Riboflavin Transporters and Breast Cancer Resistance Protein: Cimetidine-Riboflavin Interactions in the Mammary Gland

by

Liana Dedina

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Pharmacology and Toxicology

University of Toronto

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ABSTRACT

Mother's milk provides multiple benefits to the offspring. However, xenobiotics transferred into breast milk may pose a risk to the nursing infant. The breast cancer resistance protein (BCRP) actively transports xenobiotics into breast milk. BCRP also transports nutrients, like riboflavin, and together with recently identified riboflavin transporters (RFT), may provide a mechanism for riboflavin secretion into breast milk. Expression of RFT in the mammary gland remained unknown. Our objective was to characterize Bcrp and Rft mRNA expression in the mammary gland of FVB/N mice, and investigate a strategy to decrease excretion of BCRP-transported xenobiotics into the milk using riboflavin intervention. Rft and Bcrp mRNA were upregulated in the mammary gland of lactating mice. An intravenous riboflavin administration significantly reduced the levels of BCRP-transported cimetidine in milk. This study demonstrates the use of riboflavin to exploit the function of mammary BCRP in order to reduce xenobiotic secretion into breast milk.
ACKNOWLEDGEMENTS

I would like to thank Dr. Shinya Ito, my supervisor, without whose zealous support and guidance none of my thesis work would have been possible. Our weekly meetings with Dr. Ito have been of the most productive nature during my time at the Hospital for Sick Children. He has always been patient in answering my questions, and always happy to share his knowledge and wisdom with me. For this, I will remain forever grateful.

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My journey to the Land of Graduate Studies has been an experience never to be forgotten.
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<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
<td></td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
<td></td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>ESCC</td>
<td>Esophageal squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
<td></td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
<td></td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and Eosin</td>
<td></td>
</tr>
<tr>
<td>LCM</td>
<td>Laser Capture Microdissection</td>
<td></td>
</tr>
<tr>
<td>MADD</td>
<td>Multiple acyl-CoA dehydrogenation deficiency</td>
<td></td>
</tr>
<tr>
<td>MIQE</td>
<td>Minimum Information for Publication of Quantitative Real-Time PCR Experiments</td>
<td></td>
</tr>
<tr>
<td>MP ratio</td>
<td>Milk-to-plasma ratio</td>
<td></td>
</tr>
<tr>
<td>MMLV-RT</td>
<td>Moloney Murine Leukemia Virus reverse transcriptase</td>
<td></td>
</tr>
<tr>
<td>NBF</td>
<td>Nucleotide-binding folds</td>
<td></td>
</tr>
</tbody>
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## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RFT</td>
<td>Riboflavin transporter</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCP</td>
<td>Toronto Centre for Phenogenomics</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. THESIS OVERVIEW AND STATEMENT OF PROBLEM

Mother's milk provides a multitude of benefits to the offspring. However, if the lactating mother receives medication, the breastfed infant may be at risk of developing drug toxicity through breast milk exposure.

Mechanisms of drug excretion into milk are complex, involving passive diffusion and carrier-mediated transport. One of the transporters, breast cancer resistance protein (BCRP), is apparently upregulated in the lactating mammary gland, playing a key role in excretion of its substrate drugs and toxins into milk. Importantly, BCRP has been shown to function as a mammary transporter for riboflavin (vitamin B\(_2\)). The latter observation suggests a dual role of mammary BCRP, both as a xenobiotic and a nutrient transporter.

In this thesis, I describe a proof-of-principle study, exploring the potential of BCRP-based nutrient (riboflavin) – cimetidine interaction as a method to reduce drug excretion into milk.

2. BACKGROUND

2.1. Riboflavin

2.1.1 Discovery of Riboflavin

In 1879, an English chemist Alexander Wynter Blyth described the composition of cow's milk (Blyth, 1879). One of the chemical components isolated was a yellow pigment, which Blyth named “lactochrome”.

For over 30 years after Blyth’s description, the yellow pigment remained uncharacterized. Then, an observation made by McCollum and Kennedy initiated a flurry of research in the field. In their studies on prevention of the deficiency state of pellagra, McCollum and Kennedy noted the effectiveness of a tissue extract on pellagra (McCollum and Kennedy, 1916). Further examinations revealed that the tissue extract contained two separate fractions: a heat-labile fraction, and a heat-stable one. The latter fraction was shown to contain a yellow factor, which was shortly purified and termed riboflavin or vitamin B\(_2\) (Emmett and Luros, 1920).

The physiological importance of riboflavin remained unknown until the breakthrough discovery made by Otto Warburg and Walter Christian in 1932 (Massey, 2000). They isolated an enzyme, dubbed “old yellow enzyme”, containing yellow factors
- flavin nucleotides - acting as co-enzymes for the protein function. This “old yellow enzyme” is now known as a flavoprotein NADPH dehydrogenase, which requires flavin mononucleotide (FMN) a cofactor.

In the late 1930’s chemical structures of both FMN and flavin adenine dinucleotide (FAD) were identified (Rivlin and Pinto, 2001). Since then many enzymes were identified to employ FAD and FMN not only as cofactors, but also covalently binding flavins to gain functional activity (McCormick, 1994).

### 2.1.2 Structure and Properties

Riboflavin (7,8-dimethyl-10-ribityl-isooxazine) is a weak base, with a pKa of about 10.2 (Nardiello et al., 2003). The name “riboflavin” comes from the ribityl side chain and the isooxazine ring system (Figure 1), which imparts a yellow colour to the vitamin (from Latin flavus for “yellow”), (Massey, 2000). The planar isooxazine ring provides a structure to both riboflavin and its coenzymes (Rivlin and Pinto, 2001).

When excited with UV light, flavins exhibit a high degree of fluorescence, a property that can be used to detect riboflavin and its coenzymes in various assays, including quantification by high-pressure liquid chromatography (Zempleni, 1995; van Herwaarden et al., 2007).

In addition to FAD and FMN, there are other naturally occurring flavins derived from riboflavin (McCormick, 1994). Lumichrome and lumiflavin are photodegradation products of riboflavin formed under acidic and basic conditions, respectively. Both, however, are biologically inactive. Therefore, one of the key physicochemical properties of riboflavin is its sensitivity to UV light. For this reason, phototherapy of neonatal jaundice may result in deficiency of riboflavin, requiring supplementation of this vitamin (Sisson, 1987).

### 2.1.3 Synthesis of FMN and FAD

Flavin coenzymes are synthesized from riboflavin via a chain of enzymatic reactions (Rivlin, 1970). First, flavokinase phosphorylates riboflavin to produce FMN. Some FMN can be used directly; however, the major fraction of FMN produced is converted to FAD via a phosphorylation reaction catalyzed by FAD synthetase. These reactions are reversible: phosphatases can dephosphorylate FAD to FMN, and in turn, to riboflavin. Interestingly, FAD, rather than FMN, is the most common coenzyme used by flavoproteins (Rivlin and Pinto, 2001).
Figure 1. Riboflavin and its coenzymes
2.1.4 Riboflavin Sources, Absorption, Protein Binding and Excretion

2.1.4.1 Dietary Sources and Safety

Riboflavin is an essential water-soluble vitamin, necessary for normal cellular function, development and growth (Rivlin, 1986). Riboflavin and its coenzymes are crucial for carbohydrate and fat metabolism, and thus energy production within the cell (Buehler, 2011).

Humans and other vertebrates cannot synthesize riboflavin de novo, and therefore, must obtain it from the diet, or in case of a fetus and neonates from maternal sources (Swaan, 2011). Riboflavin and its coenzymes can be found in organ meats, green vegetables and dairy products. It is known that riboflavin is excreted into milk (Thomas, 1980), with levels of 0.36 mg/L and 1.75 mg/L reported in human and cow milk, respectively. In the case of humans, milk riboflavin concentrations are about 100-fold higher than maternal plasma concentrations (Lawrence, 2005a). In addition, riboflavin can be acquired from the intestinal bacteria, indigenous to human colon (Swaan, 2011).

The recommended daily allowance of riboflavin in an adult is about 1.1 to 1.3 mg per day. The official Health Canada recommendations for daily intake of vitamin B$_2$ are summarized in Table 1. Riboflavin appears to have no known toxicity (Ames et al., 2002). Doses as high as 400 mg/day of riboflavin were used for migraine prophylaxis, without any adverse events being recorded (Shoenen et al., 1998; Boehnke et al., 2004).

2.1.4.2 Absorption

The majority of dietary sources of riboflavin come in the form of its derivatives and flavoproteins, with principal absorption happening in the proximal part of the small intestine (Rivlin and Pinto, 2001). Absorption half-life is reported to be about 1.1 hour (Zempleni et al., 1996). Before absorption can occur, FMN and FAD are hydrolyzed to riboflavin, in a non-specific phosphatase-catalyzed reaction on the brush border membrane of enterocytes. It has been reported that the upper limit of intestinal absorption of riboflavin is about 25 mg, given at a single time (Zempleni et al., 1996).

Based on studies conducted in HepG2 human-derived liver cells (Said et al., 1998), intestinal brush border membrane vesicle (Said and Arianas, 1991) and human trophoblast-derived BeWo cells (Huang and Swaan, 2001), it was generally accepted that riboflavin transport across the cellular membrane was carrier-mediated. However,
Table 1. Recommended Dietary Allowance Values for Riboflavin
(Health Canada values as based on IOM 2006)

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Riboflavin (mg/day)</th>
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<tbody>
<tr>
<td>Children</td>
<td></td>
</tr>
<tr>
<td>1-3 y</td>
<td>0.5</td>
</tr>
<tr>
<td>4-8 y</td>
<td>0.6</td>
</tr>
<tr>
<td>Adolescent males</td>
<td></td>
</tr>
<tr>
<td>9-13 y</td>
<td>0.9</td>
</tr>
<tr>
<td>14-18 y</td>
<td>1.3</td>
</tr>
<tr>
<td>Adult males</td>
<td></td>
</tr>
<tr>
<td>≥ 19 y</td>
<td>1.3</td>
</tr>
<tr>
<td>Adolescent females</td>
<td></td>
</tr>
<tr>
<td>9-13 y</td>
<td>0.9</td>
</tr>
<tr>
<td>14-18 y</td>
<td>1.0</td>
</tr>
<tr>
<td>Adult females</td>
<td></td>
</tr>
<tr>
<td>≥ 19 y</td>
<td>1.1</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
</tr>
<tr>
<td>14-50 y</td>
<td>1.4</td>
</tr>
<tr>
<td>Nursing females</td>
<td></td>
</tr>
<tr>
<td>14-50 y</td>
<td>1.6</td>
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the exact mechanism of riboflavin transport within the intestinal and other epithelia remained unknown until recently (van Herwaarden et al., 2007; Yonezawa et al., 2008), when riboflavin transporters were identified. More on the specific transporters involved will follow in the subsequent sections. Receptor-mediated endocytosis of riboflavin internalization was proposed as an alternative mechanism for this vitamin’s entry into the cell (Huang et al., 2003). However, the significance of this observation is still to be determined.

Intestinal absorption of riboflavin can be affected by various factors. For instance, in experimental animals with riboflavin deficiency or ariboflavinosis, riboflavin absorption was elevated (Said and Mohammadkhani, 1993). Also, alcohol may negatively impact both absorption and digestion of dietary flavins (Pinto et al., 1987). Other drugs and metals, forming complexes with riboflavin, may also have an impact on the vitamin’s bioavailability (McCormick, 1990). Examples of such include iron and zinc metals, as well as caffeine and ascorbic acid; however, the clinical significance of these is not well understood.

After its systemic absorption, riboflavin is converted to FMN and FAD inside the tissues (Swaan, 2011). One third of total body riboflavin is contained within the liver, while excess amounts of this vitamin are excreted in urine.

2.1.4.3 Protein Binding

In human blood, riboflavin is non-covalently bound to protein, with strong and weak associations reported for immunoglobulins and albumin, respectively (Innis et al., 1986). Specific riboflavin binding proteins are present in pregnancy and are important for fetal development. For example, in hens lacking the expression of avian riboflavin carrier protein, riboflavin deficiency occurs in eggs, shortly followed by embryonic death (White and Merrill, 1988). Studies conducted in other species have indentified similar riboflavin binding proteins in humans, rats, cows and moneys, as well as the importance of these proteins for proper development (Krishnamurthy et al., 1984; McCormick, 1994).

Within the tissues, FAD and FMN are bound to enzymes, both covalently and non-covalently (Singer and Kenney, 1974; McCormick, 1994). Free flavins are quickly hydrolyzed to riboflavin, which is readily excreted. Therefore, it seems that phosphorylation of riboflavin derivatives is critical for capturing of FAD and FMN within the cells (Gastaldi et al., 2000).
2.1.4.4 Excretion

Most riboflavin excretion occurs through urine, with only a minor part present in the stool (Rivlin and Pinto, 2001). Urinary excretion of flavins occurs mainly in the form of riboflavin, which contributes to about 60% to 70% of urinary flavins (Chastain and McCormick, 1987). In addition, other derivatives are also present in urine, including 7-hydroxymethylriboflavin, 10-hydroxymethylriboflavin, lumiflavin and others. These represent metabolites from both microorganismal degradation of riboflavin and flavoproteins.

2.1.5 Biological Functions of Flavins – Riboflavin, FAD and FMN

Riboflavin’s main function in the body is to serve as a precursor for FAD and FMN (Rivlin and Pinto, 2001). These coenzymes are essential for many of biological oxidation-reduction reactions. For instance, FAD is an important component of cellular respiration, as it functions in the electron transport chain reaction, making riboflavin critical for energy metabolism.

Other roles of riboflavin include drug and endobiotic metabolism via cytochrome P450 enzyme systems (Massey, 2000). Specifically, it is the NADPH-cytochrome P450 reductase that utilizes both FAD and FMN as its coenzymes. Furthermore, FAD and FMN are required for function of various other flavoproteins, including Acyl-CoA dehydrogenases, disulphide reductases and mono-oxygenases. Together, flavoproteins catalyze a wide range of biologically vital reactions.

Although riboflavin itself is not a strong antioxidant, its phosphorylated derivatives have a notable antioxidant potential (Rivlin and Pinto, 2001). Within the cell, the glutathione redox cycle provides a powerful protection against lipid peroxides and some drug metabolites (Forman et al., 2009). These can be deactivated by glutathione peroxidase, an enzyme requiring reduced glutathione (GSH) for proper function. GSH is regenerated from its oxidized form (GSSG) by glutathione reductase, a FAD-containing enzyme. Therefore, riboflavin and its coenzymes are also imperative for cell protection against oxidative stress.

2.1.6 Riboflavin Deficiency and Relevance in Disease

Clinical features of riboflavin deficiency include angular stomatitis, glossitis and dermatitis (Rivlin and Pinto, 2001). However, these are not unique characteristics, and may be due to multiple vitamin deficiencies. Nonetheless, numerous studies have
associated riboflavin deficiency with various abnormalities and diseases.

2.1.6.1 Developmental Abnormalities

Abnormal fetal development was documented in rodents with maternal riboflavin deficiency. Both skeletal and soft tissue malformations were described (Warkany and Nelson, 1972). In a human case-study, maternal riboflavin and folic acid deficiencies were implicated in cleft lip and palate abnormalities in two newborns (Faron et al., 2001); however, because riboflavin levels were not established in the study, this association remains elusive.

2.1.6.2 Iron Status and Gastrointestinal Development

Early studies of riboflavin deficiency in both humans and animals pointed to this vitamin’s involvement in proper function of the hematopoietic system. Foy and Kondi described a case of pure red cell aplasia, which was successfully rescued by riboflavin treatment (Foy and Kondi, 1953). In the follow-up studies of non-human primates fed a riboflavin-deficient diet, abnormalities in both red blood cell production and iron status were described (Foy et al., 1964).

In the past decades, possible mechanisms of riboflavin deficiency-induced iron handling alterations were proposed. Flavins, in their reduced form, were noted to mobilize ferritin iron (Sirivech et al., 1974; Crichton et al., 1975). Moreover, in tissues of rats fed riboflavin-deficient diets, iron mobilization was inefficient as compared to control animals (Sirivech, 1977; Powers, 1987). Further support that riboflavin levels may influence iron status comes from human studies. Here, an enhanced response to iron supplementation was seen when riboflavin deficiency was corrected (Decker et al., 1977; Powers et al., 1983). Taken together, these findings suggest the importance of riboflavin and its coenzymes in iron mobilization and absorption, as well as hematologic status.

In addition, riboflavin deficiency is associated with gastrointestinal abnormalities in weanling rodents (Powers et al., 1991; Williams et al., 1995). Weanling rats fed a riboflavin-deficient diet showed early morphologic changes in the gastrointestinal tract. The exact implications of these findings for human populations remain to be established; however, dietary deficiency of riboflavin and potential effects on gastrointestinal maturation should not be disregarded.
2.1.6.3 Cancer

The precise role and importance of riboflavin status in carcinogenesis is not quite clear. Some studies show a protective effect of riboflavin deficiency in some cancers, while others indicate a reduced risk of cancer development with adequate riboflavin levels (Rivlin, 1973; Webster et al., 1996). In epidemiological studies of esophageal cancer, low dietary riboflavin was associated with elevated risk of cancer development (Van Ransberg, 1981; Foy and Kondi, 1984). Furthermore, reduced incidence of esophageal cancer was observed in Linxian, China, after a combined daily regimen of riboflavin and niacin supplementation (Blot et al., 1993).

On the molecular level, riboflavin deficiency was implicated in an increase in DNA strand breaks in rats exposed to carcinogens (Webster et al., 1996). This increased breakage was shown to be reversible on riboflavin supplementation. Nonetheless, more studies are needed to evaluate the mechanisms by which riboflavin can modulate carcinogenesis.

