to lysinuric protein intolerance (Borsani et al., 1999).

Tissue, Cellular Distribution, and Structure of y^+LAT1 and y^+LAT2

Primary expression of y^+LAT1 is in the epithelial cells of the kidney proximal tubule and small intestine (Wagner et al., 2001). Protein expression of y^+LAT1 is also found in leukocytes, lung, erythrocytes and placenta (Wagner et al., 2001). In the mouse small intestine, greatest gene and protein expression of y^+LAT1 is in the jejunum and ileum (Dave et al., 2004). No expression was observed in the colon. There are species differences in the regional expression of y^+LAT1. In broiler chickens, gene expression

was not different among the segments of the small intestine (Gilbert et al., 2007b). As with many transporters in expressed in the epithelial cells in the small intestine, there is a gradient of expression of y^+LAT1 along the crypt-villous axis (Dave et al., 2004). Little or no expression of y^+LAT1 is found in cells in the crypt region. Expression y^+LAT1 increases towards the tip of the villous and is greatest at the tip. Human y^+LAT1 protein is 511 amino acids long and y^+LAT2 is 515 amino acids long (Wagner et al., 2001).

While expression of y^+LAT1 is fairly limited, expression of y^+LAT2 is widespread (Verrey et al., 2004). Expression of y^+LAT2 is in epithelial as well as non-epithelial cells. Besides the small intestine, y^+LAT2 is also expressed in brain, testis, parotids, heart, kidney, lung, and liver (Wagner et al., 2001). In chickens, expression of y^+LAT2 does not differ between intestinal segments (Gilbert et al., 2007b) and is upregulated between embryonic day 18 and day 14 post-hatch (Li et al., 2008). A more detailed analysis using qPCR revealed in chickens, expression changed cubically with peaks of expression at day of hatch and day seven post-hatch.

Transport Properties of y^+LAT1 and y^+LAT2

As with System L, y^+LAT1 and y^+LAT2 require heterodimerization with heavy chain 4F2hC to be incorporated into the basolateral membrane (Bauch et al., 2003). Transport of dibasic amino acids by y^+LAT1 occurs independent of Na^+, while transport of neutral amino acids by y^+LAT1 requires Na^+ (Wagner et al., 2001). The y^+LAT1 operates as an obligatory amino acid exchanger, which transports extracellular and intracellular amino acids at a ratio of 1:1 (Wagner et al., 2001). In the case of transport of neutral amino acids by y^+LAT1, protons may be cotransported in place of Na^+

(Wagner et al., 2001). However, cotransport of protons does not occur under normal physiological conditions.

The y^+LAT2 also operates as an exchanger, transporting cationic and neutral amino acids across the basolateral membrane at a ratio of 1:1 of intracellular to extracellular amino acids (Wagner et al., 2001). Like y^+LAT1, transport of neutral amino acids is dependent on Na^+ (Wagner et al., 2001). Both the influx of neutral and cationic amino acids by y^+LAT2 is very efficient. However, the efflux of cationic amino acids is more efficient than neutral amino acids (Wagner et al., 2001).

Summary

Transports systems exist on the brush border and basolateral membranes for the assimilation of free dietary amino acids in the enterocyte. Generally, like the peptide transporters, they are expressed in mature enterocytes located towards the tip of a villous. Most free amino acid transporters are high affinity low capacity transporters, unlike the peptide transporters, indicating a potential role for fine-tuning of specific amino acid concentrations within the body as well as in the enterocytes. Further, many of these systems are interconnected by substrates forming a complex system for amino acid assimilation.

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CHAPTER III.
NUTRIENT TRANSPORTER GENE EXPRESSION IN MOUSE SMALL INTESTINE EPITHELIAL (MSIE) CELLS

Introduction

Today, *in vitro* model systems are a cornerstone in laboratories, providing a reasonable balance between economy, safety, reliability, and scientific discovery. Although scientists recognized long ago the potential of cell culture to transform research methods in the life sciences, it was not until the last half of the 20th century that cell culture became mainstream and routine. The discovery of HeLa cells made cell culture a success. The HeLa cell line was developed by researchers at John's Hopkins from cells isolated from an aggressive cervical cancer tumor from a woman named Henreitta Lacks. (Skloot, 2010). The main feature of HeLa cells that launched the era of cell culture was that they were immortal, meaning that the cells grew in culture over many passages without the need to start a new culture or harvest new cells. This meant that a single culture could be worked in for years rather then a couple of days. Almost overnight, cell culture became the workhorse of life science labs around the world.

While HeLa cells and similar lines are suitable for testing a plethora of hypotheses, they are not without limitations. Importantly, immortality comes at a cost. Cells, like the beings their combinations create, are limited to a finite amount of resources, including energy, nutrients, and machinery avaliable to carry out all the cellular processes necessary for life. Two major processes conducted by cells are growth and differentiation. These two processes are equally demanding on a cell, and insofar as the end objective of each process are remarkably different, cells manage the demand for resources by undergoing growth and differentiation at different times. For example, the

cell turns off genes that are not important for differentiating when growing and vice versa. Therefore, if a cell is growing and proliferating, it is not differentiating. A specific consequence of this resource allocation is that immortal cells generally do not express characteristics of specialized differentiated cells. An example of this phenomenon specific to this dissertation research is found with the enterocyte. This is reviewed in greater detail in Chapter II. Briefly, enterocytes begin their life in the crypt region of a villus. The cells found in the crypt region do not express nutrient transporters or other distinguishing characteristics typical of a mature enterocyte found at the villus tip. Cells in the crypt that are growing and proliferating, and therefore are not differentiated. As the cells migrate up the villus towards the tip, these cells begin to differentiate as evidenced by the expression of nutrient transporters, *inter alia*, and their inability to grow in culture.

Scientists recognized this problem and resorted to developing primary culture techniques, stimulating immortal lines to differentiate with chemicals and compounds, or isolating cells from cancer tumors from various tissue types, hoping to generate a tissue type immortal line in similar fashion to HeLa. While some researchers have prevailed using these methods with some tissues, no small intestinal line was developed. The closest cell model of the small intestine is the human colon carcinoma cell line known as Caco-2. This model has proved very useful and is the "gold standard" *in vtiro* model for the small intestine. While Caco-2 cells express several nutrient transporters and form a polarized monolayer with a brushborder membrane (Delie and Rubas, 1997), it is prudent to point out that these are cancer cells and with respect to the nutrient transporter profile it is not an exact model of the small intestine. For example, PepT1 is normally not found

in the large intestine (See Chapter II for a review). In fact, aberrant PepT1 expression in the colon is linked to disorders such as inflammatory bowel disease and other immune dysfunctions of the large intestine. Thus, PepT1 is aberrantly expressed in the Caco-2 line, possibly due to tumorgenic roots of Caco-2 cells. Therefore, when considering PepT1 characteristics in these cells, it must be considered that although expressed in Caco-2, the regulation and function of PepT1 may represent a pathologic physiology and not what is normal in the small intestine.

The overall objective of the research presented in this dissertation is to examine the interaction between the peptide transporter PepT1, other peptide transporters and free amino acid transporters in the small intestine. As PepT1 expression in Caco-2 cells is likely the result of abnormal physiology and regulation of the gene, Caco-2 cells may not be the best model system to examine the iteraction of PepT1 and other transporters in. To this end, several attempts to isolate and culture primary small intestinal chicken cells were conducted with no obvious success. Generally, the fibroblast population would quickly take over and the epithelial-like cells in the population would disappear within a couple of days.

Researching various attempts at developing small intestinal models resulted in the discovery of an intestinal line generated from a transgenic mouse known commercially as the "Immortomouse" (U.S. Patent Number 5866759, the "'759 patent"). A key goal of the Immortomouse, in addition to studying cancer, was to develop cell lines that solved problem of the trade off between growth and differentiation suffered by traditional immortalized cell lines. The Immortomouse ubiquitously expresses a mutant SV40-T-Antigen gene (Temperature Sensitive-A58). Normal T-antigen is an oncogene, which

stimulates non-stop growth of cells that express it. The A58 mutation causes misfolding of T-Antigen protein at higher temperatures, rendering the T-antigen inactive. Therefore, cell lines produced from the tissues of the Immortomouse, in theory, would be conditionally immortal. At lower temperatures the cells grow and divide and at higher temperatures, which deactivate T-antigen, the cells differentiate. As described in the '759 patent, several cell lines corresponding to terminally differentiated cell and tissue types, such as neurons, that have been isolated and characterized from the Immortomouse, appear to be conditionally immortalized, and useful research tools. Whitehead et al. (1993) isolated small intestinal cells from the Immortomouse to be a potential small intestinal model. These cells are called Mouse Small Intestinal Epithelial (MSIE) cells. Whitehead et al. (1993) demonstrated that the MSIE cells were conditionally immortal, were epithelial in nature, and expressed the brushborder enzymes sucrase, which is limited to the intestinal brushborder of mature enterocytes, and the more ubiquitously expressed enzymes alkaline phosphotase and dipeptidyl peptidase IV. However, this group did not analyze the expression of any nutrient transporters, which are hallmarks of differentiated enterocytes.

Therefore, the specific objective of this experiment was to determine the nutrient transporter gene expression profile of the conditionally immortalized MSIE cells to determine if MSIE cells express a panel of transporters similar to the small intestine and if MSIE cells are a small intestinal better model than Caco-2 cells to carroy out the *in vitro* characterization of the interaction of PepT1 and other peptide and amino acid transporters in.

Materials and Methods

Cell Culture

Mouse small intestine epithelial cells (MSIE) (gift from Dr. Robert Whitehead) are only conditionally immortalized. This means that they will only grow at the permissive temperature of 33ºC. These cells express a mutant T-antigen, which is active at the permissive temperature and keeps the cells growing. When grown at the non-permissive temperature of 37ºC, the mutant T-antigen is no longer active and the cells can then differentiate. The MSIE cells were cultured in standard culture media (DMEM, 5% Fetal Bovine Serum, 1µg/mL insulin, 10^{-5} M α-thioglycerol, 10^{-6} M hydrocortisone and 5 units per mL of mouse gamma interferon). Cells were split when confluent using Trypsin-EDTA at 1:2 or 1:3. Cells were periodically frozen in the culture medium plus 20% FBS and 10% Dimethylsulfoxide (DMSO) at $2x10^6$ cells per mL. To test that the cells were conditionally immortalized, cells were plated in 24-well plates at $5x10^4$ cells per mL in DMEM plus 0.5% FBS 1µg/mL insulin, 10^{-5} M α-thioglycerol, 10^{-6} M hydrocortisone minus the interferon gamma, and grown at 33ºC. After plating, the cells would become senescent within three to five days and be dead by seven days. This result confirmed that the T-antigen gene had not transformed the cells into a malignant line.

Nutrient Transporter Gene Expression Analysis

To evaluate nutrient transporter gene expression MSIE cells were plated in 12 well plates at 2 X 10^5 cells per well. There were 3 groups of cells. The first group was grown entirely at the permissive temperature (33°C), the second group was grown entirely at the non-permissive temperature (37°C) and the third group was placed at the

permissive temperature for 24 h then moved to non-permissive temperature for the remainder of the experiment (Figure. 3.1).

Cells from duplicate wells from each group (except the 24 h time) were collected at 24, 48 and 72 h post seeding (Figure 3.1). Total RNA was extracted (RNeasy Kit, Qiagen). Concentration and quality of RNA was determined using a Bioanalyzer. One-hundred nanograms of RNA were reverse transcribed into cNDA using the random priming method (cDNA High Capacity Archive kit, Applied Biosystems, CA). The cDNA was then diluted 1:6 and nutrient transporter gene expression was examined using Real-Time qPCR in an ABS 7300 Real Time qPCR machine (Applied Biosystems). Each 25 µL reaction contained the following: 2 µL diluted cDNA, 12.5 µL 2X SYBR Green PCR Master mix (Applied Biosystems), 0.5 µL 5 µM Forward primer, 0.5 µL 5 µM reverse primer, and 9.5 ddH_20. All expression was determined relative to GAPDH (Δ Ct), normalized to 24h PepT1 (ΔΔCt), and transformed to $2^{-\Delta\Delta Ct}$. The transformed $2^{-\Delta\Delta Ct}$ was analyzed for effects of temperature (experimental group) and time and the interaction of temperature x time using ANOVA followed by a contrast analysis where appropriate in JMP (SAS, Cary, NC). Statistical significance was considered at $P < 0.05$. Primer pairs for Real-Time qPCR were designed to span exons and not to include minor splice variants using the Primer Express software (Applied Biosystems) and were validated by examining expression of these genes in mouse intestinal tissue. Table 3.1 shows the genes examined and the primers used.

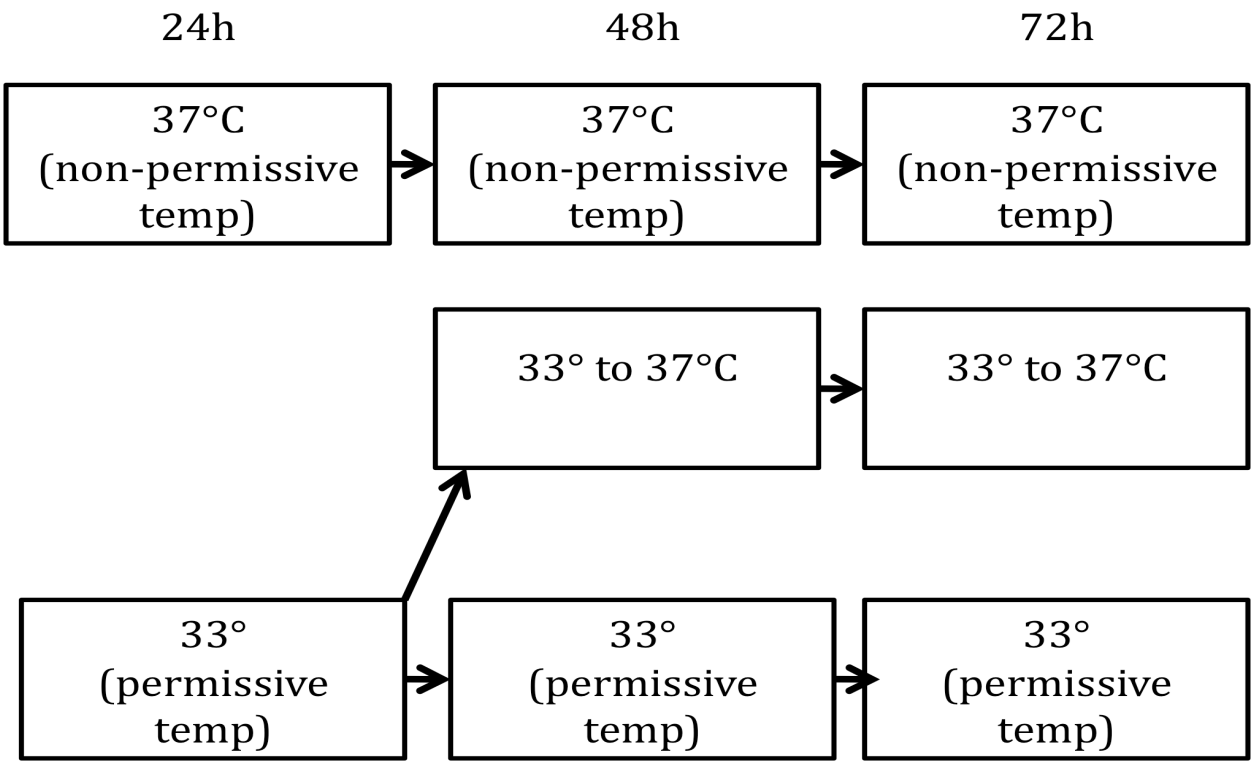

Figure 3.1. **Experimental design for evaluation of nutrient transporter gene expression in MSIE cells.** Cells were seeded and placed at 33 or 37°C. Cells were collected 24h post seeding. Some cells were moved from the permissive temperature to the non-permissive temperature to induce differentiation. Cells from all three groups were collected at 48 and 72h.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
PepT1	AAGTGGCCAAGTGCATTGGT	TCCCTCTTGGGATATGCCTTAC
EAAT3	TCTGGTCCAAGCCTGTTTTCA	ATCGCCCACAGGCTTCAC
rBAT	CCGAAATCCTGCTGTTCAAGA	GCATCAAAACTAAAACCCCATCAACAC
$B^{0,+}AT$	GGGCCTACGATGGTTGGAA	GGGCAGGTTTCTGTAAGGGTTT
$B^{0}AT1$	CCATCCACCCTGCTCTGAAG	CAGGCCCACCATGAAGGA
CAT1	GCAGCTCACGGAGAAAAATTTC	TCCTCACCGTATTTCACGTT
CAT2	TGCCGTGTGCCTTGTATTACTC	CCCAAGCAGACTCTTTTACTCCAA
LAT1	TGTCACGGAGGAGATGATCAAC	TGGGCAAGGAGATGATGATG
LAT2	TCTGGAGGTGATTACTCTTATGTGAAG	AATCCACAGCCGCAGGAA
y+LAT1	TGGCCAGTGACGCTGTTG	CGCTACTGGAATTATCCAATTGAA
y+LAT2	CACCCGTGCCTGCGTTAC	CGTCCTTCACCACGAGGTAGA
GAPDH	CGTGGAGTCTACTGGTGTCTTCAC	TTTTGGCTCCACCCTTCAAGT

Table 3.1. Genes and Real Time qPCR Primers for Mouse Intestinal Nutrient Transporters.

Results and Discussion

The purpose of this experiment was to characterize MSIE cells in order to determine if they were a suitable *in vitro* model to evaluate the effect of reducing PepT1 on amino acid and other peptide transporters in. Interestingly, MSIE cells generally express a panel of nutrient transporters that are found on the basolateral membrane of enterocytes. Specifically, MSIE cells do not express EAAT3, $B^{o,+}AT$, LAT2, and, most importantly for this research, PepT1. The amplification of their genes occurred at cycle 38-39 of a 40-cycle PCR routine, which corresponds to background amplification. Therefore, it was determined that these genes are not expressed in MSIE cells For reference, GAPDH and all other genes studied showed amplification from cycles 15-26.

Further, it was hypothesized that by switching MSIE cells to culture at the non-permissive temperature (37ºC) would stimulate the cells to switch from growing to differentiating, based on the inactivation of T-Antigen. However, this was not observed. In fact, gene expression of all the transporters expressed in MSIE cells decreased over time in the cells cultured at 33ºC then switched to 37ºC. In particular, gene expression of BoAT1, CAT2, and y^{+}LAT1 decreased to almost negligible amounts in cells cultured at 33ºC then switched to 37ºC. Based on the theory of the conditional immortalization, a reasonable mind could argue that the time allowed for differentiation to occur was too short. However, cells incubated constantly at the non-permissive temperature (37ºC) had an extra 24h to differentiate, and gene expression had not increased significantly for any of the nutrient transporters already expressed by 72h post seeding. Further, no increase in

gene expression of any of the non-expressed brush-border membrane genes (EAAT3, PepT1, $B^{o,+}AT$) was seen in the two groups cultured at the non-permissive temperature.

The decrease in expression of all expressed transporters may be due to increased cell sickness and death. By 72h in cultre the cells had formed a monolayer and had begun to appear unhealthy and detached from the wells. The Ct values of GAPDH remained constant over time in all samples, remaining within a cycle in contemporary groups across time. The GPADH transcript may be stable enough to withstand cell sickness, whereas the transcripts of the transporters are not. This would account for no change in GAPDH values despite cell sickness and death.

Conclusion

The MSIE cells do express several amino acid transporters found in the small intestine, but not PepT1. With the exception of $B^{o}AT1$, all the transporters expressed in MSIE cells are found on the basolateral membrane of the small intestine. Further, MSIE cells do not differentiate with respect to the profile of intestinal peptide and amino acid transporters when cultured at the non-permissive temperature. Based on these characteristics it was concluded that theory did not translate into reality with respect to MSIE cells. Therefore, MSIE cells were not a suitable cell line to test the overarching hypothesis of this dissertation research in.

Gene	Location	Expression ? (yes/no)
PepT1	BBM	No
EAAT3	BBM	No
B0+AT	BBM	No
BOAT1	BBM	Yes
CAT1	BL	Yes
CAT2	BL	Yes
LAT1	BL	Yes
LAT2	BL	No
y^+LAT1	BL	Yes
y^+LAT2	BL	Yes
GAPDH	cytosol	Yes

Table 3.2. Summary of nutrient transporter gene expression in MSIE cells.
In this experiment, MSIE cells express a panel of nutrient transporters that resembles that of the basolateral membrane, rather than a polarized cell line. Specifically, MSIE cells did not express EAAT3, $B^{o,+}$AT, LAT2 and, importantly, PepT1.

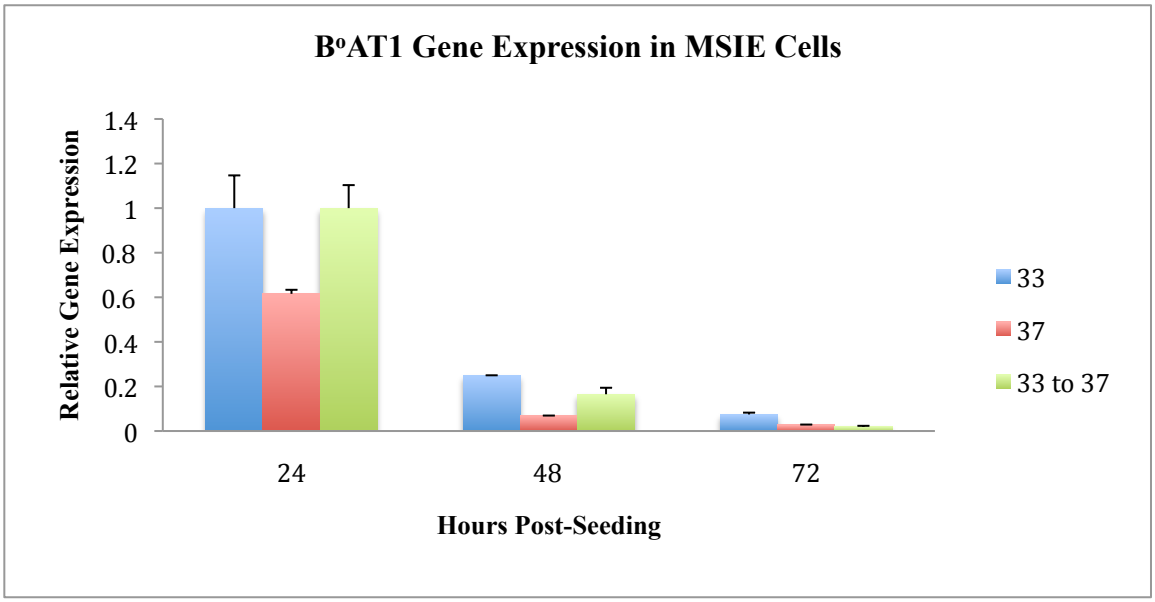

Figure 3.2. Relative gene expression of B^{0}AT1 in MSIE cells. Relative gene expression of B^{0}AT1 decreased significantly in all experimental groups over time, with greatest expression observed at 24h post-seeding and least expression observed at 72h post-seeding ($P < 0.05$). There was a significant effect of culture temperature on B^{0}AT1 relative gene expression. Overall, the cells maintained at 37ºC throughout the experiment had the least B^{0}AT1 gene expression ($P < 0.05$). Overall, There was no statistical difference between B^{0}AT1 gene expression in the cells raised at 33ºC or switched from 33ºC to 37ºC. There was a significant time x temperature interaction. At 24h and 48h post-seeding, there was no difference between cells raised at 33ºC or switched from 33ºC to 37ºC, however by 72h post-seeding, cells grown at 33ºC and switched to 37ºC had lower gene expression than cells grown at 37ºC ($P < 0.05$), which was not statistically different from cells grown only at 37ºC.

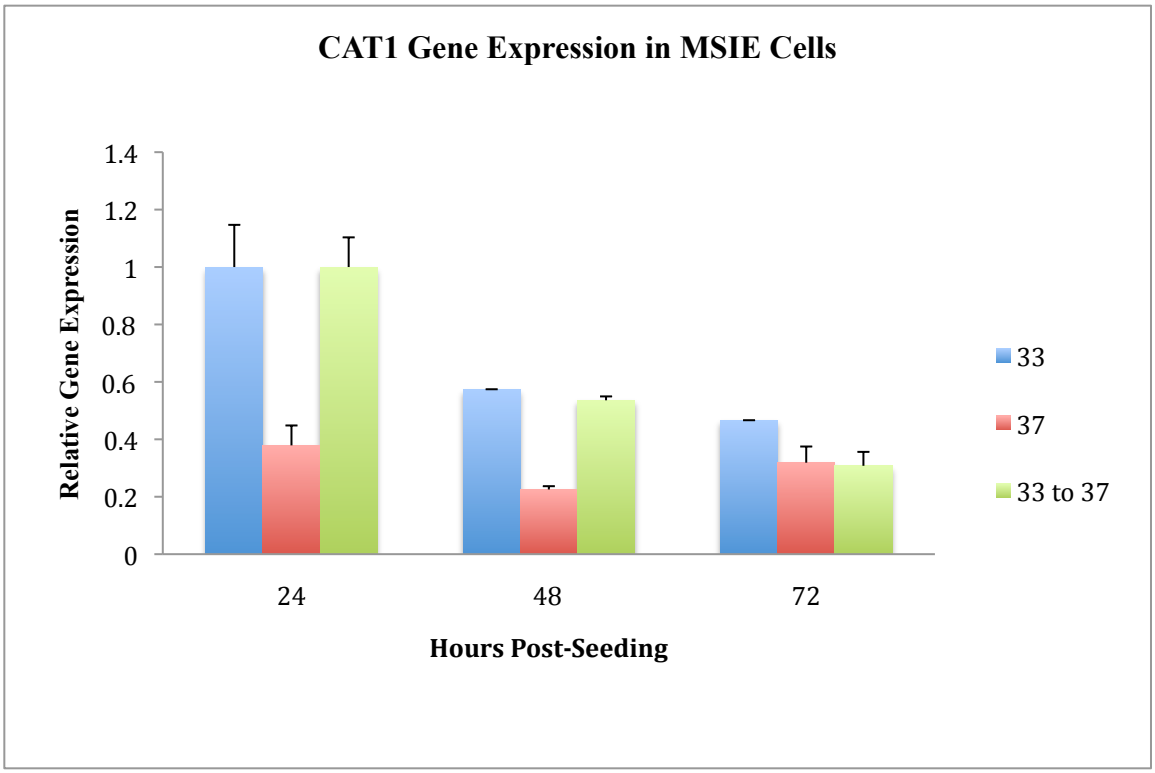

Figure 3.3. Relative gene expression of CAT1 in MSIE cells. Relative gene expression of CAT1 decreased significantly in all experimental groups over time, with greatest expression observed at 24h post-seeding and least expression observed at 72h post-seeding ($P < 0.05$). There was a significant effect of culture temperature on CAT1 relative gene expression. Overall, the cells maintained at 37ºC throughout the experiment had the least CAT1 gene expression ($P < 0.05$). Overall, there was no statistical difference between CAT1 gene expression in the cells raised at 33ºC or switched from 33ºC to 37ºC. There was a significant time x temperature interaction. At 24h and 48h post-seeding, there was no difference between cells raised at 33ºC or switched from 33ºC to 37ºC, however by 72 h post-seeding, cells grown at 33ºC and switched to 37ºC had lower gene expression than cells grown at 37ºC ($P < 0.05$), which was not statistically different from cells grown only at 37ºC.

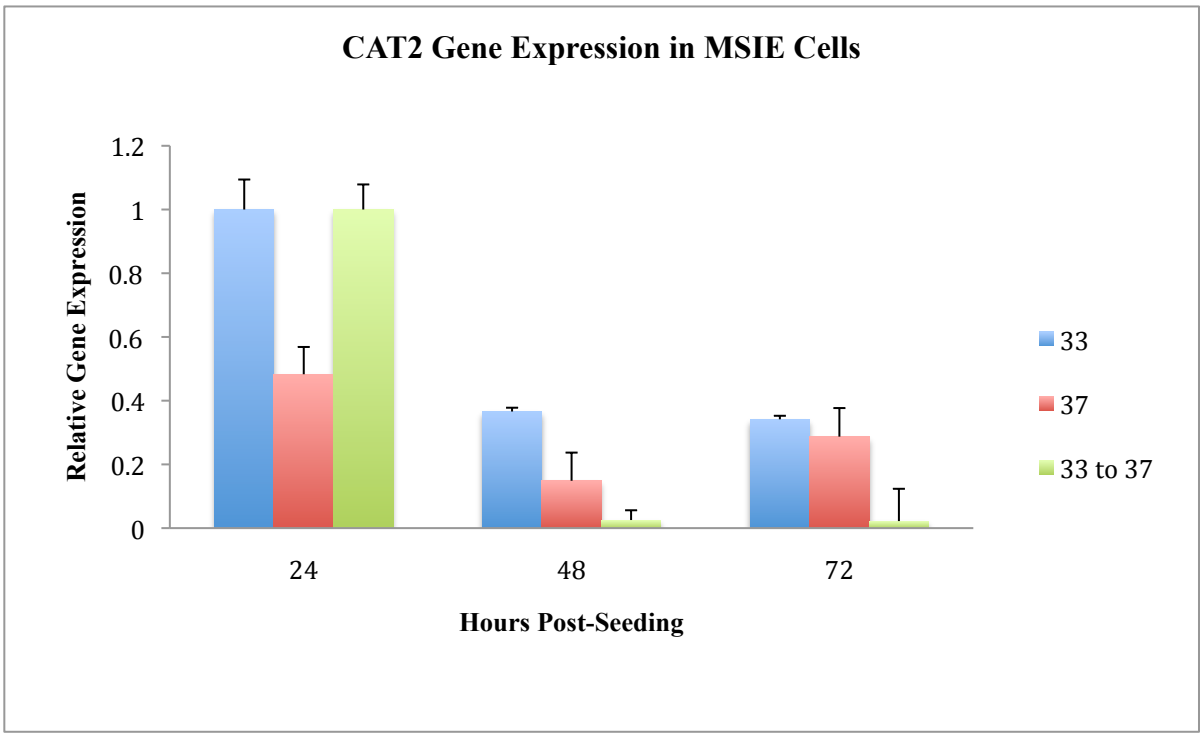

Figure 3.4. Relative gene expression of CAT2 in MSIE cells. There was a significant effect of time on CAT2 gene expression in MSIE cells. Gene expression of CAT2 decreased in all treatment groups from 24h to 48h post-seeding ($P < 0.05$), then did not change from 48h to 72h post-seeding. There was a significant time x cell culture temperature interaction. At 24h post-seeding expression of CAT2 in MSIE cells cultured at 37ºC had the least CAT2 expression ($P < 0.05$), while there was no difference in CAT2 expression in either cells cultured at only 33ºC or at 33ºC then switched to 37ºC. However, at 48h post-seeding, cells cultured at 33ºC showed greatest CAT2 gene expression ($P < 0.05$), cells cultured at 37ºC had an intermediate level of expression ($P < 0.05$), and cells cultured at 33ºC then switched to 37ºC had the least amount of CAT2 expression ($P < 0.05$). At 72h post transfection, expression in cells cultured at 37ºC increased slightly such that there was no significant difference in CAT2 gene expression between cells cultured at 37ºC and those cells cultured at 33ºC. Expression of CAT2 was least in cells cultured at 33ºC then switched to 37ºC at 72h post-seeding ($P < 0.05$).

