UNIVERSITY OF CINCINNATI

Date: 14-Aug-2009

I, Aaron Marshall, hereby submit this original work as part of the requirements for the degree of:

Doctor of Philosophy

in Molecular & Cellular Physiology

It is entitled:

The Biology of Mammary Gland Serotonin Synthesis and Transport

Student Signature: Aaron Marshall

This work and its defense approved by:

Committee Chair: Nelson Horseman, PhD

Yana Zavros, PhD

Karen Gregerson, PhD

Nira Ben-Jonathan, PhD

Michael Behbehani, PhD
The Biology of Mammary Gland Serotonin Synthesis and Transport

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the Department of Molecular and Cellular Physiology of the College of Medicine 2009 by

Aaron M. Marshall

B.S. 2005, Millersville University, Pennsylvania Committee Chairman: Nelson D. Horseman, Ph.D.
ABSTRACT

Significance: Understanding and identifying the mechanisms surrounding secretory activation and involution in the mammary gland are important to aid mothers who experience breastfeeding difficulties and to aid the dairy industry in their production of important nutritional products.

Background: Recently our lab discovered that serotonin is synthesized by the mammary gland epithelium in response to lumenal distension. This and other findings implied that serotonin acted as a negative feedback inhibitor to lactation, thus regulating the balance between secretory activation and involution. The onset of secretory activation is initiated by the assembly of functional tight junctions. It was therefore hypothesized that serotonin regulates tight junctions in order to regulate lactation. However no suitable models for studying mammary epithelial tight junction regulation were available. In addition, the physiological consequences of altering serotonin activity remain unexplored.

Goals: Goals that are met in this dissertation are as follows: (1) Create and characterize an in vitro model for studying mammary epithelial tight junction regulation, (2) Determine whether drugs that alter intrinsic serotonin turnover can recapitulate the effects of exogenously added serotonin and (3) Document the physiological consequences of altering serotonin bioactivity in vivo.

Results: An in vitro multipotential, barrier-forming model was developed and displays robustness, predictability, differentiation, and expected genetic changes throughout the development of high-resistance tight junctions. In parallel with characterization of this model, serotonin was shown to regulate tight junction permeability when added exogenously. In addition, drugs that increase serotonin bioavailability (fluoxetine, phenelzine, or dextfenfluramine) affect tight junction permeability in a biphasic manner, similar to exogenous serotonin. When serotonin bioavailability is increased in vivo in lactating mice, precocious involution occurs. Conversely, mouse mammary glands void of serotonin synthetic capacity (i.e. decreased bioavailability) display hyper-secretory activation, which presents as ductal ectasia, and lumenal distension.

Conclusion: Serotonin secreted by the breast acts to regulate the balance between secretory activation and involution, and drugs affecting serotonin bioavailability may dysregulate lactation.
ACKNOWLEDGEMENTS

There is no shortage of people to thank for helping me financially, physically, intellectually and emotionally through the process of this work. I would to thank my dissertation committee for their helpful suggestions and guidance toward the completion of this work.

Thank to my fellow graduate students in the Physiology program and to Bette Young, program coordinator, for being our surrogate mother. She is the best at taking care of her students. Thanks to Stuart Handwerger, MD, for his training grant support and believing I was worthy of it.

To my lab mates over the past years, a great many thanks: Malinda Stull PhD, Archie Vomachka PhD, Melinda Reilly, Melissa Orr, Laura Hernandez PhD, and especially fellow lab mate and past graduate of Physiology program Vaibhav Pai PhD. He is good friend and excellent source for bouncing ideas off of. Of course, thanks to my mentor and committee chair Nelson Horseman PhD. I appreciate the balance between involvement and independence he granted me toward the completion of this project. It has allowed me to flourish as a scientist and independent thinker; skills that will serve me well no matter my career path.

At last, but certainly not least, to my family (including my awesome dog, Izzy); thank you, thank you, thank you. Most especially to my beautiful wife Reneé. Thank you for being by my side and unconditionally supporting me, no matter how random and intense my time in the lab was. It means the world to me to hear how proud you are of me.
# TABLE OF CONTENTS

**Chapter I: Literature Review**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary Epithelial Homeostasis</td>
<td>2</td>
</tr>
<tr>
<td>Mammary Gland Development in the Peripartum &amp; the Initiation of Lactation</td>
<td>2</td>
</tr>
<tr>
<td>Weaning and Involution</td>
<td>8</td>
</tr>
<tr>
<td>Serotonin: A Little Monoamine with Large Responsibilities</td>
<td>11</td>
</tr>
<tr>
<td>Biosynthesis</td>
<td>11</td>
</tr>
<tr>
<td>Regulation</td>
<td>12</td>
</tr>
<tr>
<td>Physiological Function</td>
<td>15</td>
</tr>
<tr>
<td>Intraductal Fluid Homeostasis: A Common Serotonergic Mechanism Among Diverse Epithelia</td>
<td>17</td>
</tr>
<tr>
<td>Introduction</td>
<td>17</td>
</tr>
<tr>
<td>Historical Perspective on Classification</td>
<td>18</td>
</tr>
<tr>
<td>Lung</td>
<td>19</td>
</tr>
<tr>
<td>Pancreas</td>
<td>20</td>
</tr>
<tr>
<td>Prostate</td>
<td>21</td>
</tr>
<tr>
<td>Salivary Glands</td>
<td>22</td>
</tr>
<tr>
<td>Liver</td>
<td>23</td>
</tr>
<tr>
<td>Mammary Gland</td>
<td>24</td>
</tr>
<tr>
<td>Serotonin Receptor Patterning</td>
<td>26</td>
</tr>
<tr>
<td>References</td>
<td>29</td>
</tr>
</tbody>
</table>

**Chapter II: Characterization of a novel barrier forming in vitro multipotential model**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>42</td>
</tr>
<tr>
<td>Methods</td>
<td>43</td>
</tr>
<tr>
<td>Results</td>
<td>46</td>
</tr>
</tbody>
</table>
Differentiation and development of barrier forming cells.................................46
Humoral factors affecting tight junction patency..............................................52
Gene expression microarray of Transwell model.............................................55
Discussion........................................................................................................60
References........................................................................................................64

Chapter III: Effects and possible alternate ligands of 5-HT7 signaling in the
mammary epithelium......................................................................................71
Introduction....................................................................................................72
Methods........................................................................................................75
Results..........................................................................................................76
Immunolocalization of 5-HT7..........................................................................76
Downstream effector of 5-HT7 dependent tight junction disruption....................77
Stable expression of 5-HT7α in MCF10A cells...............................................77
5-HT7 antagonists effects on MCF10A cells..................................................78
Development of system to screen for alternate 5-HT7 ligands..........................79
Discussion.....................................................................................................80
References.....................................................................................................81

Chapter IV: Serotonin transporter in the mammary gland modulates secretory
activation and involution .............................................................................84
Introduction....................................................................................................85
Methods........................................................................................................87
Results.........................................................................................................90
Contribution of SERT and MAO to Intrinsic 5-HT Activity in MCF10A cells........90
Conservation of Mechanisms in Human Breast and Bovine Mammary Cells......93
Chapter V: Perspectives and Future Directions

Expression and Localization of SERT in vivo ................................................................. 94

In Vivo Blockade of 5-HT Reuptake During Lactation .................................................. 95

Assessment of SSRI as risk factor for delayed onset of lactogenesis .......................... 97

Quantification of 5-HT .................................................................................................... 100

Discussion .................................................................................................................... 101

References .................................................................................................................... 105

MCF10A Transwell model ........................................................................................... 111

Evolutionary conservation of 5-HT feedback system ............................................... 113

Serotonin’s role in the involution process ................................................................. 114

Serotonylation of milk proteins ................................................................................ 114

Lactation timing and performance effects of serotonergic drugs ......................... 115

References .................................................................................................................... 117
FIGURE CONTENTS

Chapter I
Figure 1: Mammary gland whole mounts representing four stages of development...............................2
Figure 2: Molecular control of alveolar development..................................................................................5
Figure 3: Mammopoesis in the mammary gland during secretory activation..............................................7
Figure 4: Proteins involved in the regulation of serotonin action.................................................................11
Figure 5: Alternate access mechanism for serotonin transport....................................................................14
Figure 6: Permeability of mammary epithelium during pregnancy and lactation........................................24
Table 1: Serotonin receptor expression profiles of various 5-HT producing ductal organs............................27

Chapter II
Figure 8: Diagram of Transwell® model......................................................................................................43
Figure 9: Localization of tight junctions and adherens junctions in MCF10A Transwell®.........................47
Figure 10: Barrier formation in MCF10A Transwell® cultures..................................................................48
Figure 11: Effect of medium conditioning on MCF10A polarization..........................................................49
Figure 12: Differentiation of MCF10A cells into multiple phenotypes.....................................................50
Figure 13: Development of MCF10A epithelial membranes from a clonal subpopulation..........................51
Figure 14: Effects 9-cis-retinoic acid on MCF10A Transwells®.................................................................52
Figure 15: Effects sRANKL on MCF10A Transwells®................................................................................53
Figure 16: Effects of prolactin and concomitant removal of EGF on MCF10A Transwells®......................53
Figure 17: Effects of basement membrane proteins on MCF10A Transwells®..........................................54
Figure 18: Effects of milk in the top chamber of MCF10A Transwells®....................................................55
Figure 19: Venn diagram representing gene expression changes in MCF10A Transwells®.......................56
Figure 20: Clustered expression profiles over MCF10A Transwell® differentiation..................................57
Figure 21: Immunolocalization of GABA A, B in MCF10A Transwells®..................................................60
Figure 22: Potential, resistance and current measurements of MCF10A Transwells® following
GABA, picrotoxin or isoguvacine treatment............................................................................................61

Table 2. Gene expression profiles of gene ontology categories and individual genes of MCF10A
Transwells®............................................................................................................................................58

Chapter III
Figure 23: Proposed model for serotonin system in mammary epithelium................................................73
Figure 24: Immuno-staining for 5-HT A, in MCF10A Transwell®..............................................................76
Figure 25: Effects of IL-1β on barrier function in MCF10A Transwells®....................................................77
Figure 26: Visualization of GFP representing exogenously expressed 5-HT A in MCF10A cells................77
Figure 27: Sensitivity if MCF10A-5HT A stable clones to 5-HT..................................................................78
Figure 28: MCF10A Matrigel® culture enhanced fluid secretion by metergoline........................................79
Figure 29: Luciferase assay of CHOK1 transfected 5-HT$_7$a plasmid following 5-HT treatment...