2.1.6.4 Vision

Occurrence of cataracts in riboflavin-deficient animals has been observed (Hughes et al., 1981; Wintrobe et al., 1994). However, the implication of riboflavin deficiency for cataract etiology in humans is not yet understood.

Cases of riboflavin-responsive night blindness were reported (Venkataswamy, 1967). It is thought that riboflavin-dependent photoreceptors in the retina are involved in dark adaptation (Miyamota and Sancar, 1998), and therefore riboflavin deficiency may be associated with night blindness.

2.1.6.5 Cardiovascular Diseases

As indicated by animal studies (Hultquist et al., 1993; Betz et al., 1994; Mack et al., 1995), riboflavin may be protective in ischemia-reperfusion injury. Coupled with its antioxidant properties and lack of known toxicity, riboflavin may become a potential candidate for tissue protection against oxidative damage.

In the Framingham Offspring Cohort (Jacques et al., 2001), riboflavin intake was indicated to reduce plasma homocysteine levels. Plasma homocysteine is a risk factor for developing cardiovascular disease (Boushey et al., 1995; Humphrey et al., 2008). Not surprisingly, methylenetetrahydrofolate reductase, an enzyme metabolizing folate in a reaction later leading to homocysteine formation, uses FAD as its cofactor (Horigan et al., 2010). Moreover, a common mutation 677C→T in methylenetetrahydrofolate
reductase leads to elevated plasma homocysteine levels (Kang et al., 1991), which are reported to be modulated by riboflavin status in the individuals with this polymorphism (Hustad et al., 2000).

2.1.7 Riboflavin Overdose

There is no known toxicity attributed to riboflavin overdose. Excess riboflavin is readily excreted in the urine (Rivlin and Pinto, 2001). For migraine prophylaxis, riboflavin doses as high as 400 mg/day were used without any adverse events being recorded (Shoenen et al., 1998; Boehnke et al., 2004).

2.2. RIBOFLAVIN TRANSPORTERS

For decades, riboflavin transport has been studied using human-derived cell lines (Said and Ma, 1994; Huang and Swaan, 2000; Huang and Swaan, 2001; Kansara et al., 2005; Said et al., 2005). This research suggested a carrier-mediated transport of riboflavin, saturable in nature. It was not until 2008 that the first report of a mammalian riboflavin transporter was made (Yonezawa et al., 2008).

2.2.1 RFT1

2.2.1.1 Structure

Using an mRNA expression database selected from a rat kidney cDNA library, Yonezawa and colleagues searched among functionally unknown genes, and identified rat and human riboflavin transporters (rRFT1 and hRFT1, respectively). rRFT1 and hRFT1 are 450 and 448-amino acid proteins, respectively, which are predicted to have 10 putative transmembrane domains and a N-linked glycosylation site. Both transporters have predicted sequences for protein kinase COOH-dependent phosphorylation. rRFT1 and hRFT1 have 81.1% amino acid identity and 96.4% similarity. In addition, an inactive splice variant of hRFT1 was identified (Yonezawa et al., 2008). It encodes a 167-amino acid protein.

2.2.1.2 Tissue Distribution

Yonezawa and colleagues demonstrated that hRFT1 mRNAs were expressed strongly in small intestine and placenta, while moderately so in prostate, lung, uterus, colon and kidney. Weak expression of hRFT1 was demonstrated in liver, stomach, heart, whole brain, adrenal gland, skeletal muscle and spleen. Likewise, mRNA expression was assessed in rat tissues. Organs of high rRft1 mRNA expression in rats were small intestine, brain, colon, ovary, placenta, spleen and testis, while low expression was
detected in skeletal muscle, pancreas, kidney, liver and lung. Currently, nothing is known about RFT1 distribution in the mammary gland.

2.2.1.3 Cellular Localization

To study cellular localization of these proteins, Yonezawa and colleagues transfected HEK-293 cells with GFP-tagged RFT1. Both human and rat RFT1 proteins localized to the plasma membrane. Further evidence supporting hRFT1 and rRFT1 localization in plasma membrane came from a Western blot analysis performed in crude membrane and cell lysate preparations, probing for GFP-tagged RFT1. A strong signal was detected in crude membrane fraction for both proteins; however, only a slight signal was observed in cell lysate. In a later study, it was demonstrated that GFP-hRFT1 was localized basolaterally in human intestinal Caco-2 and canine kidney MDCK polarized epithelial cells (Veendamali et al., 2011). Together, these findings indicate that plasma membrane is the primary site of RFT1 localization.

2.2.1.4 Functional Characterization

Yonezawa and colleagues showed that overexpression of hRFT1 and rRFT1 in HEK-293 resulted in increased cellular accumulation of riboflavin. Riboflavin uptake was reduced in the presence of riboflavin analogues, specifically FAD, FMN and lumiflavin. Neither probenecid nor cimetidine had an influence on riboflavin uptake by the cells. Riboflavin uptake in HEK-293 and Caco-2 cells was significantly reduced after hRFT1 siRNA transfection. Moreover, siRNA treatment of cells did not alter uptake of other chemicals, including cimetidine. These observations underscored the involvement and likely specificity of RFT1 in riboflavin transport.

Riboflavin transport by hRFT1 was also concluded to be independent of sodium, pH and membrane potential. In addition, Michaelis-Menten kinetic analysis revealed the apparent $K_m$ values for the uptake at pH 7.4 by HEK-293 and Caco-2 cells to be 28.1 nM and 63.7 nM, respectively (Yonezawa et al., 2008). Another set of experiments determined the apparent $K_m$ value of hRFT1 in HEK-293 cells to be 1380 nM at pH 7.4 (Yao et al., 2010). The reason for the large discrepancy between the apparent $K_m$ values reported by Yonezawa et al. and Yao et al. is unknown. Neither of the authors commented on the sequence of the cDNA and whether any mismatches or mutations were encountered.

2.2.1.5 RFT1 in Other Species

Homologues of hRFT1 were identified in other species by BLAST search in the
GenBank database (Moriyama, 2011). These include: mouse, chimpanzee, macaque, and others. These RFT1 proteins exhibit over 69% identity and 91% similarity to hRFT1. More specifically, mRFT1 shows 82% identity and 96% similarity to hRFT1. mRFT1 is a 450-amino acid protein, with inferred structure consisting of 11 putative transmembrane domains (Figure 2).

2.2.1.6 Summary

In conclusion, RFT1 is likely to play a role in riboflavin transport, specifically in the process of intestinal absorption and mother-to-fetus transfer of this vitamin. Furthermore, research suggests that RFT1 shows no significant similarity to bacterial riboflavin transporters; and therefore, RFT1 is currently classified as belonging to a novel family of mammalian riboflavin transporters.

2.2.2 RFT2

Soon after Yonezawa et al. functionally characterized RFT1, a homologous transporter was identified and termed RFT2 (Yamamoto et al., 2009).

2.2.2.1 Structure

Yamamoto et al. focused on characterization of rRFT2, which was identified by BLAST searches of amino acid sequence of a novel bacterial candidate riboflavin transporter impX (Vitreschak et al., 2002). rRFT2 consists of 463 amino acids, and contains 11 potential transmembrane domains and a putative N-glycosylation site. It is also 57% similar to rRFT1 (Yamamoto et al., 2009). Yamamoto and colleagues also found a cDNA coding for an orthologous protein in humans, which was termed hRFT2. hRFT2 has 469-amino acids with 83% similarity to rRFT2, and 42.9% identity with hRFT1. Its function was later characterized by Fujimura et al. (2010).

2.2.2.2 Tissue Distribution

Analysis by Northern blot revealed high rRft2 mRNA expression in ileum, jejunum and testis (Yamamoto et al., 2009). Moderate expression was observed in lung, kidney, stomach and colon, while other organs - brain, heart, liver and skeletal muscle – showed a low expression. Further analysis by RT-PCR detected rRft2 mRNA expression in all of the above tissues; however small intestine, kidney and testis stood out as the organs with the greatest expression.

RT-PCR analysis of hRFT2 mRNA revealed small intestine to have among the highest expression, suggesting potential of this transporter in riboflavin absorption.
Figure 2. Inferred Structure of Mouse Riboflavin Transporter 1 (mRFT1). Hydropathy analysis was performed using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), based on predicted mRFT1 protein sequence (NP_083919.1).
(Fujimura et al., 2010). Other organs, including placenta and testis, also showed high mRNA levels of hRFT2. Moderate to low expression was noted in skeletal muscle, kidney and stomach, while brain, lung, liver and colon showed no detectable signal. In a different study, RT-PCR analysis confirmed the above pattern of hRFT2 tissue distribution (Yao et al., 2010). In addition, prostate was identified as a tissue of high expression, while spleen, thymus and uterus showed little to no expression. Currently, no data are available about RFT2 distribution in the mammary gland.

2.2.2.3 Cellular Localization

Cellular expression of rRFT2 was examined via GFP-tagged rRFT2 (Yamamoto et al., 2009). The signal localized to the plasma membrane of HEK-293 cells. Strong signal was detected in crude membrane fractions using Western blot, indicating rRFT2 expression in cellular membranes. Interestingly, transfection with GFP-tagged rRFT2 revealed enhanced riboflavin uptake activity when compared to GFP-tagged rRFT1 transfected cells. These results suggest that rRFT2 may have a greater activity in riboflavin transport than rRFT1.

Expression of GFP-rRFT2 in the polarized MDCKII canine renal tubular cell line showed localization at the apical membrane (Fujimura et al., 2010). This observation suggests the likely expression of rRFT2 at the brush border membrane in the small intestine. In addition, rRft2 mRNA expression in the small intestine was upregulated in rats fed a riboflavin-deficient diet (Fujimura et al., 2010), suggesting an adaptive response to increase riboflavin absorption from the gut. Together, these observations further implicate rRFT2 in riboflavin absorption from the intestinal lumen.

To visualize cellular expression of hRFT2, EGFP-tagged hRFT2 was transfected into HEK-293 cells (Yao et al., 2010). Fluorescence was detected in the plasma membrane. Additionally, Western blot indicated a strong signal for EGFP-tagged hRFT2 in crude membrane fractions. Subramanian et al. (2011a) showed GFP-hRFT2 to be exclusively expressed at the apical membrane of the polarized human intestinal Caco-2 and canine kidney MDCK epithelial cells, implying a functional role and involvement of hRFT2 in riboflavin uptake form the intestinal lumen.

Surface expression of hRFT2 was found to be determined by C-terminal sequence, specifically by the conserved cysteine residues C463 and C467 (Subramanian et al., 2011b). Mutation of these residues resulted in significant reduction of riboflavin uptake. This was further explained by retention of the mutated hRFT2 in the
endoplasmic reticulum. Furthermore, mutating an additional cysteine C386, predicted to be involved in disulfide bridge formation with C463, resulted in intracellular retention of hRFT2 (Subramanian et al., 2011b). Taken together, these findings suggest a crucial role of specific cysteine residues in the C-terminus of hRFT2. Mutations in these residues may be associated with loss of cell surface localization and function of hRFT2.

2.2.2.4 Functional Characterization

Riboflavin transport via rRFT2 was found to be Na\(^+\)-independent and unchanged by the cell potential. However unlike hRFT1, rRFT2-mediated transport was moderately altered by pH, being more efficient under ambient acidic conditions (Yamamoto et al., 2009). Kinetic analysis showed that rRFT2 transport was saturable (\(V_{\text{max}}\) 11 pmol/min/mg protein), and had an apparent \(K_m\) of 210 nM in transiently transfected HEK-293 cells (Yamamoto et al., 2009).

Substrate specificity of rRFT2 was also assessed (Yamamoto et al., 2009). It was found that riboflavin derivatives (lumiflavin, FAD and FMN) significantly lowered riboflavin transport. Moreover, lumiflavin was found to have the highest affinity among the riboflavin analogues for rRFT2, followed by FMN and then FAD. Nevertheless, lumiflavin’s affinity was still about 5-fold lower than that of riboflavin, indicating that this vitamin is likely the preferred substrate for rRFT2. Other chemicals, including folate and cimetidine were found to have no inhibitory effect on riboflavin transport by rRFT2.

Similar characterization was performed for hRFT2 (Fujimura et al., 2010). Efficiency of riboflavin transport was affected by pH, being optimal in the acidic range. Replacement of sodium chloride with choline chloride in the medium did not result in changes of hRFT2 riboflavin transport, suggesting a Na\(^+\)-independent mechanism. A slight reduction in riboflavin transport was noted with cellular membrane depolarization; however, the biological significance of this observation remains unclear. Kinetic analysis in HEK-293 showed riboflavin transport by hRFT2 to be saturable (\(V_{\text{max}}\) 22 pmol/min/mg protein), with an apparent \(K_m\) of 770 nM at pH 6. At pH 7.4 apparent \(K_m\) was reported to be 980 nM (Yao et al., 2010).

Similar to rRFT2, riboflavin uptake by hRFT2 was significantly reduced in the presence of riboflavin derivatives, and unaffected by 1mM cimetidine and imipramine (Fujimura et al., 2010). However, Yao et al. (2010) reported about 15% reduction in riboflavin uptake in the presence of 1 mM cimetidine. In addition, non-riboflavin derivatives ethidium and methylene blue also inhibited riboflavin transport in HEK-293
cells, yet to a lower extent (Fujimura et al., 2010).

Yamamoto et al. (2009) also showed that riboflavin transport activities of hRFT2 and rRFT2 exceeded those of hRFT1 and rRFT1. Similarly, Subramanian et al. (2011a) reported that an increase of riboflavin uptake in HuTu-80 cells was significantly higher than that seen with hRFT1 and hRFT3. These observations may indicate that RFT2 possesses a more dominant role in riboflavin transport and absorption than RFT1.

### 2.2.2.5 RFT2 in Other Species

RFT2 appears to be well conserved across different species, including mouse and panda (Moriyama, 2011). In mouse, two isoforms of mRFT2 are present: 460- and 250-amino acid in length, respectively. These are thought to be produced by alternative splicing, with the longer isoform being chosen as a canonical sequence. mRFT2 shares 90% identity with rRFT2.

### 2.2.2.6 Summary

RFT2 is likely another player in riboflavin transport within the cells and organisms. Its expression and cellular localization suggest RFT2 involvement in riboflavin homeostasis, particularly the absorption of this essential vitamin from the small intestine. Overall, given the structural and functional similarities between RFT1 and RFT2, RFT2 is classified as a member of the RFT family of mammalian riboflavin transporters.

### 2.2.3 RFT3

Identification and functional characterization of hRFT3 followed shortly after the other two members of the RFT family were discovered.

#### 2.2.3.1 Structure

By searching for the human homolog of hRFT1, Yao et al. identified a sequence, later termed hRFT3 (Yao et al., 2010). hRFT3 cDNA is 1451 base pairs long, and encodes a 445-amino acid protein. Structurally, hRFT3 is predicted to have 10 putative membrane-spanning domains. Furthermore, hRFT3 has 86.7% amino acid identity with hRFT1, 44.1% with hRFT2, 81.5% with rRFT1, and 43.1% with rRFT2.

#### 2.2.3.2 Tissue Distribution

RT-PCR analysis for hRFT3 expression in various human tissues revealed strong expression in both fetal and adult brain, as well as salivary gland (Yao et al., 2010). Tissues such as small intestine, liver, kidney and lung were found to have low expression of hRFT3 mRNA. These observations suggest that hRFT3 may be more
important in brain homeostasis of riboflavin, rather than the absorption of this vitamin from the gut. Currently, nothing is known about RFT3 distribution in the mammary gland.

2.2.3.3 Cellular Localization

Cellular localization of hRFT3 was investigated by two different groups. Yao et al. (2010) introduced EGFP-tagged hRFT3 into HEK-293 cells. Subsequent fluorescence was observed mainly in the plasma membrane of transfected cells. When localization of GFP-hRFT3 was examined by Subramanian et al. (2011a) in polarized epithelia – intestinal Caco2 and renal MDCK, hRFT3 was found predominantly within the intracellular vesicles, with some expression detected at the basolateral membrane. Such observations suggest that regulatory processes may be in place, which mediate hRFT3 translocation to and from the basolateral membrane, thereby maintaining riboflavin homeostasis and transport within the epithelial cells.

2.2.3.4 Functional Characterization

Riboflavin uptake was significantly increased following HEK-293 transfection with hRFT3. In addition, riboflavin transport by hRFT3 was found to be Na\(^+\)-independent. Similar to hRFT1, no pH dependency for hRFT3-mediated riboflavin uptake was observed (Yao et al., 2010).

Uptake studies conducted in HEK-293 cells revealed an apparent K\(_m\) for hRFT3 to be 330 nM, and a V\(_{\text{max}}\) value of 5.2 pmol/min/mg protein (Yao et al., 2010). Similar to other RFT, riboflavin uptake by hRFT3 was significantly reduced in the presence of riboflavin and its analogues, while 1mM folate and probenecid had no effect on this vitamin’s uptake in HEK-293 cells. Interestingly, both thiamine and cimetidine at 1 mM were found to reduce riboflavin uptake by about 20% (Yao et al., 2010).

2.2.3.5 RFT3 in Other Species

Unlike with the other RFT, no homologues of hRFT3 are identified in rodent species. The only reported species to possess these homologues are chimpanzee and gibbon (Moriyama, 2011).

2.2.3.6 Summary

hRFT3 is the most recently identified member of the RFT family of mammalian riboflavin transporters. Its high mRNA expression in the brain suggests that hRFT3 may have a role in brain riboflavin homeostasis. Moreover, hRFT3 is similar in sequence to hRFT1, but shows less similarity to hRFT2.
2.2.4 Differential Expression and Function of RFT

Subramanian et al. (2011a) investigated the expression patterns and functional activity of the three human RFT in polarized epithelia. Their findings are summarized below.