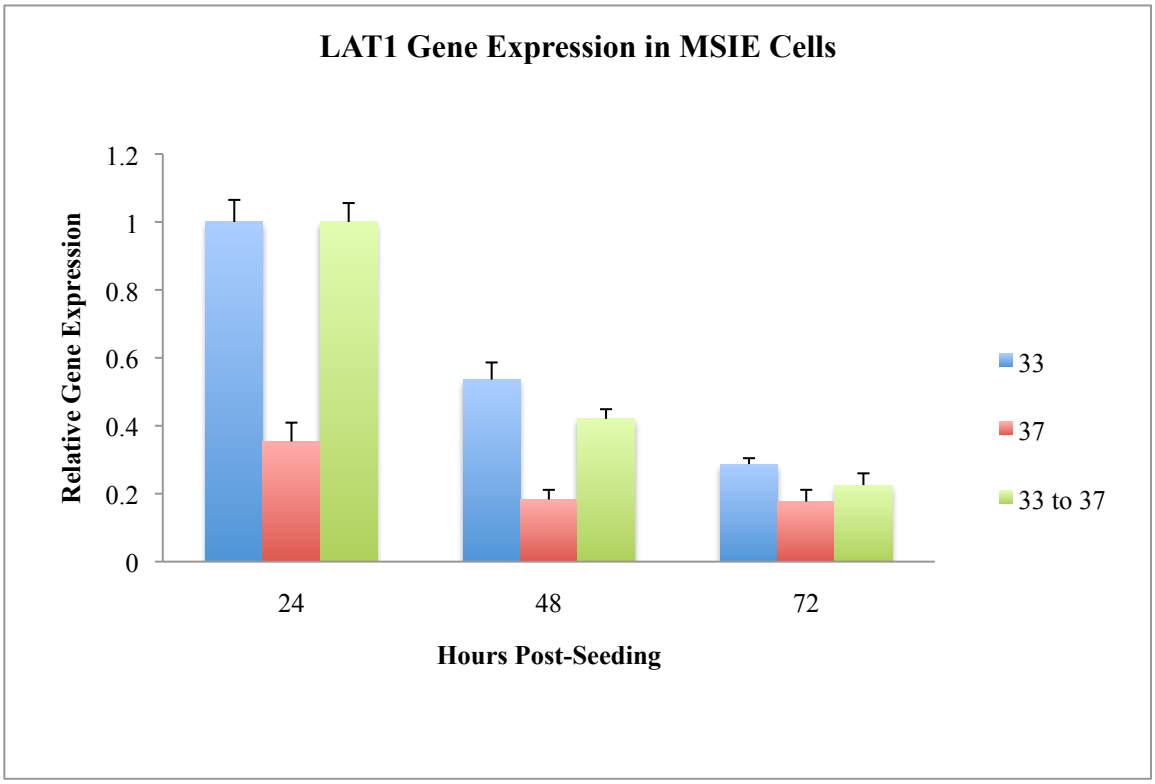

Figure 3.5. Relative gene expression of LAT1 in MSIE cells. Overall, there was no statistical difference in LAT1 relative gene expression in cells cultured at 33ºC or cells cultured at 33 then switched to 37ºC. Gene expression of LAT1 was least in cells cultured at 37ºC ($P < 0.05$). There was a significant time x temperature interaction. Gene expression of LAT1 decreased linearly with time in cells cultured at 33ºC or 33ºC then switched to 37ºC ($P < 0.05$). However, LAT1 gene expression decreased from 24h to 48h post-seeding and was not statistically different from 48h to 24h post-seeding in cells cultured at 37ºC.

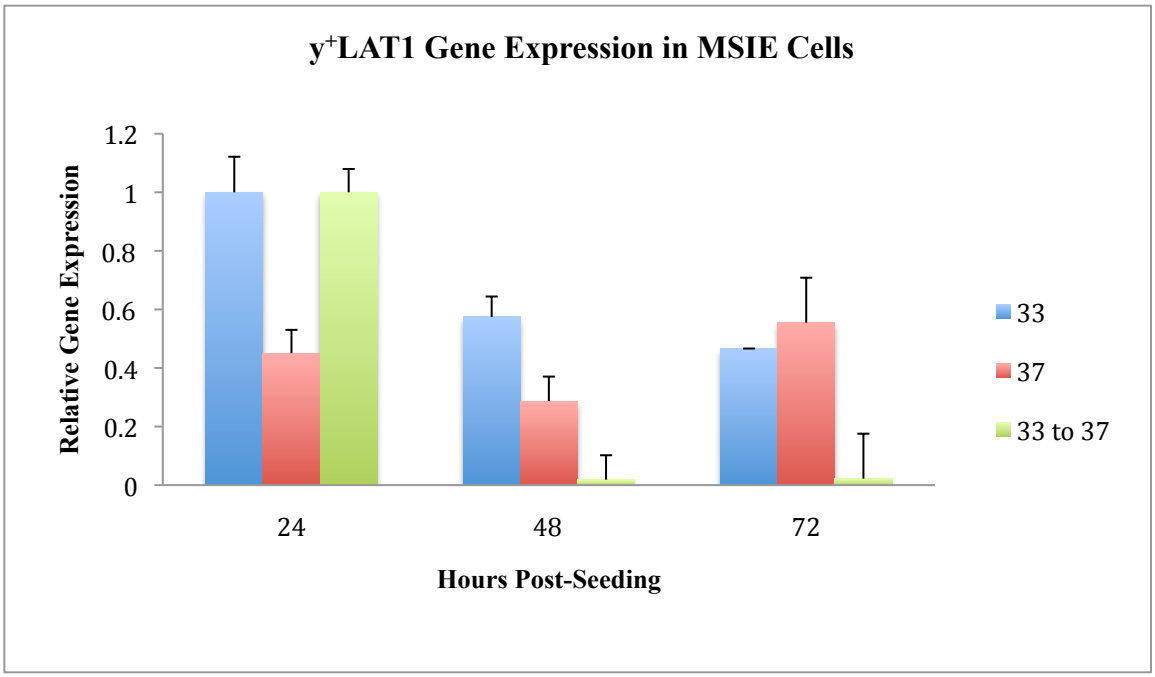

Figure 3.6. Relative gene expression of y^+LAT1 in MSIE cells. Figure 3.6 shows the relative gene expression of y^+LAT1 in MSIE cells. There was a significant time by temperature interaction. Overall, y^+LAT1 gene expression decreased linearly with time in MSIE cells cultured at 33ºC. Gene expression of y^+LAT1 in cells cultured at 37ºC did not significantly change between time points. Gene expression of y^+LAT1 decreased to almost nothing after by 48h post seeding and did not significantly change from that by 72h post-seeding. At 24h post seeding, cells cultured at 37ºC had the least amount of y^+LAT1 gene expression when compared to the other two culture groups ($P < 0.05$), which did not significantly differ from one another. Gene expression of y^+LAT1 was greatest in cells cultured at 33ºC ($P < 0.05$), intermediate in cells cultured at 37ºC ($P < 0.05$), and least in cells that were cultured at 33ºC then switched to 37ºC at 48h post-seeding ($P < 0.05$). At 72h post seeding, there was no difference in the level of y^+LAT1 gene expression between cells cultured at 37ºC and those cultured at 33ºC. Like the 48h time point, y^+LAT1 gene expression was least in cells cultured at 33ºC then switched to 37ºC ($P < 0.05$).

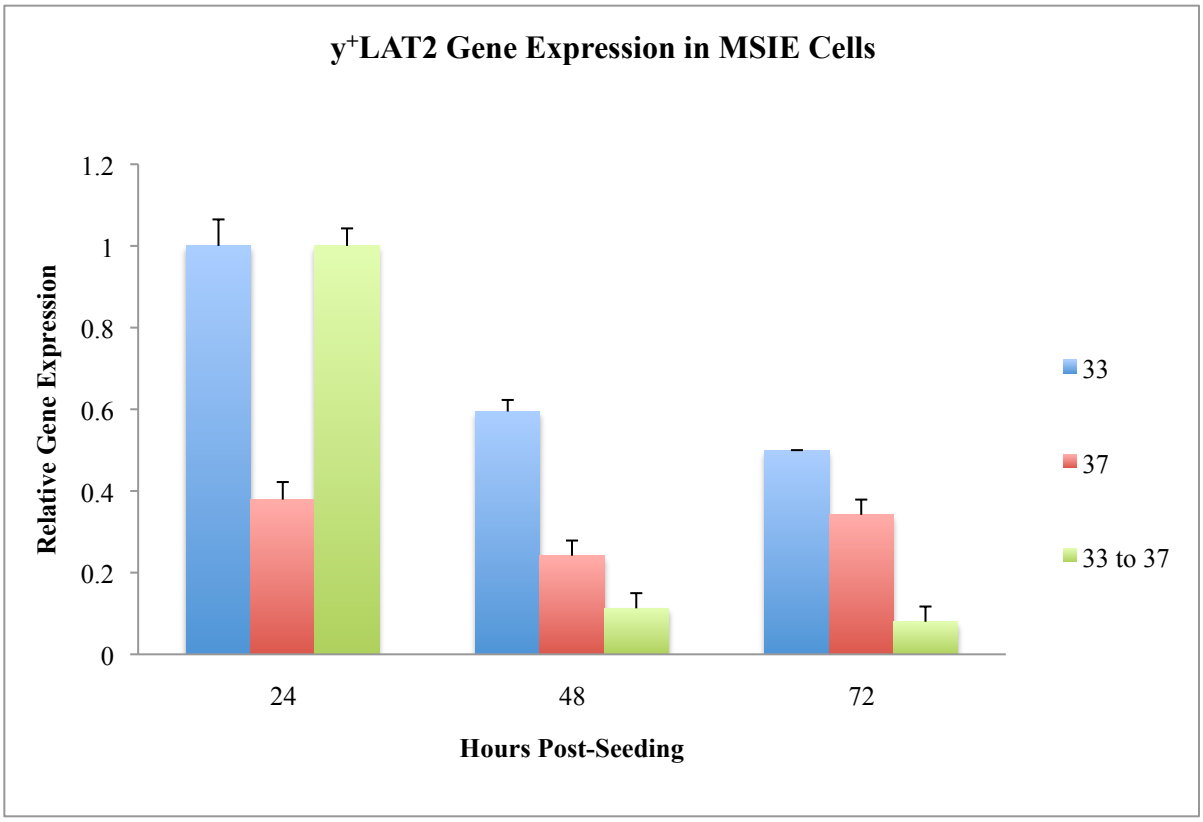

Figure 3.7. Relative gene expression of y^+LAT2 in MSIE cells. Figure 3.7 shows the relative gene expression of y^+LAT2 in MSIE cells. There was a significant time by temperature interaction with y^+LAT2 gene expression decreasing linearly over time. At 72h post-seeding, gene expression of y^+LAT2 in cells cultured at 37ºC decreased from 24h to 48h post-seeding then increased back to gene expression levels seen at 24h. In cells cultured first at 33ºC then switched to 37ºC, y^+LAT2 gene expression significantly decreased form 24h post-seeding to 48h post-seeding. There was no difference in y^+LAT2 gene expression in these cells from 48h to 72h post seeding. At 24h post-seeding, cells cultured at 37ºC had the least amount of y^+LAT2 gene expression ($P < 0.05$). There was no significant difference in y^+LAT2 gene expression between the other two groups of cells. At 48h and 72h post-seeding, gene expression was greatest in cells cultured at 33ºC ($P < 0.05$), intermediate in cells cultured at 37ºC ($P < 0.05$), and least in cells first cultured at 33ºC then switched to 37ºC ($P < 0.05$).

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CHAPTER IV.
NUTRIENT TRANSPORTER GENE EXPRESSION IN HUMAN COLON CARCINOMA (Caco-2) CELLS

Introduction

Human colon carcinoma (Caco-2) cells are frequently used as an *in vitro* model of the intestine (small and large). This cell line is used extensively for drug permeability and metabolism studies, in particular studies involving peptidiomimentic substrates of PepT1 (Hidalgo et al., 1989, Adson et al., 1995). In contrast to animal experiments, cell culture models provide a relatively inexpensive and reproducible tool to characterize drug transport and metabolism by intestinal cells. The Caco-2 cells are reasonably suited for these types of studies as they are immortal and express several nutrient transporters found in cells throughout the intestine (Delie and Rubas, 1997).

Variability from laboratory-to-laboratory with respect to drug permeability of Caco-2 cell monolayers suggests that different culture conditions in different labs have affected the profile of transporters expressed. It is known that Caco-2 cell cultures are influenced by a number of cell culture-related factors, including cell origin (Walter and Kissel, 1994; 1995) and passage number (Lu et al., 1996; Briske-Anderson et al., 1997). Further, a difference in gene and protein expression of PepT1 and HPT1 in Caco-2 cells derived from different subcultures has been observed (Behrens et al., 2003). As it is well established that different subcultures of Caco-2 have different expression and transport capabilities, the objective of this experiment was to determine the nutrient transporter gene expression profile of the Caco-2 cells received from the lab of Dr. Jiang to determine their suitability to examine *in vitro* the overarching objective of this dissertation research.

Materials and Methods

The human colon carcinoma cell line (Caco-2) (gift from Dr. Jiang) was cultured in standard media (DMEM, glucose, L-glutamine, penicillin-streptomycin, amphotericin B, 10% Fetal Calf Serum) split every four to seven days. To examine gene expression of nutrient transporters endogenously expressed in Caco-2 cells, Caco-2 cells were plated in 24-well plates and grown to confluency under standard culture conditions. Cells (n = 4) were then collected for RNA extraction at days 0 (confluency), 4, 7, 10, 14 and 21post-confluency. This test period (0 – 21 d post-confluency) was based on conditions used for PepT1 substrate transport studies, which are normally carried out at 18-21 d post-confluency. The RNA was extracted using the RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturers' instructions for Animal Cells. Concentration and quality of RNA was determined using a bioanalyzer.

Two µg of RNA was used in each reverse transcription reaction to prepare cDNA using the random priming method (High Capacity cNDA Archive kit, Applied Biosystems). The cDNA was then diluted 1:30 and nutrient transporter gene expression was examined using Real-Time qPCR in an ABS 7300 Real Time qPCR machine (Applied Biosystems). Each 25 µL reaction contained the following: 2 µL diluted cDNA, 12.5 µL 2X SYBR Green PCR Master mix (Applied Biosystems), 0.5 µL 5 µM Forward primer, 0.5 µL 5 µM reverse primer, and 9.5 ddH_20. Relative gene expression was calculated using the ΔΔCt method as previously described in Chapter III, with GAPDH as the endogenous control and d0 PepT1 as the calibrator sample. The relative gene expression was then log base 2 transformed ($2^{-\Delta\Delta Ct}$), analyzed for statistical significance by ANOVA using JMP (SAS) and graphed. Statistical significance was consider at $P <$

0.05. Primers for the human nutrient transports were designed as previously described in Chapter III and are shown in Table 4.1.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
PepT1	CCTGGAGAGATGGTGACACTTG	GTTTATCCTTGTCAGTTTGTTTACATCAA
HPT1	GAAGGCTCAGTAAGGCAGAACTCT	GGTCTGTGGCATTGACATACAAG
PTR3	TTGGTTTTACCTCCGTCATTGTG	CGTTGTGGTGGATGTAGTGTAAGC
EAAT1	ACAGCTGCAGATGCCTTCCT	GCAGGCTTCTACCAGATTTGGA
EAAT3	CGCGCTGTCGTGTATTATTTCT	TCACCACCAGCACAATACCTAGA
rBAT	CCCATGAAAATGGCAAAACC	CCAACTGGAGTTTCCATACACACT
$B^{o,+}AT$	TCCTCAAATCGTTGTGAAATGC	CCGCACGCTCAGTGAGTTC
$B^{o}AT1$	CCACCCGGCCCTGAA	TGTTGTAATACAGTCCCACCATGAA
CAT1	TCATCACCGGCTGGAACTTAA	GCCCTCGCTACGCTTGAAG
CAT2	CGTCGCAGCTGGTTCTCT	TGGGAAAATGGATCCAAGAAGA
LAT1	CATCCTGCTGGGCTTCGT	AAGTTGGGATCTAGATTGGACACAT
LAT2	CCTGGCTGCCATCTGCTT	ACCGCACACTGGAACAGTTG
y+LAT1	CGTTGCAGGCATTGTTAGACTT	GAACCCTCAAAGGAATTCTCAAAA
y+LAT2	TGTCATGGGCCTTGTTAAACTG	CTCAAAGGCGTCCTGAAAGTG
GAPDH	GACCCCTTCATTGACCTCAACT	GAATTTGCCATGGGTGGAAT

Table 4.1. **Genes and Real Time qPCR primers for human intestinal nutrient transporters.**

Results and Discussion

The purpose of this experiment was to evaluate the profile of nutrient transporter gene expression in this culture of Caco-2 cells to determine if it was a suitable *in vitro* model to determine the effect of PepT1 reduction on gene expression of amino acid and other peptide transporters in. As predicted, this specific Caco-2 line expresses a panel of peptide and amino acid transporters. In agreement with Behrens et al., 2003, HPT1 appears to be expressed in greater relative amounts than PepT1. As stated previously, Caco-2 cells are the model system used to study transport of pharmaceutically important substrates of PepT1 (Buyse et al., 2001 and Brodin et al., 2002). In these studies, Caco-2 cells are plated out on trans-well plates and are allowed to become confluent . Once the monolayer is formed, the cells are allowed to differentiate for about 18 days (PepT1 (Buyse et al., 2001 and Brodin et al., 2002). This is to allow the cells to differentiate and gain function. This is the rational behind conducting drug transport studies on about day 21 post confluency.

In agreement with other reports from drug uptake studies and the principle driving their methodology, a linear increase in several transporters, including PepT1 and HPT1 was observed in this study. The overall results suggest that these Caco-2 cells would be suitable as an *in vitro* model to test the overarching hypothesis. These results also suggest that while cells could be examined as early as day of confluency for transporter expression, it would be ideal to wait until at least day 7 to examine the effect of PepT1 knockdown. This is due to the significant change in transporter expression, including PepT1, from d0 to d7, and change less from d 7 to d21. With that being said, if an RNAi

approach is utilized, which requires transfection and thus induces a transient effect, it may be difficult to carry out the experiment beyond a couple of days.

There have been reports of chicken intestinal cell lines lasting up to a week (communicatin with Dr. Dalloul). However, these cells require the addition of fibroblast inhibitors and growth factors to maintain their epitheial nature. This line and others like it would not be appropriate as growth factors impact the regulation of PepT1 (See Chapter II for a review). Further, these lines have not been fully characterized with respect to small intestinal markers such as brush border enzymes, nutrient transporters, and their ability to form tight junctions.

Conclusion

This culture of Caco-2 cells express a panel of peptide and amino acid transporters and because of the lack of another suitable cell model, they were chosen to examine the effect of PepT1 gene silencing on amino acid and other peptide transporters.

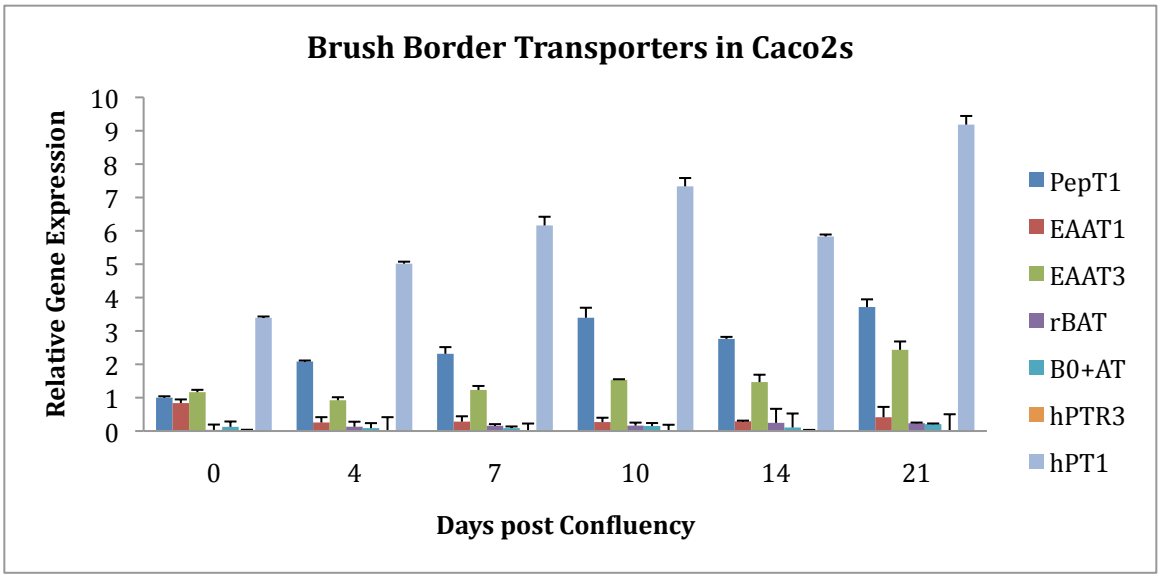

Figure 4.1. Gene expression of brushborder nutrient transporters in Caco-2 cells. Expression of PepT1, HPT1, and EAAT3 significantly increased linearly from d0 to d21 post confluency. Expression of EAAT1 significantly decreased from d0 to d4, and then remained constant, with relatively little expression from d4 to d21. The peptide transporter PTR3, rBAT, $B^{o,+}AT$, and EAAT1 (after d4) were expressed in negligible amounts in these cells.

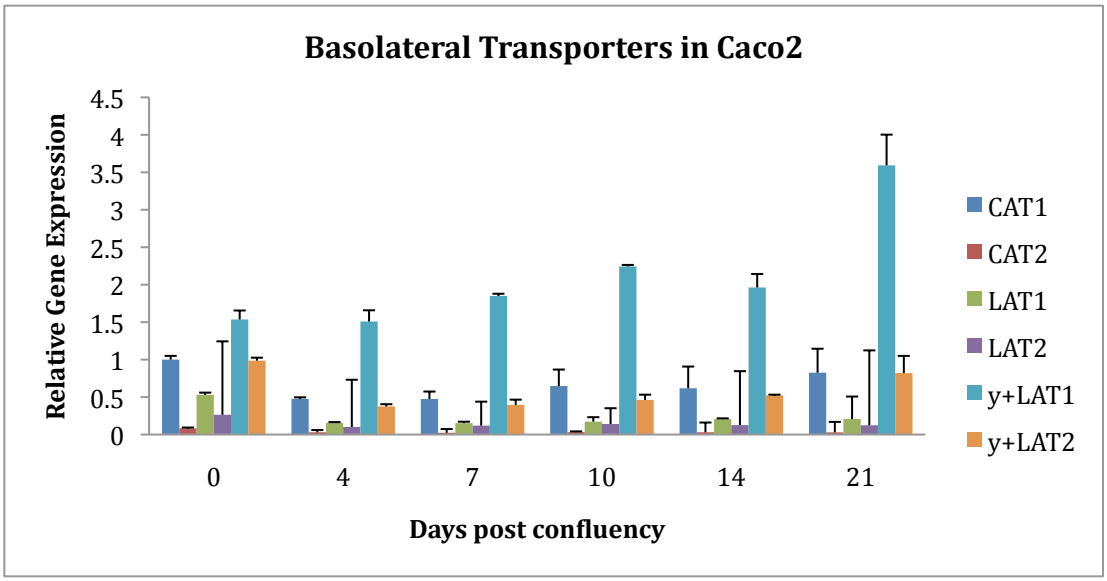

Figure 4.2. Basolateral nutrient transporter gene expression in Caco-2 cells. Expression of y^+LAT1 significantly increased linearly from d0 to d21 post confluency. Expression of CAT1, LAT1 and y^+LAT2 significantly decreased from d0 to d4 and then remained constant through d21. Expression of CAT2 and LAT2 were negligibly expressed at all time points.

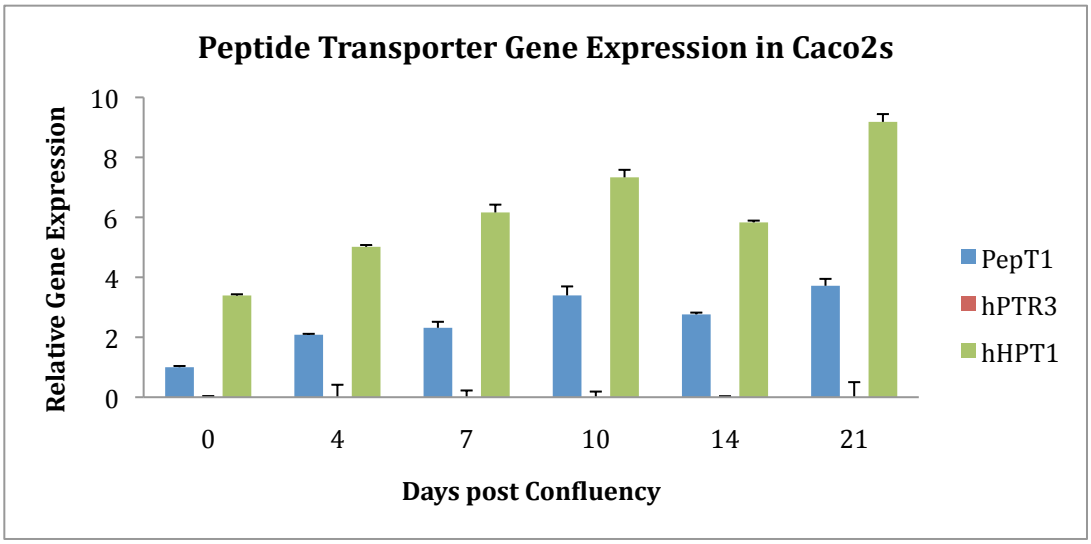

Figure 4.3. Gene expression of brushborder peptide transporters in Caco-2 cells.

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CHAPTER V.
REGULATORY BALANCE BETWEEN PEPT1 AND AMINO ACID TRANSPORTER GENE EXPRESSION IN CACO-2 CELLS

Introduction

Assimilation of nutrients across the brushborder membrane of the small intestine is conducted by several transport systems including those for carbohydrates, free amino acids, and peptides (Thomson et al., 2001). Di- and tri- peptides are transported across the brushborder membrane by the low affinity, high capacity peptide transporter, PepT1 as well as other peptide transporters (See Chapter II for a complete review of peptide transport in the enterocyte). Peptide transport also occurs on the basolateral membrane by peptide transporters that are still being characterized (See Chapter II for a review on basolateral membrane peptide transport). Additionally, several substrate-specific amino acid transporters are located on the brushborder and basolateral membranes of the enterocyte (See Chapter II for a complete review of amino acid transport in the enterocyte). These transporters work in connection to regulate the pool of amino acids within the enterocyte and as well as the animal as a whole.

Absorption of amino acids in the form of peptides is a more energy efficient pathway for the animal to assimilate amino acids, as two or three amino acids are being absorbed for the same energy cost as one free amino acid (Daniel, 2004). Despite the presence of other brushborder peptide transporters, PepT1 is generally regarded as the major peptide transporter in the small intestine as it has a large repertoire of substrates and operates as a low affinity, high capacity transporter. Reviewed in detail in Chapter

II. Therefore, the overall objective of this experiment was to examine the interaction between PepT1 and other peptide and free amino acid transporters in the enterocyte.

RNA interference (RNAi) is a method of post-translational gene silencing in which double stranded RNA is cleaved into short interfering RNA (siRNA) molecules, resulting in the specific knockdown of a target mRNA molecule. Without the gene transcript the cells are no longer capeable of producing the target protein and thus gene function can be ascertained. Many companies offer siNRAs to human and mouse genes that have been characterized according to their ability to effectively cause a decrease in target mRNA and not cause aberrant or "off-target" effects. These advances have lead to the use of RNAi as a more versatile, timely, and economical approach to elucidating gene function as compared to the generation of knockout models. In view of these reasons as well as the absence of a chicken knockout model or access to the mouse knock out model, the specific objective of this research was to examine the effect of PepT1 knockdown in Caco-2 cells on the gene expression of other nutrient transporters.

Within the enteroctye there is an intracellular pool of amino acids. It was hypothesized the cells would act to keep the content of the amino acid pool constant and that a reduction of PepT1 will lead to an alteration in the gene expression of other peptide or amino acid transporters to regulate the influx and efflux of amino acids into or out of the enterocyte. The null hypothesis is that a reduction of PepT1 will not result in changes in the gene expression of any of the transporters examined, indicating that the compensatory mechanism is not at the level of transporter gene expression.

Materials and Methods

Transfection, RNA, and Protein Collection.

Caco-2 cells were maintained in culture as before (See Chapter IV) and one day prior to transfection were seeded at a density of $1x10^5$ cells per well in 24 well tissue culture plates (for RNA collection) or at $4x10^5$ cells per well in 6-well tissue culture plates (for protein collection). Twenty-four hours post seeding, cells were transfected with either 100 μM or 200 μM of validated siRNA targeting human PepT1 (Cat No. 4392420:, Ambion) or Negative control siRNA 1 (Cat No. 4392420; Ambion) using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturers' instructions at a 1:2 nucleic acid: Lipofectamine ratio. Cells were harvested at either 48h or 72h post transfection for gene and protein analysis.