Chapter IV

Figure 30: Barrier disruption of MCF10A Transwell® cultures with fluoxetine..........................91
Figure 31: Conservation of 5-HT metabolic regulatory mechanisms in human and bovine mammary epithelial cells..............................................................................................................93
Figure 32: Expression and localization of SERT in mouse mammary glands.................................95
Figure 33: Effects of Elvax® fluoxetine pellet implants in lactating mammary glands......................96
Figure 34: Timing to onset of lactogenesis stage II in primiparous women..................................98
Figure 35: Quantification of 5-HT by ELISA in MCF10A Transwells®........................................100
Figure 36: Quantification of 5-HT by RIA in mouse milk after 5-HT spike....................................100
Figure 37: Quantification of 5-HT by RIA in mouse milk after fluoxetine injection.......................101
Table 3: Characteristics of the SSRI and non-SSRI primiparous mothers in the cohort............99
CHAPTER I

LITERATURE REVIEW

Aaron M. Marshall

Systems Biology and Physiology Program
Department of Molecular and Cellular Physiology
University of Cincinnati, Cincinnati, Ohio 45267-0576
Mammary Epithelial Homeostasis

Mammary Gland Development in the Peripartum & the Initiation of Lactation

The mammary gland is an exocrine organ. Most mammalian exocrine organs actively secrete or are in a readiness state for future secretion. The mammary is unique in that the preponderance of its development occurs postnatally. For the majority of its existence the mammary gland is quiescent and may be throughout life if gestation never occurs. Post-puberty, during adulthood the mammary gland is a dichotomous ductal network that lacks the differentiated functional structures (alveoli) (Fig 1).

In terms of milk-producing epithelial tissue, the majority of expansion and differentiation occurs during gestation and early post-partum. The reversal of the mammary gland back toward a pre-pregnant state following the cessation of nursing is termed involution. Because the vast majority of development occurs postnatally, it is experimentally tractable to study hormonal and structural

Figure 1. Panels display whole mounts representing four stages of mammary gland development in an adult mouse. Notice the rudimentary ductal tree in virgin is expanded upon by increasing side-branching during pregnancy. Although involution closely represents a pre-pregnant virgin state, the two stages are morphologically and functionally different. From: http://mammary.nih.gov/atlas/wholemounts/normal/index.html
requirements for this development. What is known about peripartum and postpartum development processes will be explored here, especially highlighting endocrine hormone influences. Much of what is known about mammary gland development was investigated using rodents (predominantly mice) as a model. Overall, mice serve as a very useful and valid model for human mammary gland development although differences do exist and will be highlighted where appropriate.

Prepartum development in mice can be divided into two parts: alveolar development and secretory differentiation. Alveolar development is the proliferation and arborization of the epithelial cells within the fat pad of the mammary. This increased side-branching is rooted within the less expansive ductal network that develops during puberty (reviewed in ref (1)). This stage of development occurs early in pregnancy and following its completion, the mammary gland is almost entirely filled with glandular tissue, replacing adipose tissue (Fig 1). At this point it is important to highlight that, conceptually alveolar development precedes secretory differentiation during gestation, however in reality these two stages overlap, with latter becoming predominant by the end of gestation. Although these same two stages are described in women, the anatomy of the human breast is different. The timing of secretory differentiation in women (measured by an increase in urine lactose) ranges from mid pregnancy (20 weeks) to late pregnancy (30 weeks) (2). In addition, miniaturized alveolar development occurs in non-pregnant women and is associated with the luteal phase of the menstrual cycle, when progesterone surges (3). Thus, the menstrual cycle represents short, “mini” periods of alveolar development. Normally, secretory differentiation does not occur without gestation however.

The positive endocrine influences in alveolar development have long been established. During pregnancy an increase in circulating progesterone and prolactin binding to their respective receptors synergize to increase proliferation, thus expanding the epithelium (4). Recently, two progesterone receptors (PR) have been identified as splice variants from the same gene; PR-A and PR-B. It seems that the pregnancy-associated alveolar expansion is a result of PR-B signaling, which is highly expressed during pregnancy (5, 6). Although not discussed here progesterone also seems to be the hormone responsible for suppressing milk production during gestation (7,
8). Also an important endocrine member, circulating estrogen is important to maintain expression
of progesterone receptor during pregnancy (9). Prolactin receptor (PRL-R) is transcribed from
a single gene, but again with multiple isoforms resulting. The number of receptor variants and
differential function of each receptor isoforms varies between species and has been reviewed
elsewhere (10).

The origin of the side-branches during alveolar development is in part controlled by the
hormone TGFβ1. TGFβ1 inhibits proliferation in steroid receptor positive cells and thus may
dictate where this branching originates (11, 12). TGFβ1 heterozygote mice display accelerated
alveolar development in response estrogen and progesterone following ovariectomy (exogenous
hormones mimic peripartum), supporting the role of TGFβ1 in mammary arborization (13).

Interestingly however, not all cells within the developing mammary express the progesterone
and prolactin receptors. In fact there is a clearly documented delineation between the cells that
express progesterone receptor (and prolactin receptor) and the cells that enter mitosis (5). The
mitotic cells are adjacent to the receptor positive cells. In addition, it has been shown that pre-partum
PRL-R and PR are uniformly expressed, but during gestation the receptors are expressed only in
a subset of epithelial cells (14). This hinted at a paracrine mediated mechanism of proliferation
during alveolar development. A few candidate proteins seem to fit the bill for paracrine mediators
of proliferation in the peripartum. Receptor activator of NF-κB ligand (RANKL) is a membrane
bound ligand for the its receptor RANK. However, RANKL ligand can be cleaved to a soluble
form (sRANKL) by certain matrix-metalloproteases (MMP) and an enzyme called TACE (15, 16).
Cells expressing progesterone receptor also express RANKL, and the neighboring cell becomes
cyclin D1 positive (proliferating) in response to progesterone (5) (Fig 2). Furthermore, RANKL
expression in mammary epithelial cells is dependent on progesterone and JAK2-STAT5 signaling
downstream of PRL-R (17). RANKL-/- mice display normal mammary gland development through
adulthood, with comparable ductal tree structures and similar numbers of side-branches. However,
RANKL-/- mothers failed to provide milk to their young because of an alveolar proliferative
defect during gestation (18). The summative hypothesis is that progesterone and prolactin
Figure 2. Molecular control over alveolar development. Heterogeneous receptor expression of the progesterone receptor (PGR) and prolactin receptor (PRLR) results in neighboring cells becoming mitotic via a paracrine mechanism. Two possible paracrine mechanisms are receptor activator of NF-κB ligand (RANKL) and wingless-type protein 4 (WNT4). RANKL signals to its receptor RANK causing NF-κB translocation and cyclin D1 expression. Wnt4 signaling in the mammary is presently unknown. Wnt4 is induced by PG signaling and RANKL is induced by a synergism between PG and PRL.

signaling through their receptors causes expression of RANKL or cleavage of membrane bound RANKL to sRANKL, which then binds to its receptor RANK on the neighboring cell causing it to proliferate. A second candidate mediator of paracrine stimulated alveolar proliferation is Wnt4. Wnt4 expression is regulated by progesterone, and its ectopic expression in PR-/- mammary tissue was able to rescue the side-branching defect observed in PR-/- mice (19). Recombinant Wnt4 expressed in normal, virgin transplanted mammary tissue displayed increased side branching and pregnancy like growth pattern (20). It is not known by what signaling mechanism Wnt4 signals to affect mammary development. Wnt proteins in general have four identified signaling modalities: the canonical Wnt/beta-catenin pathway in which Wnt proteins bind to ‘frizzled’ receptors, which leads to downstream activation of gene transcription by several signaling pathways (21). Taken together RANKL and Wnt4 play an integral role in mediating the proliferation response observed
in the peripartum (Fig 2).

Secretory differentiation (aka lactogenesis I) occurs late during gestation and involves the endocrine hormones important for alveolar development. Also permissive to secretory differentiation are insulin, glucocorticoid, and growth hormone. Secretory differentiation is the process by which luminal epithelial cells become capable to synthesize and secrete milk. It is characterized by the transcription of milk protein mRNA such as β-casein and whey acidic protein, and by the expression of lactose synthesizing enzymes galactosyltransferase and α-lactalbumin (together lactose synthase). The control of proliferation versus differentiation with many of the same endocrine influences is not completely understood. One possible explanation involves epithelial-mesenchymal interactions and physical forces placed on the alveolus. These interactions and signaling systems have been demonstrated to be important for secretory differentiation, but are not reviewed here (22). Although the endocrine mediators of secretory differentiation (versus alveolar development) remain poorly delineated, a plethora of research identifying the important transcription factors does exist (23)(Fig 3). Of particular importance is the transcription factor Gata-3, which is involved in commanding lineage-specific progenitors to the ductal cell lineage and/or alveolar cell lineage (24, 25). Down the mammopoeisis tree from Gata-3 is Elf5, a member of the Ets transcription factor family, identified in the mammary gland as being important for establishing the secretory differentiated lineage (26, 27) (Fig 3). Whether Gata-3 and Elf5 cooperate to establish secretory differentiation remains to be determined.

The initiation of lactation, termed secretory activation (aka lactogenesis II), is different from secretory differentiation mainly due to the volume and composition (discussed later) of the milk being produced. As referenced above, secretory differentiation can be detected by lactose in the urine, meaning that the mammary gland is indeed making milk, albeit little. Secretory activation normally occurs 24-40 hours after parturition, and a pathological delay in activation in humans is defined as <9.2 g of milk/feeding at 60 hours post partum (28). After secretory activation commences, copious amounts of milk are produced and will continue to be produced given frequent emptying of the glands. The main hormonal requirement for secretory activation is the removal
of a hormone, progesterone (7). Since progesterone is synthesized by the placenta in humans late in gestation, incomplete removal of placental tissue can lead to delayed secretory activation (29). The presence of prolactin, growth hormone, and IGF ligands are important positive regulators of milk production during the process (4, 30). Oxytocin, most well known for its function in the process of milk ejection, also enhances milk production. The energy burden on the mother to produce milk is tremendous, and the remobilization of energy stores and nutrients to the gland are extensive (31). Because of all these shifts in energy physiology, metabolic hormones such as thyroid hormone, insulin, and leptin can alter milk production and/or delay secretory activation (32, 33).
The changes in milk composition that accompany secretory activation are a function of changes in barrier function within the epithelium (34). Before parturition, the mammary epithelium is leaky, and therefore proteins and electrolytes comprising the milk resemble the components in plasma. After secretory activation the composition of milk gradually changes to eventually reflect the composition of “true milk”. Some of the important changes are decreases in Na⁺, Cl⁻ and serum proteins, and concomitant increases in Ca²⁺, lactose, citrate and milk proteins (34). Many of the hormones referenced above have been studied with respect to their ability to alter tight junction permeability in the mammary gland. Progesterone withdrawal facilitates tight junction closure with synergism from glucocorticoids (35). Parathyroid hormone related peptide (PTHrP) is known to have a role in Ca²⁺ mobilization at the onset of secretory activation, however it has also been shown to be important for maintaining a high resistance barrier in mammary epithelial cells (36). It is probable that other autocrine/paracrine factors synthesized in the mammary are secreted to affect tight junction closure and secretory activation.