Cellular localization of hRFT1 was primarily to the basolateral membrane in Caco-2 and MDCK cells, while hRFT2 was exclusively found at the apical domain of these cells. Distinctively, hRFT3 was mainly expressed in the intracellular vesicular structures, with some expression at the basolateral membrane of some cells (Subramanian et al, 2011a). These observations suggest RFT alone may form a transporter network necessary for absorption and trans-epithelial transport of riboflavin, thereby regulating vitamin B2 homeostasis.

Assessment of relative amounts of hRFT mRNA in differentiated Caco-2 cells and native human small intestine revealed that hRFT2 mRNA levels were significantly higher than those of hRFT1 and hRFT3 (Subramanian et al, 2011a). Coupled with the observations that riboflavin uptake in HuTu-80 cells was significantly higher in the presence of hRFT2 than with either hRFT1 or hRFT3 (Subramanian et al, 2011a), it is possible that hRFT2 plays a chief role in riboflavin absorption and transport, at least in the small intestine.

2.2.5 RFT and Disease

Although very little is known about association of RFT with diseases, a few studies have linked RFT with certain conditions.

2.2.5.1 Esophageal Squamous Cell Carcinoma and hRFT2

A genome-wide association study of esophageal squamous cell carcinoma (ESCC) was conducted in a Chinese population (Want et al., 2010). The study identified a novel locus of susceptibility, corresponding to single nucleotide polymorphisms (SNPs) in the hRFT2 gene.

Riboflavin deficiency may contribute to the risk of developing certain cancers, specifically increasing the risk of ESCC (Siassi and Ghadirian, 2005). In addition, riboflavin supplementation has been reported to reduce the risk of ESCC (He et al., 2009).

Therefore, an association between hRFT2 and ESCC seems plausible; especially given the information that hRFT2 may be predominantly apically-located in
the intestinal cells and more effective in transporting riboflavin than its orthologs. Nevertheless, functional characterizations of hRFT2 and ensuing associations are needed to be explored further.

2.2.5.2 Maternal riboflavin deficiency and hRFT1

Ho and colleagues (2010) have described a case of a newborn female, who presented with biochemical and clinical symptoms of multiple acyl-CoA dehydrogenase deficiency (MADD). Riboflavin supplementation corrected this deficiency. Further investigations have revealed that the mother was riboflavin deficient, suggesting the likely reason for newborn’s MADD.

Investigators went further, and screened the mother for possible mutations in hRFT1 and hRFT2 (hRFT3 was not characterized at the time). Two missense variations were identified in hRFT1; however, in vitro functional analysis showed that riboflavin transport was not affected by these variations. Additional analysis revealed a deletion in the hRFT1 gene in one of the maternal alleles. It was concluded that maternal haploinsufficiency may result in riboflavin deficiency in both the nursing mother and the newborn.

Although this is a case-report study, it may lead to further investigations of the reasons behind riboflavin deficiency and potential associations with MADD.

2.2.5.3 Brown-Vialetto-Van Laere Syndrome and hRFT2

The hRFT2 mutations were identified in patients with a rare neurological disorder - Brown-Vialetto-Van Laere syndrome, characterised by progressive ponto-bulbar palsy and bilateral sensorineural hearing loss (Bosch et al., 2010). In a different study, the same syndrome was also associated with hRFT2 (Green et al., 2010). However, both studies neglected to assess hRFT1 and hRFT3 in their genetic analyses, and possible contributions of these genes to disease etiology. In order to further validate these claims, a functional analysis of the identified mutations is necessary.

2.3. BREAST CANCER RESISTANCE PROTEIN

2.3.1 The ABC Transporter Superfamily

The ATP-binding cassette (ABC) transporters are a superfamily of active transport proteins (Higgins, 1992). They are present and plentiful in genomes of both eukaryotes and prokaryotes (Davidson and Chen, 2004; Annilo et al., 2006; Robey et al., 2009). Interestingly, in eukaryotic cells ABC transporters are almost always efflux
transporters, transporting substrates from cytoplasm out of the cell (Robey et al., 2009). Also, the function of almost all ABC transporters is thought to involve ATP hydrolysis.

In the human genome, there are 48 ABC transporter genes (Robey et al., 2009). The transporters are classified into subfamilies, based on the conservation of ATP-binding domains or nucleotide-binding folds (NBF) (Robey et al., 2009). Seven of eight eukaryotic subfamilies (A through G) are present in the human genome.

ABCG2, or breast cancer resistance protein (BCRP), is a highly conserved member of the ABC transporter superfamily. This gene has been found in all sequenced vertebrates, including fish and chicken (Annilo et al., 2006).

Other ABC members include p-glycoprotein (ABCB1, Pgp) and multidrug resistance-associated proteins (ABCC, MRP). Pgp is known to function as a drug transporter, assisting in xenobiotic elimination (Ito and Alcorn, 2003). Pgp was reported to be expressed in both non-lactating and lactating mammary glands (van der Valk et al., 1990; Alcorn et al., 2002). Interestingly, lactation seems to down-regulate Pgp mRNA levels (Alcorn et al., 2002).

MRPs comprise another subfamily of transporters in the ABC superfamily (Dean et al., 2001). Several proteins, specifically MRP1, MRP2 and MRP5 have been reported to be expressed in the mammary gland. However, the precise localization of MRPs in the mammary gland is unclear (Ito and Alcorn, 2003).

2.3.2 BCRP Structure
The human BCRP gene is located on chromosome 4 and contains 16 exons and 15 introns (Robey et al., 2009). BCRP is a 72 kDa protein, consisting of 665 amino acids. BCRP is predicted to have six transmembrane segments and an extracellular loop between the fifth and sixth segment. The BCRP gene encodes a “half transporter”, since only one NFB and ATP-binding domain are present in BCRP, compared to two each in most other ABC proteins. It is suggested that BCRP may homodimerize or oligomerize in order to function (Ozvegy et al., 2001; Kage et al., 2002; Henriksen et al., 2005; McDevitt et al., 2006).

2.3.3 BCRP Substrates
To date, many BCRP substrates have been identified (Table 2). However, no clear structure-function relationship is observed among these chemicals (Robey et al., 2009). One of the first discovered substrate groups for BCRP was chemotherapeutic
<table>
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<tr>
<th>Drug Class</th>
<th>Substrate</th>
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<tr>
<td>Antivirals</td>
<td>Zidovudine, Abacavir</td>
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<tr>
<td>Dietary carcinogens</td>
<td>Aflatoxin B1, PhIP, Dipyridamole</td>
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<tr>
<td>Antibiotics</td>
<td>Ciprofloxacin, Erythromycin, Nitrofurantoin</td>
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<td>Chemotherapeutic drugs</td>
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<td>Other drugs</td>
<td>Cimetidine, Riboflavin, Glyburide</td>
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agents, including imatinib, mitoxantrone, topotecan and others (Polagar et al., 2008). Other drug classes have also been shown to be BCRP-substrates, such as antivirals, carcinogens and flavins (Jonker et al., 2005; Robey et al., 2009). Therefore, BCRP is considered to be a promiscuous cellular efflux pump with a broad spectrum of xenobiotic and endobiotic substrates.

2.3.3.1 Cimetidine

Because cimetidine was used as a model drug to investigate Bcrp transport of riboflavin in vivo (see Hypotheses and Rationale), a brief summary of pertinent information is provided below.

Cimetidine is a histamine H₂-receptor antagonist, used to treat peptic ulcers and gastric hypersecretory conditions. Oral bioavailability of cimetidine is 65-80%, with 20% plasma protein binding and an apparent volume of distribution of 1-2 L/kg. Cimetidine clearance occurs mainly via the kidney, with about 20% undergoing metabolism, and its half-life in adults is about 2 hours (Bennett et al., 1996). In humans, metabolites of cimetidine include sulfoxide, hydroxymethyl and guanyl urea derivatives (Figure 3).

In both rodents and humans cimetidine is transported into milk. In FVB mice, milk-to-plasma (MP) ratio at 30 minutes was reported to be about 13 (Jonker et al., 2005). In Sprague Dawley rats milk-to-serum ratios of about 29 were recorded (McNamara et al., 1996), or in other words drug concentration in the milk is 29 fold higher as compared to plasma levels (See section 5. Mammary Gland). In humans, MP ratios range from 3 to 11, depending on the time since last dose administration (Somogyi and Gugler, 1979). Comparable mean observed milk-to-serum ratio for humans is reported to be 5.77 (Oo et al., 1995).

2.3.4 Cellular Localization, Tissue Expression and Function

Upon its discovery, cellular localization and tissue expression of BCRP were investigated. It was determined that BCRP was expressed on the apical membrane of epithelial cells, and involved in an efflux transport of substrates out of the cells Vlaming et al., 2009). Northern blot analyses revealed high to moderate levels of BCRP mRNA in placenta, brain, prostate, testis, kidney, liver, small and large intestine. Low or absent expression was reported for heart, lung, pancreas, skeletal muscle, spleen, peripheral blood leukocytes and thymus (Doyle et al., 1998; Fetsch et al., 2006).
Figure 3. Cimetidine and its Metabolites
A number of studies have investigated the functional role of BCRP in the organs of high expression. Below follows a brief summary of these investigations.

2.3.4.1 Placenta

Placental BCRP is thought to have a protective effect for the fetus, transporting drugs and other potential toxins from the fetal to the maternal side (Jonker et al., 2000; Zhang et al., 2007). Placental perfusion studies in both rats and humans suggest that BCRP removes toxins from the fetal space (Staud et al., 2006; Myllynen et al., 2008). In addition, studies have shown that BCRP limits fetal penetration of glyburide (Gedeon et al., 2008; Zhou et al., 2008), an antidiabetic drug used in treatment of gestational diabetes (Moore, 2007).

2.3.4.2 Gastrointestinal Tract

Further investigations of BCRP expression in small and large intestines revealed highest expression in the duodenum, followed by decreasing levels along the gastrointestinal tract, from ileum to the rectum (Gutmann et al., 2005).

Multiple studies suggest that BCRP in the gastrointestinal tract can limit the oral absorption of its substrate xenobiotics. For instance, Bcrp knock-out mice were shown to have increased intestinal uptake of quercetin (Sesink et al., 2005), sulfasalazine (Zaher et al., 2006), dietary carcinogen PhIP (Jonker et al., 2005), and nitrofurantoin (Merino et al., 2005) as compared to wild-type mice.

2.3.4.3 Brain and Testis

BCRP functions in both blood-testis barrier and blood–brain barrier. A study conducted in BCRP-deficient mice suggests a protective role of this transporter for germ cells (Enokizono et al., 2008). In the brain, BCRP was shown to localize to the microvessel endothelium (Cooray et al., 2002), suggesting brain to blood transfer of BCRP-substrates. For example, penetration of imatinib, a BCRP-substrate xenobiotic, through the brain-blood barrier was enhanced in Bcrp knock-out mice (Oostendorp et al., 2008).

2.3.4.4 Kidney and Liver

Due to the major drug elimination activities of liver and kidney, BCRP functional variation in those tissues may affect the pharmacokinetics of many drugs by changing their clearance.

In the kidney, BCRP was reported to localize to the kidney cortical tubule (Fetsch et al., 2006) and the proximal tubule brush border membrane (Huls et al., 2008). In addition, findings that sulfates (Mizuno et al., 2004; Mizuno et al., 2007) have an
impaired renal clearance in Bcrp knock-out mice suggest the possible role of this transporter in renal drug elimination.

In the liver, BCRP localized to the canalicular membrane (Maliepaard et al., 2001). In addition, BCRP expression was noted in the bile ducts and endothelium (Vander Borght et al., 2006), as well as luminal membrane of gall bladder epithelial cells (Aust et al., 2004). Biliary excretion of many xenobiotics was impaired in BCRP-deficient animal models (Hirano et al., 2005; Merino et al., 2005; Enokizono et al., 2007), providing support for a potential role of BCRP in hepatic drug clearance.

2.3.4.5 Mammary Gland

Recent studies indicated that BCRP is also expressed in mammary glands of human, mouse, sheep and cow (Jonker et al., 2005). Mammary expression of BCRP is induced in pregnancy and lactation, with levels decreasing after weaning (Jonker et al., 2005). BCRP is found on the apical side of alveolar epithelium, where it secretes its substrates into milk, thereby exposing suckling newborns to various xenobiotics (Jonker et al., 2005; van Herwaarden et al., 2006).

BCRP upregulation in the lactating mammary gland suggested a potential endogenous role for this transporter. More specifically, it was hypothesized that BCRP may play a role in nutrient transfer from mother to infant (Vlaming et al., 2009).

Van Herwaarden and colleagues (2007) were able to show that milk secretion of riboflavin was reduced by 60-fold in Bcrp −/− mice as compared to wild type mice. Furthermore, levels of FMN were reduced 6-fold, suggesting that this cofactor may also be a substrate for BCRP. Yet, pups from Bcrp −/− mothers did not show any signs of riboflavin deficiency. The authors suggested that the most likely explanation for this observation is the fact that the transport into the milk of FAD, the other riboflavin-derived cofactor, was not perturbed in Bcrp −/− mothers.

Levels of other vitamins in milk of Bcrp −/− mice were also analyzed (van Herwaarden et al., 2007). Biotin (Vitamin B7) was decreased in the milk of knock-out animals by 3.5 fold. Interestingly, folic acid levels were found unchanged in both wild-type and Bcrp −/− mice (van Herwaarden et al., 2007), even though folate was shown to be transported by BCRP in vitro (Assaraf, 2006).

Overall, BCRP transport of riboflavin in milk appears to be the most affected in Bcrp −/− mice. The lack of developmental abnormalities associated with riboflavin deficiency in the pups fed by Bcrp −/− mothers suggests that there may be multiple
pathways available for transporting vitamin B\textsubscript{2}-derived cofactors into the milk. Nevertheless, as a transporter initially thought to have no known endogenous role, Bcrp may be essential for mammary transfer of nutrients into the milk.

2.3.5 Cancer and BCRP

BCRP expression in cancer cell lines has been associated with drug resistance (Robey \textit{et al.}, 2009). Therefore, various studies have tried to determine the role of BCRP in cancer patients. It has been noted that BCRP may confer drug resistance in leukemia (Ross \textit{et al.}, 2000; Benderra \textit{et al.}, 2004). However, this issue is still controversial (Robey \textit{et al.}, 2007). It has been suggested that BCRP may have a role in chemotherapeutic resistance of solid tumours in the digestive tract, endometrium and lung (Diestra \textit{et al.}, 2002). Although breast cancer has been the most researched, BCRP expression is usually reported to be low (Robey \textit{et al.}, 2007).

2.4. THE MAMMARY GLAND

Mammary gland consists of two components: the parenchyma and the stroma. The parenchyma is a collective term applied to the alveoli and branching ductal system within the gland. Two main cell types are found in the mammary ducts: inner epithelial cells and outer myoepithelial cells. The stroma, made mainly of adipose, serves as both the support and the substrate for parenchymal growth (Howard and Gusterson, 2002; Richert \textit{et al.}, 2000).

2.4.1 Human Breast

2.4.1.1 Anatomy and Development of the Human Breast

2.4.1.1.1 Prenatal Stage

In both males and females, breast development starts pre-natally at about 7 to 8 weeks post-conception. At this point, only tissue thickening could be observed. At 12 to 16 weeks breast components begin to emerge: some cells become the foundation for mammary ducts and glands, while others differentiate into muscles of nipple and areola. In late pregnancy, maternal hormones stimulate formation of ducts in the fetus. These hormones even stimulate the lobules, the milk producing glands, to secrete colostrum. This phenomenon subsides shortly after birth (Howard and Gusterson, 2002; Lawrence, 2005b; Hens and Wysolmerski, 2005).
2.4.1.1.2 Postnatal Stages

Until puberty, no difference can be observed between male and female breasts. However at puberty, release of estrogen followed by progesterone stimulates further development of female breast. Mature female breast is then comprised of four main structures of lobules (glands), fat, milk ducts and connective tissue (Lawrence, 2005b, Watson and Khaled, 2008).

Mammary alveoli are the basic units of mammary gland. They are lined with cuboidal cells (milk-secreting cells) and surrounded by contractile myoepithelial cells (similar to structures described in Section 2.4.2). Alveoli are grouped together to form lobules, which are further grouped into larger structures are known as lobes. The lobes empty into lactiferous ducts. These structures lined with non-secreting columnar epithelium. Lactiferous ducts dilate within the areola to form lactiferous sinuses, structures which allow for milk accumulation during lactation. The lobes are surrounded by stroma, a structure consisting of adipose, connective tissue, vasculature and lymphatics (Howard and Gusterson, 2002; Lawrence, 2005b, Watson and Khaled, 2008).

With age and at menopause, estrogen levels drop in women. As a result, the lobules involute and are replaced with fatty tissues, thereby loosing their support (Howard and Gusterson, 2002).

2.4.1.1.3 Human Breast during Pregnancy and Lactation

Elevated levels of hormones, such as progesterone and estrogen, have a dramatic effect on breast development during pregnancy (Page and Anderson, 1987). The mammary gland enlarges due to an increased proliferation of parenchymal (epithelial) cells, as well as due to the distension of the alveoli. And although the alveoli are able to secrete milk proteins in mid-gestation, this event is delayed by high levels of progesterone and ensues at parturition (Neville, 2001).