Briefly, RNA was extracted from cells using the RNAeasy kit (Qiagen) according to the manufactures Animal Cell protocol using 300 μL of Buffer RLT plus β-Mercaptoethanol and QiaShredder spin columns (Qiagen). Concentrations were determined spectrophotometricaly and RNA quality was determined using formaldehyde-agarose gel electrophoresis. Cells for protein analysis were lysed in 100 μL of lysis buffer (1 M tris pH 7.4, 20% Triton X-100, 5M NaCl, 0.5 M EDTA, 0.5% PMSF, 50 μg/mL Protease Inhibitor Cocktail (Cat No. P8340: Sigma-Aldrich, St. Louis, MO) and centrifuged at 18,000 x g at 4ºC for 30 min. The supernatant was collected and frozen at -20ºC for analysis. Protein concentration was determined using the Peirce BCA assay (Cat No. 23225:, Perce, Rockford, IL) according to the manufacturers instructions.

Real-Time qPCR

One-hundred nanograms of RNA was used in each reverse transcription reaction to prepare cDNA using the random priming method (High Capacity cNDA Archive kit, Applied Biosystems). Nutrient transporter gene expression was examined using Real-Time qPCR in an ABS 7500 Fast-Real Time qPCR machine (Applied Biosystems). Each 10 µL reaction included the following: 5 µL 2X Fast-SYBR Green PCR Master mix, 0.5 µL 8 µM Forward Primer, 0.5 µL 8 µM Reverse primer, 2 µL diluted cDNA, and 2 µL ddH_20. Relative gene expression was calculated using the ΔΔCt method as previously described in Chapter III, with GAPDH as the endogenous control. Relative gene expression was then normalized within a time point to replication 1 of cells transfected with 100 nM PepT1 siRNA. The relative and normalized gene expression was then log base 2 transformed ($2^{-\Delta\Delta Ct}$), and analyzed for statistical significance by ANOVA using JMP (SAS). Statistical significance was considered at $P < 0.05$. Primers for the human nutrient transporters were designed as previously described in Chapter III and are shown in Table 5.1.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
PepT1	CCTGGAGAGATGGTGACACTTG	GTTTATCCTTGTCAGTTTGTTTACATCAA
HPT1	GAAGGCTCAGTAAGGCAGAACTCT	GGTCTGTGGCATTGACATACAAG
PTR3	TTGGTTTTACCTCCGTCATTGTG	CGTTGTGGTGGATGTAGTGTAAGC
ABCC3	GGCTCCGTGGCCTATGTG	GTTTTCCTGAAGAGTGCAGTTCTG
ABCC4	AGCTGGTGCTCACTGGATTGT	GCAACCTGAGCTGCAGTGTTT
EAAT1	ACAGCTGCAGATGCCTTCCT	GCAGGCTTCTACCAGATTTGGA
EAAT3	CGCGCTGTCGTGTATTATTTCT	TCACCACCAGCACAATACCTAGA
rBAT	CCCATGAAAATGGCAAAACC	CCAACTGGAGTTTCCATACACACT
$B^{0,+}AT$	TCCTCAAATCGTTGTGAAATGC	CCGCACGCTCAGTGAGTTC
$B^{o}AT1$	CCACCCGGCCCTGAA	TGTTGTAATACAGTCCCACCATGAA
CAT1	TCATCACCGGCTGGAACTTAA	GCCCTCGCTACGCTTGAAG
CAT2	CGTCGCAGCTGGTTCTCT	TGGGAAAATGGATCCAAGAAGA
LAT1	CATCCTGCTGGGCTTCGT	AAGTTGGGATCTAGATTGGACACAT
LAT2	CCTGGCTGCCATCTGCTT	ACCGCACACTGGAACAGTTG
y+LAT1	CGTTGCAGGCATTGTTAGACTT	GAACCCTCAAAGGAATTCTCAAAA
y+LAT2	TGTCATGGGCCTTGTTAAACTG	CTCAAAGGCGTCCTGAAAGTG
GAPDH	GACCCCTTCATTGACCTCAACT	GAATTTGCCATGGGTGGAAT

Table 5.1. Primer sequences for human nutrient transporter genes examined.

hPepT1 Protein Detection

Protein samples were diluted to 4 μg per mL in lysis buffer prior to addition of an equivalent of volume of 2X Lamelli Sample buffer (.005% Bromophenol blue, 20% glycerol, 4% w/v SDS, 126 mM Tris-HCl, pH 6.8, 3% β-Mercaptoethanol). Samples containing 30 μg of protein were incubated for 15 min at room temperature then 5 min at 95ºC. Samples, including a molecular weight marker (2 μL Novex Sharp prestained standards, Cat. No. LC5800, Invitrogen) were then loaded onto a discontinuous (4% stacking, 10% separating) polyacrylamide Tris-HC1 gels containing 0.1% SDS along with 2μL of molecular weight maker. Gels were run at 200V in 1X running buffer (25mM Tris-HCl, 200mM Glycine, 0.1% w/v SDS) until the dye line was about ¼ inch from the bottom of the gel. Immobilon-FL PVDF membranes (Millipore, Billerica, MA) were pre-wetted in 100% methanol and placed in transfer buffer for 10 min prior to transfer. Transfer sandwiches were set up for wet transfer using the Mini-Protean Tetra tank system (Bio-Rad, Hercules, CA). Transfer was then performed in ice-cold transfer buffer at 100 V (300 mAmps) for 1 hour). Marker positions were then marked onto the membrane with pencil to ensure visualization upon imaging. Membranes were then rinsed in PBS, blocked in Odyssey blocking buffer (Li-Cor, Lincoln, NE) for 1 hour at room temperature, and incubated in primary antibodies for 3h hours at room temperature. Human PepT1 was detected using an anti-PepT1 antibody (Cat. No. sc-19917, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution and an anti-GAPDH antibody (Cat No. CB1001, Calbiochem/EMD, Rockland, MA). Antibody solutions were removed and membranes were rinsed in PBS plus 0.1% Tween-20 3 x 10 min at room temperature with rocking. Secondary antibodies (IRDye 680RD donkey anti-goat (Li-Cor) and

IRDye 800CW goat anti-mouse (Li-Cor)) were diluted 1:20,000 in PBST. Membranes were then incubated in appropriate secondary antibodies for 1h at room temperature with gentle rocking. Post incubation, antibody solution was removed and membranes were washed in PBS plus 0.1% Tween-20 3 x 10 min. Membranes were then rinsed and stored in PBS away from light. Membranes were imaged on an Odyssey (Li-Cor) infrared imager at the appropriate channels. The image was then analyzed using the imager software to calculate the relative band density, which is representative of protein quantity. The determined quantities of PepT1 were normalized to the quantity of GAPDH and percent knockdown was calculated by dividing relative quantity of PepT1 in lysate from cells transfected with 200 nM PepT1 siRNA by the relative quantity of PepT1 in lysate from cells transfected with 200 nM Control siRNA.

Results and Discussion

Within an enterocyte there is a pool of amino acids. These amino acids are used by the enterocyte for general cellular processes, energy, and co-substrates for nutrient transporters. Amino acids from the intracellular pool are also transported out of the enterocyte into the bloodstream to supply the rest of the animal with the essential protein building blocks. Flucuations in the content of the intracellular amino acid pool may stimulate the enterocyte to alter amino acid influx across the brushborder membrane or efflux across the basolateral membrane to restore the content of the intracellular amino acid pool to some basal level. Many of the peptide and amino acid transporters examined in this experiment have amino acid response elements in their promoter regions (See Chapter II for a review). Thus, altering the intracellular amino acid pool can lead to alteration in gene expression of these transporters. The exact molecular pathway by

which the amino acid singnaling occurs remains to be characterized. Through this amino acid singaling mechanism, the enterocyte can respond to amino acid demands by both the cell and the whole animal.

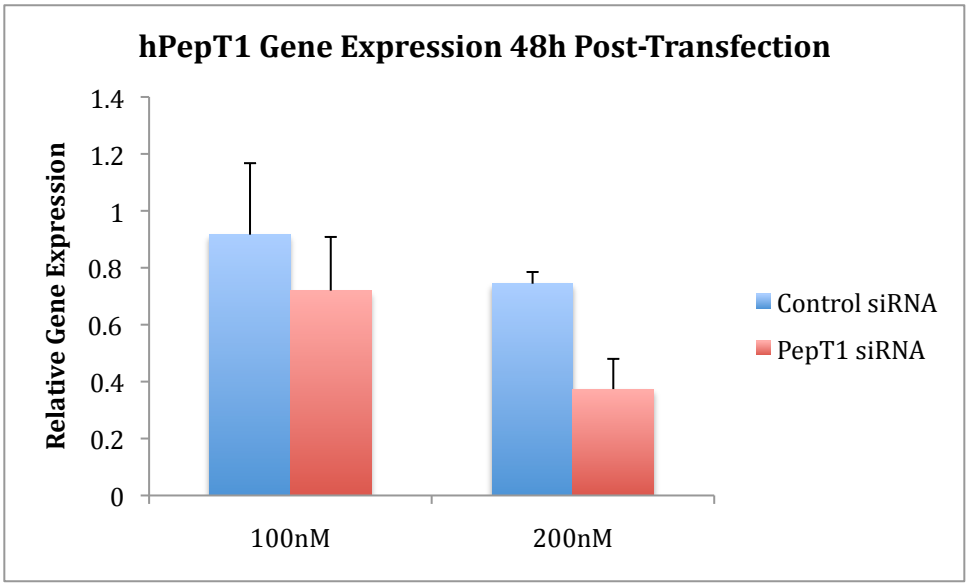
Insofar as PepT1 is the primary peptide transporter responsible for peptide influx, it was hypothesized that PepT1 gene silencing would result in an alteration of the content of the intracellular amino acid pool through a reduction of peptide influx, causing a compensatory change in the gene expression of other peptide and/or amino acid transporters. Therefore, the objective of this experiment was to ascertain if a reduction of PepT1 is compensated for through alteration of the gene expression of other peptide transporters and free amino acid transporters in the enteroctye. To this end, transient PepT1 gene silencing was induced by siRNA in the widely used Caco-2 intestinal model and peptide and amino acid transporter gene expression was subsequently evaluated.

Gene and Protein Silencing of PepT1

A reduction of 82% in PepT1 gene expression (Figure 5.1), which correlated to a reduction of 96% in PepT1 protein expression (Figure 5.2), was obtained 72h post transfection of Caco-2 cells with 200 nM of PepT1 siRNA. There were no observed cytotoxic effects of the siRNA on the cells such as changes in cell morphology, cell death or alterations in GAPDH gene expression. To account for generalized effects of siRNA the results from cells transfected with PepT1 siRNA were compared to cells transfected with a Control siRNA, which does not target any annotated human gene, rather than mock (no siRNA) trasfected cells. Greatest gene silcencing was seen at the highest concetration of siRNA transfected at 72h post-transfection. Although all the data

obtained is presented in this dissertation, the discussion of results will relate only to the results from cells treated with 200nM siRNA at 72h post-transfection.

a.



b.

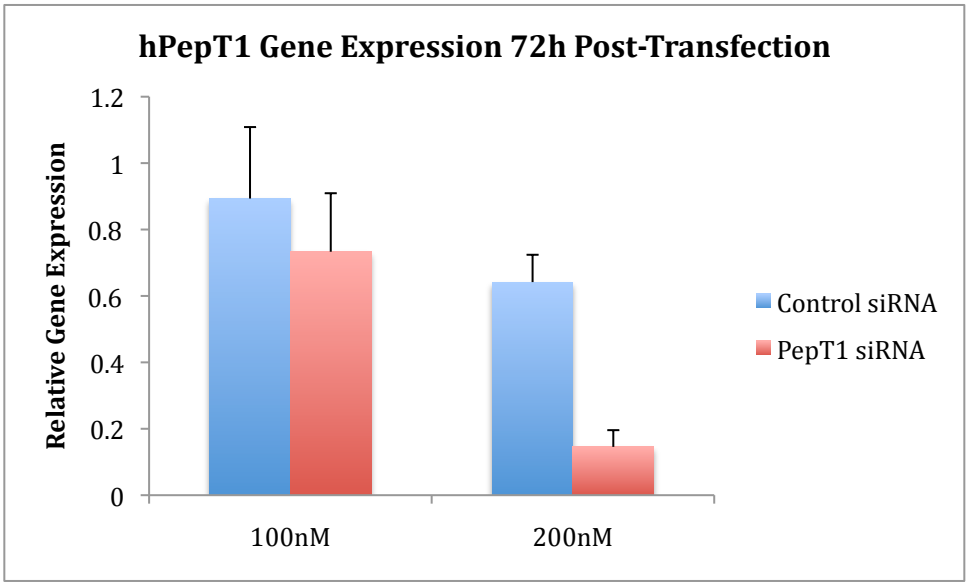


Figure 5.1. Effect of PepT1 siRNA on PepT1 gene expression in Caco-2 cell*s* 48h (a.) and 72h (b.) post transfection with varying concentrations of siRNA. In cells transfected with 200 nM of PepT1 siRNA had approximately 50% decrease in PepT1 gene expression at 48h ($P < 0.05$) and 82% at 72 h post transfection ($P < 0.05$).

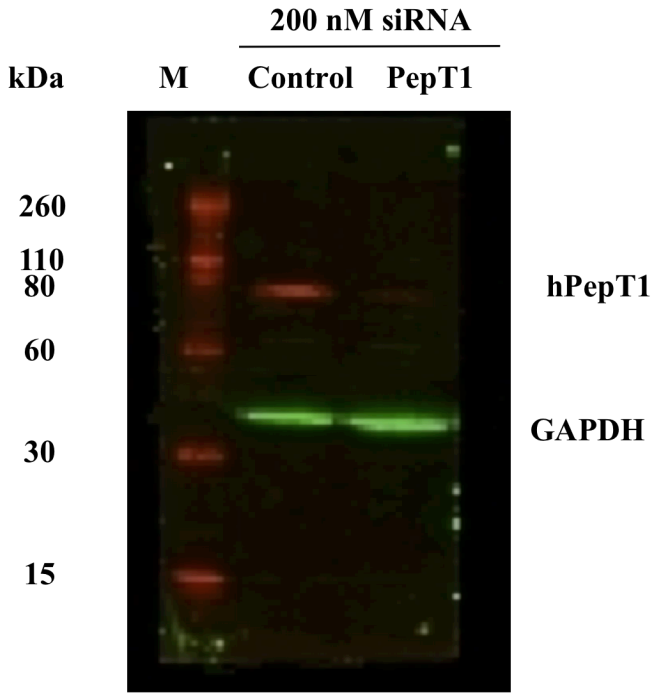


Figure 5.2. Western Analysis of hPepT1 Protein Knockdown. Caco-2 cells were transfected with 200 nM of control or hPepT1 siRNA. 72h post-transfection whole cell lysate was collected from 4 repetitions for each siRNA, pooled and analyzed for PepT1 expression using an hPepT1 polyclonal antibody targeting the C-Terminus. GAPDH was analyzed as loading control. Infrared imager software (Li-Cor) was used to calculate hPepT1 protein concentration relative to GAPDH. A 96% knockdown in relative hPepT1 protein expression was observed in lysate from cells transfected with 200 nM hPepT1 siRNA at 72h post-transfection.

No Significant Changes in Gene Expression of Other Peptide or Amino Acid Transporters were observed in Response to Transient PepT1 Gene Silencing.

Despite a 96% reduction of PepT1 protein, no significant differences were observed in the gene expression of any nutrient transporter examined at either time point (Tables 5.2 and 5.3). The level of GAPDH remained constant despite the siRNA treatment and there were no observable cytotoxic effects. First, it may be possible that the PepT1 reduction did not result in a change in function was not altered. In other words, a 4% level of PepT1 protein expression can adequately sustain uptake of peptides equal to that of 100% PepT1 protein expression. If uptake of peptides is not disrupted by a 96% reduction in PepT1 protein, then there would be no need for the cell to engage a compensatory response. This would explain that lack of any significant changes in transporter expression. Evaluation of Gly-Sar uptake in Caco-2 cells treated with PepT1 siRNA would shed light on this hypothesis.

Transient gene silencing of of PepT1 may not have impacted the physiology of the cell enough to induce any compensatory effects on gene expression of other amino acid and peptide transporters in Caco-2 cells. The effect of siRNA is transient as the cell degrades it rapidly. The silencing of a specific gene is dynamic but typically peaks around 2-3 days following transfection with siRNA and may persist up to 1 week (Personal communications with Ambion and TechNotes 15(3)). Greatest PepT1 gene silencing was achieved at 3 days following transfection. At this time PepT1 gene expression is currently being upregulated by Caco-2 cells (See Results, Chapter IV).

Therefore, evaluation of the effect of PepT1 reduction at 72h post transfection was reasonable and there is no reason to believe that examining experession at 96h or greater post transfection would lead to different results. On the contrary, it is more likely

that the percent reduction of PepT1 would have decreased due to the degredation of PepT1 siRNA combined with an increase in the expression of PepT1 in Caco-2 cells. In order to address this issue Caco-2 cells could have been transduced or stably transfected with a PepT1 shRNA construct to allow a longer-term experiment to be conducted. This would allow the effect of PepT1 reduction to be examined at day 21 post-seeding, which is a time point coterminus with drug uptake studies involving PepT1 (Brodin et al., 2002).

Another way the cell could have achieved the equivalent amount of peptide uptake with only 4% of PepT1 protein is that the cell may have reduced the turnover rate of PepT1. At first blush this hypothesis seems counterintuitive insofar as a reduction in PepT1 protein would not be ovserved in response to PepT1 siRNA if PepT1 turnover in the membrane was reduced. The PepT1 protein is not only found within the membrane of cells. It is also found in storage vesicles inside the cytoplasm of cells (Anderle et al., 2006). Therefore, the reduction of PepT1 protein in response to PepT1 siRNA may be accounted for by a depletion of PepT1 protein in these storage vesicles. In this scenario, the amount of membrane associated PepT1 protein remains unchanged, yet the total amount of PepT1 protein in the cell is reduced to 4%. To test this hypothesis, total PepT1 protein could be compared to the amount of PepT1 protein in brushborder membrane extracts prepared from Caco-2 cells exposed to PepT1 siRNA.

Caco-2 Cells May Not Reflect Normal Physiological Regulation of PepT1

Caco-2 cells may work well as an intestinal model to study PepT1 in isolation, such as tranport kinetics, structure, and substrate specificity. However, Caco-2 cells may

be an inappropriate model to study regulatory mechanisms regulating PepT1 or regulated in response to PepT1 activity. Caco-2 cells are fist cancer cells, and second colon cells. What makes a cell cancerous by nature is that they are dysregulated. The abnormally express genes and their respecitive prodcts and do not behave as normal cells, including their responses to environmental factors. Therefore, the mechaninisms resulting in the tumorgenic properties may include dysregulation of the cancer cells response to alterations in intracellular substrates such as amino acids.

Further, PepT1 is only found in human colon cells under two conditions. First, in humans with short-bowel syndrome, PepT1 expression is increased in the colon to compensate for the loss in absorptive capabilites in the small intestine (Ziegler et al., 2002). Second, PepT1 gene mutations and expression and in the colon is found in patients with inflammatory bowel disease and intestinal autoimmune diseases (Wojital et al., 2009 and Zucchelli et al., 2009). As PepT1 is abbarently expressed in Caco-2 cells in comparison to a normal human colon enterocyte, it follows that it is dysregulated in the Caco-2 model. Therefore, the results reported in this dissetation may not accurately reflect what occurs *in vivo* in the small intestine and a compensatory mechanism at the gene expression level may actually occur. Further evaluation of the mouse PepT1 knockout model should address this concern.

A Compensatory Mechanism May Exist at the Level of Protein Regulation

In this study, no change in the gene expression in any of the peptide or other free amino acid transporters was observed in response to almost complete ablation of PepT1 protein. The ultimate function of a gene product is susceptible to regulation at many points *besides* gene expression. For example, proteins may be translocated from

intracellular storage vesicles to the membrane, which may thus compensate for the reduction of PepT1. This hypothesis could be confirmed by evaluating protein concentrations of these transporters in the respective membranes in Caco-2 cells treated with PepT1 si- or shRNA or in intestinal tissues from PepT1 null mice. The amino acid and other peptide transporters may compesate for a reduction in PepT1 by transporting amino acids and peptides at a greater efficiency. This would also lead to compensation for a reduction of PepT1 without a necessitating a chage in the level of gene expression of the amino acid and other peptide transporters. This could be tested by evaluating transport kinetics of the various transporter proteins in Caco-2 cells transfected with PepT1 si- or shRNA or in intestinal tissues from PepT1 null and wild-type mice using Ussing chambers.

Peptide and Amino Acid Transporters May Constitue a Redundant System

Even in light of these arguments, it is more likely that a 96% reduction in protein would cause a decrease of peptide uptake by PepT1, which is consistent with the central dogma of biology and with the knockout mouse model (Hu et al. 2008). The existence of a PepT1 knockout mouse demonstrates that PepT1 ablation is not lethal nor do these mice show any overt physiological deviations such as altered body weight or growth rate (Hu et al., 2008). These facts suggest that PepT1 plays a very minor role in nutrient uptake, however the vast body of research regarding PepT1 suggests that the role of PepT1 in peptide uptake is far from minor (Gilbert et al., 2008). Insofar as it is accepted that PepT1 plays a significant role in nutrient uptake, then it is logical that there is another mechanism to account for the loss of PepT1 in the knockout mouse and in this *in vitro* study.

Redundant systems are common for vital processes. For example, food intake is governed by a plethora of hormones, neurotransmitters, and other chemicals (Broberger, 2005). Although the search for the silver bullet that would end the need for any exercise program to look like Madonna continues, it is unlikely that one will be found. This is because food intake is governed by a redundant system (Broberger, 2005). If one neurotransmitter fails to stimulate food intake, another neurotransmitter or hormone (or both) effectively compensates, as food intake is vital to life. This example is greatly oversimplified to for illustration purposes. Similarly, nutrient uptake from digesta in the small intestine is a vital process for the continuation of growth, development, and life as we know it. Therefore, it is logical that a redundant system is in place to ensure nutrient uptake despite a loss of a single transporter.

HPT1 is a Likely Candidate to Compensate for PepT1

In addition to the results of this experiment, which demonstrate no compensatory change in the gene expression of the most complete panel of small intestinal transporters reported anywhere to date, studies in the knockout mouse further support this mechanism (Hu et al. 2008). Like what was observed in this *in vitro* study, there is no alteration in PHT2 (PTR3) expression in PepT1 knockout mice (Hu et al., 2008). However, in Caco-2 cells there is relatively little PHT2 (PTR3) expression. This is a difference between an *in vitro* model and *in vivo.* Therefore, a comparison between the two studies in this regard is inappropriate.

Interestingly, Hu et al. (2008) did not evaluate expression of HPT1. In the study presented in Chapter IV of this Dissertation as well as other reports reviewed in Chapter II of this Dissertation, HPT1 is expressed at levels equal to, if not greater than, PepT1.

Further, HPT1 is observed to be able to transport substrates of PepT1 more efficiently than PepT1. Uptake of Gly-Sar is not completely abolished in PepT1 knockout mice and the remaining uptake presents transport kinetics consistent with transporter-mediated uptake (Hu et al., 2008 and Ma et al., 2012). Therefore, it is possible that other brush border peptide transporters, such as HPT1, serve to function in a redundant fashion to PepT1.

From the available research as well as the present experiment, HPT1 is a likely candidate to serve a redundant role to PepT1. No alteration in gene expression was observed in the present experiment. However, the lack of change in gene expression in HPT1 does not render a potential connection between these transporters invalid. Under normal physiological conditions, transporters are not transporting at V_{max}. This would waste resources and limit the adaptability of the cell for obvious reasons. Taking this into consideration, HPT1 may be able to account for the loss of PepT1 by altering its ability to transport substrate at a level closer to its V_{max}, rather than increasing the amount of HPT1 protein present. Along this line, more HPT1 may be incorporated into the membrane of cells from HPT1 protein residing in the cytoplasm. This would increase the amount of peptides through HPT1 in the membrane without a necessitating a change in HPT1 gene expression. Further, an alteration in turnover rate of HPT1 in the brushborder membrane may account for no apparent change in gene expression. Any of the suggested mechanisms would lead to an ability of HPT1 to compensate for the lack of PepT1 without causing a change in HPT1 gene expression.

A Compensatory Mechanisim May Exist on the Macroscopic Level

Although the enterocyte utilizes amino acids for energy and other cellular processes, these cells bear the responsibility for assimilating amino acids for the whole animal as well. Therefore, the enterocyte contributes directely to the overall finite source of resourses available for the animal to carry out all functions. If the overall pool of amino acids available to the aminal is altered it may affect other functions of the animal, such as immune function or growth rate. For example, if decreasing PepT1 gene expression results in a decrease in overall amino acids available to the animal, under normal conditions the animal may grow normally, but may not respond as well to an immune challenge. Examining the gene expression in an *in vitro* system would not yield insight on these potential macroscopic effects and necessarily justifies further analysis of the mouse PepT1 knockout model. In the PepT1 knockout mouse model, no examination into other macroscopic effects other than growth rate, bodyweight, reproduction fitness, and appetite were examined. Therefore, it would be prudent to examine other phenotypes such as immune response, muscle deposition, and energy expenditure.

Conclusion

While a 96% knockdown of PepT1 protein was attained, no significant changes in the gene expression of a panel of brushborder or basolateral membrane peptide and amino acid transporters was observed. These results suggest that the loss of PepT1 is not accounted for by compensatory changes in the gene expression of amino acid or other peptide transporters. Other possible levels of regulation may exsits but are beyond the scope of the data from this *in vitro* experimet. First, peptide uptake by PepT1 is part of a redundant system and that HPT1 is a likely candidate as a major player in that redundant

system. Second, compesation in response to PepT1 reduction or ablation may be at a regulatory level other than the level of gene expression, such as at the protein or whole animal level. Third, the cell may not function to regulate the pool of intracellular amino acids, such that a steady, basal level of amino acids is maintained, therefore alterations in PepT1 would not generate wide spread changes in transporter expression or function.

Average Relative Gene Expression ± SEM 48h Post-Transfection				
	Control siRNA		PepT1 siRNA	
Gene	**100nM**	**200nM**	**100nM**	**200nM**
HPT1	0.65 ± 0.18	0.58 ± 0.24	0.70 ± 0.20	0.61 ± 0.20
PTR3	1.02 ± 0.11	0.82 ± 0.18	0.88 ± 0.10	0.95 ± 0.16
ABCC3	0.68 ± 0.31	0.61 ± 0.19	0.70 ± 0.22	0.75 ± 0.15
ABCC4	0.80 ± 0.14	0.79 ± 0.20	0.80 ± 0.14	0.71 ± 0.15
EAAT1	0.42 ± 0.24	0.55 ± 0.24	0.67 ± 0.21	0.41 ± 0.21
EAAT3	0.69 ± 0.22	0.64 ± 0.14	0.81 ± 0.13	0.59 ± 0.11
B^0AT1	0.34 ± 0.06	0.25 ± 0.14	0.45 ± 0.16	0.32 ± 0.11
$b^{0,+}AT$	0.86 ± 0.14	0.56 ± 0.06	0.76 ± 0.16	1.09 ± 0.35
rBAT	0.80 ± 0.18	0.68 ± 0.18	0.82 ± 0.13	0.68 ± 0.19
CAT1	0.77 ± 0.16	0.69 ± 0.14	0.94 ± 0.07	0.73 ± 0.19
CAT2	1.80 ± 0.42	1.64 ± 0.48	1.10 ± 0.37	1.62 ± 0.52
LAT1	0.59 ± 0.22	0.54 ± 0.22	0.72 ± 0.20	0.64 ± 0.25
LAT2	0.73 ± 0.34	0.66 ± 0.10	0.75 ± 0.21	0.71 ± 0.25
y^+LAT1	0.36 ± 0.10	0.38 ± 0.27	0.68 ± 0.23	0.30 ± 0.09
y^+LAT2	0.70 ± 0.19	1.13 ± 0.22	0.85 ± 0.26	0.77 ± 0.19

Table 5.2. Relative Gene Expression of Peptide and Amino Acid Transporters in Caco-2 cells Transfected with PepT1 siRNA 48h Post-Transfection. Caco-2 cells were transfected with various concentrations of Control or PepT1 siRNA and gene expression of peptide and amino acid transporters was measured 48h post-transfection. Significance was considered at $P < 0.05$. None of the genes examined showed significantly altered gene expression levels at 48h post-transfection in cells transfected with PepT1 siRNA when compared with cells transfected with the same amount of Control siRNA.

Average Relative Gene Expression ± SEM 72h Post Transfection				
	Control siRNA		PepT1 siRNA	
Gene	**100nM**	**200nM**	**100nM**	**200nM**
HPT1	1.09 ± 0.18	0.83 ± 0.06	0.99 ± 0.22	0.74 ± 0.19
PTR3	0.81 ± 0.11	0.63 ± 0.15	0.91 ± 0.09	0.48 ± 0.18
ABCC3	1.32 ± 0.15	0.91 ± 0.10	1.19 ± 0.24	0.83 ± 0.20
ABCC4	1.12 ± 0.21	0.90 ± 0.11	1.01 ± 0.13	0.66 ± 0.24
EAAT1	1.05 ± 0.31	0.81 ± 0.06	0.97 ± 0.12	0.64 ± 0.17
EAAT3	1.25 ± 0.20	0.90 ± 0.10	1.04 ± 0.13	0.75 ± 0.19
B^0AT1	0.65 ± 0.23	0.73 ± 0.25	1.08 ± 0.23	0.54 ± 0.27
$b^{0,+}AT$	0.90 ± 0.29	0.72 ± 0.11	0.95 ± 0.15	0.59± 0.14
rBAT	1.43 ± 0.41	0.92 ± 0.15	1.12 ± 0.21	0.79 ± 0.16
CAT1	1.42 ± 0.50	0.79 ± 0.71	1.03 ± 0.13	0.61 ± 0.15
CAT2	1.03 ±0.10	0.60 ± 0.16	0.82 ±0.13	0.51 ± 0.15
LAT1	0.78 ± 0.38	0.86 ± 0.14	0.89 ± 0.17	0.62 ± 0.32
LAT2	1.06 ± 0.27	0.72 ± 0.04	0.91 ± 0.15	0.50 ± 0.11
y^+LAT1	1.13 ± 0.25	0.83 ± 0.13	0.84 ± 0.13	0.56 ± 0.15
y^+LAT2	0.82 ± 0.33	0.66 ± 0.11	1.10 ± 0.26	0.50 ± 0.11

Table 5.3. Relative Gene Expression of Peptide and Amino Acid Transporters in Caco-2 cells transfected with PepT1 siRNA 72h Post-Transfection. Caco-2 cells were transfected with various concentrations of Control or PepT1 siRNA and gene expression of peptide and amino acid transporters was measured 72h post-transfection. Significance was considered at $P < 0.05$. None of the genes examined showed significantly altered gene expression levels at 48h post-transfection in cells transfected with PepT1 siRNA when compared with cells transfected with the same amount of Control siRNA.