**Weaning and Involution**

Normally the cessation of lactation is a gradual process. In mice, pups begin eating solid food occasionally around 14-18 days of age and nurse less frequently and by 21 days of age are independent from the need for milk. A similar scenario normally occurs in breastfeeding women under the direction of a doctor. Gradual weaning is recommended for infants at 6 months of age gradually replacing milk with solid food. Full weaning is not achieved until 12 months of age in most cases. Because of this natural, gradual weaning process the involution process is also gradual. It is probable that some less used lobuloalveolar units have already regressed/involuted before full weaning, while more active ones remain producing milk. However, to study the process of involution using mice as a model, a forced weaning model is employed, where the pups are removed during peak lactation (day 7-10 postpartum). This does not normally occur in women, except in cases where women suddenly decide to stop breastfeeding, or if the infant suddenly dies. Regardless of the timing, it is believed that the molecular mechanisms are the same, the difference being the duration with which the process occurs.
The process of involution has been divided into two main stages, reversible and irreversible phases (37). In the forced weaning model, the reversible phase lasts approximately 48-72 hours and if the pups are returned within that time the gland is able to achieve full lactation again. The irreversible phase follows the reversible phase, and is characterized by total tissue remodeling and another pregnancy is required to achieve lactation again. The reversible phase can be subdivided into early and late stages. Early stage (0-36 hours) is described by filling of the alveoli, resulting in milk stasis which then causes a decrease in milk synthesis and secretion by epithelial cells and disruption of tight junctions. The late stage (36-72 hours) is accompanied by initiation of apoptosis and the leakage of milk into the interstitial space. The disruption of tight junction initiates the late stage events. Because of this compromise in the barrier, lactose can once again be observed in the urine following tight junction disruption. The initiating event for the total process is milk stasis. Miniaturization of comparative gene expression technology has enabled the identification of a plethora of molecules transcribed following milk stasis (38). Most of these factors promote cell death by apoptosis. Apoptotic pathways thought to be involved in mammary involution are mitochondrial pathways (Bcl family of proteins, caspases and cytochrome c), death receptor pathways (activation of Fas by local factors), and endocrine pathways influencing the two other pathways. For example leukemia inhibitory factor (LIF) is induced hours after milk stasis and causes phosphorylation of STAT3, which is known to be activated during involution (39). What had not been known previously is what the earliest events induced by milk stasis are. To answer this question, recently a paper from our lab described the upregulation of \textit{TPHI} the gene encoding for tryptophan hydroxylase 1 (40). This gene was found in a genomic wide screen comparing PRL knockout mammary glands from a virgin animal to hyperstimulated (by PRL) virgin tissue. The results of this study (detailed later) revealed that serotonin was induced by milk stasis and not by PRL signaling directly \textit{per se}. However, despite identification of novel factors involved in early involution it is not known what determines/delineates the transition from the reversible phase to the irreversible phase.

The irreversible phase of involution morphologically resembles a muted immune response
Inflammation is limited, however macrophages increase their presence and participate in clearance of tissue. As mentioned previously, the transition from the reversible to irreversible phase is incompletely understood, and may not be a distinct point in time, but a gradual transition. During this phase of involution, the architecture of the gland is remodeled requiring the action of many proteases such as matrix metalloproteases (MMPs), and other non specific proteases such as kallikrein (42-45). The MMPs are expressed in the stromal tissue, and it is hypothesized that even though in some genetically-manipulated mice the reversible phase of involution is dysfunctional (e.g. TPH1−/−), the irreversible phase is able to compensate. Specifically, the MMPs facilitate detachment-induced apoptosis or anoikis (albeit later) through release of the epithelia from their basement membrane (46). Phagocytosis plays an important role in the tissue’s ability to remodel. The appearance of macrophages increases dramatically by day 4 post weaning (47). Interestingly however, the bulk of apoptotic cell clearance occurs prior to day 4. Recently it was demonstrated that mammary epithelia themselves can act as phagocytes and that this occurs early in involution and as a result of normal cell turnover during full lactation (48). Epithelial cells express many receptors associated with professional phagocytes such as structures that recognize phosphatidylserine, CD36, etc (48). Overall, epithelial phagocytosis serves to clear apoptotic cells during lactation and early involution, while macrophages are recruited and take over in late involution.

Another part of the remodeling process, other than cell death in the epithelium and matrix changes, is the repopulation of adipose tissue. Few studies have sought to understand the timing and factors regulating the proliferation and differentiation of pre-adipocytes into mature adipose following weaning. One study demonstrated the importance of serum proteases to adipose regeneration in the mammary by showing that inhibition of the plasminogen protease system retards the repopulation of adipose (45). Interestingly, instead of adipose repopulation, involuting mice treated with this inhibitor displayed increasing presence of collagen fibers. Serotonin has been shown in other adipose literature to suppress adipocyte differentiation (49). Whether this action of serotonin is conserved in the mammary gland and is responsible for maintaining low
amounts of adipose during lactation remains to be determined.

Serotonin: A Little Monoamine with Large Responsibilities

Biosynthesis

5-Hydroxytryptamine (5-HT), commonly known as serotonin, is a monoamine synthesized from the essential amino acid L-tryptophan in a two step reaction (Fig 4). Tryptophan is hydroxylated to 5-hydroxy-L tryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). Oxygen and tetrahydrobiopterin are required cofactors for this first step. TPH is the rate limiting step, and it is found as two isoforms, transcribed by separate genes (TPH1 & TPH2) (50, 51). TPH1 is expressed peripherally and is responsible for the bulk of serotonin that circulates, while TPH2 is localized to neurons, and synthesizes serotonin used in neurotransmission. 5-HTP is decarboxylated by L-amino acid decarboxylase (AADC) to create 5-HT. Necessary cofactors for this step are vitamin B6, vitamin B3, and magnesium (not shown) (52). In certain tissues such as the pineal gland a third

![Figure 4](image-url.png)

Figure 4. Overview of proteins involved in regulating or mediating serotonin function. Not all proteins involved in synthesis, degradation or vesicular release are shown.
enzyme, serotonin-N-acetyltransferase (SNAT), is present converting serotonin into melatonin. In most other tissues, not expressing SNAT, 5-HT is oxidized to an inactive form (5-hydroxyindole acetylateddehyde) by the ubiquitous enzyme monoamine oxidase (MAO) (Fig 4). 5-hydroxyindole acetylateddehyde is acted upon by aldehyde dehydrogenase to yield 5-hydroxyindole acetic acid (5-HIAA). 5-HIAA circulates in plasma, is excreted in the urine and thus used as a quantitative indicator of serotonin synthesis in the body (53). Although TPH1 and 2 are compartmentalized in terms of their expression, 5-HTP is able to freely cross the blood-brain barrier, while 5-HT cannot (54). In addition, amino acid transporters carry L-tryptophan across the blood-brain barrier, however tryptophan must compete with other large neutral branched-amino acids such as valine, leucine and isoleucine (55).

Regulation

The regulation of serotonin action, like many factors, is a balance between several competing kinetic systems. The key players in this regulation are tryptophan hydroxylase, serotonin transporter (SERT, 5-HTT), monoamine oxidase, vesicular monoamine transporter (VMAT), and the receptors themselves (Fig 4). TPH, as mentioned previously, is the rate limiting enzyme in the biosynthesis of serotonin. It is a member of the aromatic amino acid hydroxylases (AAAH), which includes tyrosine hydroxylase, phenylalanine hydroxylase, TPH1 and TPH2. All members have an amino-terminal regulatory domain (R-domain), a central catalytic domain and a carboxy-terminal tetramerization domain. The carboxy-terminus contains a leucine zipper (427-444aa) that when mutated in tyrosine hydroxylase results in dimers that are inactive (56). On the contrary, although deletion of the leucine zipper in either TPH protein results in dimers and monomers only, activity was indistinguishable from the tetramer (57). This led to the hypothesis that tetramerization of TPH serves a regulatory role, rather than catalytic. Furthermore, since the R-domain has the greatest sequence variability among the enzyme family, it determines substrate specificity. However, a chimeric protein containing the TPH catalytic domain and the tyrosine hydroxylase regulatory domain was still only able to hydroxylate tryptophan, indicating the catalytic domain determines specificity (58). The R-domain of AAAH members has been shown to affect enzyme activity.
and thermostability through various mechanisms including phosphorylation (59). TPH2 differs from TPH1 at its N-terminus (R-domain), which contains 41 additional amino acids, including an additional phosphorylation site. Phosphorylation of TPH2 at this additional site (serine 19), has been shown to facilitate interaction with the 14-3-3 protein, increasing TPH2 stability (60). By implication, it could be inferred that TPH1 is relatively less stable, and indeed it is (61, 62). The discovery of the second isoform of TPH occurred in 2003 and thus data published prior to that are confounded by the unknown identification of TPH isoforms.

All monoamines, including serotonin, are released into extracellular spaces in large quantities, and often act within a microenvironment. Thus, the concentration of those chemicals in the microenvironment is much higher than in circulation (i.e. four orders of magnitude in synaptic cleft). Therefore, to effectively terminate the signal quickly, plasma membrane monoamine transporters (such as SERT) require energy to recycle monoamine intracellularly. The energy is obtained by coupling the flow of monoamines to that of sodium. The ubiquitously expressed Na⁺/K⁺ ATPase generates an inwardly directed electrochemical sodium gradient utilized by SERT to drive uphill transport of serotonin. Although research is still uncovering the mechanism of transport and stoichiometry, the dogma currently is that Na⁺, Cl⁻ and serotonin bind and cause a conformational change resulting in transport into the cell (Fig 5). Potassium then binds and is transported out setting the transporter back to its original conformation (Fig 5). The overall stoichiometry is a 1:1:1:1 electroneutral exchange of K⁺ with Na⁺, Cl⁻ and 5HT⁺ (63-65). The structure-function relationship of SERT and other neurotransmitter transporters was the subject of speculation due to lack of a suitable model until recently. In 2003, a tryptophan transporter, with properties similar to neurotransmitter transporters was crystallized (66). This has begun the process of identifying binding sites and extrapolating altered function from site-directed mutagenesis (67). One recently explored area of research with regard to SERT is its trafficking into and out of the membrane. The C-terminal end of the peptide is essential for its trafficking, as a 15 amino acid deletion completely abolished membrane bound SERT (68). Interestingly, it was recently shown that serotonin is covalently attached to Rab4, which locks it in its GTP bound form. GTP-bound Rab4, then binds to the C-
Figure 5. Possible alternate access mechanism for serotonin transport. Overall 5-HT requires cotransport of Na⁺ and Cl⁻. The order of binding is not strictly sequenced.


terminal of SERT and locks the protein intracellularly, “paralyzing” it (69). In addition, a recombinant baculovirus system study suggested N-linked glycosylation was important for membrane localization of SERT, however another group recently demonstrated a low molecular weight, endogenous, human SERT (unmodified) is found in the plasma membrane of B-lymphocytes (70, 71). Other proteins have been identified as affecting SERT distribution into and out of the membrane such as PKC, PKG, t-SNARE and SCAMP2 (72-74). PKC decreases the abundance of SERT in the membrane, therefore decreasing 5-HT uptake, while PKG does the opposite (72-74).