The lactating breast is composed of more epithelial cells, as a result of proliferation in pregnancy, and less adipose tissue surrounding the lobes (Lawrence, 2005b). At weaning, the suckling stimulus is terminated, and the breast undergoes involution. Here, the excess secretory epithelia are removed by apoptosis, and replaced by fat. The breast returns to its pre-pregnancy state: few acini and ducts remain and are dispersed throughout the adipose tissue (Howard and Gusterson, 2002).
2.4.1.2 Breastfeeding and Human Breast Milk

2.4.1.2.1 Benefits of Breastfeeding

Numerous advantages of breastfeeding are documented in the literature. Studies have shown that breastfeeding can positively impact infants’ health and development. For instance, breast milk was reported to lower the risk of infections associated with diarrhea (Dewey et al., 1995), otitis media (Owen et al., 1993), pneumonia (Write et al., 1989), meningitis (Cochi et al., 1986) and necrotizing enterocolitis (Lucas and Cole, 1990). In addition, higher cognitive function has been reported in individuals breastfed as children (Lucas et al., 1992; Mortensen et al., 2002; Kramer et al., 2008). Moreover, breast milk may modulate immune function, and has been noted to lower the risks of type 1 diabetes mellitus (Mayer et al., 1988; Samuelsson et al., 1993) and Crohn’s disease (Koletzko et al., 1989). Additionally, breastfeeding is associated with reduced incidence of breast cancer among women (Stuebe et al., 2009).

2.4.1.2.2 Human Breast Milk Composition

Breast milk is composed of numerous components, including proteins, lipids, carbohydrates, water-soluble vitamins and cellular components (Table 3) (Picciano, 2001; Lawrence, 2005c). Milk composition is not fixed and varies among women depending on the stage of lactation and maternal nutrition (Picciano, 2001).

Following parturition, the initial breast milk is known as colostrum, a thick yellowish fluid. Colostrum is high in proteins and water-soluble minerals and vitamins, while fat content is low. As breast milk matures, levels of fat, lactose and fat-soluble vitamins increases, while protein levels drop (Hibberd et al., 1982; Lawrence, 2005c).

2.4.1.2.3 Cells in Human Breast Milk

Mammary secretions collected at different stages of lactation contain various cell populations, including neutrophils, macrophages, lymphocytes and epithelial cells (Ho et al., 1979; Brooker, 1980; Boutinaud and Jammes, 2002). The highest number of cells is present in colostrum, and decreases as lactation continues. Similarly, the cells of the immune system show a decreasing pattern as nursing progresses. However, the proportion of epithelial cells in relation to total cell content increases from colostrum to mid-lactation (Ho et al., 1979; Brooker, 1980; Boutinaud and Jammes, 2002).

Epithelial cells and their fragments are shed into milk during the lactation (Boutinaud and Jammes, 2002; Maningat et al., 2009). Most of these epithelial cells can be identified as alveolar cells (Boutinaud and Jammes, 2002). As a result, numerous
Table 3. Composition of Human Breast Milk

<table>
<thead>
<tr>
<th>Category</th>
<th>Components</th>
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<tbody>
<tr>
<td>Lipids</td>
<td>Fatty acids</td>
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<tr>
<td></td>
<td>Triglycerides</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Lactose</td>
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<tr>
<td></td>
<td>Oligosaccharides</td>
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<tr>
<td>Minerals</td>
<td>Calcium</td>
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<tr>
<td></td>
<td>Chlorine</td>
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<tr>
<td></td>
<td>Sodium</td>
</tr>
<tr>
<td></td>
<td>Potassium</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Vitamin C</td>
</tr>
<tr>
<td></td>
<td>Choline</td>
</tr>
<tr>
<td></td>
<td>B vitamins</td>
</tr>
<tr>
<td></td>
<td>Fat-soluble vitamins</td>
</tr>
<tr>
<td>Nitrogen compounds</td>
<td>Carnitine</td>
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<tr>
<td></td>
<td>Urea</td>
</tr>
<tr>
<td>Cells</td>
<td>Leukocytes</td>
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<tr>
<td></td>
<td>Epithelial cells</td>
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<tr>
<td></td>
<td>Neutrophils</td>
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<tr>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td>Proteins</td>
<td>Caseins</td>
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<td></td>
<td>Peptide hormones</td>
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<tr>
<td></td>
<td>Growth factors</td>
</tr>
<tr>
<td></td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td></td>
<td>Lactalbumins</td>
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</tbody>
</table>
studies were able to isolate RNA from the milk cells in order to describe potential gene regulatory processes in the mammary gland (Smith-Kirwin et al., 1998; Alcorn et al., 2002; Maningat et al., 2009). Because studying the human breast during lactation is exceedingly difficult (Howard and Gusterson, 2002), this method is currently the least invasive way in trying to understand gene expression and regulation in the human mammary gland epithelia.

Until recently, RNA was isolated from the cell pellet obtained from centrifuged milk samples (Smith-Kirwin et al., 1998; Alcorn et al., 2002). However, the result interpretation of results was complicated by the presence of various cell types in the pellet. A novel method of studying human mammary epithelial cells was proposed by Maningat et al. (2007). It was known that milk fat was secreted through a budding mechanism, which could potentially bring along cytoplasmic portions of mammary epithelial cells enveloped in plasma membrane (Huston and Patton, 1990). In other words, some milk fat globules (mainly triglycerides surrounded by lipid bilayer) were found to contain epithelial cell cytoplasmic fractions. Based on these observations, Maningat and colleagues (2007) were able to show that RNA isolated from the milk fat globules was likely coming from the mammary epithelial cells, as suggested by the expression of mammary epithelial specific genes, such as milk-protein related genes.

2.4.1.2.4 Breastfeeding and Maternal Drug Therapy

Studies have shown that drugs can be excreted into breast milk via different mechanisms, including passive diffusion (Miller et al., 1967; Rasmussen, 1958) and carrier-mediated transport (Oo et al., 1995; McNamara et al., 1996; Gerk et al., 2001). The amount of drug excreted depended on the ionization of the drug, plasma protein binding, lipophilicity, molecular weight, and maternal pharmacokinetics. In general, drug excretion into milk is enhanced for low plasma protein bound and low molecular weight drugs, as well as lipophilic and cationic drugs (Ito, 2000). Lower pH and higher lipid content of milk as compared to plasma contribute to these observations (Ito and Lee, 2003).

Milk-to-plasma (MP) ratio is the ratio of drug concentration in breast milk as compared to drug concentration in maternal plasma. MP ratio can vary significantly over post-dose time, and therefore, is often represented as a time-averaged ratio (Ito, 2000). Most drugs have an MP ratio of 1 or less, and only about 15% of drugs have MP ratios greater than 2 (Ito and Koren, 1994). Although an MP ratio greater than 1 indicates that
the drug is accumulated in breast milk, the implications of such an observation may be clinically insignificant. For instance, if the infant ingested amount of drug is substantially lower than that required for therapeutic purposes, then regardless of the MP ratio of the drug, the level of exposure would be considered minimal (Ito and Lee, 2003). Therefore, the evaluation of drugs' MP ratios should be conducted in reference with other factors in order to gain clinical insight.

While many drugs are known to be safely taken by nursing mothers, few epidemiological studies have looked at adverse effects in breastfed infants as a consequence of maternal drug therapy. In a follow-up study of 838 breastfed infants exposed to a variety of drugs through maternal milk, 1 in 10 women reported alterations in their infants' condition. Nonetheless, none of these infants required medical attention (Ito et al., 1993). However, numerous case reports have indicated possible infant toxicity as a result of maternal drug therapy (Matheson et al., 1985; Chasnoff et al., 1987; Schimmel et al., 1989; Lester et al., 1993; Frey et al., 1999; Madadi et al., 2009). It is, therefore, not surprising that breastfeeding may reduce maternal adherence to drug therapy (Ito and Lee, 2003). Currently, there are no intervention strategies to decrease drug excretion into milk.

2.4.2 Anatomy and Development of the Mouse Mammary Gland

Among different mammals, anatomy and development of mouse mammary gland is perhaps the best understood and characterized (Hovey et al., 1999; Richert et al., 2000). It bares noteworthy similarities with human mammary gland, which also make mouse a suitable model.

2.4.2.1 Prenatal Development

At embryonic days 10-11 mammary streak appears. It extends from the posterior to anterior limb bud, and is a result of the ectodermic enlargement. By day 12, an epithelial bud, a mammary rudiment, becomes visible. It increases in size and takes on a bulb-like shape. On day 16, rapid epithelial proliferation occurs, forming a mammary sprout, which penetrates the mammary fat pad precursor tissue. This growth continues until birth, forming a rudimentary ductal system (Richert et al., 2000).

2.4.2.2 Postnatal Development

At approximately three to six weeks of age, mouse mammary ducts begin to grow rapidly. Highly proliferative structures, known as terminal end buds (TEB), are
located at the ends of ductal branches, and are responsible for this fast expansion. TEBs are composed of two cell types. The cap cells, found on the outside, interact with the surrounding stroma, and later differentiate into myoepithelial cells of the mature duct. The body cells, found inside the TEBs, differentiate into ductal epithelial cells. Ductal development is slowed down, as the mouse matures, and TEBs form terminal ducts. By 10-12 weeks, the majority of postnatal mammary development has occurred, and alveolar buds are visible (Figure 4, A). These alveolar buds will fully differentiate in pregnancy (Richert et al., 2000; Sternlicht, 2005).

2.4.2.3 Pregnancy, Lactation and Involution

Initially, pregnancy induces rapid proliferation of ductal branches and formation of additional alveolar buds. In mid-pregnancy, alveolar buds differentiate into alveoli, the ultimate structure of milk secretion. By late pregnancy, alveoli are visible throughout the mammary fat pad and the ratio of epithelia to adipocytes increases drastically (Richert et al., 2000).

Mammary gland continues to grow in early phases of parturition. As lactation establishes, mammary adipocytes are metabolized and alveolar epithelia expand within the mammary gland (Figure 4, B) (Rillema, 1994; Richert et al., 2000).

After weaning, the mammary gland goes through a process termed involution. Involution is a process of cell death and remodeling (Quarrie et al., 1996). Initially, involution is reversible (Li et al., 1997; Furth, 1999). However, 2 days after suckling cessation the process is irreversible. Here, the apoptosis of secretory epithelial cells is followed by the degradation of lobular structures and mammary basement membrane (Strange et al., 1992). By day 6 of involution, epithelium and stroma are being rearranged (Figure 4, C) (Richert et al., 2000).

2.4.2.4 Species Differences

As described above, there are many similarities between human and mouse mammary gland development, structure and function. In addition, relative ease of manipulation and short gestation term (18-21 days) make mouse an acceptable and representative model to study human mammary gland physiology and pathology.

Nevertheless, there exist species differences in mammary gland morphogenesis that are worth mentioning. For example, human mammary parenchyma undergoes a more profound lobulation during development when compared to that of a mouse (Hovey et al., 1999). In addition, cellular composition and stromal architecture is specie-
Figure 4. Hematoxylin and Eosin Staining of Mammary Glands: (A) 10-week-old virgin mouse, (B) 1-week lactating mouse, (C) 1-week involuting mouse; magnification, x10. Mammary glands were isolated from FVB/N mice and fixed in 10% PFA overnight. Tissues were stained with hematoxylin, followed by washing and eosin counterstain. Tissues were dehydrated in 95% and absolute alcohols, to remove excess eosin. Samples were mounted on the slides and scanned using Aperio ScanScope. Mammary epithelial cells are stained in deep purple.
specific, providing a unique regulatory pattern of mammary development in humans and mice (Hovey et al., 1999). Furthermore, unlike the identical in utero mammary development in human males and females, in male mice testosterone induces condensation of mammary epithelium, resulting in irregular mammary shape, and therefore, a distinction between developing male and female mice (Kratochwil and Schwartz, 1973; Richert et al., 2000).

Another obvious difference is the number of mammary glands: humans have one pair, while most mouse strains have 5 pairs, 3 thoracic and 2 inguinal (Cardiff and Wellings; 1999). The ductal system is also more complex in humans: a single nipple may contain five to ten lactiferous ducts, which branch in a radial pattern. In contrast, a single lactiferous duct marks the termination of mammary structure in rodents (Cardiff and Wellings; 1999).

Overall, mice are suitable models for studying mammary development and functions. However, caution is advised when directly extrapolating from mice to humans, as multiple species differences exist.

3. STATEMENT OF RESEARCH HYPOTHESES

Central Hypothesis: Riboflavin inhibits BCRP-mediated drug excretion into the milk.

Specific Hypotheses:

1. RFT are expressed in the mammary gland, and their expression is upregulated in lactation.
2. RFT are expressed on the plasma membrane of mammary epithelia.
3. Riboflavin reduces the excretion of BCRP-substrate xenobiotic cimetidine into the milk in FVB/N mice.

4. OBJECTIVES

4.1 Characterize temporal and spatial profiles of mammary RFT in:

1. Mouse and human mammary epithelial cell lines
2. Human mammary cells (cells from the breast milk)
3. Mouse mammary glands and epithelial cells

4.2 Characterize riboflavin-cimetidine interaction in the mouse mammary gland in vivo:

- Using high dose riboflavin intervention as treatment, evaluate the levels of radiolabelled cimetidine in milk and plasma of treated and control mice
5. RATIONALE

Riboflavin is one of the most concentrated vitamins in the milk, by about 100-fold compared to plasma levels (Lawrence, 2005a; Hustad et al., 2000). Given this vitamin’s substantial milk transfer, the lactating mammary gland is likely to possess a riboflavin transport system. Indeed, luminally expressed mammary BCRP was shown to pump riboflavin into milk, in addition to its other substrate drugs and toxins (cimetidine, PhIP, aflatoxin B<sub>1</sub>, etc.). At present, the riboflavin uptake system on the basal side of the mammary gland is unknown. Recently characterized riboflavin transporters or RFT are possible candidate proteins for this role. Therefore, temporal and spatial profiles of RFT in the mammary gland and its models are explored in this thesis.

BCRP plays a major role in excretion of drugs and toxins into the milk. Therefore, an intervention strategy to decrease BCRP-transported xenobiotic excretion into breast milk may be possible. One approach might be to inhibit BCRP function systemically. However, this may not only reduce milk levels of BCRP substrates, but also increase systemic levels of these drugs. The latter outcome may not be clinically desirable, although dose alterations may evade the problem. A different approach is a mammary-selective competition between BCRP substrates, using a high-dose riboflavin intervention. In this scenario, the mechanism of mammary accumulation of riboflavin is exploited to saturate BCRP transport function with riboflavin; thereby, reducing BCRP-mediated xenobiotic transport into the breast milk. This approach is investigated in vivo in this thesis. If this concept is shown to be valid, this may open a door to a clinical strategy for reduction of drug and toxin transfer into breast milk.

Overall, understanding riboflavin transport in the mammary gland is in itself important. Acquiring further knowledge on potential manipulation of mammary xenobiotic transport in order to reduce excretion of xenobiotics into milk may have potential implications for breastfeeding mother-infant pairs.
MATERIALS AND METHODS

1. MATERIALS

Tritiated cimetidine was purchased from American Radiolabeled Chemicals (St. Louis, MO). All secondary antibodies were purchased from Invitrogen (Burlington, ON). Microvette 0.3 mL lithium-heparin blood collection tubes were purchased from Braintree Scientific (Braintree, MA). All other chemicals were purchased from Sigma-Aldrich Canada, unless otherwise specified.

2. CELL CULTURE

2.1 Cell Lines and Maintenance

All cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). EMT6 cells were grown in DMEM and 15% fetal bovine serum. HC11 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 5ug/mL insulin, and 10ng/mL epidermal growth factor. T47D cells were grown in RPMI 1640 medium containing 10% BSA. MCF10A cells were grown in a 1:1 mixture of DMEM and Ham’s F12 medium containing 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone and 5% horse serum.

All cells were maintained at 37ºC under atmospheric mixture of 5% CO₂ and 95% air. Medium was replaced 2-4 times per week. The cells were subcultured using 0.05% trypsin when reaching 75-100% confluency.

Because the effect of underlying cellular pathology on RFT expression could not be ruled out, both tumourigenic (EMT6 and T47D) and non-tumorigenic (HC11 and MCF10A) mammary epithelial cell lines were used. In addition, these cell lines vary in their degree of cellular differentiation, thereby providing the means for the evaluation of the potential differences in RFT expression, based on the state of mammary differentiation. For instance, T47D cells represent a fully differentiated mammary ductal epithelial cell model, while HC11 cells are undifferentiated epithelia used as a model of mammary lactogenic differentiation.

2.2 Total RNA Isolation

Total RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen; Valencia, CA). The procedure was conducted according to the RNeasy Mini information handbook. In brief, cells were grown in a monolayer (70% - 80% confluency) on a 6-well plate. Cell-culture medium was aspirated, and buffer RLT was added to lyse the cells.
350 uL of cell lysate was pipetted into a QIAshredder spin column placed in a 2 mL collection tube, and centrifuged for 2 min at full speed. 350 uL of 70% ethanol was added to homogenized lysate. 700 μl of the sample was transferred to an RNeasy spin column. Flow-through was discarded. Next, 700 μl of buffer RW1 was added to the RNeasy spin column and centrifuged for 15 s at 8000 x g, to wash the column membrane. Then, 500 μl of buffer RPE was added to the RNeasy spin column, and centrifuged for 15 s at 8000 x g; flow-through was discarded. This step was repeated once more, to ensure that no ethanol remained in the column. RNA was eluted into a new 1.5 ml collection tube with 50 μL of RNase-free water. Samples were stored at -80ºC until needed.

2.3 Primer design for conventional PCR

The parameters outlined below were used to design primers used in this project. The primers were highly specific for the each individual gene. Primer length was set to be between 19 to 25 base pairs, with a GC content of 55% - 60%. Primer melting temperatures ranged from 60ºC to 65ºC. For gene expression studies (excluding primer design for cloning experiments) the annealing position of primers was chosen to be on different exons, so that there would be an intron-exon boundary to distinguish the RT-derived cDNA from possible contamination by the genomic DNA. All primers were commercially synthesized by the Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON). Each primer set generated a single band of expected size on 2% agarose gel stained with 0.5 μg/μL of ethidium bromide.