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CHAPTER VI.
DEVELOPMENT OF TRANSGENIC CHICKEN MODELS FOR THE IN VIVO INVESTIGATION OF THE REGULATORY BALANCE BETWEEN PEPT1 AND AMINO ACID TRANSPORTER GENE EXPRESSION

Introduction

Some experts believe that amino acid absorption is not rate-limiting for growth. Although this has not be tested, in species that are not growing at rapid or close to maximal rates, such as the common lab mouse, this may very well be true. However, chickens, specifically broilers, have undergone long-term selection for rapid growth and protein accretion. Fifty to sixty years ago chickens were sent to market in 8 weeks or more. Today, broilers are market weight around 6 weeks or less. In 1957, a 6 week old broiler weighed about 0.54 kg with a feed conversion ratio of 2.35 kg feed/ kg gained. In 2010 a 6 week old broiler wighs 2.8 kg and has a feed conversion ratio of under 1.70 (Feket, 2010). Therefore, it is possible that in chickens, which are growing at rapid rates, amino acid absorption is rate limiting for growth and development. This hypotehesis cannot be tested in an *in vitro* model as described here or in the PepT1 knockout mouse model (Hu et al., 2008) and necessarily warrents generation of transgenic chicken models. Therefore, the overall objective was to generate transgenic chickens that over or underexpress PepT1 to allow for the characterization of potential compensatory mechanims for PepT1 on the molecular to the whole-aimal level, as well as to ascertain if absorption of peptides by PepT1 is rate limiting for growth and development of chickens.

Targeted gene disruption is the gold standard for characterizing the impact of individual genes *in vivo*. However, current transgeneic chicken technology does not allow for targeted gene insertion and disruption in chickens. The primary roadblock is the lack of a promoter that is active in primordial germ cells or chicken embryonic stem

cells. Without an active promoter, the cells that have the specific integration due to homologous recombination cannot feasibly be selected for. Therefore, this project used lentiviral vectors to randomly introduce transgenes into the genome of primordial germ cells to lead to the production of founder (G0) transgenic chickens. The G0 chickens could then be used to generate populations of transgenic chickens that ubiquitously overexpress cPepT1 or shRNA genes that knockdown endogenous cPepT1. Figure 6.1 depicts the overall steps in the production of a transgenic chicken model system. This figure in particular references the steps used to create a LacZ expressing chicken created by a team of researchers at North Carolina State University (Mozdziak and Petitte, 2004). Drs. James Petitte and Paul Modziak of North Carolina State University (NCSU) were consulted for this portion of this dissertation. Drs. Petitte and Modziak are leaders in the world with respect to this technology. Under their tutelage, blastodermal injection and *ex ovo* culture system techniques were learned and brought to Virginia Tech.

Two approaches were designed to test the hypothesis that PepT1 interacts with the free amino acid transporters to regulate amino acid flux in the enterocyte. The first approach was to generate a line of transgeneic chickens, which ubiquitously overexpress cPepT1. The ideal promoter to express exogenous cPepT1 would be an intestinal specific promoter. Expression of PepT1 is mainly in the small intestine with some expression also in the kidney. Therefore, the use of the chicken PepT1 promoter to drive transgene expression would be ideal. Frazier et al., (2008) examined the strength of 1000 bp upstream of the cPepT1 transcription start site using deletion constructs, which drove expression of a reporter luciferase gene. Using this technique, they mapped several up and down regulatory regions with in this promoter region. Based on their results, two

candidate constructs were chosen to test to see if they would be strong enough to drive a less sensitive reporter gene and subsequently cPepT1 transgene expression.

Constructs using the -1024 bp cPepT1 promoter fragment and the -386 bp cPepT1 promoter fragment (Frazier et al., 2008) were used to drive green fluorescent protein (GFP) production and beta galactosidase (B-gal) production. These constructs were transfected into chicken embryo fibroblast (CEF) cells, chicken hepatic (LMH) cells and "primary chicken intestinal cells" and reporter gene expression was measured by fluorescent microscopy, or a B-gal activity assay and stain. Using the best fluorescent microscope available at the time, no GFP activity was observed in any cell line. Further, these constructs did not produce any measurable B-gal activity as determined by a B-gal activity assay and stain. The reasons for these negative results are many fold. In regards to CEF and LMH cells, these cells do not have any cPepT1 gene expression and therefore may not contain the appropriate regulatory elements to activate the cPepT1 promoter to levels strong enough to transcribe reporter genes with less sensitive detection assays compared to luciferase. It also may be that these cells contain repressor elements that may keep PepT1 from being expressed in these cells and these repressor elements are acting on the cPepT1 reporter constructs leading to no transcription of the reporter genes. Gene expression analysis of cPepT1 in "primary chicken intestinal cells" indicates that cPepT1 is expressed in these cells. Therefore the constructs were transfected into these cells that were isolated from an embryonic day 20 embryo. No expression of these reporter constructs was observed. However, significant toxicity due to the cationic liposome transfection agents used was observed. Toxicity of cationic liposome reagents is common in primary cell lines. Therefore, lack of expression due to cell line type or cell

death could not be distinguished from one another. At the time this experiment was completed there was no other alternative transfection method available, such as electroporation.

In other instances, enhancer hybrid promoters were used to drive tissue specific overexpression (Liu et. al., 2004). Therefore, an SV40 enhancer element was cloned in front of the 386 bp promoter fragment that drove B-gal expression. This construct was transfected into CEF cells and B-gal activity was measured using a B-gal activity assay. Although a slight increase in B-gal activity over the construct lacking the enhancer element was observed, the data suggested it was still not strong enough to be used for *in vivo* overexpression. In order to distinguish if the lack of expression was due to testing in an inappropriate cell line, the constructs were transfected into intestinal tissue explants using the same cationic lipisome reagent. It did appear that some transfection occurred by observing blue edges of the tissue after an X-gal stain in the segments transfected with the positive control. The same expression was not seen in any of the test constructs. Further it appeared that only the edges of the tissue were transfected and more transfection occurred in the muscle layers rather than the epithelial layers. This is not unexpected as different cell types have different transfection efficiencies. Further, it would not be efficient, based on the number of cells in a tissue explant, to go transduce explants with pseudolentiviral particles (Conversation with Dr. Petitte, January 2009). At the suggestion of my committee and Drs. Petitte and Modziak, the intestinally specific approach was abandoned in favor of ubiquitous overexpression, despite its disadvantages.

As discussed previously, targeted gene disruption in chickens is not feasible. Therefore, the second approach utilized interfering RNA. The objective was to generate

a cPepT1 shRNA expressing chicken line to knock down cPepT1 gene expression. As PepT1 is fairly tissue specific and shRNA is very target specific, this approach should minimize the ubiquitous nature of transgene expression.

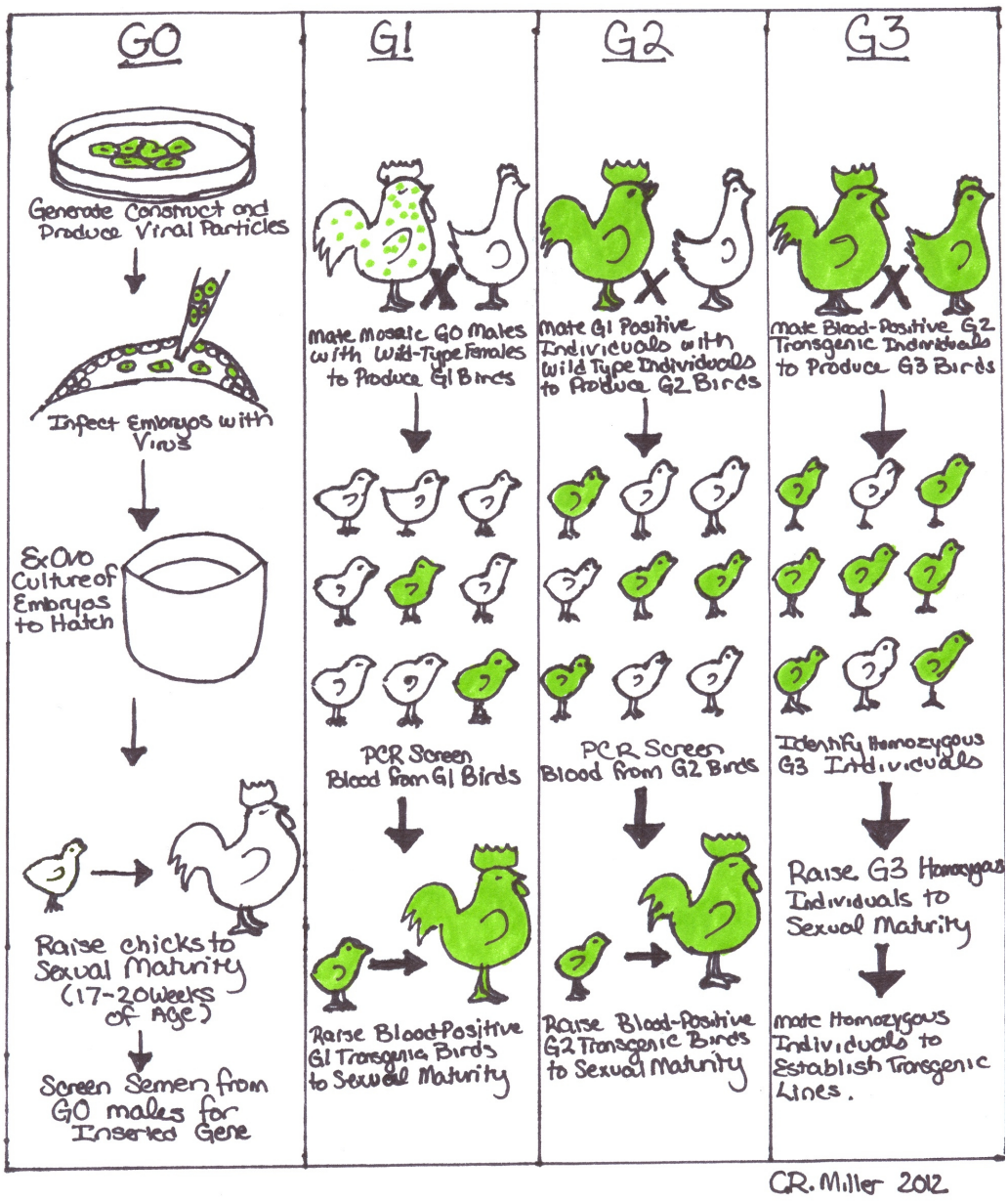


Figure 6.1. General steps in the production and establishment of a line of transgenic chickens. Generation 0 (G0) involves the construction and production of virus and the infection of embryos followed by *ex ovo* culture, hatching, rearing, and screening of putative mosaics for breeding. Generations 1–3 (G1–3) require the majority of time, cost, and facilities to establish and characterize a usable line of birds.

Materials and Methods

Exogenous cPepT1 Overexpression Lentiviral and Related Vectors Construction

FIV CAG-cPepT1 FLAG . The PepT1 cDNA was PCR amplified out of B57, a plasmid containing the whole cPepT1 cDNA, using primers that would replace the C-Terminus with a FLAG (DYKDDDDK) tag and new stop codon and 5' and 3' EcoR1 restriction endonuclease sites (Fwd: 5' AGAATTCGGCCCCATGGCTGCAAAAAGTAAGAG 3'; Rev: 5' TGAATTCCTACTTGTCGTCGTCGTCCTTGTAGTCTTTCTTCTTTTCTTCTTCATC A 3'). The cPepT1-FLAG PCR product was subcloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequence was verified using bidirectional sequencing (VBI, Blacksburg, VA). The cPepT1-FLAG cDNA was cut out of pGEM-T Easy and cloned into the EcoR1 site in the FIV based lentivector, pCDF1-MCS2-copGFP (Systems Biosciences (SBI), Mountain View, CA; Cat. # CD111B-1). This vector was called FIV-CMV-cPepT1-FLAG. Orientation and sequence was verified by sequencing. The chicken beta-actin/CMV intermediate-early enhancer (CAG) hybrid promoter from pZGS (gift from M. Capecchi) was cloned into the Spe1 and blunted BamH1 sites in FIV CMV-cPepT1-FLAG, replacing the CMV promoter with CAG, creating the vector FIV CAG-cPepT1-FLAG (Figure 6.2). Orientation and sequence was verified by sequencing (VBI).

FIV CAG-cPepT1 Endogenous

The cPepT1 cDNA, including the 3' UTR, was PCR amplified out of B57, a plasmid containing the whole cPepT1 cDNA and subcloned into pGEM-T Easy vector (Promega). The fragment sequence was verified using bidirectional sequencing (VBI). The cPepT1

was cut out of pGEMT-Easy using restriction endonuclease EcoR1 and cloned in the EcoR1 site in the FIV vector backbone (SBI) similar to how FIV CAG-cPepT1-FLAG was constructed. This vector was called FIV CAG-cPepT1 Endogenous.

FIV CAG-Empty

To generate FIV CAG Empty, FIV CAG-cPepT1-FLAG was cut with EcoR1 (Figure 6.2). The resulting backbone was gel purified from the cPepT1 FLAG insert and re-ligated back together. The resulting vector is called FIV CAG-Empty.

Rational cPepT1 siRNA and shRNA Design

The cPepT1 siRNAs were designed with the help of Ambion using their Silencer algorithm. The ultimate goal was to produce shRNAs, therefore other design criteria based on observations made by Li et al. (2007) were also taken into consideration. Li et al. (2007) identified which nucleotides in the siRNAs were preferentially certain bases in functional shRNAs versus non-functional shRNAs. Criteria considered in addition to the Ambion *Silencer* algorithm (Ambion, Grand Island, NY) for optimal shRNA design from Li et al. (2007) are shown in Table 6.1. The result was the generation of six cPepT1 targeting siRNAs, four of which that met the criteria of the Silencer algorithm and the all the criteria from Li et al. (2007) (Table 6.2). All sequences were blasted against the chicken genome and concluded to have limited potential for off-target effects based on the lack of significant homology (< 15 contiguous bases) with any annotated genes.

cPepT1 shRNA Lentiviral and Related Vectors Construction

FIV Control shRNA 1/2. The control shRNA 1/2 gene was *de novo* synthesized (GenScript, Pitscataway, NJ) and cloned into pUC-57. FIV Control shRNA 1/2 (Figure 6.4) was made by cloning the Control shRNA 1/2 gene from Control shRNA 1/2 pUC57 between the Spe1 and Not 1 sites in the FIV lentivector backbone (SBI). This results in the addition of the Control shRNA 1/2 synthetic gene and the deletion of the CMV promoter and the MCS. The chicken U6 promoter (GenBank Accession # DQ531570) was from Wise et al., 2007 and the chicken 7SK promoter (GenBank Accession # EF488957) was from Bannnister et al., 2007. The Bgl II site is unique to the shRNA gene. The control shRNA 1/2 gene is shown in detail in Figure 6.3.

FIV cPepT1 shRNA 5/6. The cPepT1 shRNA 5/6 gene was *de novo* synthesized (GenScript) and cloned into pUC-57. FIV cPepT1 shRNA 5/6 (Figure 6.5) was made by cloning the cPepT1 shRNA 5/6 gene from cPepT1 shRNA 5/6 pUC57 between the Spe1 and Not 1 sites in the FIV lentivector backbone (SBI). This results in the addition of the cPepT1 shRNA synthetic gene and the deletion of the CMV promoter and the MCS. The chicken U6 promoter (GenBank Accession # DQ531570) was from Wise et al., 2007 and the chicken 7SK promoter (GenBank Accession # EF488957) was from Bannnister et al., 2007. The Bgl II site is unique to the shRNA gene. The cPepT1 shRNA 5/6 gene is shown in detail in Figure 6.3.

cPepT1 HUSH. The complete cPepT1 cDNA was PCR amplified out of B57 with primers adding 5' SacII and 3' Xma I restricition endonuclease sites (Fwd: 5'TCCGCGGGGCCCCATGGCTGCAAAAAGTA 3'; Rev: 5'TCCCGGGGTTACAACACAAGTTTTATTCT 3'). The PCR product was subcloned

into pGEM T-Easy (Promega) and verified by sequencing (VBI). The cPepT1 cDNA was then cloned into the Sac II and Xma I restriction endonuclease sites in the RNAi validation vector HUSH Luciferase (Figure 6.6) (Origene, Rockville MD). This vector is called cPepT1-HUSH (Figure 6.7). Vector was verified by sequencing (VBI).

Validation of the Exogenous cPepT1 Overexpression Lentiviral Vector

Western Analysis

The lab of Dr. Wong (Virginia Tech) has developed a polyclonal antibody directed against cPepT1 amino acids 696-710, which are located near the end of the cytoplasmic C-terminus. The secondary structure and amino acid sequence of the C-terminus of cPepT1 are shown in Figure 6.6. This C-terminal region is highly variable among different species and thus is useful for creating species-specific anti PepT1 antisera. In order to distinguish between endogenous and exogenous cPepT1, a FLAG-tag (N-DYKDDDDK-C) was introduced into the exogenous cPepT1 cDNA replacing the amino acids that served as the epitope of the custom antiserum against endogenous cPepT1. The FLAG tag was inserted because of the availability of quality commercial antibodies directed to the FLAG sequence. The C-terminus of exogenous cPepT1-FLAG is shown in Figure 6.6.

One day prior to transfection, chicken embryo fibroblasts were seeded at 4 x 10^5 cells per well on 6-well tissue culture dishes. Cells were then transfected with 2 μg of FIV CAG-cPepT1-FLAG, FIV-Empty, or FIV CAG-cPepT1 Endogenous using Lipofectamine 2000 (Invitrogen). Three independent transfections per construct were performed. Seventy-two hours post-transfection, cells were lysed in lysis buffer (1 M tris pH 7.4, 20% Triton X-100, 5M NaCl, 0.5 M EDTA, 0.5% PMSF, 50 μg/mL Protease

Inhibitor Cocktail (Cat. No. P8340, Sigma-Aldrich) and centrifuged at 18,00 x g at 4ºC for 30 min. The supernatant was collected and pooled within a construct and frozen at -20ºC. Protein concentration was determined using the Peirce BCA assay (Cat. No. 23225, Pierce) according to the manufacturers instructions. Samples were diluted to 2 µg/µL and an equal volume of protein sample buffer (1% SDS, 50mM Tris-HCl, pH 6.8, 20% glycerol, 0.2 M DTT, 0.01% Bromophenol blue), vortexed, incubated for 15 min at room temperature. Samples were then loaded onto a discontinuous (4% stacking, 10% separating) polyacrylamide gel containing 0.1% SDS. A pre-stained molecular weight standard was loaded along with the samples (Cat. LC5800, Novex Sharp Prestained Standards, Invitrogen). Gels were run at 200V for 1.5 h and then equilibrated for 15 min in ice-cold transfer buffer (0.025 M Tris, 0.192 M glycine, 20% methanol). Immobilon-FL PVDF membranes (Millipore) were pre-wetted in 100% methanol and placed in transfer buffer for 10 min prior to transfer. Transfer sandwiches were set up for wet transfer using the Mini-Protean Tetra Tank system (Bio-Rad). Transfer was then performed in ice-cold transfer buffer at 100V (300 mAmps) for 1h. Following transfer, membranes were rinsed in PBS and blocked in Odyssey Blocking buffer (Li-Cor) for 1h at room temperature. Membranes were then incubated in primary anti-FLAG mouse monoclonal (Cat. No. F1804, Sigma-Aldrich) or anti cPepT1 rabbit polyclonal and anti-GAPDH mouse monoclonal antibody (Cat. No. Cat No. CB1001, Calbiochem/EMD) diluted in PBST (PBS, 0.1% Tween-20) for 2h at room temperature with gently rocking. The cPepT1 antibody was diluted 1:500, the FLAG antibody was diluted 1:500, and the GAPDH antibody was diluted 1:1000. Antibody solutions were removed and membranes were washed 3 x 10 min in PBST at room temperature with rocking. The IRDye 800CW

goat anti-mouse or –rabbit secondary antibodies (Li-Cor) were diluted in PBST and 1:20,000. IRdye 680RD goat anti-mouse was added to each IRDye 800CW secondary antibody dilution at a final dilution of 1:20,000. Membranes were incubated in either the secondary antibody mixture containing the anti-mouse IRDye 680 RD (detects GAPDH and FLAG) or the secondary antibody mixture containing the anti-rabbit IRDye 800 and the anti-mouse IRDye 680 (detects GAPDH and cPepT1 respectively) for 1h at room temperature with rocking. Secondary antibody solution was removed and the membranes were washed 3 x 10 min in PBST and then rinsed and stored in PBS. Membranes were imaged using an Odyssey imaging system (Li-Cor) at the 700 and 800 channels.

Immunocytochemistry Analysis

Chick embryo fibroblasts were seeded on sterile glass coverslips in complete media (DMEM, glucose, glutamine, penicillin-streptomycin, amphotericin B, 10% FCS) in 6-well tissue culture dishes. Twenty-four house post seeding cells were transfected with 2 µg with one of the following plasmids: FIV-CAG-cPepT1-FLAG, FIV-CAG-cPepT1-Endo, FIV-CAG-Empty. Lipofectaime 2000 (Invitrogen) was used at a ratio of 1:2 Lipofectamine: µg DNA according to the manufacturers directions. Briefly, per well 2 µg plasmid was added to 100 µl incomplete DMEM (no serum or antibiotics). In a separate tube, 4 µl of Lipofectamine 2000 (Invitrogen) was added to another 100 µl of incomplete DMEM. Both mixtures were incubated at room temperature for 5 min. After incubation the contents were combined and incubated at room temperature for 20 min. Afterwards, the media on the cells was replaced with transfection media (DMEM, 10% FCS, no antibiotics) and 200 µl of transfection mix was added to each well.

Seventy-two hours post transfection cells were washed in ice cold PBS and then incubated in ice-cold acetone for 10 min at -20°C to fix and permeabilize the cells. Cells were rinsed twice with ice cold PBS to remove the acetone. Acetone, although a relatively harsh fixative, was used to fix and permeabilize. Permeabilization was necessary as the antigen is intracellular. The combination of a fixative such as paraformaldehyde does not permeabilize the cells. Typically, fixation with paraformaldehyde is followed by permeabilzation with acetone or a detergent. However, the use of a detergent will result in disruption of membranes. Since PepT1 is a membrane protein the use of a detergent to permeabilize was not chosen. Therefore, acetone, as it fixes and permeabilizes was chosen. Coverslips were then transferred to new culture plates for antibody staining. Cells were incubated in PBS with 0.05% Tween-20 (Sigma-Aldrich) and 1% BSA for 30 min at room temperature with shaking. Cells were then incubated for 2 hr at room temperature with shaking in 500 µl of mouse-anti-FLAG monoclonal antibody conjugated with Cy3 (Sigma-Aldrich) diluted 1:200 (20 µg/mL) PBS with 0.05% Tween 20 (Sigma-Aldrich) and 1% BSA. Cells were then washed 3 times for 5 min each in PBS. Cells were then counter stained with 143 nM 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS for 5 min. Cells were rinsed twice in PBS. Coverslips were then mounted cell side down using Prolong Gold antifade reagent (Invitrogen, Cat. P36934). Coverslips were then sealed with nail polish and stored in the dark at 4° C.

Slides were imaged on a laser scanning inverted microscope through a 488 nM filter or UV filter was applied to image DAPI or Cy3. The NIS-Elements (Nikon, Melveille, NY) software was used to capture and analyze the images. To minimize false

positives, the slide containing the proper atigen-primary antibody pair was imaged first within that primary antibody group. The imaging software saves the imaging paramaters such as frame length and those same settings were used for the rest of the slides in which that primary antibody was used.

Glycylsarcosine Uptake Assay

The day before transfection, 2 X 10^5 CEF were plated in 12-well tissue culture plates. Twenty-four hours later, cells were transfected with 0.8 µg FIV CAG cPepT-FLAG, FIV CAG-cPepT1-Endogenous, or FIV CAG-Empty using Trans-Fast (Promega) at a 2 to 1 Transfast to DNA ratio. Glycylsarcosine (Gly-Sar) uptake was measured at 72h post transfection. Briefly, the media was aspirated and cells were washed 3 times in room temperature uptake buffer (25mM MES, pH 6.0 with Tris base; 5 mM glucose, 0.8 mM $MgSO_4$; 1.8mM $CaCl_2$, 5.4 mM KCl, 140mM NaCl). Tridiated Gly-Sar ([^{3}H]Gly-Sar) (Moravek Biochemicals, Brea CA; Cat # MT-1545) was diluted in uptake buffer to a specific activity of 110 mCi/ mmol using unlabeled Gly-Sar (Sigma-Aldrich) and a final radioactive concentration of 0.94 µCi/µL. Cells were incubated for 20 min in 1 µCi of [^{3}H] Gly-Sar in 250 µL of room temperature uptake buffer. To stop transport, cells were washed 3 times in ice-cold uptake buffer. Cells were lysed in 500 µL of 0.1% SDS. 400 µL of lysate was added to 4mL of scintillation fluid and radioactivity was measured using a scintillation counter (LS6000, Beckman-Coultier, Brea, CA). One hundred microliters of lysate was saved for protein quantification. Protein concentration was measured using a micro BCA assay (Peirce). Counts per minute (CPM) was normalized to protein concentration. Triplicate transfections performed in duplicate were performed for all constructs and the experiments were repeated twice (n = 6). Data was statistically

analyzed by ANOVA using JMP (SAS). Statistical significance was considered at $P < 0.05$.

Validation of cPepT1 siRNA and shRNA Lentiviral Vectors

cPepT1 siRNA and shRNA Target Validation Assay

Figure 6.7 shows the principle behind the validation assay. Chick Embryo Fibroblasts (CEF) were plated out on 96 well tissue culture plates (Corning, Cat. #258861) at a density of 1 X 10^4 in 200 µL of complete media (DMEM, 5% Fetal Bovine Serum, 5% Chicken serum, 100 U/mL Penicillin, 100 µg Streptomycin, Fungizone, 1 X Non-Essential Amino Acids). Twenty-four hours later cells were transfected with 25 or 50 ng of cPepT1 HUSH or HUSH Luciferase (Origene, Rockville MD) and varying concentrations (0-100 nM) of cPepT1 or control siRNAs (Ambion). As a control for general knockdown of protein expression the siRNAs were also co-transfected with 100 ng of pEM-GFP, a plasmid containing a GFP protein. All wells also contained 25 ng pRL-SV40 (Promega), a plasmid that expresses a Renilla luciferase, as a transfection control. Briefly, DNA and siRNAs were mixed in serum free DMEM and Trans-Fast (Promega) at a ratio of 1 µg DNA to 2 µL of Transfast for a final volume of 50 µL. Final siRNA concentration was based upon this 50 µL transfection volume. The transfection mix was incubated at room temperature for 15 min. Media was aspirated off cells and transfection mix was added to the appropriate well. The cells were incubated in transfection mix for 1 h at 37°C, 5% CO_2. After 1 h, 200 µL of complete CEF media was added to each well to stop the transfection reaction. All transfections were completed in duplicate and the experiment was repeated twice. Twenty-four hours post-transfection cells were viewed under a microscope for integrity and GFP expression was observed in

cells transfected with pEM-GFP using a laser scanning confocal microscope through a 488nm filter. After cell integrity and GFP expression was observed all cells were rinsed in phosphate buffered saline (PBS) and lysed in 30 µL 1X Passive Lysis Buffer (Promega). After lysis was confirmed by viewing cells under a microscope a dual luciferase assay was performed using the Dual Luciferase Reporter Assay system (Promega) in a plate reader with dual injectors (Fluostar Optima). Briefly, 100 µL of 0.25X Luciferase assay reagent was injected into a well and Firefly luciferase activity was measured over 10 s. Following the read, 100 µL of 0.25X Stop and Glo reagent was injected into the same well and Renilla luciferase activity was measured over 10 s. Normalized relative light units (RLU) were calculated by dividing firefly luciferase RLU by the renilla RLU for each sample.

To evaluate a possible additive or synergistic effect of cPepT1 siRNAs on cPepT1 knockdown, siRNAs were transfected in combinations and knockdown was evaluated using the same validation assay. For this, CEFs were co-transfected with 50 nM each siRNA in all possible combinations of two siRNAs or 100 nM of Negative control siRNA and HUSH Luciferase or cPepT1 HUSH and a renilla Luciferase vector (pRLSV40) for normalization of transfection efficiency. To compare to a comparable amount of siRNA, reactions containing 50nM of the single cPepT1 siRNA and 50nM of filler siRNA (Negative control siRNA) were also examined. The cells were analyzed using the RNAi validation assay previously described.

The FIV cPepT1 shRNA 5/6 vector was tested similarly using the luciferase assay previously described. Briefly, CEF were plated at 2 X 10^5 cells per well in 12 well tissue culture plates. Twenty-four hours later cPepT1 HUSH or HUSH Luciferase was co-

transfected with FIV cPepT1 shRNA 5/6 or FIV Control shRNA 1/2 at 1:10 HUSH vector : shRNA vector, along with pRLSV40. Triplicate transfections were completed in duplicate and the entire experiment was repeated twice. Forty-eight h post transfection, cells were imaged for GFP expression and lysed using 1X Passive lysis buffer (Promega). Twenty-five microliters of lysate was used in a dual luciferase assay described above. Normalized RLU were calculated as before. Statistical significance was considered at $P < 0.05$.

Glycylsarcosine Uptake Assay

The day before transfection, 2 X 10^5 CEF were plated in 12-well tissue culture plates. Twenty-four hours later, cells were transfected with 8 µg FIV cPepT1 shRNA 5/6 or FIV Control shRNA 1/2 and 0.8 µg of FIV CAG-cPepT1-Endogenous using Trans-Fast (Promega) at a 2 to 1 Transfast to DNA ratio. Glycylsarcosine (Gly-Sar) uptake was measured at 48h post transfection. Briefly, the media was aspirated and cells were washed 3 times in room temperature uptake buffer (25mM MES, pH 6.0 with Tris base; 5 mM glucose, 0.8 mM $MgSO_4$; 1.8mM $CaCl_2$, 5.4 mM KCl, 140mM NaCl). Tridiated Gly-Sar ([^{3}H]Gly-Sar) (Moravek Biochemicals,Brea CA; Cat # MT-1545) was diluted in uptake buffer to a specific activity of 110 mCi/ mmol using unlabeled Gly-Sar (Sigma-Aldrich) and a final radioactive concentration of 0.94 µCi/µL. Cells were incubated for 20 min in 1 µCi of [^{3}H] Gly-Sar in 250 µL of room temperature uptake buffer. To stop transport, cells were washed 3 times in ice-cold uptake buffer. Cells were lysed in 500 µL of 0.1% SDS. 400 µL of lysate was added to 4mL of scintillation fluid and radioactivity was measured using a scintillation counter (LS6000, Beckman-Coultier). 100µL of lysate was saved for protein quantification. Protein concentration was

measured using a micro BCA assay (Peirce). Counts per minute (CPM) was normalized to protein concentration. Triplicate transfections performed in duplicate were performed for all constructs and the experiments were repeated twice (n = 6). Data was statistically analyzed by ANOVA using JMP (SAS).