Monoamine oxidase is a ubiquitously expressed flavoenzyme, found on the outer membrane of mitochondria. There are two isoforms (MAOA & MAOB), originating from separate genes, each on the X-chromosome. Both possess an FAD cofactor covalently linked to a cysteine residue. MAOA and B share approximately 70% sequence homology, however they have drastically different substrate and inhibitor specificities. MAOA degrades norepinephrine, dopamine and is the only MAO able to degrade serotonin (75). MAOB degrades phenylethylamine and benzylyamine, but is also able to degrade dopamine, but with lower efficiency than MAOA (76, 77). MAOA and B knockout mice have both been created. As expected MAOA -/- mice have elevated levels of 5HT, dopamine and norepinephrine, resulting in aggressive behavior (78). MAOB knockout animals do not display aggressive behavior, but do have phenylethylamine accumulation in multiple organs.
MAOB activity increases with age, and thus the increased dopamine metabolism by glial cells in the substantia nigra is a contributing factor to Parkinson’s disease (80). MAO is also implicated in the etiologies of stress-related disorders such as obsessive-compulsive disorder, depression and panic disorders.

Vesicular monoamine transporters (VMAT) are fairly nonspecific transporters whose function is to compartmentalize monoamines. They belong to the vesicular amine transport (VAT) family (SLC18). In the nervous system, the vesicles are derived from the endosomal compartment, while in endocrine cells secretory granules originate from the Golgi. Regardless of origin, the mechanism is the same active transport process. VMAT is an antiport, exchanging H+, for a monoamine. The driving force is a concentration gradient of H+ generated by the vacuolar H+-ATPase, located on the same vesicles. There have been two isoforms of human VMAT cloned (VMAT1 and VMAT2) (81, 82). VMAT2 is the predominant isoform expressed in nervous tissue, and in the neuroendocrine cells of the stomach. Interestingly, VMAT1 was expressed in the neuroendocrine cells of the small intestine. Both isoforms are coexpressed in the adrenal medulla. Substrate specificity is largely similar between the two isoforms, especially with respect to serotonin and dopamine transport (83). VMAT1 is capable of transporting norepinephrine and histamine more efficiently (4 and 33 fold, respectively). The two isoforms are also differentially sensitive to pharmacologic agents such as amphetamines, MDMA and fenfluramine; in every case VMAT2 being more sensitive (83, 84).

Physiological Functions

Serotonin has a primary functional role in several physiological systems, and subsidiary roles in many more. The primary roles will be reviewed here, and individually have been extensively reviewed elsewhere (85-87). The most well-studied roles of serotonin involve neurotransmission, hemostasis, gut motility and secretion, bone metabolism and cardiovascular smooth muscle contraction. The range of serotonin functions within one organ or across an organism is attributable the diversity of serotonin receptors: seven families (5HT1-7) transcribed from (depending on the species) ~13 separate genes (88, 89). All serotonin receptors are G-protein coupled, with the
exception of the 5-HT3 family, which is a ligand-gated cation channel (Fig 4). Members of the 5HT1 and 5HT5 classes are G_{i/o} coupled; 5HT2 family is G_{q/11}, and 5HT4, 5HT6 and 5HT7 are G_s coupled. Further adding to the diversity, some serotonin receptor genes, such as 5HT2C and 5HT7, yield multiple isoforms from differential splicing events (89). Main differences between isoforms within one family are their relative distribution, structure and sensitivity to various agonists and antagonists. Because of the vast amount of diversity and literature on serotonin function in the central and peripheral nervous systems, it will not be reviewed here. Suffice it to say, it is widely accepted that serotonin plays central roles in mood regulation, behavioral control and cognition in a complex manner (90).

The bulk of serotonin (90+%%) found in the body is made in enterochromaffin (EC) cells, which are present at varying amounts throughout the gut. Serotonin can also be found within a small subset of enteric neurons (~1%), specifically interneurons (91). Stimulation of EC cells by intraluminal distension, vagal nerve stimulation, ingestion of a meal, or the presence of acid causes the release of 5-HT (85). The physiology of 5-HT and the gut is complicated by the presence of almost every 5-HT receptor within either the muscle, enteric nerves, lymphocytes or enterocytes (85). Specific receptor agonists have helped elucidate the specific roles of these receptors. In the upper fundus of the stomach, 5-HT_{1A, 1B, 7} receptor agonists can decrease gastric tone, thus functioning to aid the accommodation of a meal (92). 5-HT_{3} antagonism causes constipation (93). 5-HT_{2B} has also recently been shown to reduce peristaltic movement (94). Together, these two receptors and endogenous 5-HT modulate colonic motility and transit time of ingested food. 5-HT_{3} is also important for emesis (95). It is a common target of anti-emetic drugs given to cancer patients receiving chemotherapy and radiation. A lot of information about 5-HT function in the gut remains unknown, as demonstrated by unexplained side effects of a recently developed drug, tegaserod, a 5-HT_{4} and 5-HT_{2B} agonist (96).

As with the gut, the cardiovascular system expresses a large subset of 5-HT receptors. The central goal around 5-HT function in the cardiovascular system is to determine how and if it affects vascular tone, independent of its role in coagulation. Two powerful observations have
created this question: the 5-HT$_2$ antagonist, ketanserin and the 5-HT$_{1A}$ agonist, 8-OH-DPAT, lower blood pressure. However, the mechanism of action for these two drugs was later shown to be indirect via the central nervous system and $\alpha_1$-adrenoreceptors, respectively (97, 98). Despite the interest generated by these initial observations into 5-HT function and the cardiovascular system, relatively little is understood about the 5-HT effects on blood pressure. Overall, the effects are described as triphasic. Initially, there is a transient fall in blood pressure, followed by a moderate rise, and finally a longer lasting fall in blood pressure. Setting aside 5-HT indirect action on the cardiovascular system via the central nervous system (reviewed elsewhere (86)), a few receptors have been localized to cardiovascular tissue and shown to have important function. For example, 5-HT$_{1B}$, and 2A are found on vascular smooth muscle and when stimulated cause vasoconstriction (99). Counteracting this effect is 5-HT$_7$, also present in vascular smooth muscle, and causing vasodilation when stimulated (100). In the atrial wall of the heart, 5-HT$_4$ can be found, and when stimulated causes tachycardia. The contribution of these receptors to changes in circulating serotonin is unclear; especially given the input of the CNS, and nerve terminals on vascular beds. Further complicating things, the overall triphasic picture may be an artifact of experimental design, since it was not recapitulated by 5-HT delivered i.v. by a mini pump. In this case a maintained fall in blood pressure only was observed (101).

**Intraluminal Volume Homeostasis: A Common Serotonergic Mechanism Among Diverse Epithelia**

**Introduction**

Volume homeostasis is a common physiological problem that epithelial cells in various organs encounter. In some cases, such as the mammary gland and prostate, the goal of the organ is to synthesize, secrete and store (for a time) a fluid for delivery. In other organs, such as the developing lung, the goal is to clear all fluid once breathing commences. Other organs are almost continuously synthesizing and secreting fluid, and thus encounter an over-filling problem when a duct becomes occluded, as is the case with the liver, salivary glands and pancreas. The common features among all the aforementioned organs are (1) existence of partially- or fully-filled fluid
lumen, (2) the need to respond to stretch or changes in intraluminal pressure, and (3) the presence of serotonin-producing epithelial cells. Here, we review the literature related to serotonin regulation of intraluminal volume homeostasis in the lung, pancreas, prostate, liver and mammary gland and propose that serotonin secretion is a common molecular mechanism in ductal systems controlling volume homeostasis.

**Historical Perspective on Classification**

The characterization of serotonin-producing epithelial cells started with the coining of the acronym APUD (amine precursor uptake and decarboxylation) (102). This acronym was developed to describe cells in endocrine and nonendocrine organs that shared some common cytochemical and ultrastructure characteristics. The cytochemical characteristics that combine together to produce the acronym are as follows: (1) Fluorogenic amine content (5HT, catecholamine or other), (2) amine precursor uptake (5-HTP, DOPA), and (3) amino acid decarboxylase (102). The common ultrastructural criteria are high levels of smooth endoplasmic reticulum, membrane bound vesicles and high content of ribosomes. The coining of the term coincided with the identification of APUD cells in the pancreas, thyroid, lung, stomach and intestine (102). Although the term APUD is still somewhat used, more common today is the unfortunate term diffuse neuroendocrine system. This author believes it to be unfortunate because it misrepresents the classic neuroendocrine system, which is defined as the neurally-derived tissue directly producing and releasing hormones into the bloodstream. Often the neural-derived hormones affect the physiology of another endocrine organ, such as the pituitary gland. The diffuse neuroendocrine system, is not “connected” to CNS or PNS, nor is derived from neural tissue. The adaptation of the word neuroendocrine refers to two characteristics of APUD cells. First, these cells often produce and/or utilize amines commonly known as neurotransmitters to do a function (such as 5-HT, dopamine, ACh, norepinephrine etc.). Second, sometimes, but not always, cells of this classification produce endocrine hormones. Calcitonin-producing parafollicular cells of the thyroid gland are the founding members of APUD (103). The root of the discrepancy is that APUD is a histochemical classification, while neuroendocrine is a functional and developmental classification. Some cells will inevitably
be both, while some cells will be one or the other. Unfortunately, intermixing of the terms is common, and neuroendocrine has come to identify cells that immunostain for a subset of markers (neurotransmitters, chromagranin A and/or neuron-specific enolase).

One representative example of intermixing of the term is found within the prostate science community. It is common to refer to serotonin-producing cells within the prostate as neuroendocrine (also staining for chromagranin A). Originally, these cells were identified histochemically, and therefore called APUD cells of prostate (104). Over time, the neuroendocrine label has since replaced APUD in spite of the fact that no functional connection between the CNS or PNS and these cells has been established, nor have they been shown to differentiate from neural tissue (105). Their identification came as a result of histochemical analysis, and therefore would more accurately be called APUD prostate cells. There is a well understood nervous system innervation of the prostate, originating from hypogastric and pelvic splanchnic nerves, also referred to as neuroendocrine (106). However, these nerve innervations have only been shown to effect autocrine/paracrine epithelial secretions (107, 108). An overarching classification system is needed to distinguish a histochemical classification of neuroendocrine from its more accurate functional classification.

**Lung**

In the lung, scientists have defined two different amine-producing cell populations as “neuroendocrine”. Neuroepithelial bodies (NEB) are situated in intrapulmonary airways and appear to be concentrated at airway branch points (109, 110). Pulmonary NEB function as O₂ sensors, releasing serotonin and other vesicle contents locally following hypoxia (111). The second neuroendocrine cell type defined in the lung is the primary neuroendocrine cell (PNEC). These are believed to play an important role in fetal lung development (when the lungs are filled with fluid), since they are the first cells to differentiate (112). These cells are more widely distributed within the lung and come in two forms. The “open type” of PNEC, is flask-shaped and has apical cytoplasmic processes. The “closed type” does not have apical access, but rather has dendritic-like cytoplasmic extensions that extend along the basement membrane, and thus contact several surrounding epithelial cells (113). This morphology is believed to be ideally situated for response
to stretch, and interestingly the closed type is only found in fluid-filled fetal lung (114, 115).

It has been known that mechanical stretch of the fetal lung is vital to development, but the molecular mediators of that process remained poorly defined. To further understand the role of PNEC in lung development, one study cultured fetal lung tissue after dissection. The study found that 5-HT was released in the fetal lung following mechanical stretch (116). The 5-HT release was independent from NEB-derived 5-HT because it did not require functioning L-type Ca++ channels, which are known to be essential for NEB 5-HT release (111, 112, 116). In addition TPH protein expression was increased by mechanical stretch over the time course measured. The authors also hypothesized (via indirect evidence) that the 5-HT release from PNEC was from the cytoplasmic pool, not from the vesicular pool; again opposite from hypoxia-induced 5-HT release from NEB (116).