2.4 Conventional Polymerase Chain Reaction (PCR)

To check for the presence of riboflavin transporters in both human and mouse cell lines, conventional PCR was run. A 25 μL reaction mixture was set up as follows: 4 μL of reverse transcription product, 2.5 μL of 10 x PCR reaction buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 0.75 μL 50 mM magnesium chloride, 20 mM forward and reverse primers, 10 mM dNTPs, 0.05 U/ μL recombinant Taq DNA polymerase (Invitrogen, Burlington, ON), and RNase-free water.

All reactions were carried out in an automated thermocycler. A typical PCR reaction was programmed with the following parameters: (1) denaturation at 94 ºC for 30 sec to separate DNA double-strands; (2) annealing at 57 ºC for 30 sec to promote DNA-primer association; (3) elongation at 72 ºC for 30 sec to synthesize new DNA strand. Slight modifications to this protocol were applied, when necessary. These three
phases were propagated for 35 cycles to obtain required product. After the final cycle was completed, the PCR reaction was allowed to cool to 4 °C.

PCR products were visualized under UV light on 2% agarose gel (0.05% ethidium bromide) following the electrophoresis.

3. **IN VIVO MOUSE EXPERIMENTS**

The experimental procedures outlined below were approved by the animal care committee of the Toronto Centre for Phenogenomics (TCP, Toronto, Canada).

3.1 *In vivo* Expression of *mRft1* and *mRft2* mRNA in the Mammary Gland

3.1.1 Animals

Animal care and housing were provided by the TCP. Mammary expression of *mRft1* and *mRft2* mRNA was examined in five groups of FBV/N mice on standard chow: 1) virgin (9-10 weeks old), 2) mid-gestational (15th day post coitus), 3) mid-lactating (on the 7th day post delivery), 4) late-lactating (on the 14th day post delivery), and 5) involution (a week after litter-weaning). The TCP standard chow contained 15 mg/kg of riboflavin. All time-pregnant mice were first time mothers. All timed-pregnant mice were housed singly in separate cages throughout the course of the experiment. To control for the litter size between replicates, a litter size of six pups was maintained immediately after the delivery.

The reason for choosing FVB/N mice is two-fold. Firstly, previous studies evaluating the excretion of BCRP-substrate xenobiotics and riboflavin into the mouse milk were conducted in BCRP knock-out and wild-type mice with FVB genetic background. Secondly, the most extensive characterizations of mouse mammary gland development were conducted in FVB mice. C57Bl/6 mice were selected for the purpose of analyzing any strain differences in RFT expression.

3.1.2 Tissue Collection

Mice were euthanized in a sealed CO₂ chamber, followed by cervical dislocation. Collected tissues included mammary gland and small intestine. Mammary gland was the tissue of primary interest; the rest were reference tissues for positive expression of *mRft1* and *mRft2* mRNA. Tissues were preserved in RNA*later* solution (Invitrogen, Carlsbad, CA), and stored at -80°C.
3.1.3 Total RNA Isolation

Total RNA was isolated from the collected tissues using QIAzol Lysis Reagent (Qiagen; Valencia, CA). The procedure was performed according to the QIAzol information handbook, with minor modifications to the protocol. In short, 1 mL QIAzol Lysis Reagent was added to about 50 mg of tissue, followed by completed sample homogenization. Tubes containing the homogenate were incubated at room temperature for 5 min, to promote nucleoprotein complex dissociation. Next, 0.2 mL of chloroform was added to the homogenate. Tubes were shaken for 15 sec, then placed on the bench-top at room temperature for 3 min. Samples were centrifuged at 12,000 x g for 15 min at 4°C. The upper, aqueous phase containing RNA was transferred into a new tube. To precipitate RNA, 0.5 mL of 100% isopropanol was added, and mix thoroughly by vortexing. Samples were allowed to incubate at room temperature for 10 min, followed by centrifugation at 12,000 x g for 10 min at 4°C. Supernatant was aspirated and discarded. To wash the pellet, 1 mL of 75% ethanol was added to the tubes, followed by centrifugation at 12,000 x g for 10 min at 4°C. Supernatant was completely removed, and the pellet was left to air-dry briefly. RNA was resuspended in an appropriate volume (depending on the pellet size) of diethylpyrocarbonate-treated (DEPC) water. Samples were stored at -80°C until needed.

RNA concentration and purity were assessed using a NanoDrop Spectrophotometer (Thermo Scientific). OD_{260}/OD_{280} ratio ranging from 1.8 to 2.0 was indicative of high purity. Additionally, RNA integrity was determined by visualizing under ultraviolet light the intact eukaryotic ribosomal subunits (28S and 18S) after electrophoresis on a 2% agarose gel (2% w/v agarose, 1xTAE [40mM acetic acid, 40mM Tris base, 1 mM EDTA, pH 8.0] ) stained with ethidium bromide (0.5mg/mL). Also, random samples were sent to the Centre for the Applied Genomics, where RNA integrity was assessed by agilent BioAnalyzer analysis. RNA integrity numbers ranged from 8 to 9.7, indicative of high quality RNA.

3.1.4 Reverse Transcription (RT)

Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Invitrogen, Burlington, ON) was used to synthesize complementary DNA (cDNA) from mRNA. Prior to reverse transcription, 2 μg of total RNA was incubated for 3 min at 90°C in presence of 2 μL of oligo-d(T) primers (0.4 μg/μL), for the total reaction volume of 10 μL. Next, 30 μL of Master-mix were added to the RNA samples. Master-mix contained: 5x PCR
buffer, 1.75 mM MgCl₂, 10 mM dNTPs, 10 mM dithiothreitol (DTT), 10 U/μL MMLV-RT, and RNase-free water. Samples were incubated for 10 min at 20ºC, followed by incubation for 60 min at 42 ºC. Resultant cDNA samples were stored at -20ºC, until further use.

3.1.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) were followed in processing the samples and running qPCR. TaqMan probes (Table 4) for gene expression (Applied Biosystems) were used for Real-Time PCR. Mouse cDNA samples, as well as cDNA isolated from mouse and human cell lines and human breast milk, were analyzed by qPCR. Reactions were set up in a 96-well plate as follows: 2 μL cDNA, 1 μL TaqMan probe, 7 μL DEPC-treated water, and 10 μL TaqMan universal PCR master mix. Plate was centrifuged at 800 x g for 5 min. Applied Biosystems 7500 Real-Time PCR System was used to run the samples, and later obtain the results. The thermal profile used consisted of three stages. First, 1 cycle at 50ºC for 2 min. Second, 1 cycle at 95ºC for 10 min. Third, 40 cycles at 95ºC for 15 sec, followed by cooling to 60ºC for 1 min. Resultant data in form of Ct values were saved to Excel files for later analysis.

3.1.6 Laser Capture Microdissection (LCM)

LCM was performed by the Histology department at the Toronto Centre for Phenogenomics. All reagents and Arcturus Paradise Plus Quality Assessment kits were purchased from Applied Biosystems.

In brief, mammary glands from virgin and 1-week lactating FVB/N female mice were surgically removed and incubated for 6 hours at room temperature in paraformaldehyde (PFA). Tissues were then paraffin-embedded, and later sectioned and mounted on RNase free membrane slides. RNA was isolated using Arcturus Paradise Kit according to the manufacturer’s instructions. The quality of the RNA was analyzed using an Agilent Bioanalyser (The Hospital for Sick Children, Toronto). Reverse transcription protocol to generate cDNA was performed according to the manufacturer’s instructions, using the reagents provided in the Arcturus Paradise kit. The samples were quantified using a NanoDrop spectrophotometer. The samples were stored at -80 ºC until further analysis by qPCR.
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3.1.7 Hematoxylin and Eosin (HE) Staining

Mammary glands were isolated from FVB/N mice and fixed in 10% PFA overnight. Tissues were then processed by the Pathology Department at the Hospital for Sick Children. In short, tissues were paraffin embedded and stored for future use. For HE stating, tissue preparations were sectioned, deparaffinized and hydrated. Hematoxylin was applied for 15 minutes, followed by washing. To counterstain, eosin was applied for 2 minutes. Tissues were dehydrated in 95% and absolute alcohols, to remove excess eosin. Samples were then mounted on the slides and scanned using Aperio ScanScope.

3.2 In Vivo Riboflavin-Cimetidine Interaction Study

3.2.1 Animals

Animal care and housing were provided by the TCP. Animals received standard chow and water ad libitum. Lactating FVB/N mice at 7-9 days after delivery were used for the experiment. Within one day after the delivery, the litter size was controlled to six pups. Mice were not allowed to recover from the anesthesia, and were euthanized by conducting cervical dislocation.

3.2.2 Drug Doses

Doses of ketamine/xylazine at 100/10 mg/kg, respectively, were used, with maintenance doses of about 30/3 mg/kg. Riboflavin dose was optimized by quantitatively assessing the levels of the vitamin in both milk and plasma by high-performance liquid chromatography. Riboflavin was dissolved in phosphate buffered saline, and injected at a dose of 5 μg/g body weight. To enhance milk secretion, 250 μL of a 1 IU/ml oxytocin solution was injected subcutaneously (Jonker et al., 2005). Radiolabeled cimetidine [N-methyl-3H] (Figure 5) was injected at doses of 5 mg/kg body weight.

3.2.3 High-performance liquid chromatography (HPLC)

Levels of riboflavin, FAD and FMN were determined as described previously (van Herwaarden et al, 2006; Hamilton and al, 2009). During the procedure, samples were protected from light. 35 μl plasma or 25 μl of 1/10 dilution milk samples were transferred into Eppendorf tubes and spiked with 5 ng of Lumiflavin (internal standard). 250 μl of methanol were added to each tube to precipitate proteins. The samples were then mixed for 1 minute, and placed on ice for 1 hour. Samples were centrifuged for 15 min
Figure 5. [N-Methyl-3H] Cimetidine
at 10,500 x g. Supernatants were carefully removed and transferred to a new set of tubes. 250 µl methanol were added to original tubes, and the procedures were repeated. Combined methanol fractions were evaporated to dryness at 40°C under a stream of nitrogen. Samples were reconstituted in 100 µl of methanol / 50 mM ammonium acetate (pH = 5.0) (25 / 75), and transferred to 200 µl inserts. 10 µl aliquots were injected into the HPLC system.

The chromatographic system consisted of a Dionex Ultimate 3000 series pump and autosampler. Chromatographic separation was performed on a Nova-Pak C18 column (150 x 3.9 mm (inside diameter); 4 µm particle size) (Waters Corporation, Milford, MA). The mobile phase consisted of a mixture of 50 mM ammonium acetate (pH = 5.0) and methanol (75 / 25). The flow rate was 0.8 ml / minute and the detection was performed fluorimetrically using a Dionex RF 2000 fluorescence detector with excitation and emission wavelengths set at 372 and 520 nm, respectively. Elution of the flavins followed the order of polarity (FAD>FMN>Riboflavin>Lumiflavin) with approximate retention times of 2.2, 3.1, 5.0 and 8.4 minutes, respectively. Calibration curves spanned 0.1 to 50 ng / 100 µl and were spiked with 5 ng Lumiflavin. A representative chromatogram of riboflavin, FAD and FMN is shown in Figure 6.

3.2.4 Experimental Timeline
A previously described protocol (Jonker et al., 2005) was adapted for the experiment. The timeline of the experimental procedures was as follows:

- 2 min: ketamine-xylazine injection (IP)
- 0 min: riboflavin or vehicle control injection (IV)
- 30 min: 3H-labelled cimetidine injection (IV)
- 50 or 80 min: oxytocin injection (SQ)
- 60 or 90 min: milk and blood sampling

Note, milk was collected from the inguinal mammary glands, followed by blood sampling 1-2 minutes later.

3.2.5 Milk and Blood Sampling
Milk was collected as per the method described by Parr et al. (1995), with minor modifications. In short, at 7-8 days post-partum, the pups were removed from the mothers for 3 h to allow for milk accumulation in the mammary glands. The dams were anesthetized with ketamine-xylazine, as per TCP protocol described above. At time 50 or 80 minutes, the females received a subcutaneous injection of oxytocin (250 µL of a 1
Figure 6. A representative chromatogram of riboflavin, FAD and FMN in samples spiked with an internal standard lumiflavin.
IU/ml oxytocin). The breast tissue was massaged lightly for about 30 sec. 10 min later the mice were placed on their backs. Nipples were pulled back slightly and cut off. Milk flowed to the surface. About 200 μL was collected into amber tubes (Eppendorf Canada) to protect the samples from light.

Blood samples were collected from the heart using cardiac puncture. The mice were fully anesthetized at the time of the procedure. Briefly, the animal was placed on its back. The 23 gauge needle was inserted slightly left of and under the sternum, directed toward animal’s cranial end. The needle was held 20-30° off horizontal, and inserted into the heart. Negative pressure was gently applied on syringe plunger. Needle was withdrawn upon blood collection. About 200 μL of blood was injected into a heparin-coated Microvette tube (BRAINTREE SCIENTIFIC; Braintree, MA). The Microvette tube was sealed with a plastic cap, and inserted into the outer case designed for centrifugation. Whole blood was centrifuged at 2000 x g for 10 min at room temperature. Plasma (upper aqueous layer) was pipetted into new tubes.

3.2.6 Sample Analysis

Levels of radioactivity in plasma and milk samples were determined by liquid scintillation counting. In short, 50 μL of milk or plasma were added to 2 mL of scintillation fluid, and measured over a 2-minute range in a scintillation counter. DPM values were obtained and later converted to concentration values in ng/mL.

4. HUMAN BREAST MILK

A waiver from the Research Ethics Board at the Hospital for Sick Children (Toronto, ON) was obtained to perform the following study.

4.1 Milk Collection

Mature breast milk (15 mL), 1-2 months post-partum, was collected from a donor via a standard breast pump. The sample was immediately stored at 4°C, and processed within the next twelve hours. Approximately 15 mL of milk was transferred into sterile tubes, tightly sealed, and then centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant fat layer was transferred into a new tube using a sterile spatula. The supernatant aqueous layer was discarded, and cell pellet was preserved. 1 mL of TRIzol (Invitrogen) was added to the fat and pellet samples, prior to storage at -80°C.
4.2 RNA Isolation and Sample Processing

RNA was isolated from TRIzol-treated samples according to manufacturer’s guidelines. In short, 200 μL of chloroform were added to the samples. The samples were centrifuged at 12,000 x g for 15 min at 4°C. The upper, aqueous phase containing RNA was transferred into a new tube. To precipitate RNA, 0.5 mL of 100% isopropanol was added. Samples were allowed to incubate at room temperature for 10 min, followed by centrifugation at 12,000 x g for 10 min at 4°C. Supernatant was aspirated and discarded. The resultant pellet was washed with 1 mL of 75% ethanol, followed by centrifugation at 12,000 x g for 10 min at 4°C. Supernatant was removed, and the pellet was left to air-dry. RNA was reconstituted in DEPC water.

Reverse transcription reactions and qPCR were performed according to the procedures outlined in sections 3.1.4 and 3.1.5, respectively.

5. WESTERN BLOTTING

Whole tissue and cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS), and then centrifuged at 10,000 rpm for 10 min at 4°C to isolate the supernatant. Bradford assay was used to measure protein concentration of the resultant preparations. Reduced protein samples were loaded onto a 4-12% NuPAGE Novex bis-tris gel (Invitrogen), and later transferred to a nitrocellulose membrane (Amersham Biosciences, NJ). Blots were blocked with 5% skim milk in 1X PBST buffer (137mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ • 2 H₂O, 2 mM KH₂PO₄, 0.05% Tween-20, pH 7.4) overnight. Primary antibodies (working concentrations) were used: rabbit polyclonal anti-RFT2 (1:200) (Santa Cruz Biotechnology, CA); mouse polyclonal anti-GAPDH (1:20000) (Santa Cruz Biotechnology, CA). To ensure equal loading for the protein preparations, GAPDH was probed for on the same, stripped blot membranes, used initially for detecting target RFT2 protein.

6. CUSTOM ANTIBODY DESIGN

Custom design for anti-mouse mRFT1 antibody was performed utilizing the services provided by Open Biosystems. A unique antigenic peptide was selected, based on mRFT1 (NP_083919) protein sequence. BLASTp analysis of the antigenic peptide RRLARGKGEQVPIR revealed no similarities to other mouse proteins. A 90 day Rabbit
Protocol was applied to raise the antibodies. In short, a control serum sample was collected on day 0. Primary immunization occurred on day 1, with antigenic peptide emulsified with Freund's complete adjuvant. The first booster was given on day 14, and serum was collected shortly after on day 28. The second booster with antigenic peptide emulsified with Freund's incomplete adjuvant occurred on day 42, and serum was collected on day 56. The third booster shot was given on day 56, followed by two additional bleeds on days 70 and 72. The latter serum was affinity purified. The antibodies were tested in a Western blot protocol as described above.