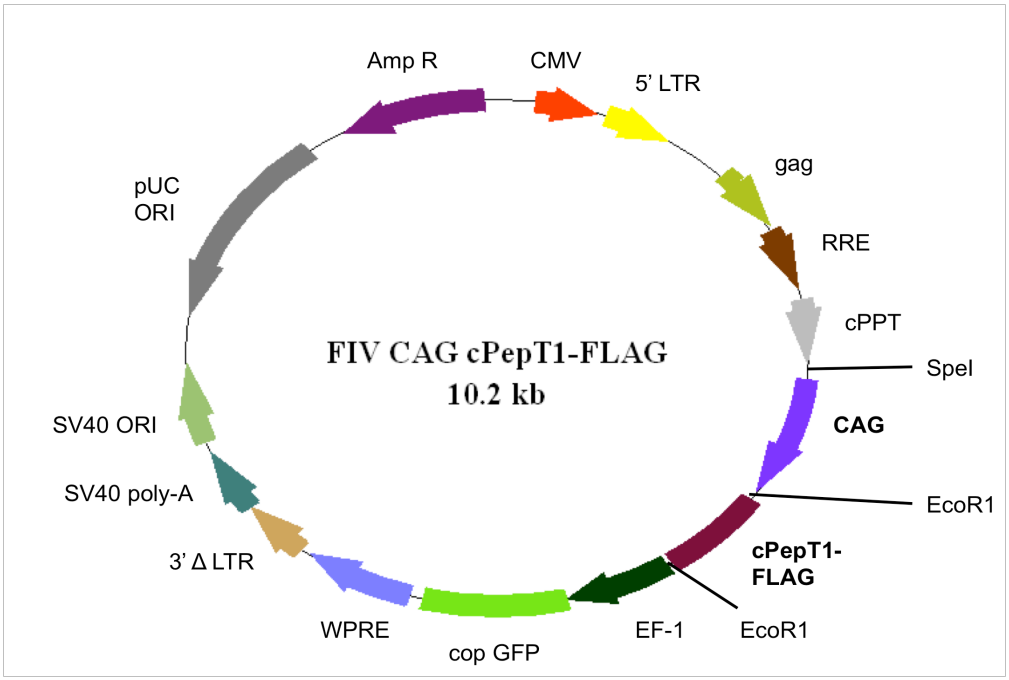


Figure 6.2. Diagram of the exogenous cPepT1 lentiviral overexpression vector.

Design Criteria (all are in reference to sense strand)	**Absolutely Required?**
No strings of 4 or more consecutive nucleotides	Yes
Last two nucleotides must NOT be a TT or UU	Yes
Start with a purine (A/G)	Yes
Nucleotide 11 is a C or G	Yes
Nucleotide 9 is an A or U	Yes
A/U rich in nucleotides 3-7	No
G/C rich in nucleotides 14-16	No
A/U rich in nucleotides 17-19	No
Nucleotide 18 is an A or U	Yes

Table 6.1. cPepT1 siRNA additional design criteria. Li et al. (2007) identified which nucleotides in the siRNAs were preferentially certain bases in functional shRNAs versus non-functional shRNAs. The criteria listed below are the criteria given to siRNA designers at Ambion to consider in addition to their own *Silencer* siRNA design algorithm. Although there was a preference for these characteristics, not all criteria were considered absolutely necessary for functional shRNA design as functional shRNAs were identified that did not meet those particular criteria (Li et al., 2007).

siRNA #	Sense 5'→ 3'	Target Position in cPepT1	Target location
1	GGUGCUUUUCCUUUACAUCtt	Not used	Not used
2	CCAGAAGAAGGAAAGAAUCtt	1537	ORF
3	CCACCAAGAAUAAAACUUGtt	Not used	Not used
4	AGACAGUCAACAUCACUAUuu	1581	ORF
5	GAAGUAGAUUCUUCUCUAUuu	561	ORF
6	AAUAGUAAUUGAAGCCAAAuu	2578	3`UTR

Table 6.2. cPepT1 siRNAs. This shows the sequences of the cPepT1 siRNAs designed. Although six were made, only four (2, 4, 5, and 6) met all of the functional siRNA/shRNA design criteria from the Ambion Silencer algorithm and observations made by Li et al. (2007), Table 6.1.

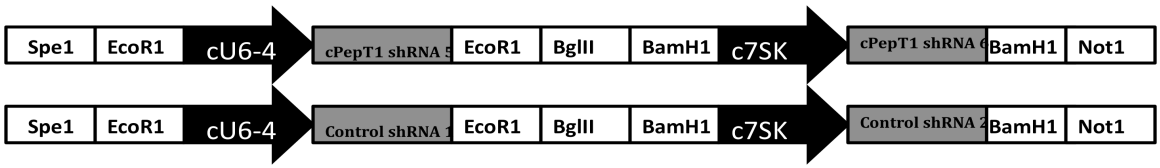


Figure 6.3. Design of cPepT1 shRNA 5/6 and Control shRNA 1/2 synthetic genes. The cPepT1 shRNA 5 and Control shRNA 1 are expressed from the chicken U6-4 promoter (GenBank Accession # DQ531570). The cPepT1 shRNA 6 and Control shRNA 2 are expressed from the chicken 7SK promoter (GenBank Accession # EF488957). Restriction endonuclease sites were included to facilitate cloning. The Bgl II site is unique to the shRNA gene to facilitate Southern blotting.

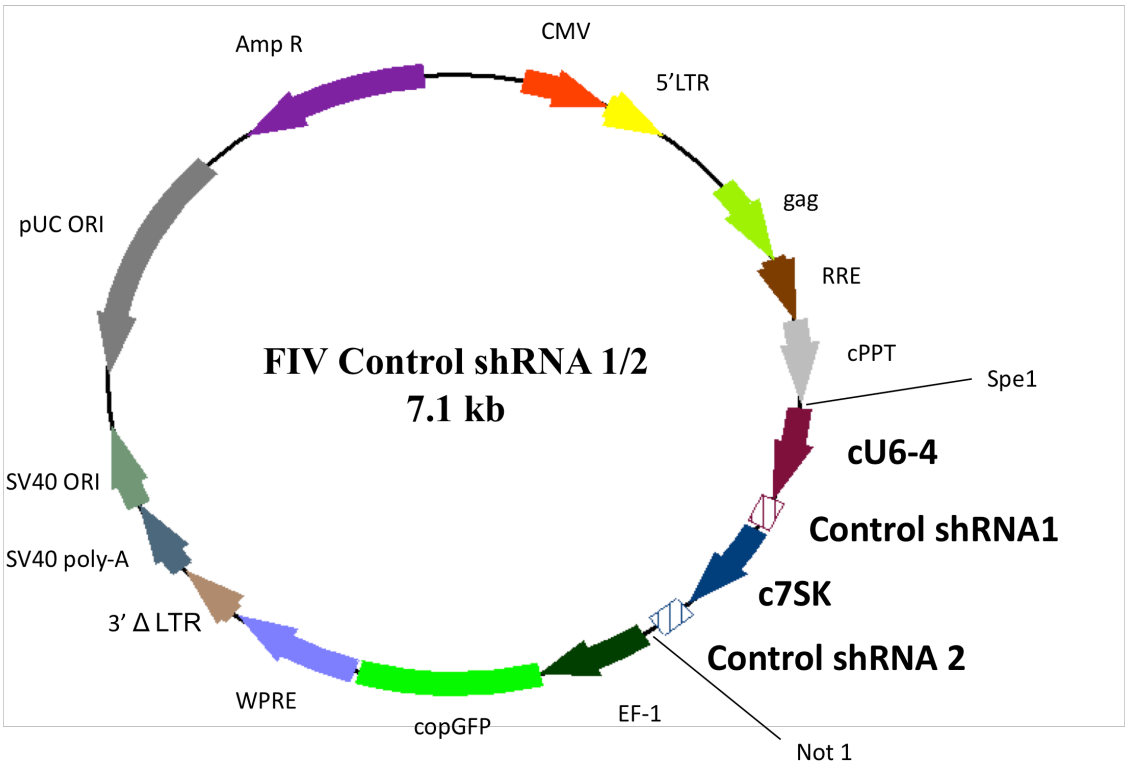


Figure 6.4. Vector map of FIV Control shRNA 1/2. FIV Control shRNA 1/2 was made by digesting Control shRNA 1/2 pUC57 with Spe1 and Not 1 and inserting the 770bp fragment between the Spe1 and Not 1 sites in the FIV lentivector backbone (SBI). This resulted in the addition of the cPepT1 shRNA synthetic gene and the deletion of the CMV promoter and the MCS.

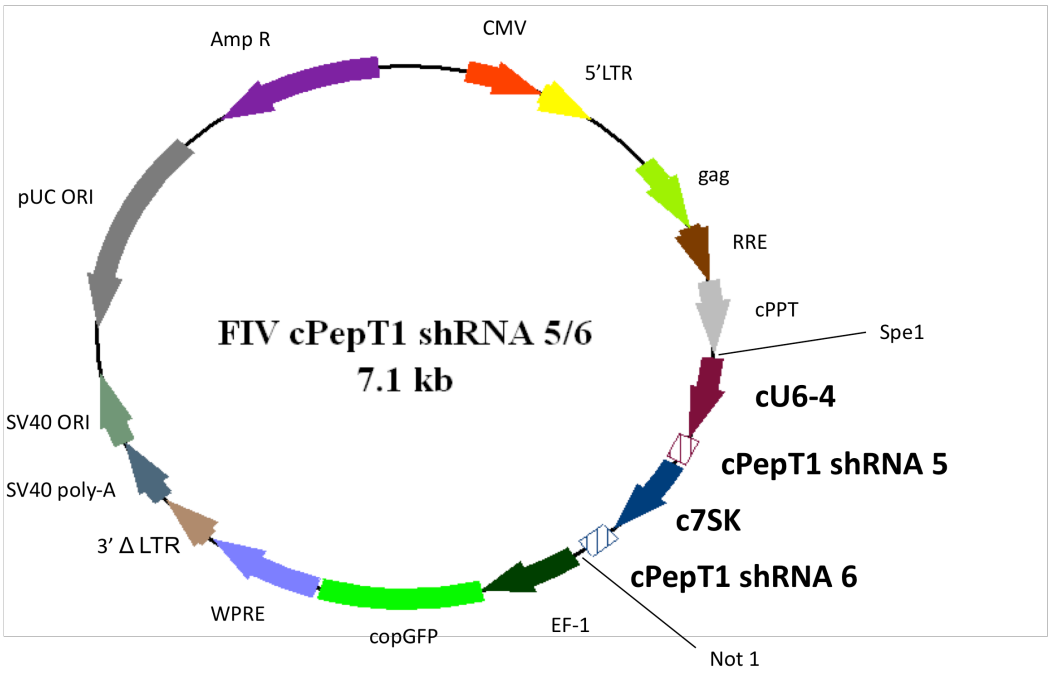
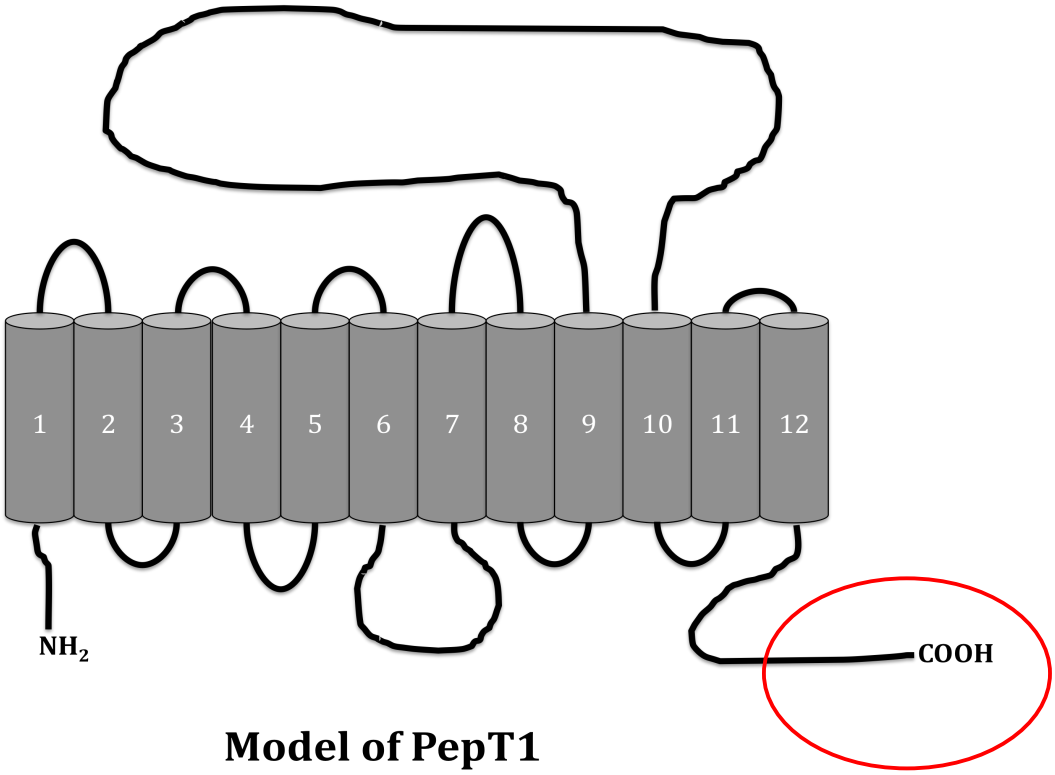


Figure 6.5. Vector Map of FIV cPepT1 shRNA 5/6. FIV cPepT1 shRNA 5/6 was made by digesting cPepT1 shRNA 5/6 pUC57 with Spe1 and Not 1 and inserting the 770bp fragment between the Spe1 and Not 1 sites in the FIV lentivector backbone (SBI). This resulted in the addition of the cPepT1 shRNA synthetic gene and the deletion of the CMV promoter and the MCS.



Endogenous chicken PepT1
N-**E**$_{690}$**EEKKKQIKQDPDLHGKESEAVSQM**$_{714}$-C
Exogenous chicken PepT1-FLAG
N-**E**$_{690}$**EEKKKDYKDDDDK**$_{703}$-C

Figure 6.6. Epitopes in exogenous and endogenous cPepT1. Shown is the secondary structure of rabbit PepT1 (Fei et al., 1994) showing approximate location of the epitope used for generating a chicken PepT1 antiserum (left). Chicken PepT1 has a similar proposed structure based on rabbit PepT1. The C-terminal sequences of endogenous and exogenous chicken PepT1 are shown above.

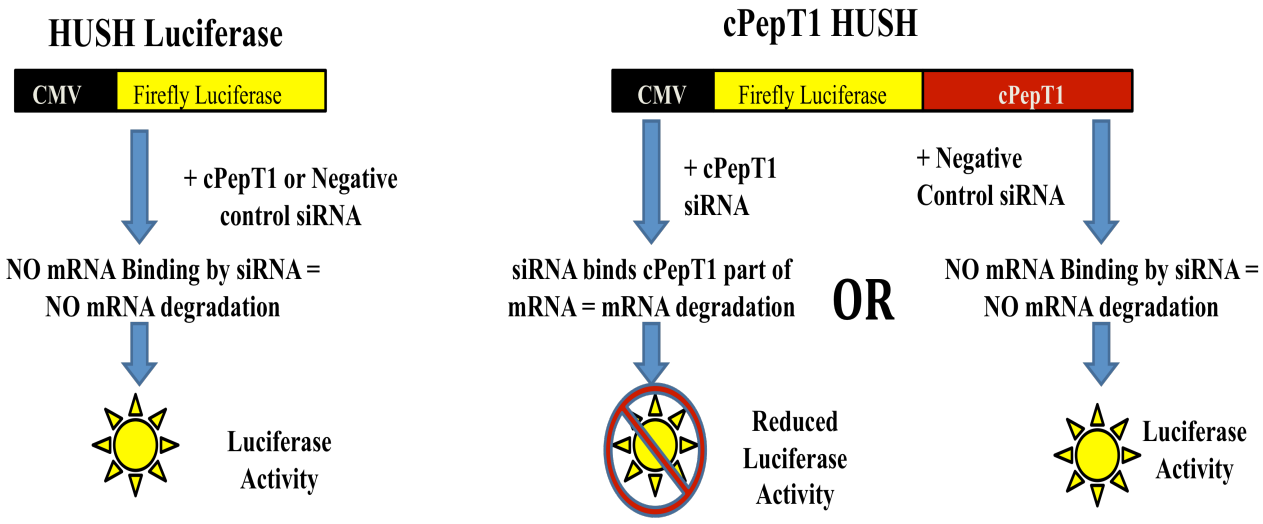


Figure 6.7. RNAi target validation assay. To rapidly test the ability of cPepT1 siRNA and shRNA to degrade cPepT1 mRNA, a luciferase based assay was developed. The cDNA sequence through the 3'UTR of cPepT1 was cloned directly downstream of a firefly luciferase gene under the control of a CMV promoter in the vector pCMV-LUC (Origene). This vector is called cPepT1 HUSH. If the siRNA targets cPepT1 the entire cPepT1/Luciferase fusion transcript will be degraded, resulting in a reduction of luciferase activity. The control vector, HUSH Luciferase, does not contain the cPepT1 transcript fragment and therefore will not bind cPepT1 or Negative control siRNA.

Generation of G0 Transgenic Chickens

Production of Pseudovirus Particles.

Pseudo-virus production was contracted out to System Biosciences (SBI) as well as made in house according to the same protocol used by SBI. For the virus particles made in house first the packaging plasmids pVSVG and pFIV-34n (SBI) were individually cloned out from the pPACK vector mix (SBI). To produce the virus, 3 X 10^6 low passage (2-20) HEK293TN cells (SBI) were seeded onto 100mm tissue culture dishes with 10 mL DME with 10% FBS and without antibiotics. The next day, cells were transfected with 2 μg of FIV-transgene construct, 3 μg pVSVG and 6 μg pFIV-34N per 100 mm plate using Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection cell morphology was examined as cells producing virus should look sick, fluorescence at 488nm was observed as a measure of transfection efficiency. The media was collected and centrifuged at 3000 x g for 15 min. The supernatant was collected and filtered over a 0.45 μm PVDF membrane (Millipore). The virus was further concentrated by PEG precipitation. For PEG precipitation, one volume of PEG-IT solution (SBI) was added directly to four volumes of virus containing supernatant. This solution was allowed to complex overnight (>12h) at 4°C. After complexing, the solution was centrifuged in a swinging bucket centrifuge at 1500 x g for 30 min at 4°C. The supernatant was aspirated off and the pellet was re-centrifuged at 1500 x g for 5 min at 4°C. The remaining supernatant was aspirated off and the pellet containing the virus was re-suspended in 1/100 of the original volume.

Virus tittering was carried out in NIH 3T3 cells (gift from Dr. Corl). Briefly, for each viral stock, NIH 3T3 cells were plated in 24 well tissue culture plates at 5 x 10^4 cells

per well in complete medium (DMEM, penicillin, streptomycin, 10% FBS). One day post-seeding, the culture medium was replaced with 0.5 mL transduction medium (DMEM, 10% FBS, 5 µg/mL Polybrene). Viral stock was then added to the wells for a 1:500, 1:50, and 1:5 dilution of virus. A control well containing no virus was included to determine the effect of polybrene on cells. After an overnight incubation, trasnduction medium was replaced with complete medium and cells were cultured and split as necessary for 24 more hours so that titer was evaluated at 72h post transduction. To determine titer, a PCR-based protocol and tittering kit were used according to the supplied protocol (SBI). The protocol measures the copy numbers of integrated lentiviral constructs directly from lysates of the cells infected with a SBI lentiviral vector. The protocol is based on amplification of a small fragment from the lentivector-specific WPRE (Woodchuck hepatitis virus Post-transcription Regulation Element).

Blastodermal Injection and Ex Ovo Culture

Pseudo-lentiviral particles were diluted 1:1 to 1:4 in DME containing phenol red and polybrene (Sigma-Aldrich) to a final concentration of 1X phenol red and 5 µg/mL polybrene and 5 µL (50,000-200,000 pseudo-lentivirus particles) were injected into the blastoderm of a freshly laid (< 24h) egg (~ Stage X embryo) (Figure 6.11). The embryos were then incubated using the *ex ovo* surrogate system (Borwornpinyo et al., 2005) (Figure 6.8)**.** For System II, surrogate eggshells that were 3-4 g heavier than the donor embryo were selected and cleaned thoroughly with 70% ethanol. A 32 mm window was cut in the small end using a Dremel tool (Figure 6.9), contents were removed and the shell was rinsed with ddH_20 to completely remove albumin. The fertilized donor embryo was poured into a cut plastic drinking cup, air bubbles in the albumin were removed with

a glass pipette and the embryo was poured into the surrogate shell (Figure 6.10) and injected (Figure 6.11) with pseudo-lentiviral particles. Extra albumin was then added to fill the surrogate shell and a 5 cm X 5 cm piece of Saran wrap was placed over the window and secured with PVC rings and rubber bands (Figure 6.12). Embryos were then incubated window side down at 37°C and 60% relative humidity with rocking through 90° every hour (45° of vertical every half hour) for 3 days (Figure 6.13). For System III, turkey surrogate eggshells that were 35-40 g heavier than the donor embryo were selected and cleaned thoroughly with 70% ethanol. A 42 mm window was cut in the blunt end of the egg using a Dremel tool, contents were removed and the shell was rinsed with ddH_20. Gently, the day 3 embryo was transferred to the surrogate turkey shell and excess air bubbles were removed using a glass pipette (Figure 6.14). The window was covered with a 6 cm X 6 cm piece of Saran Cling Plus wrap and secured with albumin, PVC rings and rubber bands (Figure 6.14). The embryos were incubated window side up at 37.5°C and 60% relative humidity with rocking through 30° every hour (15° of vertical every half hour) for 15 days (Figure 6.15). On embryonic day 18, embryos were moved to the hatcher, which was maintained at 37.5°C and ≥ 60% relative humidity. Cling Plus wrap was gently loosened and holes were punctured in it when the embryo was observed to be about to internally pip to allow for adequate air-flow. The eggs were set on and angle in a ring and a petri dish was placed over the exposed embryo (Figure 6.16). The chicks were then allowed to hatch independently. The cPepT1 overexpression founder transgenics were made at N.C. State University and the cPepT1 and control shRNA founder transgenics were made at Virginia Tech.

Sexing and MHC Genotyping of G0 chicks

A nuclear pellet from blood was made from each bird (Petitte et al., 1994). Briefly, whole blood was collected and suspended in potassium EDTA in a microfuge tube. Twenty to fifty microliters of whole blood was diluted in 1 mL phosphate buffered saline and mixed by inversion. In a new microfuge tube, 600 µL of diluted blood was mixed with 800 µL of lysis buffer (10 mM Tris-HCl, pH 7.5, 5mM MgCl2, 0.32 M sucrose, 1% Trition X-100) and vortexted and spun for 15 s at 10,000 x *g*). The supernatant was discarded and this was repeated using 1 mL of lysis buffer. After the final supernatant was removed the nuclear pellet was stored at -20°C. DNA was extracted using the DNeasy Tissue extraction kit (Qiagen) according to the manufacturers protocol. Five hundred nanograms of DNA was used in a PCR reaction containing primers to amplify a 384bp PepT1 fragment (negative control) and a 1200bp W chromosome fragment. Males were identified by the amplification of only the PepT1 fragment and females were identified by the amplification of both the PepT1 and W chromosome fragment. Barred Rock males are also observed to have double the white barring compared to female Barred Rocks. For the G0 chickens generated at Virginia Tech, the leghorn lines that have undergone selection for high (HAS) or low (LAS) antibody response to sheep red blood cells were used as donor embryos, as the VT Barred Rock flock was not reproductively fit. To determine if a G0 chicken was HAS or LAS, the chicks were screened using a PCR assay (Zheng et al., 1999) for the presence of either the B13 (corresponding to the LAS) or B21 (corresponding to the HAS) majorhistocompatability complex class II allele. Primers used were: B21 Forward 5' TTCTTCTACGGTAAGATAGG 3'; B21 Reverse 5' CCTGTCCACTTCATTCATTA

3’; B13 5’ ATCTACAACCGGCAGCAGTTA 3’; B13 5; CGTGTCCACCTCATTCCTTTTTA 3’.

Transgene Screening of G0 Chicks

Tissue, blood, or semen samples of chicks were screened using a standard PCR assay or Real-Time qPCR assay. DNA was extracted from tissue or a prepared nuclear pellet using the DNAeasy Tissue kit (Qiagen). For the standard PCR assay, 100 ng of DNA was included in the following 50 µL reaction: 25 µL 2X TaqMan PCR Master mix (Promega), Forward and Reverse primers. The following primers were used to detect the overexpression construct: cPepT1-FLAG Forward 5’ CCGTTCTCAGGAGGAAGAAC 3’; cPepT1-FLAG Reverse 5’ TTGTCGTCGTCGTCCTTGTA 3’. The following primers were used to detect the copGFP of the shRNA constructs: copGFP Forward 5’AGATGAAGAGCACCAAAGGC 3’; copGFP Reverse 5’ TGTTGTCGGTGAAGATCACG 3’. For the Real-Time qPCR assay a standard curve was generated using plasmid containing the transgene construct diluted 100 ng of negative control genomic obtained from blood or semen 1:1, 1:10, 1:100, and 1:1000 based on copy number per ng of DNA. An example standard curve calculation is shown below.

The haploid chicken genome has 1×10^9 bp
The diploid chicken genome has 2×10^9 bp

The FIV cPepT1 shRNA vector is 7.1kb = 7.1×10^3 bp

Calculating molecule equivalents based on DNA size

If looking in diploid cells: $\frac{2 \times 10^9 \text{ bp}}{7.1 \times 10^3 \text{ bp}}$

$= 2.8 \times 10^5$ bp genomic DNA: 1 plasmid

Calculate ng genomic DNA per ng of plasmid

$$\frac{x}{100\text{ng genomic DNA}} = \frac{1 \text{ plasmid}}{2.8 \times 10^5 \text{ bp genomic DNA}}$$

x = 357 fg plasmid

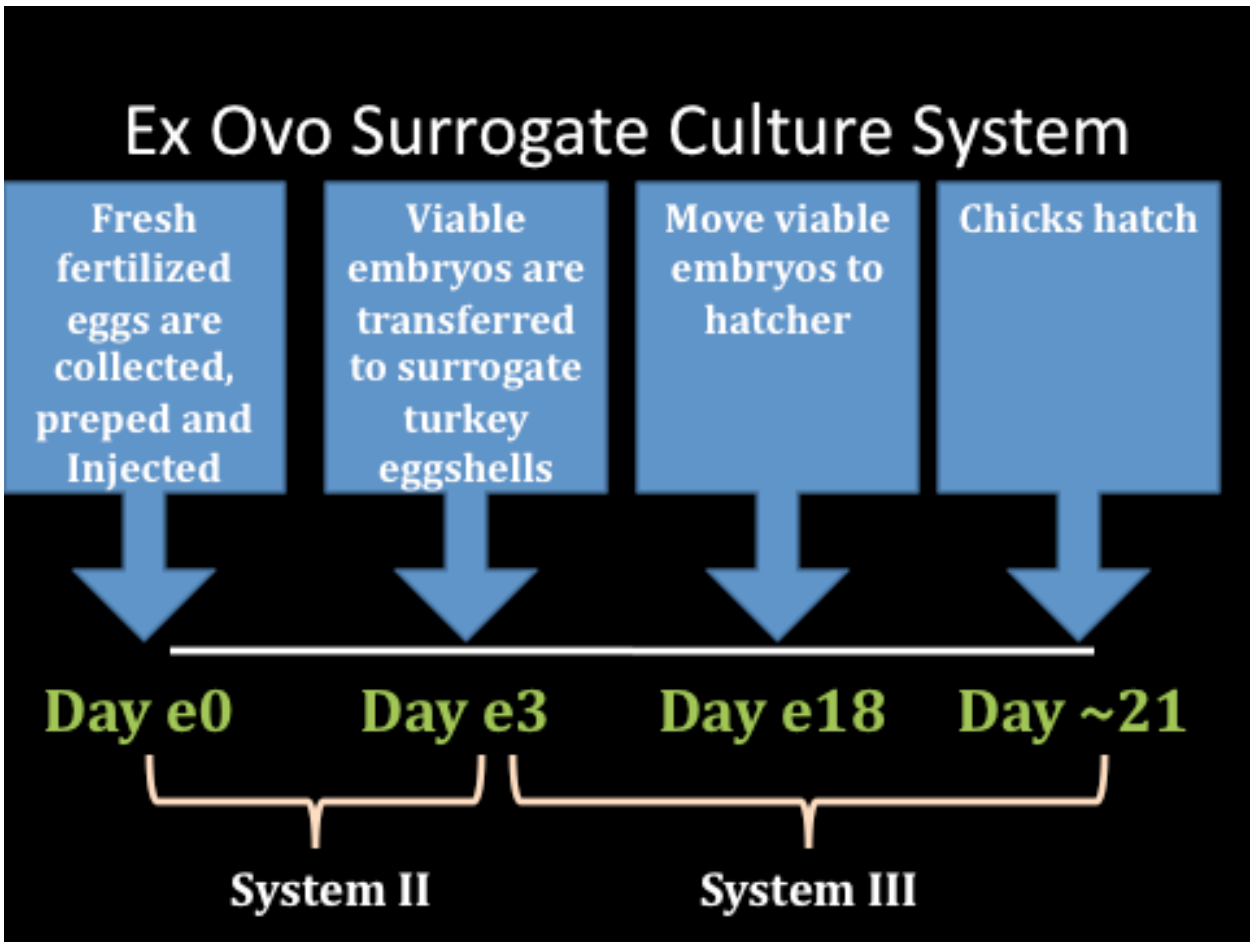
Standard Curve

Genomic DNA : plasmid	fg plasmid	ng Genomic DNA
1:1	357	100
1:10	35.7	100
1:100	3.57	100
1:1000	0.357	100
1: 0	0	100
Positive Control	20 ng	100

If there is a positive response in the test sample that corresponds to 1:100 on the standard curve, then one out of every 100 diploid cells is caries a copy of the transgene.