Complementing the above study, one group cultured guinea pig lungs removed from late gestation fetuses and administered exogenous 5-HT. 5-HT caused a dose-dependent decrease in the rate of fluid secretion in the lung, and activated reabsorption of fluid (117). Previously, somatostatin also had been shown to decrease the rate of fluid secretion, but not increase absorption (118). The effects on secretion and absorption were enhanced in older gestational age lung explants (60 days vs 66 days), indicating that 5-HT action is important for preparing the lungs to breath air. Furthermore, the authors demonstrated that 5-HT receptor antagonism blocks the effects, using cyproheptadine. Cyproheptadine blocks 5-HT2 receptors, but also affects Ca++ channels, complicating interpretation of these results. Regardless, taken altogether, serotonin is induced by stretch, and causes fluid clearance in the late gestation developing lung. In other words, 5-HT is secreted in response to stretch, functioning as a volume homeostatic factor in the developing lung.

Pancreas

The positive players involved in pancreatic secretions have longed been established and are reviewed elsewhere (119). Originally, it was believed that the pancreas only operated under positive influences, and the removal of the influences returned it to basal secretion state (120).
Now, it is clear that negative influences to pancreatic secretion do exist. Moreover, these negative factors act on two levels. There have been recent studies and reviews elucidating the role of intestine and gastric-derived factors in limiting pancreatic secretion (letdown) into the lumen of the GI tract (120, 121). This represents an indirect mechanism controlling secretion of fluid into the pancreatic lumen. The second level, which will be detailed here, includes the directly acting autocrine/paracrine factors within the ductal epithelium that regulate the rate of synthesis and secretion of fluid (volume homeostasis). 5-HT has been identified as one of these factors, and the most potent (122). Other factors that have similar effects to 5-HT in the pancreatic ductal epithelium include arginine vasopressin, substance P, ATP, and EGF (121).

The pancreas was identified early on as having APUD cells (123). The pancreas science community has since adopted the term enterochromaffin cell (ECC) because of some histochemical, biochemical, and functional similarities to ECC of the stomach and intestine. In addition, it was shown that APUD cells of the pancreas are not derived from neural crest and thus should not be considered neuroendocrine (124). The function of the ECC of the pancreas remained elusive until 2001. Isolated pancreatic ducts from guinea pigs responded to exogenous application of 5-HT by decreasing their HCO$_3^-$ dependent basal fluid secretion. The effect was more dramatic when the explants were first primed with a positive secretion factor (acetylcholine or secretin) (122). Using receptor specific agonists, 5-HT$_3$ was shown to be responsible for the effect, and it was specific to application to the basolateral side. Moreover, the authors determined that intraductal pressure decreased secretion rate as well, and this decrease could be attenuated by a 5-HT$_3$ receptor antagonist (122). Although it has not been directly determined that 5-HT is secreted in response to stretch, the indirect evidence hints at it. Overall, 5-HT is secreted by a subset of ductal epithelium in the pancreas, and acts as a homeostatic feedback for fluid secretion via the 5-HT$_3$ receptor.

*Prostate*

The literature about APUD (neuroendocrine, NEC) cells in the prostate focuses almost entirely on their prevalence among different prostatic states: normal, benign hyperplasia, and cancerous. The central hypothesis surrounding these cells is that factors secreted by NEC in the
prostate are responsible for facilitating cancer progression, especially in androgen-independent tumors (125). These cells are known to secrete 5-HT and other peptides (105). All studies that attempted to elucidate the role of secreted peptides and factors from NEC focused on proliferation of adjacent epithelium, with varying results (126-129). It is also known, analogous to the lung, there are “open” and “closed” types of NEC (130). This observation alone hints at a stretch-activated mechanism of secretion, although that possibility has not been investigated directly.

Supporting this idea indirectly, is evidence that prolactin increases the number of NEC in the prostate (131). This increase occurs in normal prostates, or prostates where androgen action has been blocked. Androgen blockade significantly lowers the NEC count, but this is rescued by prolactin (131). Extending this observation, it is probable that prolactin (and androgen) are acting as positive fluid secretory factors, that would require a homeostatic feedback (5-HT), and thus more NEC are present (132). Supporting this notion, an increase in NEC populations is seen during puberty, when the prostate gland increases in size and becomes more secretory (133). Overall, NEC function in the normal prostate is poorly understood, especially the local control of prostate secretion. This area needs to investigated further in light of some commonalities it shares with other NEC/APUD containing organs.

Salivary Glands

Like the prostate, innervation of the salivary glands has been anatomized, and the function well studied. Norepineprine release by nerve terminals in the submandibular glands causes cAMP accumulation and decrease in fluid secretion. In addition, vasoactive intestinal peptide and prostaglandins have been identified as affecting fluid secretion via a cAMP receptor mechanism (134, 135). Serotonin also affects fluid secretion in the salivary glands, with more potency than VIP, similar potency to prostaglandins and less than norepinephrine (136). This study used in vivo cannulation of rat submandibular glands to determine that 5-HT inhibited acetylcholine stimulated fluid secretion. This result is in contrast to what was reported for the rat parotid gland in a study using in situ preparations (137). This may represent differences in experimental techniques or a difference between the parotid and submandibular glands. Further studies to rectify this discrepancy
Interestingly, 5-HT–mediated decreases in fluid secretion were shown to utilize cAMP as a second messenger (136). Subsequent to this initial study the investigators reported that the submandibular gland expresses two 5-HT receptors coupled to $G_s$ ($5-HT_4$ and $5-HT_7$) (138). Overall, they concluded that 5-HT causes decreased fluid secretion in the submandibular gland via 5-HT receptor mediated cAMP accumulation. This cAMP-driven mechanism is conserved in the mammary gland (detailed later), after 5-HT synthesis and secretion is induced by fluid stasis. Additional studies are needed to determine the definitive source of 5-HT and the induction mechanism (fluid stasis?) in the salivary glands.

Liver

Epithelial cells lining intrahepatic and extrahepatic ducts are called cholangiocytes. There is considerable interest in understanding the physiology of fluid secretion/flow in the biliary tree. Cholangiopathies (diseases of the biliary tree) are characterized by cholestasis (block of bile flow) and result in liver failure. They are responsible for more than 20% of adult liver transplantations and 50% of pediatric transplantations (139). The pathophysiology of cholangiopathies commonly share an impaired balance between proliferation and apoptosis (140). Because of this observed impaired balance, many studies focus on factors that effect proliferation and apoptosis of cholangiocytes. Normally cholangiocytes are mitotically dormant. A common experimental and pathological cause of proliferation is bile duct ligation (BDL). The cells within the bile ducts that proliferate after BDL have been described as a neuroendocrine niche, responding to various factors affecting cell turnover (141).

Serotonin was initially discovered to be taken up by the liver and stored there in 1966 (142). It was not until recently that 5-HT was shown to be synthesized in the cholangiocytes and have important cellular functions (143). Again, the focus has been on 5-HT affecting the proliferation of cholangiocytes following BDL. 5-HT through binding to basolateral 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors decreased proliferation after BDL (143). Although not the focus of the paper, the authors also observed decreased bicarbonate and bile flow to normal animals administered 5-HT$_{1A,1B}$ agonists.
This observation indicates that serotonin is involved in the normal fluid homeostasis within the liver, in addition to its anti-proliferative role following BDL. Another group observed changes in tight junction (TJ) protein localization following BDL (144). They hypothesized the redistribution of TJ proteins affects barrier function, in an effort to contain bile intraluminally. 5-HT was not investigated as the cause of TJ protein redistribution and expression changes. However that mechanism has been observed in 5-HT–producing mammary gland epithelia (145, 146).

**Mammary Gland**

The mammary gland is the newest member of the 5-HT–producing epithelial tissues. Ductal epithelia of the mammary gland remain relatively quiescent throughout adult life. During pregnancy, massive proliferation and differentiation of the lobuloalveolar epithelium occurs in a coordinated endocrine/paracrine effort (see Fig 1). Although tight junction proteins are expressed in a virgin and pregnant state, tight junctions are not assembled, and thus the epithelium is highly permeable as indicated by the diffusion of a tracer, FITC-albumin (Fig 6) (35). During pregnancy (Fig 6A,C), tracer injected in the lumen or exposed to the basolateral surface was able to diffuse across the epithelia. Conversely, during lactation, tracer was confined to the compartment in which it originates, indicating the presence of impermeable tight junctions. As with the other
aforementioned organs, the mammary gland must regulate luminal volume. Different from the other organs however, leakage of luminal fluid (milk) does not constitute a pathology, but rather it is part of a cyclical arrangement of processes that bring about the ordered shut down, destruction, and remodeling of the mammary gland back to a quiescent state. Therefore, extended fluid stasis within the mammary gland leads to tight junction leakage, and mammary gland involution.

The discovery that the mammary gland synthesizes and utilizes 5-HT did not come about by histochemical methods, but rather by a comparative genomic approach. Messenger RNA transcripts from mammary glands between prolactin knockout mice (non-secretory) and hyperprolactinemic mice (hypersecretory) were compared using subtraction cloning. TPH1, the rate limiting enzyme in the synthesis of serotonin was highly induced in the hyperprolactinemic mice (40). Further study revealed that TPH1 gene expression (and 5-HT synthesis) was induced by fluid stasis. Using pharmacologic approaches to antagonize 5-HT production or receptor binding, 5-HT was found to function as an autocrine/paracrine feedback inhibitor of lactation. Verification was provided by TPH1 knockout mice. Mammary glands from TPH1 knockout animals showed a hypersecretory phenotype and delayed involution. (40). Although involution was delayed in these knockout animals, tissue remodeling back to a quiescent state did occur. This solidifies the theory that 5-HT is important for proper fluid clearance in ducts in the mammary glands as well as other organs.

Since tight junctions are dynamically regulated during lactation in response to fluid stasis, 5-HT was investigated as a autocrine/paracrine mediator of tight junction permeability. A biphasic effect of 5-HT is observed in the mammary epithelium. 5-HT causes a transient decrease in tight junction permeability, followed by a precipitous increase in permeability (145, 146). The former serves a teleological role of sustaining the status quo until nursing resumes, while the latter presumably facilitates the transition from lactation to involution. Each response occurs downstream of the 5-HT7 receptor, however the former utilizes a cAMP-dependent PKA pathway and the latter a cAMP-dependent MAP kinase pathway (145). Currently, it has not been determined whether 5-HT production occurs in a subset of mammary epithelial cells, or in all epithelial cells. On the other hand, staining for 5-HT in breast tissue and phyllode tumors did identify a sub-population of
cells containing serotonin, however the origin of the 5-HT was unknown (147).