7. LOCALIZATION STUDY: GFP-hRFT2 FUSION PROTEIN

7.1 Plasmid preparation

The full length 1407 kb hRFT2 cDNA (GenBank accession no. NM_033409) was isolated by PCR cloning from the human placenta cDNA (Clontech). The hRFT2-specific primers were designed: forward primer 5’ CGCAGAGATCTATG GCCTTCCTGATGCAC 3’ with Bgl II restriction site (underlined), reverse primer 5’GTTCTAGGATCCGGCTGGACAGTGAGATTGCA 3’ with BamH I restriction site (underlined). Using QIAGEN LongRange kit, PCR reactions were set up as follows: 5 μl of 10x LongRange PCR Buffer with Mg²⁺, 2.5 μl of 10 mM dNTP mix, 0.2 μl of each forward and reverse primers, 11.7 μl RNase-free water, 0.4 μl LongRange PCR enzyme mix, 30 μl of 3 ng/μl human placenta cDNA to final volume of 50 μl per reaction. All reactions were carried out in an automated thermocycler. The cycling protocol for LongRange PCR was set as follows: (1) denaturation at 93 ºC for 15 sec; (2) annealing at 62 ºC for 30 sec; (3) elongation at 68 ºC for 2 min. These three phases were propagated for 35 cycles to obtain required product. After the final cycle was completed, the PCR reaction was allowed to cool to 4 ºC. PCR products were visualized under UV light on 1.5 % agarose gel (0.05% ethidium bromide) following the electrophoresis. The band corresponding to PCR product size 1429 bp was cut from the gel. PCR product was purified using QIAGEN gel extraction kit. PCR product was then subcloned to TOPO TA cloning kit (Invitrogen): 3 μl of PCR product, 1 μl salt solution, 0.5 μl of TOPO vector, 1.5 μl of distilled water to the final volume of 6 μl per reaction. Reaction preparations were incubated for 20 minutes at room temperature, followed by transformation of One Shot TOP10 chemically competent E. coli. To analyze transformants, white colonies were cultured over night in lysogeny broth medium.
containing 50 μl/mL kanamycin. QIAGEN Mini kit was used to isolate plasmid. To confirm presence of the insert restriction enzymes Bgl II and BamH I were used. To confirm correct insert sequence and orientation, constructs were sequenced (The Hospital for Sick Children). One mismatch was identified: C → T, and later confirmed to be a synonymous SNP: L → L (255) (rs3746805, dbSNP). To make GFP-hRFT2 fusion protein, hRFT2 insert was purified from chemically competent E. coli, and subcloned to pAcGFP1-C1 vector using Bgl II and BamH I restriction enzymes. DH5a competent cells (Invitrogen) were then transformed with pAcGFP1-C1 vector. To confirm correct insert sequence and orientation, constructs were sequenced (The Hospital for Sick Children). The presence of the same synonymous SNP was confirmed. Plasmid was prepared for later transfection using QIAGEN HiSpeed plasmid Midi kit. DNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Scientific).

7.2 Transfection and Imaging

MCF10A cells were seeded at 100 000 cells per well on 24-well plates in 500 μl of complete growth medium, and grown to confluency. Plasmid solution was diluted in OptiMEM to a final concentration of 0.02 μg/μl. 3:1 ratio of FuGENE HD transfecting reagent to DNA was added to plasmid solution, and allowed to incubate at room temperature for 15 min. Cells were transfected with 500 ng/well GFP-hRFT2 plasmid or empty pAcGFP1-C1 vector by adding 50 μL of transfection solution to the wells. Forty-eight hours after the transfection, the cells were visualized using a long-term imaging microscope (Imaging Facility, The Hospital for Sick Children).

8. STATISTICAL ANALYSIS

Statistical tests were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL). Normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA. If statistical significance of p < 0.05 was achieved, Dunnett’s post-hoc comparison test was performed to analyze the difference between the groups. Comparisons between two groups on single variable were accomplished by Student’s independent t-test. Differences of p < 0.05 were considered statistically significant.

9. DATA ANALYSIS

The ΔΔC₉ method for relative quantitation of qPCR was used. First, the C₉ target
values \((Rft, Bcrp)\) were normalized to an endogenous reference (Keratin 18, Villin or \(\beta\) Actin) to obtain the \(\Delta C_T\) values. Next, the \(\Delta C_T\) values were normalized relative to the calibrator sample (virgin group) to obtain the \(\Delta \Delta C_T\) values. Then, the amount of target was determined by the \(2^{-\Delta \Delta C_T}\) as a fold change compared to the virgin group.
RESULTS

1. mRNA Levels of \textit{RFT} in Human and Mouse Mammary-Derived Cells \textit{in vitro}

To understand whether RFT may have a potential role in mammary riboflavin transport, we investigated the relative mRNA expression of these transporters \textit{in vitro}.

1.1 Human and Mouse Mammary Gland Cell Lines

To assess the relative amounts of endogenous \textit{RFT} mRNA in human and mouse mammary epithelial cells, qPCR was performed using gene-specific primers (Table 4). There were no previous reports of \textit{RFT} mRNA expression in these cells, or any other mammary epithelial cell lines.

The mRNA expression levels of \textit{hRFT1}, \textit{hRFT2} and \textit{hRFT3} in human mammary epithelial cells MCF10A and T47D were measured and normalized to β-Actin expression (Figure 7). Multiple group comparison using one-way ANOVA, followed by Dunnett’s post-hoc comparison test revealed significantly higher (p < 0.05) levels of \textit{hRFT2} mRNA, as compared to \textit{hRFT1}. On the other hand, no statistical difference was observed between \textit{hRFT3} and \textit{hRFT1}. This observation was consistent between the two cell lines.

The expression levels of \textit{mRft1} and \textit{mRft2} mRNA in mouse mammary epithelial cells EMT6 and HC11 were also measured and normalized to β-Actin expression (Figure 8). Comparisons between the groups using Student’s independent t-test revealed significantly higher mRNA levels of \textit{mRft1} as compared to \textit{mRft2}, the relationship conserved in both cell lines.

1.2 Cellular Fractions in Human Milk

To evaluate the possible expression patterns of \textit{RFT} mRNA in human mammary gland in a non-invasive way, levels of \textit{RFT} were characterized in cells and cell fragments derived from human breast milk.

The relative amounts of \textit{RFT} mRNA were measured in cell pellet and milk fat cell fragments using qPCR. Rft expression was normalized to β-Actin (Figure 9). Multiple group comparison using one-way ANOVA did not reveal any statistically significant difference between \textit{hRFT1}, \textit{hRFT2} or \textit{hRFT3} expression, probably due to the small sample size.
Figure 7. Levels of hRFT1, hRFT2 and hRFT3 mRNA Expression in Human Mammary Epithelial Cell Lines. Cells were grown in standard media (see Materials and Methods) to 70-80% confluency. Total RNA was isolated from the cells using RNeasy Mini Kit. The expression of hRFT1, hRFT2 and hRFT3 mRNA was analyzed using Real-Time Quantitative PCR. Results were normalized to β-actin, and expressed as percent of β-actin expression. Each group value represents a mean ± standard error. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the difference between the groups. Differences of p < 0.05 were considered statistically significant. * denotes a statistically significant result as compared to hRFT1 within the same cell line.
Figure 8. Levels of \textit{mRft1} and \textit{mRft2} mRNA Expression in Mouse Mammary Epithelial Cell Lines. Cells were grown in standard media (see Materials and Methods) to 70-80% confluency. Total RNA was isolated from the cells using RNeasy Mini Kit. The expression of \textit{mRft1} and \textit{mRft2} mRNA was analyzed using Real-Time Quantitative PCR. Results were normalized to β-actin, and expressed as percent of β-actin expression. Each group value represents a mean ± standard error. Comparisons between the two groups were accomplished by Student’s independent t-test. Differences of \( p < 0.05 \) were considered statistically significant. * denotes a statistically significant result as compared to \textit{mRft2} within the same cell line.
Figure 9. Levels *hRFT1*, *hRFT2* and *hRFT3* mRNA Expression in Human Milk Cells and Cell Fragments. Approximately 15 mL of milk were centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant fat layer was transferred into a new tube using a sterile spatula. The supernatant aqueous layer was discarded, and cell pellet was preserved. 1 mL of TRizol was added to the fat and pellet samples. Total RNA was extracted from cell pellet and milk fat, and then reverse transcribed. The expression of *hRFT1*, *hRFT2* and *hRFT3* mRNA was analyzed using Real-Time Quantitative PCR. Results were normalized to β-actin, and expressed as percent of β-actin expression. Each group value represents a mean ± standard deviation. Multiple group comparisons were carried out by one-way ANOVA. Differences of p < 0.05 were considered statistically significant.
2. mRNA Expression Profiles of *Rft*, *Bcrp* and Other Main ABC Transporters in Virgin, Pregnant, Lactating and Post-Lactation Stages of Mouse Mammary Gland *in vivo*

To further evaluate the possible roles of *Rft* in mammary riboflavin transport, relative mRNA expression of these transporter was investigated in mice *in vivo*. The mRNA expression of ABC-family transporters, including *Bcrp*, were also characterized in vivo, for reasons to be discussed below.

2.1 *Bcrp*

As described in the introductory section of this thesis (page 26), previous reports have indicated that protein levels of BCRP follow a specific pattern of expression in the mammary gland, with significant upregulation in lactating mammary gland (Jonker *et al.*, 2005). It was, however, previously unreported whether this is a transcriptional event.

The levels of mammary *Bcrp* expression in five groups of FVB/N were measured using qPCR. The five groups included: virgin (9-10 weeks old); mid-gestational (15th day post coitus); lactation 1st week (on the 7th day post delivery); lactation 2nd week (on the 14th day post delivery); and involution (a week after litter-weaning). Results were standardized to *Keratin-18* at virgin stage. Significantly higher levels of *Bcrp* mRNA (> 25 fold) in lactating mice (1st and 2nd week), as compared to virgin group were observed (Figure 10). No differences were found between mid-gestational and involution groups, when compared to the virgin state. Similar statistically significant differences in mammary *Bcrp* expression between virgin and lactating stages were observed in C57BL/6 mice (Figure 11).

2.2 *Rft* (*mRft1* and *mRft2*)

As riboflavin is concentrated in breast milk (Lawrence, 2005a), it could be anticipated that mRNA levels of *Rft* would be upregulated in the lactating mammary gland, similar to the pattern of mammary *Bcrp* expression (Jonker *et al.*, 2005). There were no previous reports investigating this question.

The levels of mammary *mRft1* and *mRft2* expression in five groups of FVB/N mice at different stages of mammary gland development (virgin, mid-gestational, 1st and 2nd week of lactation and involution) were measured using qPCR. Statistical analysis revealed significantly higher levels of *mRft1* mRNA (> 10 fold) in mid-gestational and lactating mice (1st and 2nd week), with no difference in involuting animals, as compared to virgin group (Figure 12). Similarly, a 3-fold induction in *mRft2* mRNA was detected.
Figure 10. Levels of *Bcrp* mRNA Expression at Different Stages of Mammary Gland Development in FVB/N Mice. Inguinal mammary glands were excised from five groups of FBV/N mice on standard chow: 1) virgin (9-10 weeks old), 2) mid-gestational (15th day post coitus), 3) lactation 1 wk (on the 7th day post delivery), 4) lactation 2 wk (on the 14th day post delivery), and 5) involution (a week after litter-weaning). Total RNA was extracted from the mammary tissue, and then reverse transcribed. The expression of *Bcrp* mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to *Keratin-18*, and then to virgin state. The $2^{-\Delta\Delta C_T}$ method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Normality tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the differences between the virgin and the other four groups. *denotes a statistically significant result as compared to virgin group.
Figure 11. Levels of Bcrp mRNA Expression at Different Stages of Mammary Gland Development in C57BL/6 Mice. Inguinal mammary glands were excised from two groups of C57BL/6 mice on standard chow: 1) virgin (9-10 weeks old), and 2) lactation 1 wk (on the 7th day post delivery). Total RNA was extracted from the mammary tissue, and then reverse transcribed. The expression of Bcrp mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to Keratin-18, and then to virgin state. The \(2^{-\Delta \Delta C_T}\) method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Comparisons between the two groups were accomplished by Student’s independent t-test. Differences of \(p < 0.05\) were considered statistically significant.* denotes a statistically significant result as compared to virgin group.
Figure 12. Levels of *Mrtf1* mRNA Expression at Different Stages of Mammary Gland Development in FVB/N Mice. Inguinal mammary glands were excised from five groups of FVB/N mice on standard chow: 1) virgin (9-10 weeks old), 2) mid-gestational (15<sup>th</sup> day post coitus), 3) lactation 1 wk (on the 7<sup>th</sup> day post delivery), 4) lactation 2 wk (on the 14<sup>th</sup> day post delivery), and 5) involution (a week after litter-weaning). Total RNA was extracted from the mammary tissue, and then reverse transcribed. The expression of *Mrtf1* mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to *Keratin-18*, and then to virgin state. The $2^{-\Delta\Delta C_T}$ method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Normality tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the differences between the virgin and the other four groups. *denotes a statistically significant result as compared to virgin group.
in 1\textsuperscript{st} week lactating mice (Figure 13), with no difference between other groups, when compared to the virgin state. Lactation state of animals was confirmed using a milk protein gene $\beta$-Casein, which is a marker of lactogenesis (Kumar \textit{et al.}, 1994). $\beta$-Casein was significantly upregulated (>75 fold) in 1\textsuperscript{st} and 2\textsuperscript{nd} week lactating mice (Figure 14).

Significant upregulation of mammary $mRft1$ and $mRft2$ was also observed between virgin and lactating C57BL/6 mice (Figure 15), with about 17-fold and 8-fold increase, respectively.

In addition, expression of $mRft1$ and $mRft2$ mRNA in the small intestine of the FVB/N mice was examined. It was previously reported that human and rat intestinal tissues were expressing high levels of $Rft$ (Yonezawa \textit{et al.}, 2008; Yamamoto \textit{et al.}, 2009). Therefore, small intestine was selected as a reference tissue for high $Rft$ expression. The expression of $mRft1$ and $mRft2$ in the proximal third of the small intestine was measured in virgin, mid-gestational, 1\textsuperscript{st} and 2\textsuperscript{nd} week of lactation and involuting FVB/N mice using qPCR (Appendix I). $Rft$ expression was normalized to Villin, the marker of intestinal epithelium. No significant changes in the intestinal $mRft1$ expression during the different stages of mammary gland development were observed. On the other hand, a statistically significant reduction (about 2-fold) of intestinal $mRft2$ in the gestational state was observed, as compared to the virgin group.

\textbf{2.3 Mammary Epithelia-Specific mRNA Profiles of $mRft1$, $mRft2$ and $Bcrp$ in Virgin and Lactating Mice: Laser-Captured Microdissection (LCM)}

As mammary epithelial cells are involved in milk production and secretion of various milk constituents, including riboflavin, expression of $Bcrp$, $mRft1$ and $mRft2$ mRNA in these cells was investigated.

Inguinal mammary glands were excised from two groups of FVB/N mice on standard chow: 1) virgin (9-10 weeks old), and 2) lactation 1 week (on the 7\textsuperscript{th} day post delivery). Total RNA was isolated exclusively from mammary epithelial cells using LCM protocols. The expression of $mRft1$ and $mRft2$ mRNA was analyzed using qPCR. In the instances where Ct values were undetected, a detection limit value of Ct = 40 was assigned to the measurement. The results were first normalized to Keratin-18, and then to virgin state.

Comparisons between the groups revealed significant upregulation in $mRft1$ and $Bcrp$ mRNA by 4- and 52-fold, respectively (Figure 16, Figure 17). On the other hand, levels of $mRft2$ mRNA remained unchanged (Figure 16).
Figure 13. Levels of mRft2 mRNA Expression at Different Stages of Mammary Gland Development in FVB/N Mice. Inguinal mammary glands were excised from five groups of FVB/N mice on standard chow: 1) virgin (9-10 weeks old), 2) mid-gestational (15th day post coitus), 3) lactation 1 wk (on the 7th day post delivery), 4) lactation 2 wk (on the 14th day post delivery), and 5) involution (a week after litter-weaning). Total RNA was extracted from the mammary tissue, and then reverse transcribed. The expression of mRft2 mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to Keratin-18, and then to virgin state. The 2^{-\Delta\Delta C_T} method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Normality tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the differences between the virgin and the other four groups. *denotes a statistically significant result as compared to virgin group.
Figure 14. Levels of $\beta$-Casein ($Csn2$) mRNA Expression at Different Stages of Mammary Gland Development in FVB/N Mice. Inguinal mammary glands were excised from five groups of FBV/N mice on standard chow: 1) virgin (9-10 weeks old), 2) mid-gestational (15th day post coitus), 3) lactation 1 wk (on the 7th day post delivery), 4) lactation 2 wk (on the 14th day post delivery), and 5) involution (a week after litter-weaning). Total RNA was extracted from the mammary tissue, and then reverse transcribed. The expression of $Csn2$ mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to Keratin-18, and then to virgin state. The $2^{\Delta\Delta C_T}$ method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Normality tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the differences between the virgin and the other four groups. *denotes a statistically significant result as compared to virgin group.
Figure 15. Levels of $mRft1$ and $mRft2$ mRNA Expression at Different Stages of Mammary Gland Development in C57BL/6 Mice. Inguinal mammary glands were excised from two groups of C57BL/6 mice on standard chow: 1) virgin (9-10 weeks old), and 2) lactation 1 wk (on the 7th day post delivery). Total RNA was extracted from the mammary tissue, and then reverse transcribed. The expression of $mRft1$ and $mRft2$ mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to Keratin-18, and then to virgin state. The $2^{-\Delta\Delta CT}$ method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Comparisons between the two groups were accomplished by Student’s independent t-test. Differences of $p < 0.05$ were considered statistically significant.* denotes a statistically significant result as compared to virgin group.
Figure 16. Levels of \textit{mRft1} and \textit{mRft2} mRNA in the Mammary Epithelial Cells of Virgin and Lactating FVB/N Mice. Inguinal mammary glands were excised from two groups of FVB/N mice on standard chow: 1) virgin (9-10 weeks old), and 2) lactation 1 wk (on the 7\textsuperscript{th} day post delivery). Total RNA was isolated exclusively from mammary epithelial cells using laser capture microdissection (LCM) protocols. LCM was performed by the Histology department at the Toronto Centre for Phenogenomics. The expression of \textit{mRft1} and \textit{mRft2} mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to \textit{Keratin-18}, and then to virgin state. Each group value represents a mean ± standard deviation. The 2\textsuperscript{ΔΔC\textsubscript{T}} method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Comparisons between the two groups were accomplished by Student's independent \textit{t}-test. Differences of \(p < 0.05\) were considered statistically significant. * denotes a statistically significant result as compared to virgin group.
Figure 17. Levels of *Bcrp* mRNA in the Mammary Epithelial Cells of Virgin and Lactating FVB/N Mice. Inguinal mammary glands were excised from two groups of FVB/N mice on standard chow: 1) virgin (9-10 weeks old), and 2) lactation 1 wk (on the 7th day post delivery). Total RNA was isolated exclusively from mammary epithelial cells using laser capture microdissection (LCM) protocols. LCM was performed by the Histology department at the Toronto Centre for Phenogenomics. The expression of *Bcrp* mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to *Keratin-18*, and then to virgin state. Each group value represents a mean ± standard deviation. The 2^−ΔΔC_T method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Comparisons between the two groups were accomplished by Student's independent t-test. Differences of *p* < 0.05 were considered statistically significant. * denotes a statistically significant result as compared to virgin group.
It is noteworthy to mention that expression of \textit{Bcrp}, \textit{mRft1} and \textit{mRft2} was also measured in mammary adipose tissue. However, most \textit{Rft} values remained undetected, regardless of the state of mammary gland development. These values are summarized in Appendix II.