The PCR reaction was carried out using 2X SYBR green master mix, 125 nM final concentration of forward and reverse primers designed using either Primer 3 (overexpression construct) or Primer express (short hairpin constructs-primers targeted the copGFP) (See above for sequences), 100ng of negative control or unknown genomic

DNA obtained from tissue, blood, semen and run on a 7300 Real-Time PCR machine (Applied Biosystems). A standard curve containing the target construct of interest (described above) was run in tandem with each run. A positive reaction was anything definitively four fold over background. Female chickens were raised to approximately 12 weeks of age and then were killed and the following tissues were examined for transgene expression using the previously described method. Tissues examined were the duodenum, jejunum, ileum, liver, kidney, heart, spleen, pancreas, lung, proventriculus, comb, breast muscle, ovary and magnum.



C. R. Miller 2012

Figure 6.8. Ex ovo culture summary.

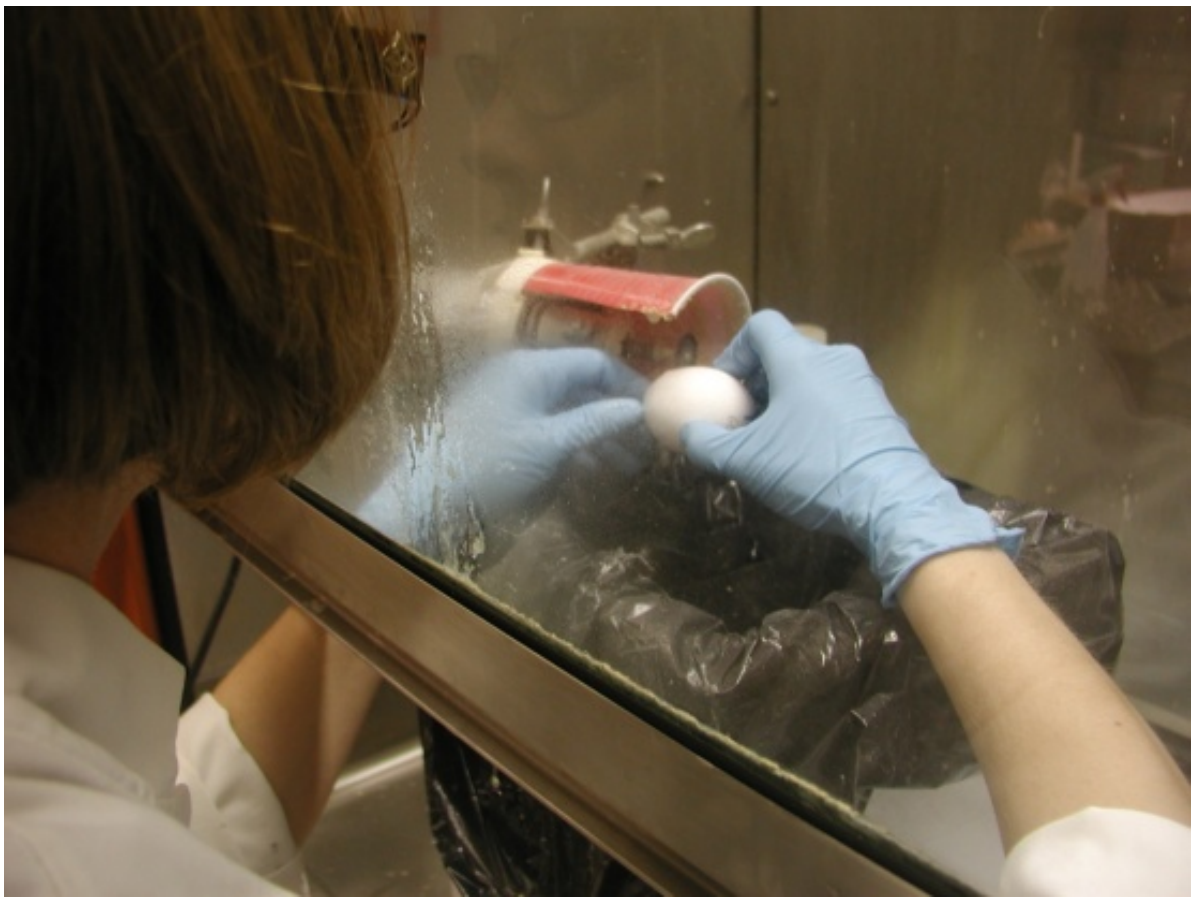


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Figure 6.9. Windowing a surrogate chicken egg. A 32mm window was cut in the small end of the surrogate egg using a Dremel tool.

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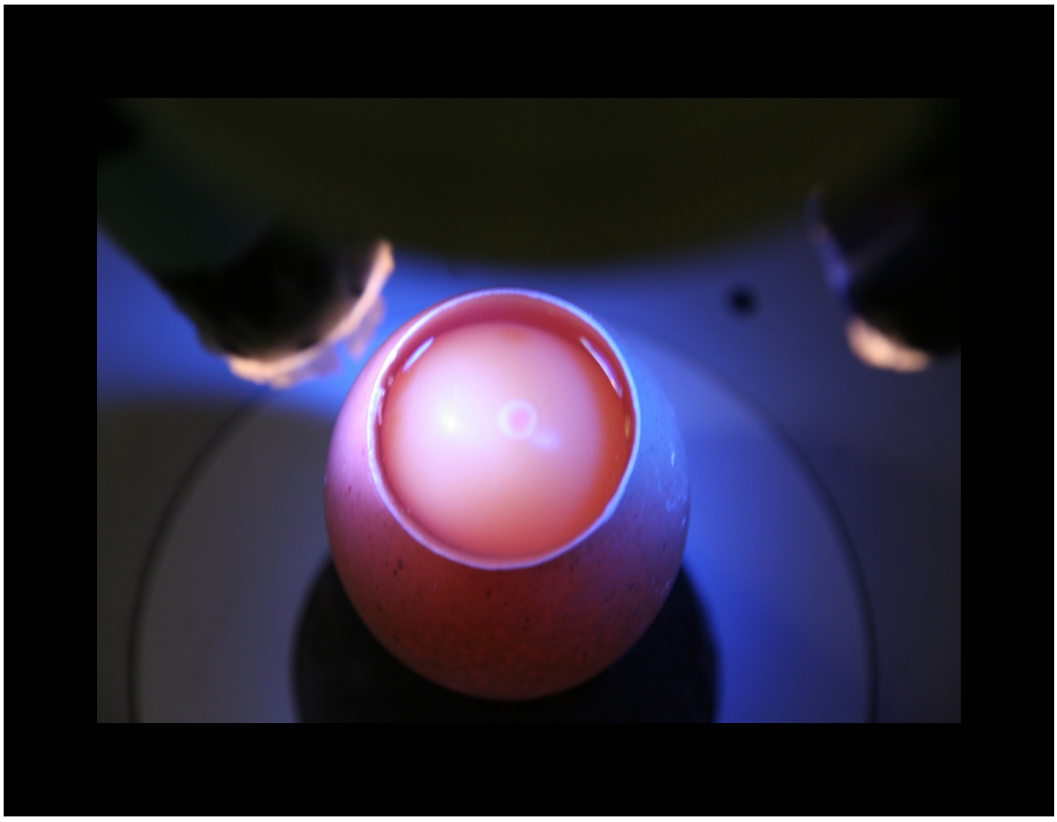
Figure 6.10. Transfer of a donor embryo into a windowed surrogate chicken egg. The fertilized donor embryo was poured into a cut plastic drinking cup, air bubbles in the albumin were removed with a glass pipette and the embryo was poured into the surrogate shell.



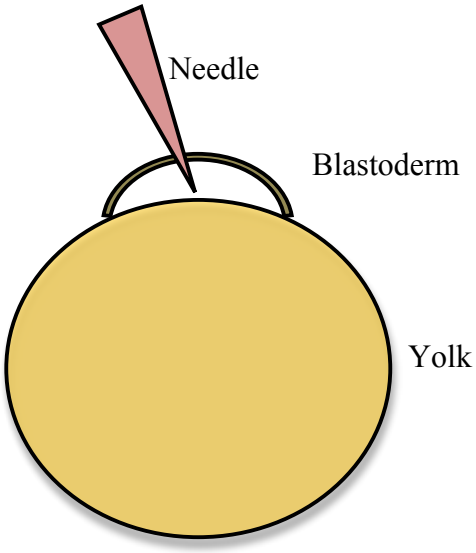


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Figure 6.11. Blastodermal injection of stage X embryo. The image above shows an injected stage X embryo. The pink color is due to phenol red being added to the injection mixture to facilitate the injection. The cartoon diagram shows the placement of the injection needle into the space in the blastoderm.

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Figure 6.12. **Preparation of injected eggs for Stage II incubation**. After blastodermal injection, extra albumin was added to the egg, air bubbles were removed and the window was sealed with Saran wrap and secured with PVC rings and rubber bands.

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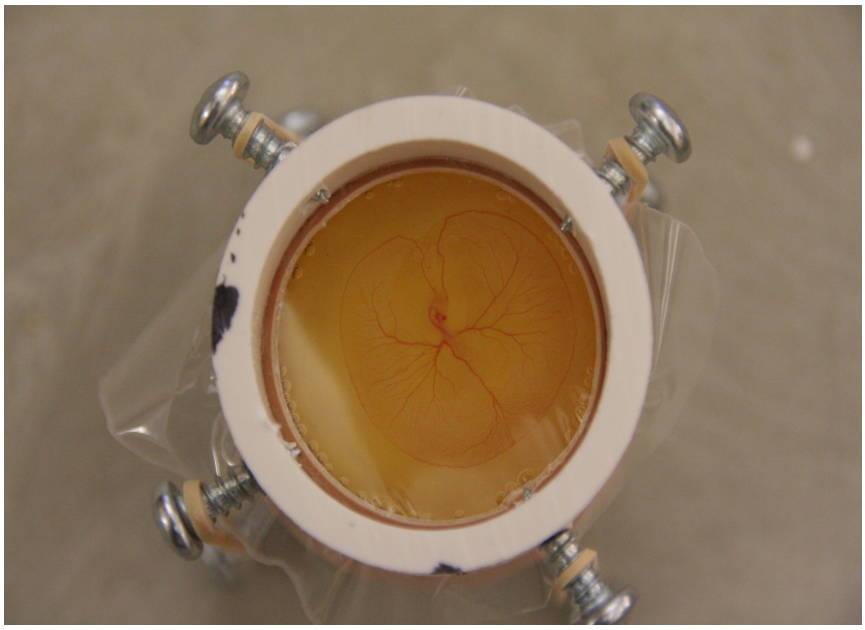
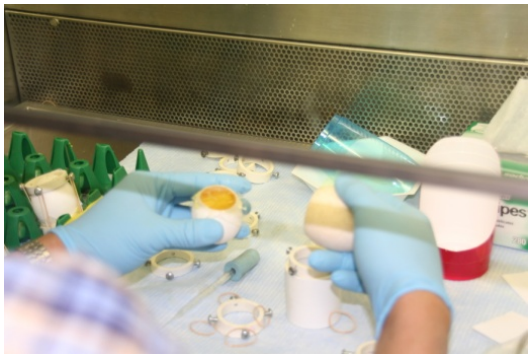
Figure 6.13. **Stage II incubation.** Eggs were incubated window side down for 3 days at 37°C, 60% relative humidity with rocking through 90° every hour.





C. R. Miller 2012

Figure 6.14. System II to System III transfer. Viable e3 embryos (top image) were transferred to turkey surrogate shells (System III), covered with Saran Cling Plus wrap, which was secured with PVC rings and rubber bands.

C. R. Miller 2012

Figure 6.15. System III incubation. System III embryos were incubated window side up at 37.5°C, 60% relative humidity with rocking through 30° every hour for 15 days.

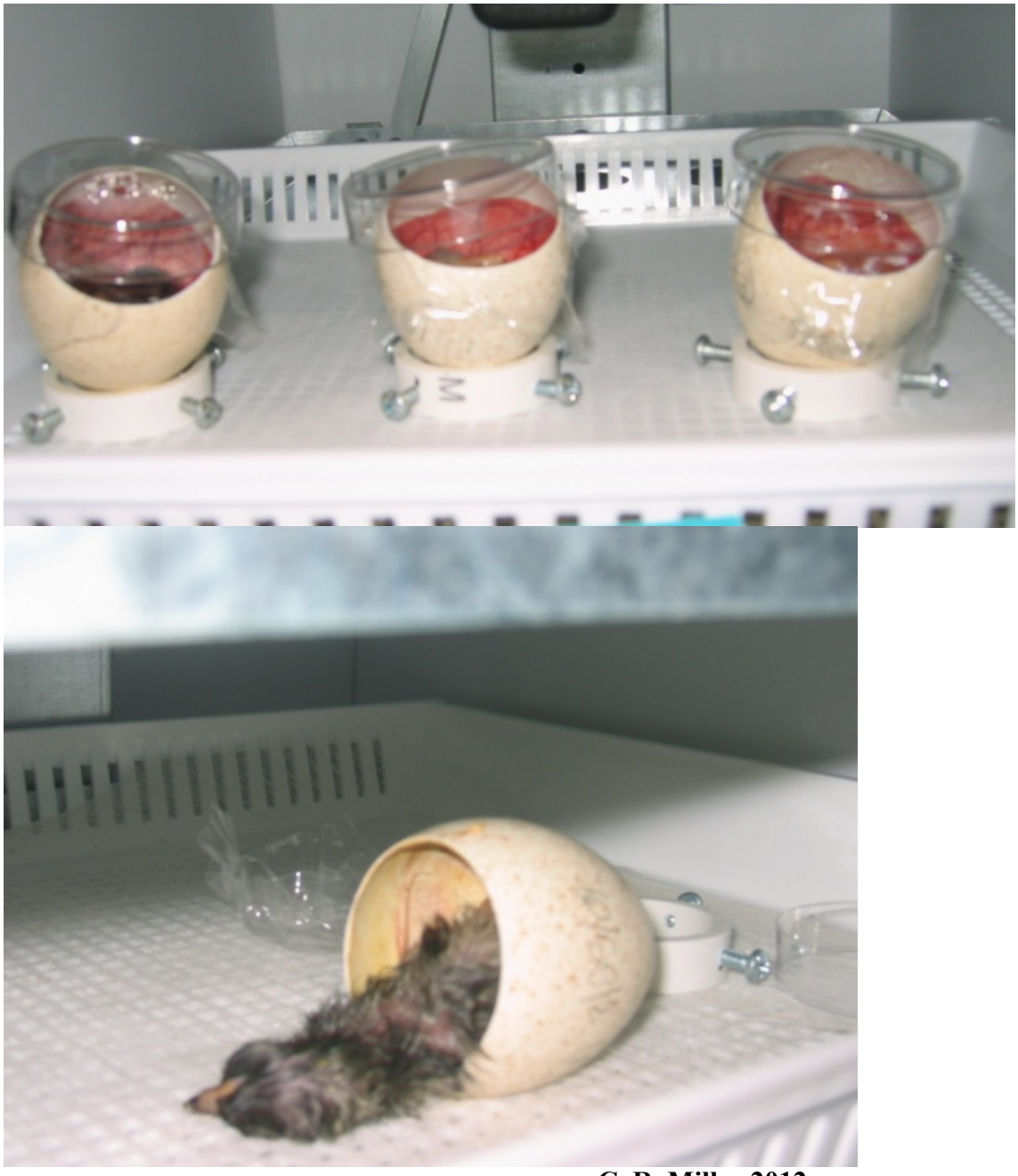


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Figure 6.16. Hatching of potential founder chickens. On embryonic day 18, embryos were moved to the hatcher, set at an angle in the bottom ring and holes were made in the cling plus covering and the opening was covered with a petri dish. The chicks were allowed to hatch independently.

Results and Discussion

The overall objective of this research was to exaime the effect of PepT1 manipulation *in vivo* and compare the results to the *in vitro* results (Presented in Chapter V of this dissertation) and knockout mouse model. These model systems would also allow for determination of macroscopic effects such as altered growth or peformance of chickens that cannot be ascertained in the aforemention models. To this end the generation of cPepT1, cPepT1 shRNA, and control shRNA expressing chickens was undertaken.

Overexpression Vector Construction and Validation

Through an elegant design, the exogenous cPepT1 contained a FLAG tag, which allowed for distinction of exogenous cPepT1 from endogenous PepT1. The exogenous cPepT1 construct was created by replacing the endogenous epitope with the FLAG sequence (DYKDDDK) and a new stop codon (Figure 6.6) and cloning into the lentiviral vector backbone (Figure 6.2). Ability of the overexpression lentivector to produce a functional protein was analyzed by western (Figure 6.17) and immunocytochemical (Figures 6.18-6.23) through analysis and a Gly-Sar uptake assay (6.24).

The results from immunoblotting and immunocytochemical techniques indicate that the exogenous vector design translated into function. Both techniques indicate that an anti-FLAG antibody specifically recognizes only the exogenous cPepT1 protein and does not cross react with the endogenous sequence. The exogenous protein is of expected molecular weight of approximately 80 kDa. Further, the anti-endogenous cPepT1 antibody specifically recognizes the endogenous cPepT1 sequence and does not

cross react with the exogenous cPepT1 protein. Unfortunately, as different antibodies operate as different affinities for their epitopes, an immuno-detection method cannot be relied upon for an estimation of quantitative differences in protein quantity.

Interestingly, an apparent difference in cellular localization was observed. Again, because different antibodies were used, the results must be interpreted with caution. Nonetheless, the observation merits comment. In CEFs, the exogenous cPepT1 protein was highly expressed throughout the cell, presenting almost a uniform presence. This expression pattern indicates the presence of the exogenous protein in the nucleus, cytoplasm and membrane. Although the images do not clearly define expression of the exogenous protein in the membrane, the functional assay confirms that it is present there. If it were not present in the membrane, no Gly-Sar uptake would have been observed. The endogenous cPepT1 appeared to be expressed in a pattern more typically seen of other membrane-expressed transporters when studied using immunocytochemical techniques.

Instead of an appearance of uniformity, as was observed with the exogenous PepT1, expression of endogenous PepT1 appeared more restricted, and gave the cell a speckled appearance. The difference in pattern of expression, if ignoring the fact that different antibodies were employed, may indicate the importance for the 14 amino acids that were removed and replaced with the FLAG sequence and/or the 3'UTR region to restrict expression to or in the membrane and/or cytosol. The speckled appearance also suggests the aggregation of endogenous cPepT1 in the membrane and/or cytosol. It was reported that PepT1 associates with lipid rafts in the membrane (Nguyen, et al., 2007), and the pattern observed by the endogenous cPepT1 protein may be due to cPepT1

association with lipid rafts in the membrane. The lack of a similar pattern of expression by the exogenous PepT1, again ignoring the influence of antibody used, may indicate that deleted amino acids and 3'UTR may influence the location of expression within the membrane itself. It has also been observed that PepT1 is contained within the cytoplasm and translocated to the membrane upon stimulation by intracellular signals (See Chapter II for a review). The aggregate pattern observed in the transfected CEF cells may reflect the cytoplasmic storage of cPepT1. The different pattern of expression of the exogenous cPepT1 may indicate that the deleted amino acids may affect the ability of PepT1 to be contained in aggregates in the cytoplasm. Expression of both constructs was also nuclear. This may indicate that ubiquitous overexpression results in aberrant expression of the construct or for some reason in this cell line, PepT1 is incorporated into the nuclear membrane as well. Although it is interesting to note, any further comment is beyond the scope of this research.

Despite an apparent difference in expression, both exogenous cPepT1 and the endogenous cPepT1 construct produced a function cPepT1 protein with respect to ability to transport substrate. This was determined by measuring the amount of uptake of Gly-Sar, a non-metabolizeable PepT1 substrate, in transiently transfected CEF cells. Normally, CEFs do not express PepT1 nor do they have any endogenous ability to transport Gly-Sar, as reflected by no uptake observed in CEF cells transfected with an empty vector. Interestingly, the endogenous cPepT1 produced almost double the amount of uptake, which was normalized to total protein concentration, as compared with the exogenous PepT1 construct. The observed difference may be due to the amount of PepT1 protein integrated into the membrane or a change in the transport kinetics between the

endogenous and exogenous PepT1. Immunocytochemical analysis suggests that the later hypothesis is likely, but due to the different antibodies employed to detect each construct, the analysis is not conclusive. Regardless of the difference in uptake between the two constructs, the exogenous cPepT1 produced uptake in the order of 20,000 fold greater than the control cells. Therefore, it is concluded that the exogenous cPepT1 produced from the lentiviral vector, is functional *in vitro* and suitable for use to make a transgenic chicken model.

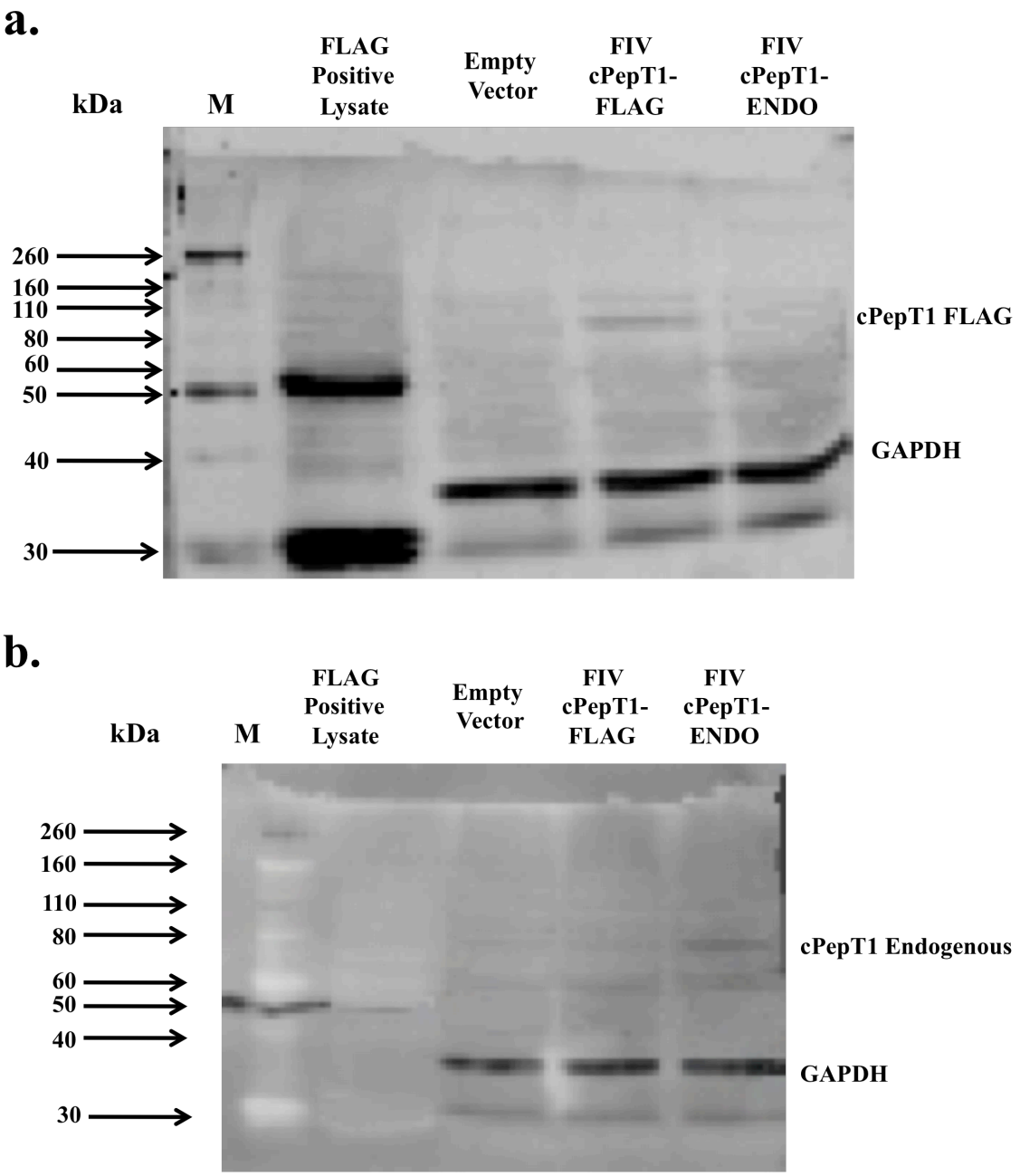


Figure 6.17. Protien Expression of Exogenous cPepT1-FLAG. This figure shows the results from a western blot for exogenous cPepT1-FLAG (a) and endogenous cPepT1 (b). The exogenous cPepT1 FLAG lentiviral vector produced a protein approximately 80 kDa that was identified using an anti-FLAG primary antibody. The anti-FLAG antibody did not cross-react with endogenous cPepT1 or the vector backbone. The cPepT1 polyclonal antibody recognized an approximately 80kDa protein in CEFs transfected with the endogenous cPepT1 lentiviral vector. The polyclonal cPepT1 antibody did not cross-react with the exogenous cPepT1 or the vector backbone.

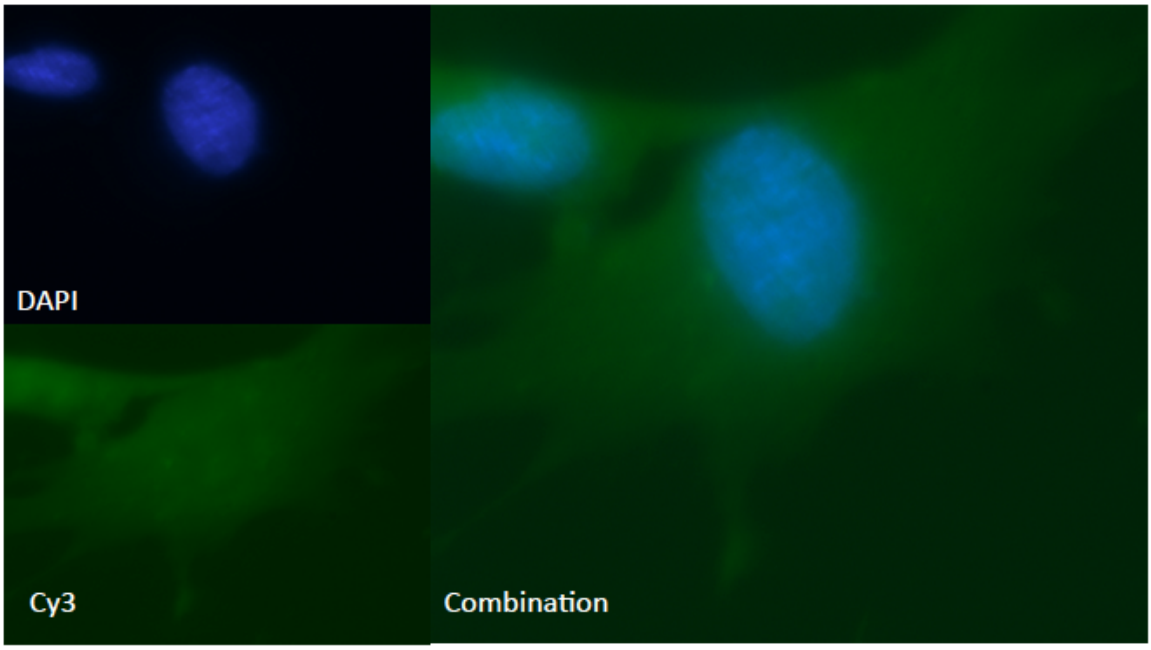


Figure 6.18. Immunocytochemistry of CEFs expressing cPepT1-FLAG incubated with an anti-FLAG primary antibody. The image in the upper left shows the nucleus of cells counterstained with DAPI. The image in the lower left shows the expression of cPepT1-FLAG as determined by staining with an anti-FLAG antibody conjugated to Cy3. The image on the right shows the superimposed images.

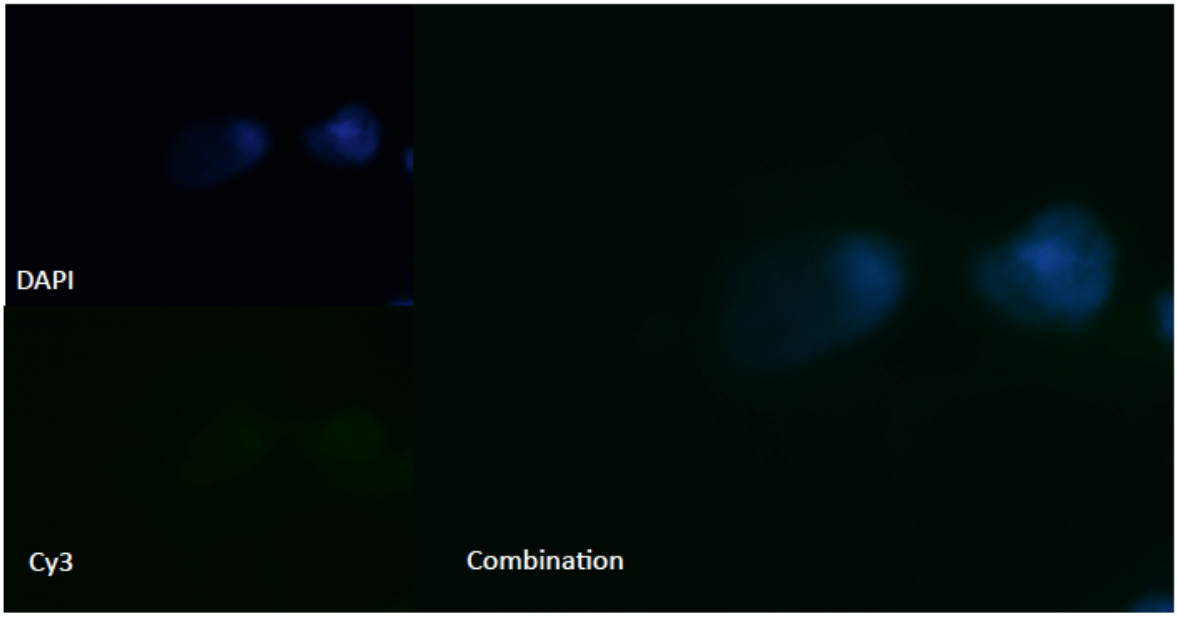


Figure 6.19. Immunocytochemistry of CEFs expressing endogenous cPepT1 incubated with an anti-FLAG primary antibody. The image in the upper left shows the nucleus of cells counterstained with DAPI. The image in the lower left shows the expression of cPepT1-FLAG as determined by staining with an anti-FLAG antibody conjugated to Cy3. The image on the right shows the superimposed images.