**Serotonin Receptor Patterning**

A pattern is emerging among different ductal organs that 5-HT is stimulated by increases in intraluminal pressure caused by fluid accumulation. While the initiating event and the ligand are conserved, what varies is the serotonin receptor expression pattern. It is plausible to assume
that variation in the response to 5-HT observed is due to receptor signaling differences and the end goal. Serotonin receptors are classified into seven families (5-HT₁–₇) with multiple isoforms of each (88)(148). Table 1 summarizes which serotonin receptors are known to be expressed in various epithelial tissues. Some receptors have simply been identified as being expressed, with no specific function known, while others have a specific function that was investigated in a normal or diseased state. In addition, some receptors have been identified pharmacologically, and others by PCR or other molecular approaches. While some progress has been made in understanding which receptors mediate 5-HT action in epithelial tissues, much more research is needed before this picture is complete.

<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>Receptor</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
</table>
| Lung
alveolar epithelial type II cells | 5HT1A, 5HT1B, 5-HT1E, 5-HT1F, 5-HT2A, 5-HT3, 5HT4, 5HT6, 5HT7 | Unknown | (149) |
| GLC-8 cells (small cell lung carcinoma) | 5HT1A, 5HT1D | Mitogenesis | (150, 151) |
| Neuroepithelial bodies | 5HT3 | Positive feedback on 5HT secretion | (152) |
| Pancreas
Isolated interlobular ducts | 5HT3 | Inhibit HCO₃⁻ secretion | (122) |
<p>| Pancreatic homogenate | 5HT4 | Unknown | (153) |</p>
<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>5-HT Receptor</th>
<th>Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic parenchyma and nerve tissue</td>
<td>5-HT1A</td>
<td>Inhibits exocrine and endocrine secretion</td>
<td>154-156</td>
</tr>
<tr>
<td>Prostate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human prostate homogenate</td>
<td>5-HT1A, 5-HT1B, 5-HT1D</td>
<td>Growth promoting in cancers</td>
<td>157, 158</td>
</tr>
<tr>
<td>BPH cells</td>
<td>5-HT2B</td>
<td>Growth promoting</td>
<td>159</td>
</tr>
<tr>
<td>High grade tumors</td>
<td>5-HT4</td>
<td>Growth promoting</td>
<td>159</td>
</tr>
<tr>
<td>Salivary Glands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submandibular gland homogenate</td>
<td>5-HT4, 5-HT7</td>
<td>Inhibit fluid secretion</td>
<td>135</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholangiocarcinoma cells</td>
<td>5-HTR1A, 5-HT2A, 5-HT2B, 5-HT6</td>
<td>Growth promoting</td>
<td>160</td>
</tr>
<tr>
<td>Mammary Gland</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells (immortal and primary)</td>
<td>5-HT7</td>
<td>Tight junction regulation</td>
<td>145, 146</td>
</tr>
<tr>
<td>Isolated epithelial cells</td>
<td>5-HT1D, 5-HT2B, 5-HT3A</td>
<td>Unknown</td>
<td>Pai V, unpublished</td>
</tr>
</tbody>
</table>
References


13. Ewan, K.B., Shyamala, G., Ravani, S.A., Tang, Y., Akhurst, R., Wakefield, L., and Barcellos-


149. Bayer, H., Muller, T., Myrtek, D., Sorichter, S., Ziegenhagen, M., Norgauer, J., Zissel,


CHAPTER II

CHARACTERIZATION OF A NOVEL BARRIER FORMING IN VITRO MULTIPOTENTIAL MODEL

Aaron M. Marshall, Vaibhav P. Pai

Systems Biology and Physiology Program
Department of Molecular and Cellular Physiology
University of Cincinnati, Cincinnati, Ohio 45267-0576
Introduction

Postnatal mammary gland development is controlled by interactions between endocrine hormones and factors that act locally to drive mammary development and maintain homeostasis (1-4) (see Chapter 1). Rodent models, i.e., rats and mice, have been the predominant experimental context in which to study mammary physiology and development. These animal models share many features with the human breast, and are experimentally tractable, but rodent models are not ideal for studying the influence of local factors on human mammary gland development. Obvious differences between rodent mammary gland and human breast are structural. The rodent mammary epithelium is associated with an extensive adipose stroma, whereas the human mammary epithelium is surrounded by fibrous connective tissue, and the human breast adipose tissue is largely remote from the epithelium. These morphologic features manifest themselves as differences in the physiological interactions between stroma and epithelium in the human and mouse (5). On a practical level, it has not been possible to grow and differentiate normal human mammary epithelial cells in the mouse mammary fat pad (6). Recent studies have attempted to circumvent this problem by co-transplanting human stroma and epithelium in mice (7, 8). This human-in-mouse model is useful, but it has some practical limitations as a platform for studying many aspects of development, homeostasis and tumorigenesis. There also are important hormonal differences between human and rodent, including the lack of a functional luteal phase in rodents (9), and poor binding of rodent prolactin to human prolactin receptors (10). Overall, there continues to be a need for studies of differentiation and signaling in well-characterized human mammary epithelial systems, including studies of local signaling events, epithelial transport ion fluxes, and cell-cell interactions.

Tight junctions in the mammary epithelium have been studied primarily in the context of pregnancy, parturition, lactation, and involution because of their integral role in those events. Although the resting gland (non-pregnant, non-lactating) has not been extensively studied, tight junction proteins are expressed in the human ductal network at this stage, but the permeability of the membrane is not known (11). At the time of parturition high resistance tight junctions
are established in the human mammary epithelium (12). Compared with true milk the first-milk (colostrum) contains higher concentrations of serum proteins, sodium and chloride, and lower concentrations of potassium and lactose. The changes that accompany the transition to true milk are attributable to barrier formation, which results from closure of the tight junctions (13). Conversely, involution is accompanied by the opening of tight junctions, and breaching the epithelial barrier resulting in leakage of milk components into the interstitium and plasma (13, 14). In the context of breast cancer, changes in tight junction protein expression correlate with tumor progression, and tight junction dysregulation is necessary for tumor cell migration and invasion (15, 16).

Given the central role of barrier formation in mammary epithelial development and pathogenesis, any useful model for studying local regulation in the mammary epithelium must be able to establish a functional tight junction barrier in a predictable manner. Advances in understanding the microenvironment of the human mammary gland are currently restricted because there have been few studies in human cells that display characteristics of a differentiated mammary epithelium (17, 18).

Methods

Cell Culture and FITC-Inulin Flux. An immortalized human mammary epithelial cell line, MCF10A was used for the present studies. The normal growth media for MCF10As was DMEM:F12 (1:1, Cellgro) with 2mM glutamine, containing 5% horse serum, insulin (10 μg ml⁻¹) (Gibco), hydrocortisone (0.5 μg ml⁻¹) (Sigma), EGF (20 ng ml⁻¹) (Upstate) and 1 I.U. ml⁻¹ penicillin, 0.1 μg ml⁻¹ streptomycin, 0.25 μg ml⁻¹ amphotericin B (Cellgro). Cells were grown in monolayer to 90-95% confluency, trypsinized and counted for seeding onto permeable supports (Transwell®, 0.4 μm pores, polyester) in normal growth medium. MCF10A cells were seeded on 12-well Transwells® (Corning) at 10⁵ cells/cm² (Fig 8). Both chambers of media were changed
strictly on a 24-hour schedule, unless otherwise noted. Where indicated, 9-cis-retinoic acid (9-cis RA, Sigma) was administered in both chambers. Transepithelial electrical resistance (TEER) was measured daily with Epithelial Volt-Ohm Meter (EVOM; World Precision Instruments), prior to media change. For inulin flux experiments, 0.5 mg/ml of FITC-inulin was included in the bottom chamber medium and allowed to equilibrate for 20-22 hours. After incubation, 100 μl of both the top and bottom chamber media was transferred to a 96-well plate for fluorescence quantification on a plate reader (Labsystems Fluoroskan II).

Unlike anchorage-dependent cells that express lineage-specific phenotypes, cells with progenitor/stem cell properties survive and proliferate when cultured in the presence of a low-adherence substrate. The resulting colonies are capable of generating both self-renewing lineage-restricted daughter cell populations (19). In the case of mammary epithelium these stem cell colonies have been referred to as non-adherent mammospheres (20). A trypsinized suspension of MCF10As were filtered through a 40 μm sieve, and then counted. The single cell suspension was transferred to a 6-well ultra-low adherence plate (Corning) at a density of 2,500 cells/well. Non-adherent culture media was composed of the following: MEBM (Cambrex), B-27 supplement (Invitrogen), hEGF (20 ng/ml), insulin (5 μg/ml), and hydrocortisone (0.5 μg/ml). After five days in culture, the surviving non-adherent mammosphere colonies were serially diluted onto normal tissue culture plastic multiwell dishes. A well containing a single colony was expanded and passaged onto T75 culture flasks (designated MCF10A-UC1), and then was cultured on Transwell® permeable membranes for analyses.

**Immunofluorescence and Imaging.** Cells were grown on permeable supports to peak TEER, fixed by a brief incubation in 4% paraformaldehyde (in phosphate-buffered saline), and were immunostained as floating sections of membranes. Floating sections were permeabilized in 0.1% Triton X-100, incubated in borate buffer pH=8.5 (80 mM boric acid, 20 mM sodium borate) overnight at 75°C for antigen retrieval. The following antibodies and fluorescent stains were used: rabbit polyclonal anti-occludin (5 μg ml⁻¹) (Zymed), mouse monoclonal anti-E-cadherin (0.5 μg ml⁻¹) (BD Bioscience), mouse anti-mucin 1 (0.8 μg ml⁻¹) (Santa Cruz), rabbit anti-CD10 (0.8 μg
ml⁻¹) (Santa Cruz), rabbit anti-human milk proteins serum (1:500) (Nordic Immunology), goat anti-rabbit Alexa Fluor 488 (2 μg ml⁻¹) (Molecular Probes), goat anti-rabbit Alexa Fluor 546 (2 μg ml⁻¹) (Molecular Probes), goat anti-mouse Alexa Fluor 488 (2 μg ml⁻¹) (Molecular Probes), goat anti-mouse Alexa Fluor 546 (2 μg ml⁻¹) (Molecular Probes), LipidTOX (1:500) (Invitrogen) and TO-PRO-3 iodide dye (1 μM) (642/661) (Molecular Probes). Images were collected using a Zeiss LSM510 Confocal Microscope: excitation source were from argon lasers at 488 nm, 543 nm and HeNe2 laser at 633 nm; emission collection at 505-530 nm, 560-615 nm and 650-720 nm respectively.

*Matrigel*® Culture. MCF10A cells were cultured in Matrigel® (BD Bioscience) as previously described with some modifications (21). The medium supplied during differentiation in Matrigel® contained 2% horse serum, insulin (10 μg ml⁻¹) (Gibco), hydrocortisone (0.5 μg ml⁻¹) (Sigma), EGF (5 ng ml⁻¹) (Upstate) and 1 I.U. ml⁻¹ penicillin, 0.1 μg ml⁻¹ streptomycin, 0.25 μg ml⁻¹ amphotericin B (Cellgro), but did not contain cholera toxin. 9-cis-retinoic acid (Sigma) was added at the first medium change (day 3) at 10 nM.