\textbf{2.4 Pgp3 (Abcb1a, Mdr3), Pgp1 (Abcb1b, Mdr1b) and Mrp2 (Abcc2)}

To validate the specific nature of \textit{Rft} and \textit{Bcrp} upregulation in the lactating mammary gland, the pattern of expression of other transporters at different stages of mammary gland development in FBV/N mice was investigated.

Previous studies have indicated that protein levels of Pgp (ABCB1) and MRP2 (ABCC2) show reduced expression in the mammary glands of lactating FVB/N mice (Jonker \textit{et al.}, 2005). It was, however, previously unknown whether the mRNA expression of these genes follows the similar pattern of downregulation.

The levels of mRNA expression of mammary \textit{Mdr3} (\textit{Abcb1a}), \textit{Mdr1b} (\textit{Abcb1b}) and \textit{Mrp2} (\textit{Abcc2}) were measured using qPCR in five groups of FVB/N mice, as outlined above. Significantly lower levels of \textit{Mrp2} mRNA (about 4-fold) in the 1\textsuperscript{st} and 2\textsuperscript{nd} week lactating mice, with no differences in other groups, as compared to virgins were observed (Figure 18). On the other hand, \textit{Mdr3} and \textit{Mdr1b} showed a trend toward reduction during lactation; however, statistical significance was not observed.

\textbf{3. Protein Levels of mRFT1 and mRFT2 in Virgin, Lactating and Post-lactation Stages of Mouse Mammary Gland in vivo}

To evaluate whether the pattern of RFT protein expression mirrored that of mRNA in mouse mammary gland, Western blots were performed using two primary antibodies: rabbit polyclonal anti-mRFT1 and anti-mRFT2.

\textbf{3.1 mRFT1}

As no anti-mouse mRFT1 antibody was commercially available, custom design for anti-mouse mRFT1 antibody was performed utilizing the services provided by Open Biosystems (see Methods). Using both crude and affinity purified sera numerous Western blots were run using both cell preparations and mouse mammary gland preparations. However, regardless of preparation, protein amount loaded and primary antibody dilutions, no strong signal was detected at or near the predicted sizes of 46 and 37 kDa of mouse mRFT1 isoforms. Rather, multiple non-specific bands appeared across blots (data not shown).
Figure 18. Levels of *Pgp3* (*Abcb1a, Mdr3*), *Pgp1* (*Abcb1b, Mdr1b*) and *Mrp2* (*Abcc2*) mRNA Expression at Different Stages of Mammary Gland Development in FVB/N Mice. Inguinal mammary glands were excised from five groups of FBV/N mice on standard chow: 1) virgin (9–10 weeks old), 2) mid-gestational (15th day post coitus), 3) lactation 1 wk (on the 7th day post delivery), 4) lactation 2 wk (on the 14th day post delivery), and 5) involution (a week after litter-weaning). Total RNA was extracted from the mammary tissue, and then reverse transcribed. The expression of *Pgp3*, *Pgp1* and *Mrp2* mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to *Keratin-18*, and then to virgin state. The $2^{-\Delta\Delta Ct}$ method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Normality tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the difference between the virgin and the other four groups. * denotes a statistically significant result as compared to virgin group.

![Graph showing mRNA expression levels for Pgp3, Pgp1, and Mrp2 at different stages of mammary gland development.](image-url)
3.2 mRFT2

Although commercially available, anti-RFT2 antibody was not previously validated. The antibody is claimed to cross react with both human and mouse RFT2 protein.

Whole mammary gland lysates from virgin, lactating and involuting FVB mice were prepared. 60 microgram of each preparation was run on the Western blot (Figure 19). Strong bands of variable intensity were detected at about 40 kDa in all three preparations. An additional band of lesser intensity was visualized for lactating mammary tissue at 50 kDa. No corresponding signal in adjacent virgin and involuting mammary samples was detected at 50 kDa. Predicted size of mRFT2 is 49.6 kDa.

4. Subcellular Localization of hRFT2 in Mammary-derived Cell Line MCF10A

To visualize the cellular localization of hRFT2, MCF10A cells were transfected with GFP-tagged hRFT2 (Figure 20, A). Fluorescence was observed in the plasma membrane of most cells transfected with GFP-tagged hRFT2. MCF10A cells transfected with GFP-only vector showed no pattern of localization, with fluorescence observed within whole cells (Figure 20, B).

5. Riboflavin-Cimetidine Interaction Study in Mice in vivo

5.1 Riboflavin Dose Finding

To identify the riboflavin dose that produces significantly higher plasma levels of riboflavin in FVB/N mice on standard chow, a dose finding study was conducted. As per the initial protocol, milk and plasma levels of riboflavin were quantified 90 min after riboflavin intravenous injection (Figure 21). Milk and plasma riboflavin concentrations in control mice, not receiving riboflavin supplementation, were 10800 nM and 160 nM, respectively. Additional supplementation with 1, 2 or 5 μg/g body weight resulted in 1.8-, 2.9- and 8.8-fold increase in plasma levels and 1.8-, 2.7- and 3-fold increase in milk levels of riboflavin at 90 minutes. Statistically significant differences in both milk and plasma riboflavin concentrations were achieved at 5 μg/g weight. Subsequently, the dose of 5 μg/g body weight of riboflavin was chosen for the in vivo experiments (see below). Additional quantification of milk riboflavin levels using 5 μg/g body weight of riboflavin were performed at 30 and 60 min post-dose (Figure 22). No significant differences in milk riboflavin levels at 30, 60 and 90 min were found.

Levels of FAD and FMN were also quantified by HPLC at 90 min after riboflavin supplementation (Appendix III). No significant changes in milk and plasma levels as
Figure 19. mRFT2 Expression at Different Stages of Mammary Gland Development in FVB/N Mice. Whole tissue lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer, and then centrifuged at 10,000 rpm for 10 min at 4°C to isolate the supernatant. Reduced protein samples were loaded onto a 4-12% NuPAGE Novex bis-tris gel, and later transferred to a nitrocellulose membrane. Blots were blocked with 5% skim milk in 1X PBST buffer overnight. Primary antibodies were used: rabbit polyclonal anti-mRFT2 (1:200); mouse polyclonal anti-GAPDH (1:20000).
Figure 20. Fluorescent Imaging of Green Fluorescent Protein (GFP)-tagged hRFT2. Human mammary epithelial cells MCF10A were transfected with 500 ng/well GFP-hRFT2 (A) plasmid or empty pAcGFP1-C1 vector (B). Forty-eight hours after the transfection, the cells were visualized using long-term imaging microscope. Fluorescent images show GFP-hRFT2 outlining the cells at the plasma membrane. Scale: A. bar 23 μm; B. bar 49 μm.
Figure 21. Milk and Plasma Levels of Riboflavin in FVB/N Mice. One-week lactating FVB/N mice were intravenously administered riboflavin at doses of 1 μg/g, 2 μg/g or 5 μg/g body weight, or phosphate buffered saline (control). To enhance milk secretion, 250 μL of a 1 IU/ml oxytocin solution was injected subcutaneously 10 min prior to milk sampling. At 90 min, milk was collected from the inguinal mammary glands, followed by blood collection via cardiac puncture 1-2 minutes later. Whole blood was centrifuged at 2000 x g for 10 min at room temperature. Plasma was transferred into new tubes. Milk and plasma samples were protected from light. Riboflavin levels were analyzed by high-performance liquid chromatography. Each group value represents a mean ± standard error. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the differences (p < 0.05) between the control and the other three treatment groups. * denotes a statistically significant result as compared to virgin group.

A. Milk

B. Plasma
Figure 22. Milk Levels of Riboflavin in FVB/N Mice. One-week lactating FVB/N mice were intravenously administered riboflavin at a dose 5 μg/g body weight. To enhance milk secretion, 250 μL of a 1 IU/ml oxytocin solution was injected subcutaneously 10 min prior to milk sampling. Milk was collected from the inguinal mammary glands at 30, 60 or 90 min. Milk samples were protected from light. Riboflavin levels were analyzed by high-performance liquid chromatography. Each group value represents a mean ± standard error. Multiple group comparisons were carried out by one-way ANOVA. Differences of p < 0.05 were considered statistically significant.
compared to control group of these flavocoenzymes were observed.

5.2 Reduction of Cimetidine Excretion into Milk by High-Dose Riboflavin

The initial set of experiments was conducted using 5 μg/g weight of cimetidine. Milk and plasma samples were collected at 90 minutes after riboflavin or PBS IV injections, or in other words, at 60 minutes after cimetidine IV injection (Refer to Experimental Time-line, page 47). Milk levels of cimetidine trended toward reduction with high-dose riboflavin treatment; however, statistical significance was not observed (Figure 23). Similarly, no significant differences in plasma levels of cimetidine were observed. MP ratios of control and treatment groups were 4.59 and 4.14, respectively.

The reported MP ratio of cimetidine in FVB/N mice at 30 minutes post-injection was about 13 (Jonker et al., 2005). As our observations indicated a 3-fold reduced MP ratio at 60 minutes post cimetidine injection, the milk and plasma sampling time-points were moved to 30 minutes after cimetidine IV injection. At this time-point and using 5 μg/g weight of cimetidine, a significant reduction in milk levels of cimetidine in the treatment group was observed (Figure 24). No significant differences in plasma levels of cimetidine were observed. MP ratios of control and treatment groups were 1.91 and 1.36, respectively. Experimental MP ratios are summarized in Figure 25.
Figure 23. Milk and Plasma Levels of 5 mg/kg $^3$H-Cimetidine in FVB/N Mice at 60 min after cimetidine IV injection. One-week lactating FVB/N mice were intravenously administered riboflavin (treatment group) or phosphate buffered saline (control group). Riboflavin was dissolved in phosphate buffered saline, and injected at a dose of 5 μg/g body weight. After 30 min, radiolabeled cimetidine [N-methyl-$^3$H] was injected into the tail vein at the dose 5 mg/kg body weight. To enhance milk secretion, 250 μL of a 1 IU/ml oxytocin solution was injected subcutaneously 10 min prior to milk sampling. At 90 min, milk was collected from the inguinal mammary glands, followed by blood collection via cardiac puncture 1-2 minutes later. Whole blood was centrifuged at 2000 x g for 10 min at room temperature. Plasma was transferred into new tubes. Levels of radioactivity in plasma and milk samples were determined by liquid scintillation counting. Each group value represents a mean ± standard deviation. Mean Milk-to-Plasma ratios are as follows: Control 4.59; Treatment 4.14. Comparisons between the groups were accomplished by Student’s independent t-test. Differences of $p < 0.05$ were considered statistically significant.
Figure 24. Milk and Plasma Levels of 5 mg/kg $^3$H-Cimetidine in FVB/N Mice at 30 min after cimetidine IV injection. One-week lactating FVB/N mice were intravenously administered riboflavin (treatment group) or phosphate buffered saline (control group). Riboflavin was dissolved in phosphate buffered saline, and injected at a dose of 5 μg/g body weight. 30 min later, radiolabeled cimetidine [N-methyl-$^3$H] was injected into the tail vein at the dose 5 mg/kg body weight. To enhance milk secretion, 250 μL of a 1 IU/ml oxytocin solution was injected subcutaneously 10 min prior to milk sampling. At 60 min, milk was collected from the inguinal mammary glands, followed by blood collection via cardiac puncture 1-2 minutes later. Whole blood was centrifuged at 2000 x g for 10 min at room temperature. Plasma was transferred into new tubes. Levels of radioactivity in plasma and milk samples were determined by liquid scintillation counting. Each group value represents a mean ± standard deviation. Mean Milk-to-Plasma ratios are as follows: Control 1.91; Treatment 1.36. Comparisons between the groups were accomplished by Student’s independent t-test. Differences of p < 0.05 were considered statistically significant. * denotes a statistically significant result.
Figure 25. Summary of Cimetidine Milk-to-Plasma Ratios. Milk and plasma samples were collected at 30 or 60 minutes after cimetidine IV injection. Levels of radioactivity in plasma and milk samples were determined by liquid scintillation counting. Comparisons between the groups were accomplished by Student’s independent t-test. Differences of \( p < 0.05 \) were considered statistically significant. * denotes a statistically significant result.
DISCUSSION

1. RFT mRNA Expression Studies in vitro

Recent discovery of RFT (Yonezawa et al., 2008; Yamamoto et al., 2009; Yao et al., 2010) provided a possible explanation and mechanism for riboflavin transport within the body. Although mRNA expression of these transporters was investigated in various tissues (Yonezawa et al., 2008; Yamamoto et al., 2009), including small intestine, nothing was known about RFT expression in the mammary gland. Given the fact that riboflavin is highly concentrated in the breast milk (Lawrence, 2005a; Hustad et al., 2000), it was reasonable to hypothesize that the mammary gland could expresses RFT.

I used human and mouse mammary epithelial cells as a model to investigate the possibility of mammary RFT mRNA expression. Two human mammary epithelial cell lines were used: T47D and MCF10A, a ductal carcinoma and a non-tumorigenic epithelial cell lines, respectively. The mRNA expression of all three human RFT was detected in these cells. Interestingly, despite the dissimilar origin of these cells and potential cell line-to-cell line differences, the relative mRNA expression profiles of RFT were remarkably comparable between these cell lines. Specifically, hRFT2 showed a predominant expression in human mammary cells. Such differential expression of RFT was also noted in human small intestine and Caco2 cells (Subramanian et al., 2011b). In both instances, hRFT2 expression was shown to be significantly higher than that of hRFT1 and hRFT3. Together these findings may suggest a principal role of hRFT2 in riboflavin transport in the human intestine and mammary gland; however, given the lack of knowledge about protein expression of RFT this point remains a speculation.

Similarly, two mouse mammary epithelial cell lines were used: EMT6 and HC11, mammary carcinoma cells and BALB/c mouse mammary cells, latter considered to retain important characteristics of normal mammary epithelia. Both were shown to express Rft in my experiments. However, unlike the human mammary epithelial cells, mRft1 expression was significantly higher than mRft2 in the two cell lines. This observation indicated that there might be species specific differences in relative expression profiles of Rft, and possibly in their contribution to riboflavin transport in a given organism. The observed patterns of relative expression in the human and mouse mammary epithelial cell lines brought forth the question about the Rft expression profiles in vivo.

Because of practical and ethical issues, obtaining human mammary tissue is
challenging. Therefore, to investigate RFT expression in human mammary gland a noninvasive method is desirable. Isolating and analyzing cells from breast milk represents such a method. However, a few important issues must be considered. First, the cellular fraction of breast milk is a complex matrix, consisting not only of mammary epithelial cells, but also of macrophages and leukocytes (Ho et al., 1979; Boutinaud and Jammes, 2002). Second, both cell count and relative cellular composition of breast milk change with time, with the proportion of epithelial cells increasing from colostrum to mid-lactation (Ho et al., 1979; Brooker, 1980). Therefore, in an attempt to enhance the contribution of mammary epithelial cells (in the cell pellet) to my planned analysis, I used mature breast milk 1-2 months postpartum. In addition to isolating RNA from the milk cells (pellet), RNA from human mammary epithelial cell fragments (milk fat) was also isolated (see page 30). It has been shown that milk fat globules contain cytoplasmic fragments of epithelial cells, and are therefore, an additional way of assessing gene expression more specific to mammary epithelial cells (Maningat et al., 2009).

Real-time PCR analysis revealed that in cell pellet and milk fat cell fragments, hRFT3 mRNA expression tended to be high, as compared to hRFT1 and hRFT2. The lack of statistical significance is potentially explained by the small sample size (n = 4). Because the exact pattern of RFT mRNA expression differs between human epithelial cell lines and cells derived from human breast milk, it is difficult to predict the true picture of RFT expression in the human mammary epithelium. As mammary cell lines are derived from a diseased tissue, the contribution of the disease state to RFT expression cannot be ruled out. Similarly, the relative contribution of non-epithelial milk cells, isolated from the cellular pellet, and its effect on characterized RFT expression is unknown. Furthermore, the epithelial cellular fragments from the milk fat may not be representative of a complete human mammary secretory epithelium, as they are only cell fragments. Nevertheless, regardless of the specific pattern of expression, RFT are likely present in the human mammary gland.

Overall, it is possible to conclude that RFT mRNAs are expressed in the mammary epithelial cell lines and potentially in the human mammary tissue. The relative expression of different RFT and their contribution to riboflavin transport may be species-specific.
2. *Rft* mRNA Expression Studies in Mice *in vivo*

The initial observation that protein levels of BCRP were upregulated in the lactating mammary glands of various species (Jonker *et al*., 2005), including humans and mice, was puzzling, because the increased levels of this transporter and its xenobiotic substrates in milk, may be detrimental to the offspring. One possible explanation to this phenomenon came with the discovery of BCRP-mediated transport of an essential vitamin, riboflavin (van Herwaarden *et al*., 2007). It was proposed that mammary BCRP is necessary for mother-to-infant nutrient transfer, hence, its dramatic upregulation in lactation.