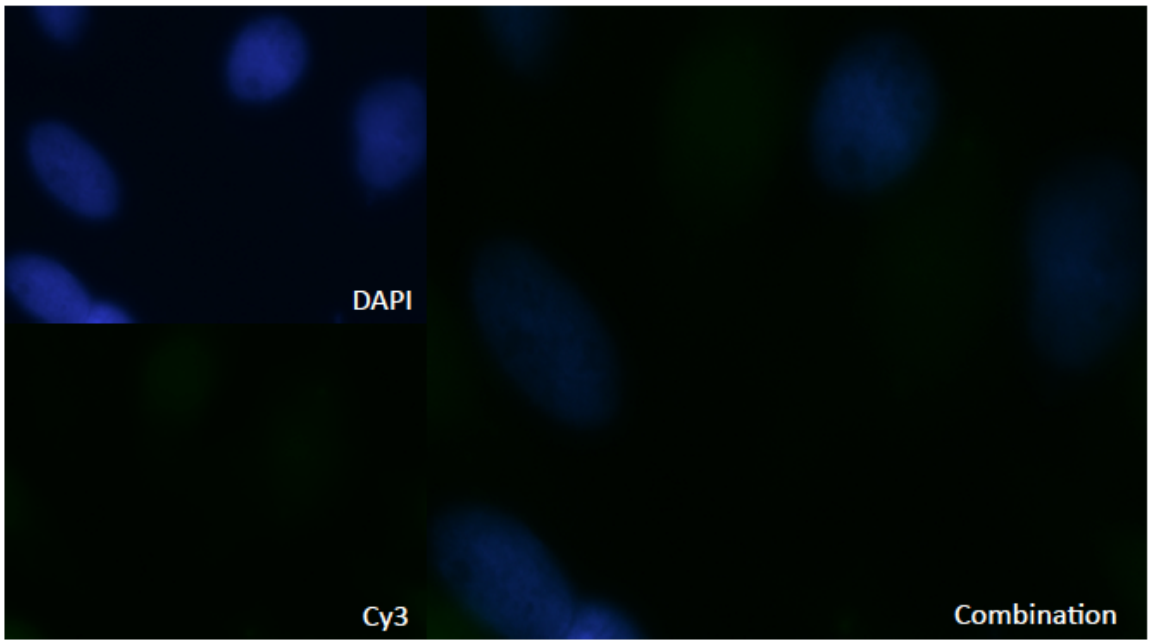


Figure 6.20. Immunocytochemistry of CEFs transfected with an empty vector control incubated with an Anti-FLAG primary antibody. The image in the upper left shows the nucleus of cells counterstained with DAPI. The image in the lower left shows the expression of cPepT1-FLAG as determined by staining with an anti-FLAG antibody conjugated to Cy3. The image on the right shows the superimposed images.

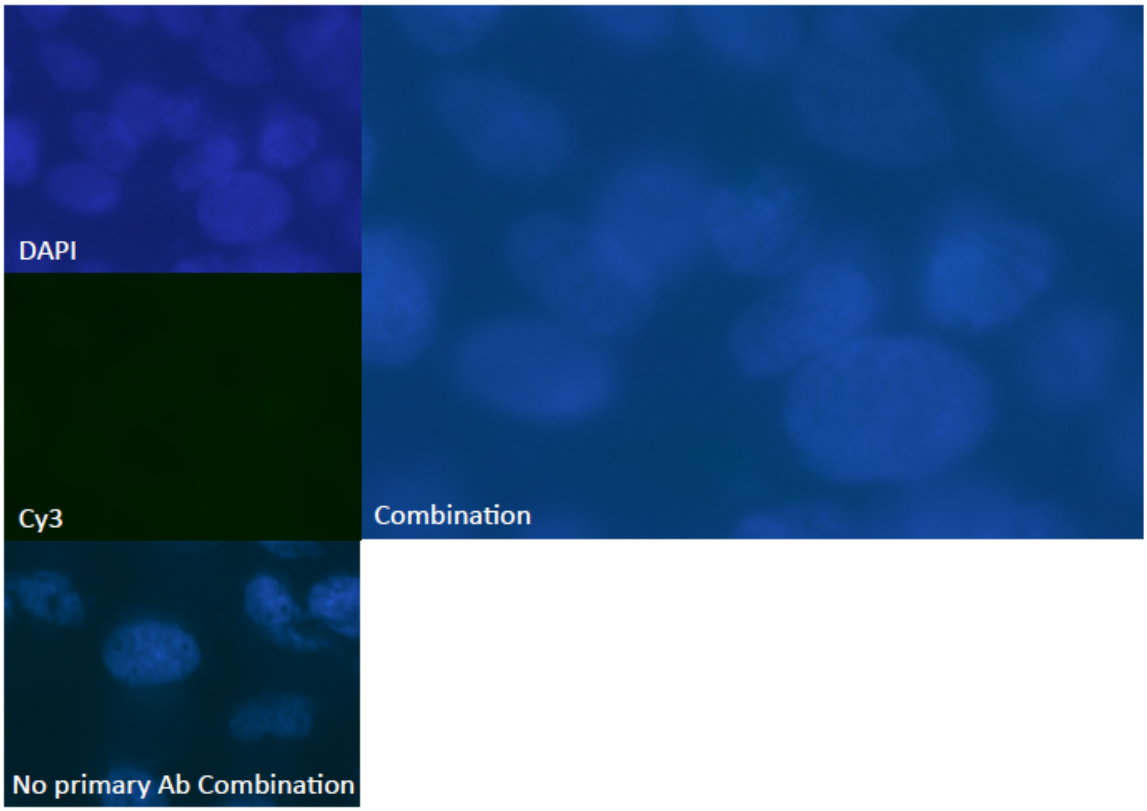


Figure 6.21. Immunocytochemistry of CEFs expressing cPepT1-FLAG incubated with an anti-cPepT1 primary antibody. The image in the upper left shows the nucleus of cells counterstained with DAPI. The image in the middle left shows the expression of cPepT1-FLAG as determined by staining with an anti-cPepT1 antibody and corresponding secondary antibody conjugated to Cy3. The image in the lower left shows cells expressing cPepT1 FLAG that were incubated in secondary antibody only and counterstained with DAPI. The image on the right shows the superimposed images of cells incubated with the primary and secondary antibody and counterstained with DAPI.

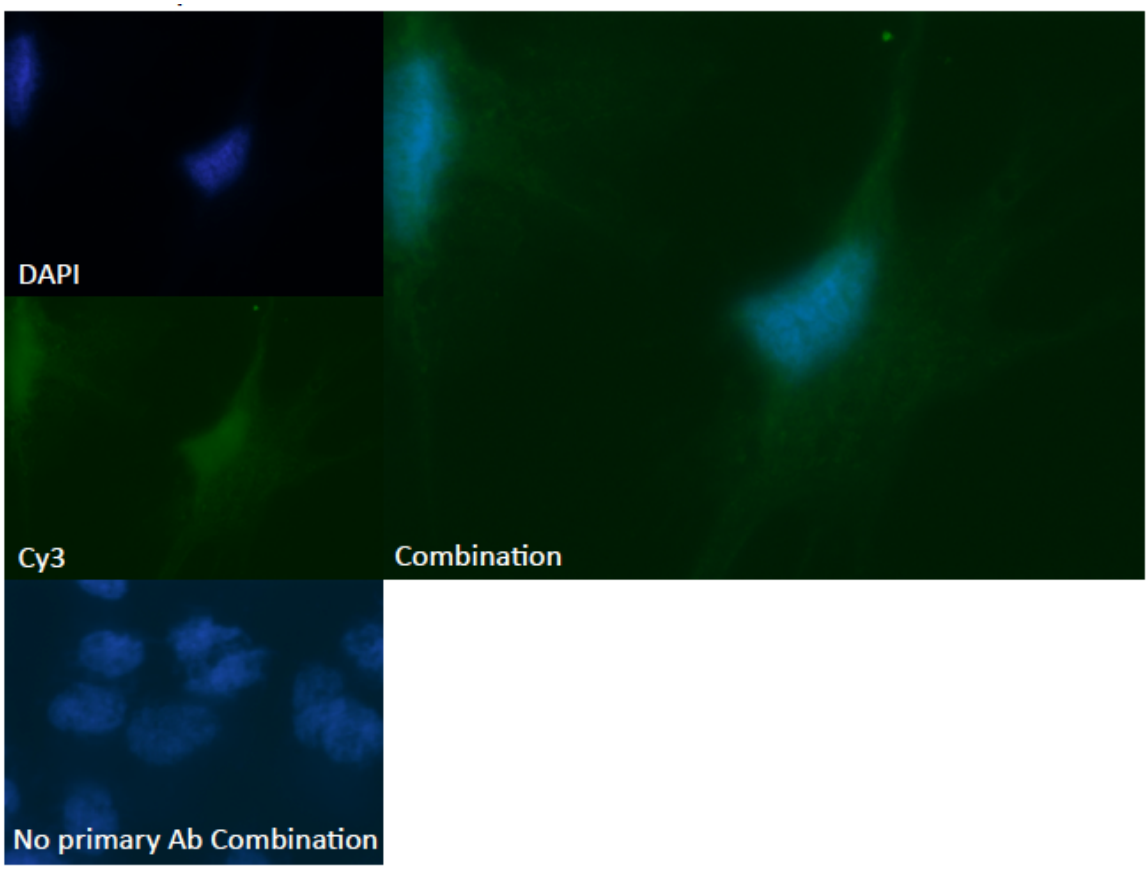


Figure 6.22. Immunocytochemistry of CEFs expressing endogenous cPepT1 incubated with an anti-cPepT1 primary antibody. The image in the upper left shows the nucleus of cells counterstained with DAPI. The image in the middle left shows the expression of endogenous cPepT1 as determined by staining with an anti-cPepT1 antibody and corresponding secondary antibody conjugated to Cy3. The image in the lower left shows cells expressing endogenous cPepT1 that were incubated in secondary antibody only and counterstained with DAPI. The image on the right shows the superimposed images of cells incubated with the primary and secondary antibody and counterstained with DAPI.

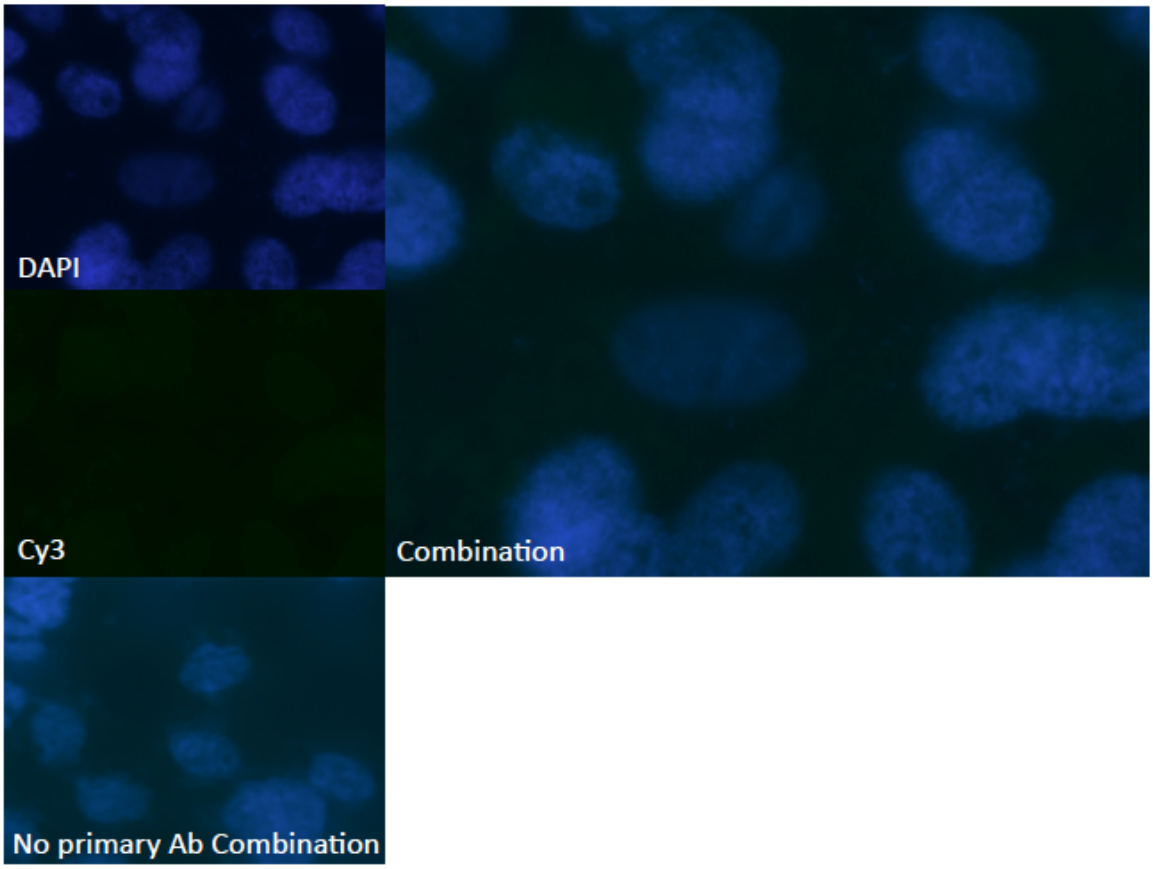


Figure 6.23. Immunocytochemistry of CEFs transfected with an empty vector control incubated with an anti-cPepT1 primary antibody. The image in the upper left shows the nucleus of cells counterstained with DAPI. The image in the middle left shows cells transfected with an empty vector control and incubated with an anti-cPepT1 antibody and corresponding secondary antibody conjugated to Cy3. The image in the lower left shows cells transfected with and empty vector control that were incubated in secondary antibody only and counterstained with DAPI. The image on the right shows the superimposed images of cells incubated with the primary and secondary antibody and counterstained with DAPI.

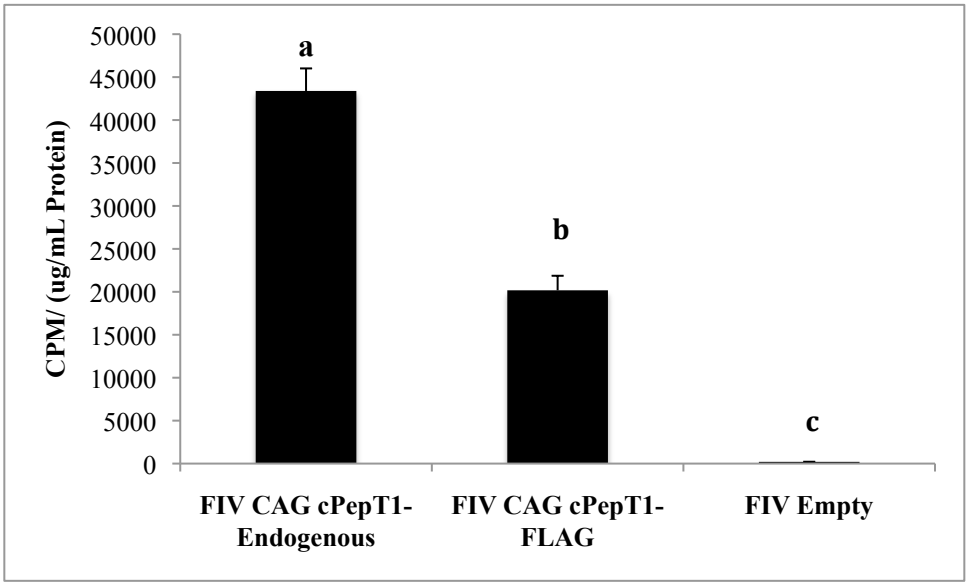


Figure 6.24. Functional transport assay by exogenous chicken PepT1. Wild type (PepT1-Endo) and FLAG tagged cPepT1 (PepT1-FLAG) under the control of the CAG promoter were transfected into CEF. Peptide transport was assayed by measuring the uptake of [3H]-Gly-Sar in transfected CEF. FIV Empty lacks the cPepT1 cDNA and served as a control. Bars with different letters are significantly different ($P < 0.001$).

shRNA Vector Construction and Validation

Working in non-mammalian species always presents a challenge, as the availability of commercial products and reagents is limited. However, this also provides the opportunity to design reagents, exactly tailored to a specific need. This exact situation presented itself with the use of an RNAi approach to knockdown cPepT1. Using a rational design approach (Discussed in detail in the Methods section), siRNAs were designed whose sequences would be acceptable also when expressed as shRNA sequences. Briefly, potential siRNA target sequences to cPepT1 were designed using an algorithm from Ambion. From the results each of the siRNA sequences were blasted against the chicken genome to assess potential off-target effects. . If 15 or more bp were an exact match for any annotated or predicted gene, these sequences were not used.

There are differences in effective siRNA and shRNA characteristics because of the different cellular processes and machinery that handle the two molecules. Therefore, the sequences with little chance of off-target effects were then assessed for their potential to make effective shRNA sequences by looking at design criteria described by Li et al. (2007) (Table 6.1). Further, considerations that the sequences would be ultimately have to be part of a transcript produced from a Pol II promoter to be packaged into a virus particle had to be taken into consideration as well. For example, when screening potential sequences, any siRNA sequence that would have a poly A when incorporated with the short hairpin loop of the shRNA were excluded as the poly A would act as a stop sequence for Pol II and inhibit the production of a viral transcript. From a list of over one

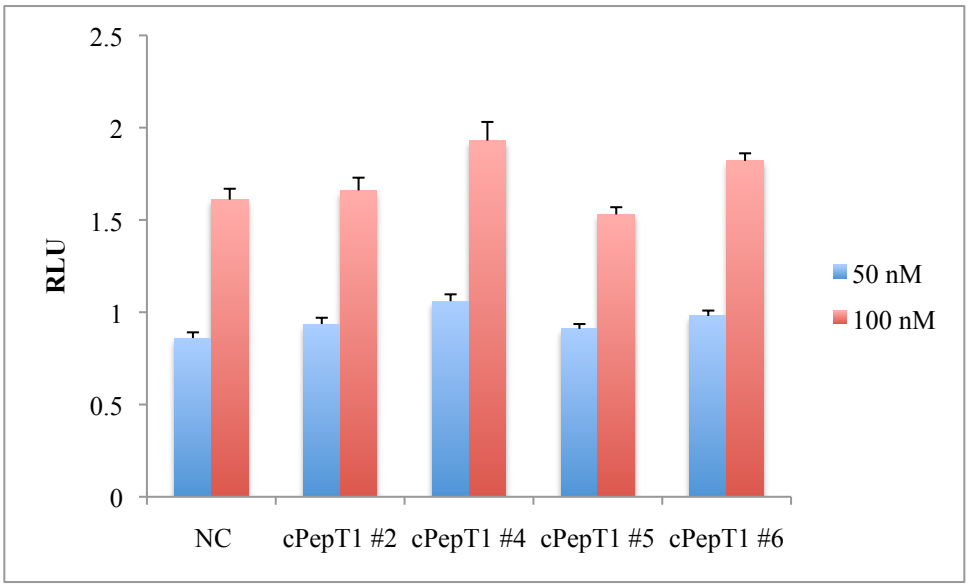
hundred potential sequences 4 were determined meet all the criteria previously described (Table 6.2).

To determine which sequence targeted cPepT1 siRNA the best, siRNAs corresponding to the designed sequences were purchased as custom siRNA sequences from Ambion, whose design algorithm was used to help develop the sequences. The siRNA sequences were validated by determining their ability to degrade a cPepT1-firefly luciferase fusion transcript. The assay works on the premise that successful targeting of a transcript by siRNA results in degradation of the entire transcript. This set up a simple and economical assay in which a reduction of luficerase activity is a result of cPepT1 degradation. Often two different siRNA sequences produced a synergistic or additive effect when used together. Therefore, combinations of the cPepT1 siRNAs were also tested by determining their ability to degrade a cPepT1-firefly luciferase transcript. The results are shown in Figures 6.25 and 6.26. Overall, all siRNA sequences produced cPepT1 mRNA degradation. However, the combination of any single siRNA sequence with siRNA sequence 5 produced the greatest cPepT1 mRNA degradation. Many microRNA targets reside in the 3'UTR of transcripts. Therefore, cPepT1 siRNA sequences 5 and 6 were chosen to make the shRNA genes.

Typical promoters used to drive shRNA sequences are the Pol III promoters U6, 7SK and H1. Chicken U6 and 7SK promoters have been characterized and successfully generated functional shRNA transcripts (Bannister et al., 2007; Wise et al., 2007). Therefore, these promoters were used to drive the cPepT1 shRNA sequences. Additionally, restriction enzyme sites to facilitate subcloning and ultimately Southern analysis were included. The gene map is shown in Figure 6.3. Also necessary was a

corresponding control shRNA gene. Ambion would not release the sequence of their control siNRA sequence so this was not a suitable option. Typically, a scrambled sequence containing all the same nucleotides of the test shRNA sequence is utilized. However, the expense of de novo synthesizing a control shRNA gene combined with the risk that it may cause deleterious off-target effects despite passing a genome blast analysis was too great. Therefore, the control sequences used by Bannister et al. (2007) and Wise et al. (2007) were chosen as they had been used in a knockdown experiment in chickens and no observable off-target effects were acknowledged. The genes were *de novo* synthesized and then subcloned into the lentiviral vector backbone. The vectors were then tested using the same luciferase based assay as was used to test the siNRA sequences. The results are shown in Figure 6.27. The cPepT1 shRNA vector caused a 64% reduction in luciferase activity. As the ultimate goal was to knockdown the cPepT1 to impair its transport of di- and tri-peptides, the cPepT1 shRNA were tested by determining their ability to knock down Gly-Sar transport by cPepT1 in CEFs. The results from this uptake assay are shown in Figure 6.28. In CEFs, Gly-Sar transport by PepT1 was almost completely abolished. Overall, this data demonstrates the generation of a lentiviral vector that produces shRNAs capable of degrading cPepT1 mRNA resulting in almost a complete loss of function *in vitro*, without any observable deleterious effects on the cells. Therefore, it is concluded that these vectors would be suitable for the production of transgenic chickens.

a. HUSH Luciferase



b. cPepT1 HUSH

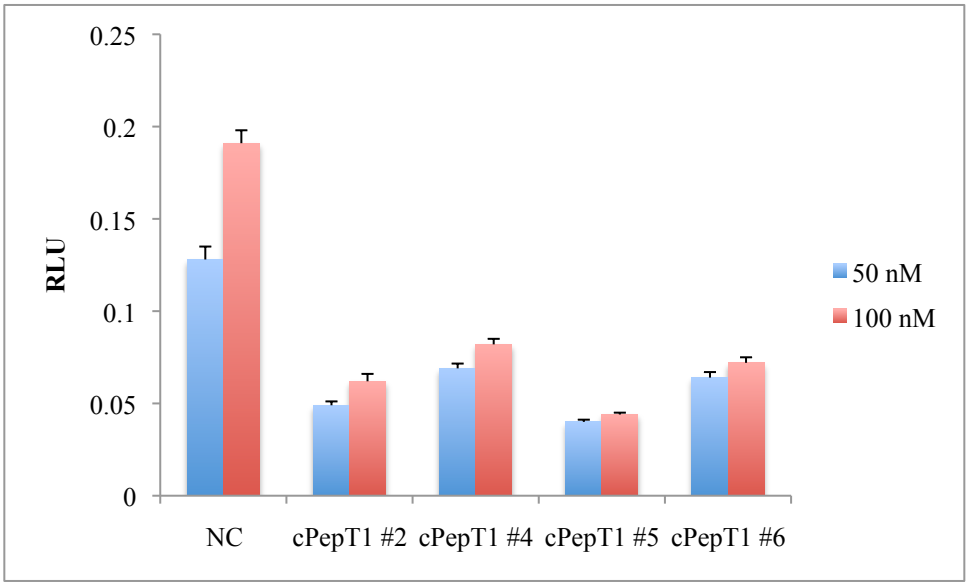
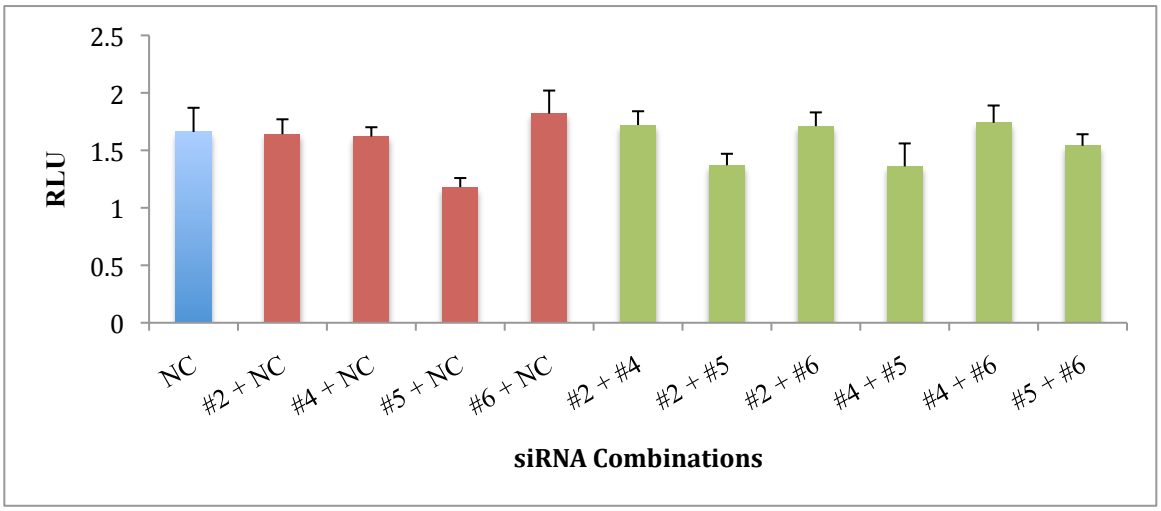


Figure 6.25. Knockdown of cPepT1 by cPepT1 siRNA. CEF were co-transfected with 100 nM or 50 nM cPepT1 siRNA or Negative control (NC) siRNA and HUSH Luciferase (a) or cPepT1 HUSH (b) and pRL-SV40. Three independent transfections were completed in triplicate. Cells were lysed and a dual luciferase assay was performed 24 h post transfection. Data shown represent the mean Normalized RLU ± SEM from two independent experiments (n=4). All cPepT1 siRNAs produced 46-77% knockdown ($P < 0.05$) as compared to the Negative Control.

a. HUSH Luciferase



b. cPepT1 HUSH

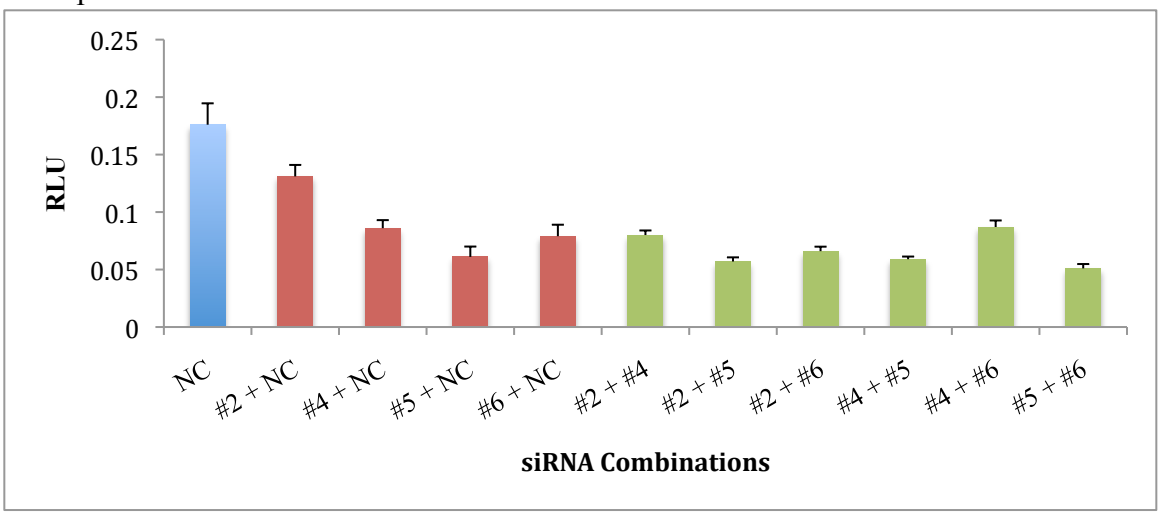
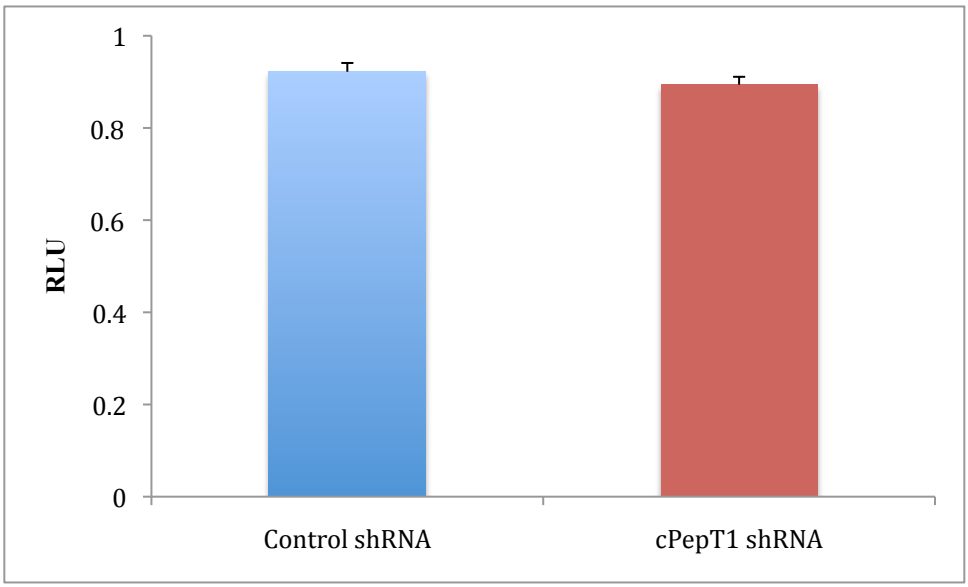


Figure 6.26. Knockdown of cPepT1 by combinations of cPepT1 siRNA. CEF were co-transfected with 50 nM each siRNA in all possible combinations of two siRNAs or 100 nM of Negative control (NC) siRNA and HUSH Luciferase (a) or cPepT1 HUSH (b) and pRL-SV40. Two independent transfections were completed in duplicate. Cells were lysed and a dual luciferase assay was performed 24 h post transfection. Data shown represent the mean Normalized RLU ± SEM from two independent experiments (n=4). All combinations produced knockdown (26 to 71%) as compared to the Negative Control ($P < 0.05$).

a. HUSH Luciferase



b. cPepT1 HUSH

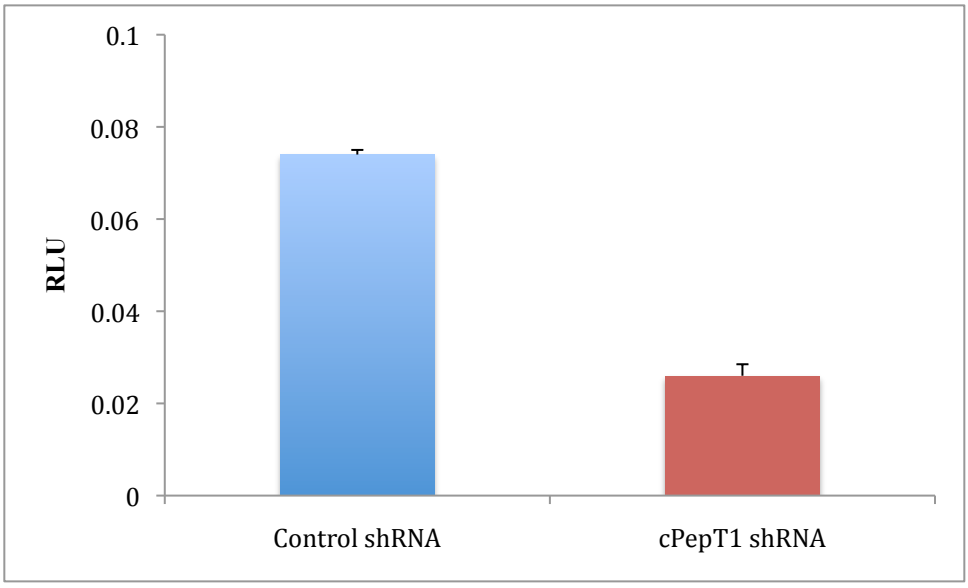


Figure 6.27. Knockdown of cPepT1 by FIV shRNA vectors. CEF were co-transfected with FIV cPepT1 shRNA 5/6 or FIV control shRNA 1/2 and pRL-SV40 as a transfection control. Three transfections were completed in duplicate. Cells were lysed and a dual luciferase assay was performed 48 h post-transfection. Data shown represent the mean Normalized RLUs ± SEM from two independent experiments (n= 6). The cPepT1 shRNA caused a 64% reduction in luciferase activity compared to the control shRNA ($P < 0.05$).