*RNA Extraction.* Total RNA from cells grown in monolayer and Transwell® cultures were extracted using Tri reagent (MRC, Cincinnati, OH) and cleaned using DNaseI (Promega, Madison, WI) followed by standard phenol/chloroform precipitation and extraction. RNA from monolayer MCF10A was extracted 5 days after the cells reached 100% confluence. Total RNA from Transwell® cultures were extracted at the following trans-epithelial resistances: 200-300 Ω cm² (Base), 1400-1600 Ω cm² (Midpoint), and 3000-3200 Ω cm² (Plateau).

*Microarray Hybridization and Analysis.* Gene expression experiments were carried out using the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). Quality of total RNA was first analyzed by Agilent Bioanalyzer 2100, and RNA samples with RIN (RNA Integrity Number) greater than 7.0 are acceptable for microarray analysis. Total RNA samples were amplified one round, and the amplified RNA (aRNA) biotinylated using Ambion Biotin-Enhanced Message Amp II kit (#1791, Ambion, Austin, Texas), following the manufacturer’s protocols. The hybridization, staining, and washing were carried out using the Affymetrix GeneChip
Hybridization Wash and Stain Kit following the manufacturer’s protocols, and the arrays were hybridized for 16 hr at 45°C. The data were analyzed to identify differentially expressed genes among the Monolayer, Base, Midpoint, and Plateau conditions, defined as above. Analysis was performed using R statistical software and the limma Bioconductor package (22). All steps of data preprocessing, including background correction, normalization, and expression set summaries, were performed using RMA (Robust Multi-chip Average). Estimated fold changes at each time point were calculated using ANOVA, and resulting t-statistics from each comparison were modified using an intensity-based empirical Bayes method (IBMT) (23) to obtain accurate significance levels. P-values were adjusted using the False Discovery Rate (FDR) method, (24) and genes with \(FDR < 0.05\) and fold change \(> 50\%\) were considered significant for differential expression, and were used in creation of the Venn Diagram and expression profile patterns, and for Gene Ontology enrichment analysis. The latter was performed with David 2007 (25) and used to separately test genes up- and down-regulated through the differentiation process. For assignment of genes to specific expression profile patterns, a fold change \(> 50\%\) alone was employed. Microarray data for the three independent replicates of Monolayer, Base, Midpoint, and Plateau are available from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) with accession GSE10070.

Results

Differentiation and development of barrier forming cells

Formation of adherens junctions and tight junctions are hallmarks of polarization and barrier function of any epithelium. The cells in this model formed a stratified epithelium consisting of multiple cell layers (Fig 9c, see also Stull, et al.,2007). Each panel of Figure 9 displays an X-Y plane image and a reconstructed X-Z-plane image. The Z-plane images (top panels) result from bisecting the X-Y plane at the indicated green line. Cells in the bottom layer (underlying cells) were tightly packed, with irregularly situated nuclei and relatively small cytoplasmic volumes. In contrast, the cells in the uppermost layer (superficial cells) had much larger cytoplasmic volumes and morphologies that varied from narrow columnar to broad squamous cells. Immunostaining for
Figure 9. Localization of tight junctions and adherens junctions. Junction localization is shown by immunostaining for occludin (a) representing tight junctions and E-cadherin (b) representing adherens junctions. Nuclei were counterstained (c) and merged image (d) shows the apical cell layer forming the barrier. Arrowheads highlight a probable third cell type identified by cytoplasmic E-cadherin, and occupying a suprabasal niche. Bottom panels show XY plane images, while top panels display Z plane image cut along the displayed line. Scale bars = 50 μm.

E-cadherin and occludin was used to visualize adherens and tight junctions, respectively (Fig 9a & 9b). The tight junction protein staining, and by implication the barrier function, was exclusively in the superficial layer cells, and concentrated in the lateral membranes. E-cadherin generally colocalized with occludin, both being primarily membrane-associated. There were numerous cells with E-cadherin in cytoplasmic organelles (arrowheads), which appeared to occupy a particular niche in a suprabasal zone, making contact with both the most basal and the most superficial...
cells.

The integrity of epithelial tight junctions can be quantified by measuring the TEER or flux of a metabolically inert molecule such as inulin. FITC-Inulin flux represents paracellular movement of a 3.5 kDa molecule, while TEER represents trans- and paracellular ionic impedance. Under control conditions (see Methods), the plateau resistance and time to plateau inflection in this cell line was 3200-3700 Ω cm² and 11-13 days, respectively (Fig 10). The flux of FITC-inulin (3.5 kDa) was significantly declined to <5% by day 8 (Fig 10). Furthermore, the decline in inulin flux precedes the rapid rise in TEER. These observations demonstrate the capacity of MCF10A cells to form a high resistance functional barrier in vitro, in contrast to previous studies that did not observe barrier formation (26-28).

In its normal microenvironment the mammary epithelium receives nutrients and distributes metabolites to the circulation through its basal side. We tested the consequence of withholding fresh medium for a period of several days while monitoring TEER. If the medium in both the upper and lower chambers was replenished every 24 h, the TEER was maintained above 3500 Ω cm², and gradually drifted upward over several days (Fig 11). In contrast, if neither chamber was changed the TEER fell precipitously on the 3rd day. The TEER also fell on day 3 if only the upper chamber was changed, however, if only the lower chamber was changed daily, TEER remained at the plateau level, although it did not increase (Fig 11). Therefore the composition of the lower chamber has a major impact on maintaining the membrane barrier function, possibly because of medium depletion, or accumulation of one or more inhibitory substances.

Various proteins have been identified as markers of mammary epithelial cell differentiation

![Figure 10. Barrier formation in MCF10A Transwell® cultures. Left Y-axis displays FITC-Inulin flux as a percentage of flux in an empty Transwell® (no cells). Right Y-axis shows the TEER (triangles) over the same time period on the X-axis. Note the decrease in Inulin (3.5 kDa) flux precedes the rapid rise in TEER. FITC-Inulin flux measures paracellular movement of a 3.5 kDa molecule, while TEER measures trans- and paracellular ionic impedance.](image-url)
Figure 11. Effect of medium conditioning and retinoic acid on MCF10A polarization. Maintenance of the barrier only when the basal chamber is replenished (a). After peak TEER was achieved only the listed chambers of medium were replenished daily. The polarized epithelium was dependent on the basal chamber being replenished. Means on the final day of the experiment were analyzed by one-way ANOVA (n = 6) followed by Holm-Sidak post-hoc test (29, 30). Among these mucin-1 (MUC1) has proven to be a useful lumenal cell marker. MUC1 is a membrane glycoprotein that is expressed on lumenal cells in the breast and other glandular organs, and is often dysregulated in cancers (31, 32). MUC1 stained exclusively in the superficial cells (Fig 12b). In contrast, an established basal cell marker is the common acute lymphoblastic leukemia antigen (CALLA), also known as cluster of differentiation-10 (CD10) (33). CD10 is expressed within the basal layer of the breast epithelium in both contractile (myoepithelial) and non-contractile cells (33). CD10 in MCF10A Transwell® cultures showed intense staining in the underlying cell layer, with the greatest staining intensity in a perinuclear pattern that suggested high protein concentrations in the endoplasmic reticulum and/or golgi apparatus (Fig 12a).

The differentiation of MCF10A cells into MUC1 expressing lumenal cells provided a rationale for determining whether the cells synthesized components of milk. LipidTOX®, a fluorescent neutral lipid stain, showed accumulation of neutral lipid droplets in Transwell® cultures of MCF10A cells (Fig 12d). There was no significant accumulation of lipid droplets in cells cultured as conventional monolayers (not shown). In addition, using a multivalent, polyclonal antibody mixture made against human milk proteins, we observed staining for milk components in the MUC1-positive superficial layer of cells (Fig 12c). Formation of milk protein-containing vesicles was also evident (Fig 12c arrowheads). It is not possible to definitively attach the phenotype of these differentiated MCF10A cells with their equivalent basal and lumenal homologues in vivo. While these cells generically express milk proteins, they do not express the caseins or aldolase C,(34) nor
Figure 12. Differentiation of MCF10A cells into multiple phenotypes. Different cell phenotypes are evidenced by CD10 (a) staining of the underlying layer of cells and MUC1 (b) staining in the superficial layer of cells. The superficial layer of cells synthesized milk proteins (c) and lipid droplets (d). Arrowheads in (c) highlight milk protein vesicles. Bottom panels show XY plane images, while top panels display Z plane image cut along the displayed line. Scale bars = 50 μm.

Do they express either the prolactin or progesterone receptors; all of which are characteristic of the most specialized lumenal alveolar cells in the pregnant and lactating mammary gland. The lack of these markers, combined with the clear basal-lumenal differentiation of the membranes suggests that the MCF10A cells may differentiate only to a stage similar to ductal epithelium.
The development of two cell phenotypes from a long term established cell line implies that MCF10A cells retain some level of multipotency. Furthermore, these cell differentiation markers validate that the model replicates several features that are reflective of mammary epithelial development in vivo. However, one obvious feature that we have not observed in this model system is the myoepithelial cell. The basal layer did not produce cells with the myoepithelial morphology and we did not detect smooth muscle actin, a marker for myoepithelium (not shown).

To validate that MCF10A cell cultures retain cells with a multipotent differentiation capability, a clonal sub-population was generated from a single progenitor cell using a method previously described for mammary epithelial stem cells (see Methods)(20). Briefly, a single cell suspension of MCF10A cells was plated into ultra-low adherence plates, and grown in a stem cell supportive medium. Approximately 1-2% of the cells survived and proliferated, forming non-adherent mammospheres (Fig 13a)(20, 35), which are anatomically and physiological distinct from 3D mammospheres grown in a semisolid matrix (36). A single non-adherent mammosphere was isolated and allowed to reattach and proliferate on normal plastic. This clonal subline (MCF10A-UC1) was cultured in Transwells® and assayed by morphological and physiological criteria. Similar to membranes derived from the original MCF10A cells, MCF10A-UC1 cell membranes were able to generate a barrier with a comparable plateau TEER (~2800 Ω x cm²) and generated both lumenal (MUC1⁺ CD10⁻) and basal (MUC1⁻ CD10⁺) cell phenotypes (Fig 13b). These results imply that MCF10A cultures retain a population of self renewing stem-like cells, as well as non-

![Figure 13. Development of MCF10A epithelial membranes from a clonal subpopulation. Panel (a) displays a phase-contrast image of 7 day old non-adherent mammospheres generated from a single cell suspension of MCF10A (Scale bar = 75μm). A single colony was subsequently proliferated on normal plastic (MCF10-UC1), and assayed for barrier formation in Transwells®. Panel (b) shows immunostaining of MCF10A-UC1 for MUC1 (red), CD10 (green) and nuclei (blue) of one of the Transwells® fixed at plateau TEER.](image-url)
stem cells. We have not determined the proliferative potential of each variety of cell under normal culture conditions, but since the stem-like cells represent only 1-2% of the population, it is likely that other cells contribute to the normal growth of the cells in culture.