In our study, we have confirmed this pattern of mammary BCRP expression at the mRNA level, which was previously unexplored. We have observed a dramatic increase in *Bcrp* in the lactating mammary glands of FVB/N and C57Bl/6 mice. As predicted, the levels of mammary *Bcrp* declined to the pre-pregnancy levels in the involuting gland of FVB/N mice. Taken together, these findings suggest lactation-specific functions of mammary BCRP, one of which may include enhanced nutrient supply to the newborn.

A few mechanisms may explain the observed upregulation in mammary BCRP expression. For instance, progesterone and estradiol have been shown to regulate BCRP expression *in vitro* (Ee *et al*., 2004; Imai *et al*., 2005; Wang *et al*., 2008). Given the significant involvement of these hormones in mammary gland development during pregnancy and lactation, the contribution of hormonal regulatory pathways to mammary BCRP upregulation should be considered. In addition, it has also been noted that heterodimerization between the retinoid X receptor and the peroxisome proliferator-activated receptor-γ resulted in an increase in BCRP expression in dendritic cells (Szatmari *et al*., 2006). Contributions of this pathway to mammary BCRP upregulation may have to be evaluated. Also, it was suggested that BCRP expression is transcriptionally upregulated through the inhibition of extracellular signal-regulated kinase pathway, providing yet another possible mechanism of mammary BCRP regulation (Imai *et al*., 2009).

It was reasonable to assume that if nutrient transport to the infant through maternal milk was essential, other nutrient transporters may also be upregulated in lactation. RFT were suitable candidates to test this hypothesis, because of their potential role in mammary riboflavin transport. By investigating their expression in the
mammary gland of FVB/N mice, I made an observation that both \textit{mRft1} and \textit{mRft2} levels were increased in lactation. Of the two, \textit{mRft1} showed a more pronounced upregulation, as indicated by > 10-fold increase when compared to the virgin state (this observation is also true for C57Bl/6 lactating mice). Although elevated in lactation, \textit{mRft2} levels were more variable and lower than \textit{mRft1}. Interestingly, the \textit{mRft1} dominant expression pattern observed in mouse mammary epithelial cell lines was also maintained \textit{in vivo}. At all stages of mammary development, mammary \textit{mRft1} mRNA expression exceeded that of \textit{mRft2}. Assuming that \textit{mRFT1} takes a primary role in riboflavin transport (compared to \textit{mRFT2}) in the mammary gland, then it is not surprising that \textit{mRft1} mRNA shows a more distinct upregulation.

Another possible explanation may rely on differential functions of RFT. Although the localization of RFT and their direction of transport in the mammary gland is unknown, it may be possible that RFT1 and RFT2 are expressed on the opposite sides of mammary epithelium, and therefore, have different roles in riboflavin transport. For instance, if RFT2 is expressed luminally and serves as a complementary mechanism to mammary BCRP excretion of riboflavin, then a dramatic upregulation in its levels may be redundant. On the other hand, if RFT1 is the sole transporter responsible for riboflavin uptake to the mammary epithelium, then its remarkable upregulation in lactation may be essential. However, without additional localization and function studies of mammary RFT, these are simply theoretical renderings.

While some transporters are upregulated in lactation, others are downregulated. Jonker \textit{et al.} (2005) have shown that certain ABC transporters, including Pgp and MRP2, are downregulated at the protein level in the lactating mammary gland of FVB mice. I have shown similar findings in mRNA expression of these proteins. Although only mammary \textit{Mrp2} mRNA has shown a statistically significant reduction in lactation, the trend towards diminished mRNA levels in lactation is clear for Pgp. Most importantly, however, is the fact that neither of these transporters are upregulated; thereby, underscoring the significance of \textit{Rft} and \textit{Bcrp} patterns of mammary expression.

Because mammary gland consists of various cell types, it was essential to confirm that \textit{Rft} and \textit{Bcrp} mRNA expression profiles, observed in the whole mammary gland, were reflective of the cell type of interest – mammary epithelial cells. By applying laser capture microdissection (LCM), RNA was isolated from mammary epithelium of FVB/N mice. It was observed that both \textit{Bcrp} and \textit{mRft1} where significantly upregulated.
in the lactating epithelium, as compared to the virgin state. Interestingly, lactating epithelial levels of \textit{Bcrp} seemed upregulated by 20-fold more when compared to the whole tissue levels. This may suggest that mammary epithelium in lactation is a predominant contributor to BCRP expression.

On the other hand, lactating epithelial levels of \textit{mRft1} were slightly less than those observed in the whole tissue. Here two explanations are possible. First, the contribution of non-epithelial cells, including adipocytes and endothelial cells, to \textit{mRft1} levels detected in the mammary gland may not be marginal. Second, the type of mammary epithelial cells may be of importance. In our experiment, longitudinal sections of mammary gland were performed. This did not allow for clear distinction between ductal and acinar mammary epithelium. Assuming that RFT are crucial for riboflavin transfer into the milk, it stands to reason that acinar epithelial cells could be of the most importance, as they mainly function in lactogenesis, and the LCM approach may have failed to capture the acinar structure. Further analysis, using cross-sections of the mammary tissue, may address \textit{Rft} sub-tissue expression.

As for \textit{mRft2}, no upregulation in mouse mammary epithelial cells was observed. The most likely explanation for this observation lies in technical issues of the LCM. LCM allows for morphological specificity at a cost of sensitivity. Although this method works quite well for highly expressed genes when relative expression is in question, the difficulty arises with low expression genes. Overall, it is likely that \textit{mRft2} expression in mouse mammary epithelial cells is enhanced in lactation; however, a more robust method of RNA isolation and further analysis is necessary to confirm this conjecture.

Lastly, the proximal small intestinal levels of \textit{Rft}, used as a positive control for \textit{Rft} mRNA expression, remain relatively unchanged during pregnancy and lactation in the FVB/N mice. Whether the statistically significant reduction of \textit{mRft2} in the small intestine during gestation stage has any biological significance is open to speculations.

3. RFT Protein Expression

Previous attempts to raise antibodies against hRFT1 have been unsuccessful (Yonezawa et al., 2008). Our endeavor to raise an antibody against mRFT1 was similarly fruitless.

No attempts to produce an anti-hRFT2 antibody have been discussed in the literature. Yet, a commercial polyclonal anti-RFT2 antibody is available from Santa Cruz
Biotechnology. The manufacturer claims this antibody to cross-react with human, mouse and rat RFT2 proteins. We have used the anti-RFT2 antibody to evaluate the levels of this protein in the mammary glands of FVB/N mice. Our mRNA studies indicated a dramatic upregulation of $mRft2$ in lactation, followed by a return to virgin-like levels during involution. It was hypothesized that the similar pattern of expression would be observed at the protein level. The predicted size of mRFT2 is 49.6 kDa. Interestingly, in the lactating state, we have observed two bands at 50 kDa and 40 kDa, with noticeably stronger signal for the latter band. The signal at 40 kDa persisted in both virgin and involuting mammary preparations, with no signal observed at 50 kDa. The signal at 40 kDa was not initially anticipated. Whether it represents a splice variant of mRFT2, or a completely different protein remains unknown.

Although it cannot be said for certain, which signal (if any) is to be attributed to mRFT2, the pattern of protein expression observed at both 40 kDa and 50 kDa closely parallels our mRNA findings. If true, this finding may suggest a lactation-specific role of mammary mRFT2 in concentrating riboflavin in the milk.

In order to make a definitive conclusion, anti-RFT2 antibody must be validated. One approach involves fusing GFP and RFT2 proteins. Here, by expressing the fusion protein in a cell line of choice, and later running Western blots incubated in anti-GFP and anti-RFT2 antibodies, the banding pattern can be analyzed. If signals recognized by these two antibodies align, it could be possible that anti-RFT2 antibody indeed reacts with RFT2. Alternately, a more robust proteomics approach can be applied, where sequencing of a specific band could be performed; thereby, identifying the protein in question.

4. Subcellular Localization of hRFT2 in Mammary-derived Cell Line MCF10A

Our mRNA studies and those of others (Subramanian et al., 2011b) indicate that relative expression of $hRFT2$ exceeds that of other $RFT$. Hypothesizing that the same observation is conserved at the protein level, and that functionally hRFT2 may play a primary role in riboflavin transport, it was decided to investigate the localization of hRFT2 in a mammary gland model.

Previous studies have shown that hRFT2 is localized primarily to the plasma membrane of the HEK-293, Caco-2 and canine kidney MDCK cells (Yao et al., 2010; Subramanian et al., 2011b). It was also shown that hRFT2 was exclusively expressed at
the apical membrane of the polarized human intestinal (Subramanian et al., 2011b). It was hypothesized that in mammary epithelial cells, hRFT2 would be similarly localized to plasma membrane, and likely to be expressed on the basal side of polarized mammary epithelial cells.

Indeed, it was observed that in MCF10A mammary epithelial cells, GFP-hRFT2 fusion was localizing to the plasma membrane, underscoring a role for hRFT2 in riboflavin transport across the cellular membrane. Although at the moment, it cannot be stated, where on the plasma membrane of mammary epithelial cells hRFT2 is expressed, we are currently exploring ways to polarize mammary epithelium, in order to answer this question.

Overall, our observations are in agreement with the others: hRFT2 is localized to the plasma membrane.

5. Riboflavin-Cimetidine Interaction Study in Mice in vivo

BCRP plays a major role in excretion of drugs and toxins into the breast milk (Robey et al., 2009), such as chemotherapeutic agents, carcinogens and multiple other drugs, including cimetidine. Implications for some of these drugs on the nursing infant are currently unknown. Therefore, an intervention strategy to decrease BCRP-transported xenobiotic excretion into breast milk may be necessary.

Cimetidine was chosen as a model drug for investigation of mammary BCRP transport in vivo. It was hypothesized that mammary BCRP transport function could be saturated with riboflavin, a vitamin with no known adverse effects; thereby, reducing the accumulation of cimetidine in the milk.

To test this hypothesis, the dose of riboflavin supplementation was initially characterized, by measuring milk and plasma levels of this vitamin 90 minutes after administration. This time point was chosen initially as the endpoint of the proposed experiment. Compared to the control FVB/N mice on standard chow, intravenous administration of riboflavin at 2 μg/g and 5 μg/g of body weight significantly enhanced the milk riboflavin levels, with only a slight increase at 5 μg/g when compared to 2 μg/g dosing. On the other hand, plasma levels were dramatically increased only at 5 μg/g of body weight of riboflavin. This observation suggested that at a dose of 5 μg/g of body weight systemic riboflavin excretion including milk secretion is saturated, resulting in an increase of plasma riboflavin. This observation is further supported by comparing MP
ratios of control and supplemented groups: 64, 62, 63, and 23 being the MP ratios of control, 1 μg/g, 2 μg/g and 5 μg/g body weight riboflavin, respectively. Therefore, it was decided to utilize a dose of 5 μg/g body weight of riboflavin to conduct the mammary BCRP competition study.

By following the experimental outline (see page 47), using the 90 minute endpoint, no significant reduction in milk levels of cimetidine was observed. It was expected that in the presence of high-dose riboflavin supplementation, cimetidine milk levels will be decreased, while plasma levels may rise. Furthermore, the observed MP ratio of cimetidine was lower than expected. Jonker and colleagues (2005) report an MP ratio of 13 at 30 minutes post-dose for cimetidine; however, we have only observed an MP ratio of about 4 at 60 minutes post-dose.

It was then decided to alter the original protocol, and move the endpoint of the experiment to 60 minutes. Additional quantifications of milk riboflavin levels at 60 and 30 minutes, using 5 μg/g body weight supplementation, were conducted in order to ensure the milk levels of this vitamin remained sufficiently high. It was observed that in the presence of high-dose riboflavin supplementation, cimetidine milk levels were significantly reduced. This finding suggests that it may be possible to manipulate mammary BCRP function, thereby reducing its substrate transport into the milk.

Having said that, it is unclear why our cimetidine MP ratios at 30 minutes post-dose were still low, about 2 as compared to the study by Jonker et al. By comparing our observations to those of Jonker and colleagues (2005), it can be seen that even though the milk levels of 3H-cimetidine are similar, it is the plasma levels of the drug that makes the difference. At 30 minutes post-dose, Jonker et al. (2005) report plasma 3H-cimetidine levels to be 150 ng/ml, while we observe 1000 ng/ml. One possible explanation is the difference in clearance between the animals used. Even though both Jonker et al. (2005) and we used mice of the FVB genetic background, some differences may be expected, as these animals are inbred.

Another explanation may relate to milk riboflavin levels in the animals on standard chow. Even though the levels of riboflavin per kilogram of chow may be identical, it is difficult to predict the eating behaviours and their differences between the animals. It may be possible, that initially milk levels of riboflavin in mice used by Jonker et al. (2005) were lower; thereby, indirectly influencing the cimetidine transport through BCRP.
6. Limitations

We have characterized *RFT* mRNA expression in mammary epithelial cells *in vitro*. Given further attempts to evaluate the possible patterns of expression in the breast milk cells, the precise expression profiles in the human mammary gland and epithelial cells *in vivo* remain unknown. Having shown that certain *RFT* are predominantly expressed in mammary epithelial cell lines, it is unclear whether higher mRNA levels relate directly to functional activity and protein expression. Specific and sensitive RFT antibodies are needed to further address some of these questions.

7. Future Directions

There are a number of directions that can follow from this project. Firstly, functional analysis and contribution of mammary RFT to riboflavin transport could be further determined, in order to understand the patterns of predominance observed in this project. Secondly, localization of hRFT-GFP fusion proteins in human polarized mammary epithelium can be evaluated. These finding may suggest the likely differences between RFT and their roles in mammary riboflavin transport. Furthermore, raising and validating specific antibodies against RFT may provide not only a way to analyze protein expression of RFT in cells and tissues, but also an alternative way of evaluating RFT localization by immunohistochemistry in whole mammary gland preparations.

In addition, the experimental protocols, described under BCRP interaction studies, could be applied to study the effect of riboflavin enhanced or deficient diet on BCRP substrate xenobiotics. Similarly, other substrates, such as chemotherapeutic agents, and their interactions with riboflavin for BCRP transport could be evaluated. If these proof-of-principle *in vivo* experiments show clinically significant differences, the next step would be to refine the dosing schedule of riboflavin for clinical studies.
CONCLUSIONS

Currently, very little is known about riboflavin transport in the mammary gland. In addressing Specific Hypothesis 1, I was able to show that RFT mRNAs are differentially expressed in human and mouse mammary cell lines, as well as cells derived from human breast milk and mouse mammary glands. In the mouse mammary tissue RFT mRNAs were shown to be upregulated in lactation, consistent with Specific Hypothesis 1. Under Specific Hypothesis 2, I have shown that hRFT2 is expressed on the plasma membrane of MCF10A mammary epithelial cells. Overall, these findings suggest a potential role of RFT in mammary riboflavin transport. Informed by these findings, the riboflavin-cimetidine interaction experiments in mice in vivo further suggested that riboflavin may be utilized to reduce milk excretion of BCRP substrate xenobiotics.

This study provides valuable insight into mammary riboflavin transport and its potential use for pharmacological intervention. It is hoped that further studies evaluating BCRP-RFT networks of riboflavin transport will open doors to novel clinical application.
REFERENCES


APPENDICES

Appendix I. Levels of intestinal mRft1 and mRft2 mRNA Expression during Pregnancy and Lactation in FVB/N Mice. Proximal third of small intestine was excised from five groups of FBV/N mice on standard chow: 1) virgin (9-10 weeks old), 2) mid-gestational (15th day post coitus), 3) lactation 1 wk (on the 7th day post delivery), 4) lactation 2 wk (on the 14th day post delivery), and 5) involution (a week after litter-weaning). Total RNA was extracted, and then reverse transcribed. The expression of mRft1 and mRft2 mRNA was analyzed using Real-Time Quantitative PCR. Results were first normalized to villin, and then to virgin state. The $2^{-\Delta\Delta C_T}$ method was used to determine relative changes in gene expression. Each group value represents a mean ± standard deviation. Statistical tests were conducted using SPSS 17.0. Normality tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test was performed to analyze the differences between the virgin and the other four groups. * denotes a statistically significant result as compared to virgin group.

![Graph showing fold change in expression of mRft1 and mRft2 mRNA during pregnancy and lactation](image)
Appendix II. Raw qPCR data of mammary fat cDNA samples extracted by LCM

<table>
<thead>
<tr>
<th>Fat</th>
<th>Sample</th>
<th>mRFT1</th>
<th>mRFT2</th>
<th>m-Csn2</th>
<th>m-Gapdh</th>
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Appendix III. Milk and Plasma Levels of FAD and FMN in FVB/N Mice. One-week lactating FVB/N mice were intravenously administered riboflavin at doses of 1 μg/g, 2 μg/g or 5 μg/g body weight, or phosphate buffered saline (control). To enhance milk secretion, 250 μL of a 1 IU/ml oxytocin solution was injected subcutaneously 10 min prior to milk sampling. At 90 min, milk was collected from the inguinal mammary glands, followed by blood collection via cardiac puncture 1 to 2 minutes later. Whole blood was centrifuged at 2000 x g for 10 min at room temperature. Plasma was transferred into new tubes. Milk and plasma samples were protected from light. Riboflavin levels were analyzed by high-performance liquid chromatography. Each group value represents a mean ± standard error. Multiple group comparisons were carried out by one-way ANOVA. Dunnett’s post-hoc comparison test did not reveal significant differences (p < 0.05) between the control and the other three treatment groups.

A. Milk

B. Plasma