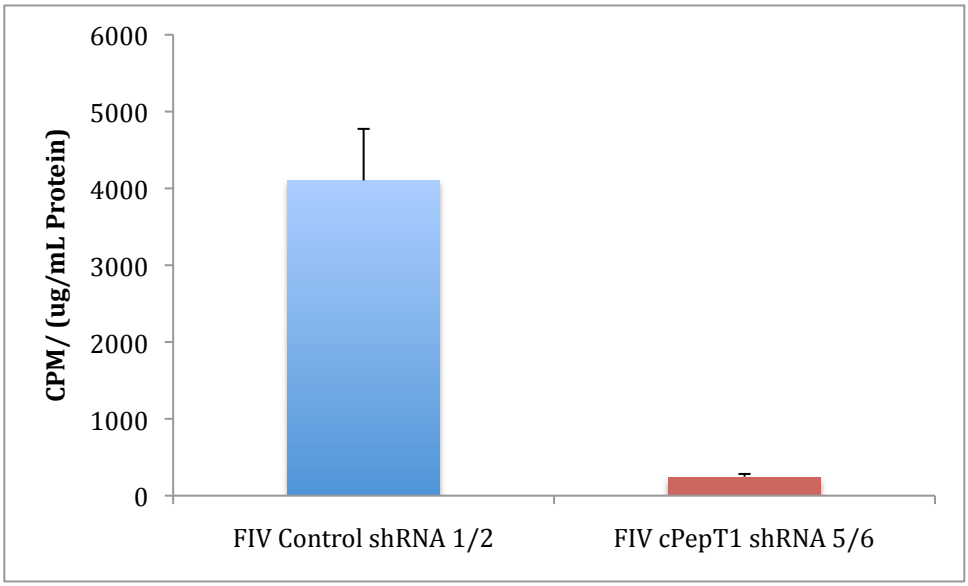


Figure 6.28. Knockdown of Gly-Sar Uptake by cPepT1 shRNA. CEF were co-transfected with FIV cPepT1 shRNA 5/6 or FIV control shRNA 1/2 and FIV CAG cPepT1 endogenous (to provide the PepT1 protein to CEF cells) Three transfections were completed in duplicate. Cells were lysed and a Gly-Sar uptake assay was performed 48 h post-transfection. Data shown represent the mean the mean CPM ± SEM / ($\mu g^{-1}mL^{-1}$ protein). The cPepT1 shRNA 5/6 caused a reduction of Gly-Sar uptake to background levels ($P < 0.001$)

Generation of G0 Mosaic Chickens

Overall, 9 potential G0 cPepT1 overexpressing chickens, 15 potential G0 cPepT1 shRNA expressing chickens, and 4 potential G0 control shRNA expressing chickens were generated at NCSU and VT. See Tables 6.3, 6.4, and 6.5 and Figure 6.29. Despite the success with the *ex ovo* culture system, the same success did not translate into production of a male a germline transgenic. However, PCR analysis indicated that the transgene could integrate as shown by one blood positive male (Figure 6.30.). Although it is uncommon for non-integrated lentivirus to be present in the cell at this stage and PCR is commonly used to screen for the transgene (McGrew et al., 2004), integration and site could be confirmed by southern analysis or splinkerette PCR. Gel electrophoresis of all the tissues from female potential G0 chickens revaled no tissue positive for any transgene.

Several methods have been used to generate transgenic chickens over the past decade. The most successful methods involve injection of replication incompetent virus particles into the developing embryo, thus generating somatic and germline mosaic founder animals. The efforts described in this dissertation followed this general methodology. Although the general methodology applied by the groups cited above, the exact techniques used across the groups varies significantly. See Table 6.3 for a summary. This indicates that many factors may contribute to the success and/or failure of generating a transgenic chicken. Some of these factors include type of virus used, virus titer, egg culture system used, parental breed, and transgene insert.

Citation	Virus	Virus Titer (IU/mL)	Injection Site	Egg Culture System	Hatch Rate	Somatic Mosaic Rate	Germline Mosaic Rate	Germline Transmission Rate
Harvey et al.,2002	Retro (ALV)	2-7 X 10^6	Subgerminal cavity, Stage X	Small window	23.1-33%	23.5-28.6%	5.4-6.2%	1.5-1.8%
Kodama et al., 2008	Retro (MSCV)	3 X 10^8 to 5 X 10^{10}	Heart of 55h old embryo	Large Window/ Surrogate Egg	27%	23-100%	0.004 to 0.07 genome equivalents	0
Kamihira et al., 2005	Retro (MSCV)	1 X10^8 to 1.8 X 10^9	Heart of 55h embryo	Large Window/ Surrogate Egg	63%	100%	At least one positive rooster obtained	3.3%
McGrew et al., 2004	Lenti (EIAV)	10^7 to 10^{10}	Subgerminal cavity, Stage X	Large Window/ Surrogate Egg	27%	70%	0.5 to 1 genome equivalents	0-45% depending on G0 bird
Chapman et al., 2005	Lenti (HIV)	10^5 to 10^7	Subgerminal cavity, Stage X	Small Window	4%	NR	3 roosters produced	0.63% from only 1 G0 rooster
Rapp et al., 2003	Retro (ALV)	2-7 X 10^6	Subgerminal cavity, Stage X	Small Window	NR	NR	0.8%	0.0668%
Kwon et al., 2004	Retro (MoMLV)	> 10^9	Subgerminal cavity, Stage X	Small Window	10%	100%	Birds too young to test	NA
Lee et al., 2007	Retro (MoMLV)	NR	Subgerminal cavity, Stage X	Small window	8.3%	100%	NR, but at least 1 as G1 positive embryos were produced	None, transgene embryonic lethal
Mozdziak et al., 2003	Retro (SNV)	2.5 X 10^6 to 2 X 10^7	Subgerminal cavity, Stage X	Large Window/ Surrogate Egg	36%	10%	8 of 15 males were positive for transgene	0.89% from the one male that produced positive offspring, overall 0.12%
Lillico et al., 2007	Lenti (EIAV)	10^7 X 10^{10}	Subgerminal cavity, Stage X	Large Window/ Surrogate Egg	Not Reported	Not reported	1 rooster with 5% of sperm positive	4%

Table 6.3. Summary of G0 transgenic chickens made by various techniques.
NR= Not Reported

Pseudovirus Type. Two types of replication incompetent virus systems have been used to produce transgenic chickens: retrovirus and lentivirus. Lentivirus has the advantage over retrovirus that it infects non-dividing as well as dividing cells. Several types of retroviruses have been used to generate germline mosaics and hemi-and homozygous offspring. These include avian leukosis virus (Harvey et al., 2002 and Rapp et al., 2003), murine stem cell virus (MSCV) (Kodama et al., 2008; Kamihira et al., 2005), spleen necorsis virus (SNV) (Mozdziak et al., 2003), and Moloney murine leukemia virus (MoMLV) (Lee et al., 2007; Kwon et al., 2004). Those using lentivirus systems used replication incompetent human immunodeficiency virus (HIV) (Chapman et al., 2005) or equine infectious anaemia virus (EIAV) (McGrew et al., 2004 and Lillico et al., 2007).

For this experiment, despite using a retroviral system to create a lac Z expressing chicken Drs. Petitte and Mozdziak suggested to use a lentiviral system due to its advantages over the retroviral vectors. The feline immunodeficiency virus was chosen based on its commercial availability and perceived safety compared to an HIV based lentiviral system. Based on the variability in reports found in the literature, it is difficult to attribute the low hatchability in this research to the virus system used. However, the low integration (as demonstrated by low number of G0 mosaics) may be reflective of the FIV based virus not being able to infect chicken cells. When titering virus preparations, mouse cells were easily transduced by the FIV vector. However, in a few pilot studies for other experiments, such as attempts to produce stable cell lines, it was casually observed that transduction rates of other types of cells varied significantly. In particular, it was noted that chicken embryo fibroblasts did not appear to transduce well. Therefore, FIV based lentivectors may not be well suited for generation of a transgenic chicken

based on a poor ability to infect chicken cells and may have contributed to not producing a germline mosaic G0 chicken.

Virus Titer. At the time a stage X embryo is injected there is an estimated 60,000 cells (Karagenc et al., 1996). Furthermore, only 20-25 of those cells (correspondance with J.N. Petitte) will become germ cells. It is generally understood that one lentivirus particle will infect one cell. Therefore, when injecting embryos, at least 100,000 virus particles were injected to maximize the chance of infecting the precursor germ cells. Generally, all reports, including our efforts try to inject as many virus particles as possible. However, Kimihira et al. (2005) reported a toxic effect of using a retroviral titer of greater than 1.8×10^9. It is difficult to extrapolate this observed effect to the research presented here because of the different virus system and injection method used. Kimihira et al. (2004) used a retroviral based system and here a lentiviral based system was employed. Further, Kimihira et al. (2004) injected the body or the heart of embryos at a later developmental stage rather than the subgerminal cavity of a stage X embryo.

However, a possible toxic effect of the virus cannot be completely dismissed. In considering a possible toxic effect, it is unlikely that titer concentration contributed to low efficiency of transgenic production reported in this dissertation. The maximum virus titer produced during this research was about 3×10^8 virus particles per mL and to be cost effective on average about 150 to 200,000 virus particles were injected per embryo. This was on par with other successful efforts reported using lentiviral-based systems that did produce chickens. When learning the *ex ovo* and blastoderm injection technique for this research, embryos were mock injected with plain cell culture media (minus virus particles). Overall, the hatchability was about the same as what was seen with virus

injected embryos. This indicates that the virus itself was not contributing to the low success rate of this project.

Type of Egg Culture System. Two basic culture systems are used to generate transgenic chickens. The first system was generated by Perry et al., (1989) and further modified by Borwornpinyo et al., (2005). This is the method used to produce the birds described in this dissertation. The other method and its variations do not involve surrogate eggshells or a large window in the egg. Breifly, fertilized eggs are collected and set with the small end up so that the embryo positions itself upward. Then a small (4 mm X 4mm) window is cut in the small end of the egg to allow access to the embryo. The embryo is then injected and the small window is sealed with medical tape akin to Scotch tape or parafilm. The eggs are then incubated and hatched out under standard conditions. Despite the latter method being less invasive, it has not proved to be a more efficient culture system (Table 6.3 and Borwornpinyo et al., 2005).

A review of the literature reveals the hatch rate achieved in this dissertation research was lower than that described by other methods using the *ex ovo* surrogate system. However, despite this when consulting with Drs. Petitte and Modziak, they did not seem that it was of concern or abnormal. The lower hatachability was probably a result with familiarity with the system or other external factors such as facility. The birds produced from this experiment hatched out in three different facitilies, one at NCSU (the overexpressing chickens) and two at Virginia Tech due to lab space reassigments. Further, different personel worked on injection and preparation of the eggs. As new personel were trained, hatachability fluctuated. Particularly, an increased loss during the transfer from System II to System III (surrogate chicken to surrogate turkey shells) was

seen when new personel learn the technique. The transfer involves pouring the embryo from one egg to the other. If this process is not done gently, significant damage to the embryo ensues. Also, at this time the yolk is particularly fragile and breaks easily. This often occurs when people are learning the technique. Therefore, the low hatch rate observed in this study was likely due to the "learning curve" associated with the *ex ovo* culture technique itself.

Parental Breed Type. The research described here attempted to generate birds with altered PepT1, an intestinal nutrient transporter. Broiler chickens have lower gene expression of PepT1 when compared to egg-layer chickens. Ideally, overexpression of PepT1 would have been conducted in a broiler line and underexpression would have been done in a layer line. However, reproductive efficiency and performance are inversely related and chickens are no exception. The reproductive efficiency of broiler chickens is lower than that of layer chickens. Therefore, broilers were not suitable to use for the production of a transgenic chicken. As layers already have increased gene expression of PepT1, they were not the ideal system to overexpress PepT1. Therefore, initially Barred Plymouth Rock chickens were used to develop the transgenic lines. They are intermediate with respect to growth rate and reproductive efficiency and have an intermediate level of PepT1 expression. This approach is also advantageous as PepT1 could be over and underexpressed in the same genetic background.

Barred Plymouth Rocks were used to generate the cPepT1 overexpressing chickens at NCSU. The cPepT1 shRNA and control shRNA expressing chickens were then completed at Virginia Tech. Due to reproductive problems with the Virginia Tech Barred Plymouth Rock flock as a result of flock downsizing and age, this breed was

abandoned for a leghorn type chicken. Specifically, the donor embryos were from lines of chickens that have undergone long term genetic selection for high or low antibody production to sheep red blood cells. Despite this long-term selection, no difference in hatchability using the *ex ovo* culture system was observed.

In the research described in this dissertation, no apparent somatic or germline mosaic for cPepT1 shRNA was observed. This may be due to the ability of the FIV to infect chicken cells. Different breeds of chickens may have different susceptibilities to viruses. This may account for why somatic mosaics were produced using the Barred Plymouth Rocks as donors but no somatic mosaics were produced using the Antibody response selected leghorn chickens. However, different transgene constructs were also used, making it impossible to do anything beyond speculate on this point.

Transgene Effect. The generation of an intestinally specific overexpression PepT1 transgenic chicken is ideal. However, a lentiviral based approach does not allow for targeted integration. This may be overcome by the use of a tissue specific promoter to drive transgene expression. There are no characterized chicken intestine specific promoters that are strong enough to drive reporter gene expression in cells. Preliminary experiments using various cPepT1 promoter fragments did not indicate that those fragments would function to drive intestine specific transgene expression in a transgenic animal. Despite the potential for deleterious effects, a ubiquitous approach was utilized for the generation of cPepT1 overexpressing chickens.

These chickens were generated at NCSU with the help of Dr. Petitte and his lab technicians who are well versed in the generation of and maintenance of transgenic birds and the Barred Plymouth Rock flock used as embryo donors. The stress of the *ex ovo*

culture system is known to produce weaker chicks and in particular leg abnormalities and weakness. The reasons for this are unknown. These abnormalities were increased in number in the cPepT1 overexperssing G0 chicks than what has been seen with the Barred Rocks and *ex ovo* culture system. This may be a direct result of ubiquitous cPepT1 overexprssion despite PepT1 having no known direct influence on bone or muscle development and growth. The yolk is a finite source of nutrients for the developing embryo. Development of the embryo occurs during the first two-thirds of gestation, with growth occurring in the last third of gestation. If the developing embryo is mosaic in the intestine, then more uptake of amino acids would be occurring, thus depleting the store in the yolk prematurely. This would potentially lead to growth and other abnormalities in the later stages of gestation, which would carry over to the hatched chick.

There was also an increased mortality of older cPepT1 G0 chickens when compared to prior transgenic and other chickens produced from the *ex ovo* culture system further suggesting that ubiquitous PepT1 overexpression is detrimental. This is not entirely unexpected since a ubiquitous promoter was chosen to drive exogenous cPepT1 expression. Peptides and their monomer amino acid components play significant roles in many other physiological functions (e.g. neuron signal transmission). Normal Pept1 expression is limited to a few tissues and cell types. Overexpression of PepT1 in, for example, the brain may cause aberrant signals between neurons by increasing the pool of amino acid precursors for neurotransmitters or the amount of amino acids, which are neurotransmitters themselves (glutamate). This hypothesis may translate to other tissues such as cardiac or skeletal muscle, which may have accounted for some of the abnormalities and sudden deaths.

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Figure 6.29. Two cPepT1 overexpression potential founder chickens at NCSU.

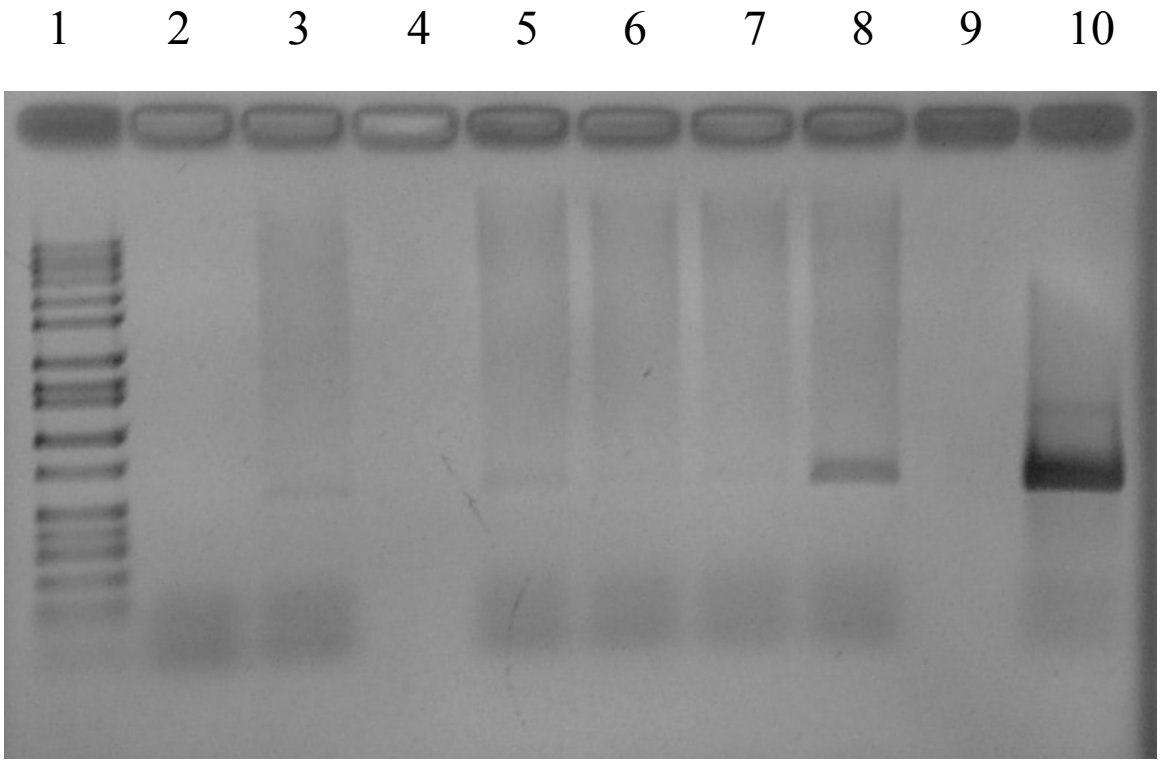

Figure 6.30. **Standard PCR test for the cPepT1-FLAG transgene in 4 male G0 chickens.** PCR reactions: Lane 1: marker; Lane 2: no DNA; Lane 3 non-transgenic barred rock DNA; Lane 4, no sample; Lanes 5-8 G0 males; Lane 9: no sample; Lane 10: plasmid control.

Injection Date	Number of Eggs Injected	Number of Chicks Hatched	Sex /Bird Number	Status	Blood	Semen or Testes
March 2009	50	5	Male/NSCU	Alive	Negative	Negative-semen
			Male/NSCU	Died, 22 wk	Negative	TBT
			Male/NCSU	Died, 13 wk pericarditis	Negative	Negative-Testes
			Male/NCSU	Died, 12 wk	Positive	Negative-testes
			ND	Died, 1 wk	ND	ND
May 2009	70	5	ND	Died, 1 d	ND	ND
			ND	Died, 2d	ND	ND
			Male/NCSU	Died, ? wk	TBT	TBT
			Male/NCSU	Died, ? wk	TBT	TBT
			Female/NCSU	Died, ? wk	TBT	TBT
June 2009	70	0				
September 2009	120	5	ND	Died, 1 d	ND	ND
			ND	Died, 1 d	ND	ND
			Female/NCSU	Killed, 7 months	TBT	TBT
			Male/NCSU	Alive	TBT	TBT
			ND	Died , 0 d	ND	ND

Table 6.4. G0 PepT1 overexpressing chickens. This table shows the results from the gnereation of G0 PepT1 overexpressing chickens at NCSU. The samples needed for the TBT were thrown out during a freezer failure at VT. It is unknown at this time if duplicates exist at NCSU. Information in this chart is current as of August 2010.

Injection Date	Number of eggs injected	Number of chicks hatched	Sex/Bird Number	Status	Line (LAS or HAS)	Blood	Semen
12/13/09	36	3	Female/VT811-812	Killed (Aug 2010)	LAS	Negative	NA
			Female/VT813-814	Killed (Aug 2010)	LAS	Negative	NA
			Female/VT815-816	Killed (Aug 2010)	LAS	Negative	NA
12/17/09	42	5	Male/VT801-802	Alive	LAS	Negative	Negative
			Male/VT803-804	Alive	LAS	Negative	Negative
			Male/VT805-806	Alive	HAS	Negative	Negative
			Female/VT807-808	Killed (Aug 2010)	LAS	Negative	NA
			Female/VT809-810	Killed (Aug 2010)	HAS	Negative	NA
1/4/2010	19	1	Male/VT821-822	Alive	HAS	Negative	Negative
1/6/2010	37	1	Female/VT823-824	Killed (Aug 2010)	HAS	Negative	NA
1/8/2010	36	1	Male/VT825-826	Alive	LAS	Negative	Negative
1/12/2010	39	2	Male/VT827-828	Alive	LAS	Negative	Negative
			Female/VT829-830	Alive Killed (Aug 2010)	HAS	Negative	Negative
1/14/2010	35	0					
1/18/2010	27	2	Male/VT831-832	Alive	LAS	Negative	Negative
			Male/VT833-834	Alive	LAS	Negative	Negative

Table 6.5. G0 cPepT1 shRNA expressing chickens. This table shows the results from the production of G0 cPepT1 shRNA expressing chickens at VT. Information in this chart is current as of August 2010.

Injection Date	Number of eggs injected	Number of chicks hatched	Sex/Bird Number	Status	Line (LAS or HAS)	Blood	Semen
2/2/2010	20	1	Male/VT835-836	Alive	TBT	TBT	TBT
2/9/2010	14	0					
2/16/2010	19	0					
3/15/10	21	0					
3/23/10	21	1	Male/VT 837-838	Alive	LAS		Not sexually mature
3/30/10	18	2	Male/VT 839-840	Alive	HAS		Not sexually mature
			Female/VT 840-841	Killed (Aug 2010)	HAS	Negative	NA
5/26/10	22	0					
6/4/10	31	0					
6/12/10	27	0					
6/20/10	46	0					
6/23/10	36	0					
6/25/10	16	0					

Table 6.6. G0 Control shRNA expressing chickens. This table shows the results from the production of G0 control shRNA expressing chickens at VT. New personel were being trained from 6/4/10 to 6/25/10. Information in this Table is current as of August 2010.

Conclusion

Exogenous cPepT1-FLAG, cPepT1 shRNA and control shRNA lentiviral vectors and pseudovirus were generated. Nine potential cPepT1 overexpressing G0 transgenic chickens were produced, one of which was a somatic mosaic. Fifteen cPepT1 shRNA and four control shRNA expressing G0 transgeneic chickens were produced, none which appeard to be somatic mosaics. None of the male G0 chickens of any construct appared to be germline mosaics.

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CHAPTER VII. EPILOGUE

This dissertation presented an overall project ambitious in scope and despite failing to produce an in vivo model, is not without scientific merit. Nutrient transporter analysis in conditionally immortalized mouse small intestinal cells offers insight to a poorly characterized but potentially quite useful in vitro model system. To date no study has analyzed the amino acid and peptide gene expression profile in the MSIE cell line. The analysis presented here characterizes these cells with respect to the expression of a panel of nutrient transporters expressed in enterocytes. To extrapolate on the data presented here would also prove useful. It would be beneficial to try and see if this line could be differentiated by possibly adding growth factors which promote epithelial cell differentiation to the media while growing at the permissible temperature. The nutrient transporters could then be used as markers as differentiation since they are only expressed in differentiated enterocytes. This could lead to the development of a very useful *in vitro* model for the study of anything related to the small intestine.

The *ex ovo* culture system and blastodermal injection combined with the A58 mutant technology could be used to develop a chicken small intestinal cell line analogous to the MSIE cell line. This would potential solve the problem of bacterial contamination that is seen when cell lines are developed from hatched chicks as the cells would be harvested from chicks before hatch. It would also solve the problem of differentiated cell death and from cells harvested from non-A58 mutant tissue, as the A58 mutant transgene renders the cells conditionally immortal. An A58 lentiviral vector was made during this project an intended to be used in the above fashion, but this project was never progressed any further.

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There is still value in developing a chicken model as it is recognized that a chicken is not merely a feathered mouse. The mouse has not been subjected to generations of intense selection pressure for rapid growth and development. Therefore, the chicken may perform much differently than the mouse does without expression of PepT1, thus indicating its significance to the chicken. As feed is the greatest expense in chicken production, this analysis would prove useful in determining feed formulations or markers for genetic selection. It is believed that using blastodermal injection of lentiviral particles carrying the construct of interest would still generate a transgeneic chicken model if enough potential G0 chickens were generated. This is because the method is strictly a game of chance and eventually a germline cell will be infected in a chick that hatches. In the effort described in this dissertation, it was demonstrated that the construct could integrate into cells as indicated by gereation of a blood positive mosaic chicken. Therefore, it is just a "numbers game" and eventually a germline mosaic would be produced.

As far as the development of a transgenic chicken line, more efforts to further develop the technology utilizing primordial germ cells should be considered. Primordial germ cells are the equivalent to mouse embryonic stem cells in terms of generating transgenic animals. Using primordial germ cells versus blastodermal injection would increase the efficiency of generating a germline transgenic many fold. By definition primordial germ cells are the progenitors of the germline, therefore by inserting the transgene into these cells, all birds produced would be germline positive for the transgene. As previously discussed, the main hurdle with primordial germ cells is the fact that no promoter seems active in the immortalized culture maintained by Dr. Petitte

at NCSU. A simple study would include running a transcriptome sequencing analysis or microarray analysis to determine what genes are normally highly expressed in the primordial germ cells. From there the promoters of highly expressed genes could be characterized and used to drive reporter gene constructs in primordial germ cells. After the development of an active promoter, transgenic chickens could theoretically be made in the same fashion as the mouse. This way targeted gene disruption could be attained. An added feature of using primordial germ cells is that the cells can be sexed, thereby allowing the production of only male G0 birds, which are germline positive. The ex ovo culture system reliably produces offspring, therefore the chances would be great that a true knock out model in the chicken could be attained by using manipulated primordial germ cells.

Most significantly, this dissertation presents for the first time *in vitro or in vivo*, evidence that transport of di- and tri- peptides by PepT1 is not compensated for by amino acid transporters but more likely by other brushborder peptide transporters. As a PepT1 knockout mouse model exists, the next logical step would be to analyze the same panel of transporters in intestinal tissue. Further investigation into mechanisms of compensation other than changes in gene expression would also prove valuable. In the knockout mouse model, the level and location of free amino acid and peptide transporters could be examined. This would aid in answering if changes in protein turnover, protein trafficking membrane incorporation were involved in compensating. To determine compensation at the functional level would be slightly more complex but could be done. For example, since PepT1 and HPT1 both transport Gly-Sar and Valcylcolvir, a PepT1 null mouse

could be bred to a HPT1 null mouse and the transport of Gly-Sar and protein and gene expression of amino acid and peptide transporters could be examined.

If one wanted to continue with the *in vitro* model, it would make sense to create a stable line of Caco-2 cells that express a PepT1 shRNA. This could be accomplished by transfecting Caco-2 with a plasmid containing a PepT1 targeting shRNA, or transducing them with a virus that will integrate a PepT1 shRNA gene into the Caco2 cells. This would create a nice *in vitro* model, which would be more high-throughput and economical for drug screening assays. The information produced from the *in vitro* or *in vivo* models would be extremely valuable for the therapeutic and nutritional industries, whose focus has been on PepT1 for many decades.

Overall, this research present in this dissertation establishes that changing the gene expression of free amino acid or other peptide transporters in enterocytes does not compensate for a reduction in the PepT1 trasnporter in the Caco-2 *in vitro* intestinal model