*Humoral factors affecting tight junction patency*

Reviewed in Chapter 1, many endocrine and paracrine factors affect mammary gland development and differentiation. Therefore, we tested the hypothesis that some known factors/hormones may affect the development of the MCF10A Transwell® model either morphologically or by affecting tight junction formation. It is not known what stage of mammary gland development the Transwell® model recapitulates, therefore we tested various factors, known to be important at different stages of mammary gland development. Retinoid signaling inhibits growth and promotes differentiation in many tissues, including the mammary gland (37), and the various isoforms of the retinoic acid receptors (RAR, RXR) are differentially expressed during the stages of mammary gland development (38). The differentiation activity of RA led to its testing as a breast cancer chemopreventative agent (39). Incubation with 9-cis RA caused a concentration-dependent increase in maximum TEER. Additionally, treatment with 9-cis RA accelerated differentiation, with the plateau inflection of TEER occurring by day 8, compared with the typical time course, which reached the plateau at 12 d (Fig 14).

During pregnancy additional proliferation and differentiation of the mammary epithelium occurs. Two important factors involved are prolactin and RANKL. The disruption of either gene results in similar mammary gland phenotypes, where mammary gland growth arrests at the pubertal stage (40, 41). While it is generally known that proliferation and subsequent differentiation are
interdependent processes, the importance of RANKL to subsequent differentiation has not been explored. To that end, the soluble form of RANKL (sRANKL) was added to the differentiating MCF10A Transwell® culture and morphology and TEER development assessed. The result was a morphology and TEER development that was indistinguishable from control (Fig 15), indicating either that RANKL does not affect differentiation for MCF10A cells or the cells are unable to respond.

During lactation, estrogen circulates at a very low levels. Also, estrogen dependent proliferative responses pre-lactation are mediated by EGF ligands. EGF is the main growth factor utilized in the culturing of the MCF10A cells. It is known from previous unpublished experiments that EGF is required for barrier formation in the MCF10A Transwell® cultures. However, I hypothesized that if the differentiation of this model represents a step toward a lactogenic state, then prolactin rather than EGF would be the preferred growth factor or at least be able to replace EGF. Therefore, in this experiment, EGF was removed and PRL added as the Transwell® cultures were approaching their predicted and reliable plateau TEER of ~3200 Ω cm². Removal of EGF resulted in a gradual and steady decline in TEER, which was not rescueable by PRL (Fig 16). This indicates one or more of several things: (1) the model cannot respond to PRL (2) EGF is required for barrier maintenance (3) EGF is important.

Figure 15. Addition of sRANKL (1 uM) and to medium on day 1 of MCF10A Transwell culture. Normal feeding conditions (24 hour cycle) were maintained.

Figure 16. Addition of prolactin (PRL) and concomitant removal of EGF from the medium on indicated day (arrow) of MCF10A Transwell culture. Normal feeding conditions (24 hour cycle) were maintained.
for cell turnover independent of PRL effects or (4) the model does not represent a lactogenic state. It was subsequently determined that MCF10A cells do not express prolactin receptor (data not shown).

The last two experiments represent an effort to optimize and characterize the MCF10A Transwell® model by recapitulating in vivo conditions. First, I attempted to model the basement membrane using three different conditions: (1) laminin coated membrane, (2) Matrigel® coated membrane and (3) inclusion of laminin nonapeptide in basal chamber. Matrigel® is a basement membrane-like substance secreted from a tumor cell line and available commercially (BD Biosciences). It is used to create mammary epithelial cysts (or mammospheres) that are thought to recapitulate some characteristics of a mammary alveolus in vitro (36). Earlier reports collectively have determined that laminin was the pertinent protein found in Matrigel® that mediated the differentiation of mammospheres (42). Laminin signaling and mammary epithelial differentiation has been extensively studied (43). The results of proteomic studies on laminin and mammary epithelia indicated that a 9-amino acid peptide region of laminin could recapitulate the signaling events, but not the physical forces associated with basement membrane attachment (44, 45). Thus, the nonapeptide would test whether the differences occurring in Transwell® differentiation are a result of laminin-dependent signaling or anchorage. The addition of Matrigel® caused a rightward shift in the TEER development, that was functionally insignificant, since it achieved a comparable plateau TEER at the same day as control (Fig 17). The coating of laminin or inclusion of a laminin nonapeptide did not affect TEER development. Moreover, the morphology of all the groups was not different from controls when assessed by confocal microscopy following immunostaining for cadherin and tight

**Figure 17.** Addition of (green) laminin coated membrane, (blue) matrigel coated membrane and (red) inclusion of laminin nonapeptide in basal chamber Normal feeding conditions (24 hour cycle) were maintained.
The second experiment performed intended to better recapitulate in vivo conditions by replacing the apical chamber medium with milk (bovine source). It had been previously documented that bovine mammary epithelial cells develop a higher plateau TEER when the apical chamber is changed to a solution electrolytically similar to milk, however milk itself was not tested (46). When milk replaced medium in the apical chamber the TEER of the Transwell® culture increased approximately 2-fold by 24 hours (Fig 18). The sustaining effects of milk replacement depended on the whether the milk solution was subsequently replenished daily or not. When the milk was replenished daily after it was initially added the TEER gradually drifted down, reaching control TEER after 6 days. If the milk was not replenished, TEER maintained the initial 2-fold increase (Fig 18). The mechanism of this increase have not been investigated. Possible mechanisms include electrolyte composition and/or growth factor(s) present in milk.

*Gene expression microarray analysis of Transwell® model*

To further understand the differences occurring in MCF10A cells as they polarize and differentiate in the Transwell® model, we performed gene expression profiling with Affymetrix Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA). Four experimental time points, each performed in triplicate, were sampled: conventional cultures of MCF10A cells cultured 5 days after reaching confluence on plastic (Monolayer) and MCF10A cells plated on Transwells® sampled at three TEER values; 200-300 Ω*cm² (Base), 1400-1600 Ω*cm² (Midpoint), and 3000-3200 Ω*cm² (Plateau). Consistent with the immunostaining results in Figure 12, the microarray assay showed MUC1 and CD10 to be significantly up-regulated in the Transwell® cultures (by
approximately 13- and 5-fold, respectively). The majority of gene expression changes observed were between confluent monolayer MCF10A cells and any of the Transwell® time points assayed. Figure 19 depicts a Venn diagram for two of those comparisons (Base vs. Monolayer and Plateau vs. Monolayer). For example, of the 1133 genes that were significantly down-regulated in base versus monolayer, 888 (78.4%) were also down-regulated in the plateau versus monolayer comparison (Fig 19). Of the 1219 genes that were up-regulated in base versus monolayer, 894 (73.3%) were also up-regulated in plateau versus monolayer. Five genes were oppositely regulated in comparing base versus plateau; three were up regulated in plateau and down regulated in base, while the other two were vice versa.

Gene clustering organizes gene expression patterns into coordinate regulatory profiles. Depicted in Figure 20 are six functionally informative clustering patterns. Large numbers of genes were downwardly or upwardly regulated comparing monolayer cultures to the Transwell® format, and did not change significantly thereafter (Clusters A and B, respectively). Genes that gradually changed as the TEER increased, ultimately reaching significant differences, are illustrated by clusters C and D respectively. Clusters E and F represent genes that changed significantly in the transition from monolayer to base, and again changed significantly back toward their initial expression level. Not all genes returned to the monolayer expression level; however the expression at plateau was significantly different from base. The hatched areas between curves in clusters C, D, E, and F represent variability in the rates of change among these genes. Groups of genes

![Figure 19. Venn diagram representing overall gene expression changes in MCF10A cells. Two separate comparisons; Base versus Monolayer (red and blue circles) and Plateau versus Monolayer (green and yellow circles) are depicted. Upwardly regulated genes are represented on the right side (blue and yellow circles), while downwardly regulated genes are represented on the left side (red and green circles). Criteria for inclusion were FDR<0.05 and ≥1.5 fold difference.](image)
within clusters generally correlated well with Gene Ontology (GO) categories (47) (http://www.geneontology.org/).

Using GO categorization, a pattern of differentiation during the Transwell® phase of culture became evident (Table 2). For example, genes regulating the progression through the cell cycle \( (p=2.3 \times 10^{-6}, \text{FDR}=9.5 \times 10^{-5}) \) and cell division \( (p=5.3 \times 10^{-6}, \text{FDR}=2.0 \times 10^{-4}) \), including p53, cyclin D1, CDK2, were downwardly regulated as the MCF10A cells differentiated onto Transwells®. It is noteworthy that the highly significant changes in cell cycle genes occurred in the context of comparing contact inhibited monolayer cultures with the Transwell® cultures, so the changes do

![Clustered expression profiles over MCF10A Transwell® differentiation. All four time points; Monolayer, Base, Midpoint, Plateau are shown on the x-axis, and total number of genes in each cluster is listed. Criteria for obtaining the total number of significantly changed genes in the figure was ≥1.5 fold difference and FDR<0.05. Criterion for stepwise comparisons between two connected x-axis time-points was ≥1.5 fold change.](image-url)
not simply reflect a difference between exponentially proliferating and static cultures.

Genes associated with extracellular matrix components such as matrix metalloproteases, mucins and collagens were upwardly regulated \((p=6.6\cdot10^{-7}, FDR=1.6\cdot10^{-4})\) (Table 2). Several pregnancy-associated gene products were changed during differentiation, including parathyroid hormone-related protein (PTHrP), steroid sulfatase, an enzyme that catalyzes the conversion of sulfated estrogen metabolites to estrogens, and pregnancy-specific glycoprotein. Tight junction proteins occludin and claudins 1,4,7,8 were also upregulated in the Transwell® format. This subset of claudins has previously been identified in the mammary epithelium of mice (48-50).

Also indicative of the luminal phenotype, the expression of GABA\(_{\lambda}\) receptor subunit pi and the transcription factor GATA3 were stimulated by 231-fold and 1.9-fold respectively. Although GABA\(_{\lambda}\) receptors have no well-characterized function in mammary tissue, GABA\(_{\lambda}\) is expressed primarily in luminal mammary epithelial cells, and is commonly dysregulated in breast cancers (51). Expression of GABA\(_{\lambda}\) was verified using semi-quantitative RT-PCR comparing monolayer to Transwell® (data not shown). GATA3 is an essential transcription factor involved in the differentiation and maintenance of a luminal epithelial cell population (52). Conditional deletion of GATA-3 results in epithelial progenitor expansion, and a reduction of differentiated luminal cells (53).

<p>| Table 2. Gene Expression Profiles of Gene Ontology Categories and Individual Genes |
|-----------------------------------------------|------------------|-----------------------------------------------|
| <strong>GO Category/Gene Name</strong> | <strong>Cluster</strong> | <strong>Maximum Fold Difference from Monolayer</strong> |
| Cell Cycle | -- | -- |
| p53 | C | -2.0 |
| CDK2 | C | -2.3 |
| cyclin E2 | A | -3.1 |
| CDC25A | A | -4.2 |
| cyclin D1 | A | -1.6 |
| Extracellular Matrix | B | 3.5 |
| Mucin 4 | D | 60.2, 2.6, 8.0, 78.5 |
| MMP1,3,9,10 | -- | -- |
| Structural Constituent of the Cytoskeleton | B | 72.4, 2.9, 33.7, 76.9, 12.0, 45.0, 8.8, 12.7 |
| Keratins 4, 6A, 6B, 13, 14, 16, 17, 23 | A | -3.2 |
| Keratin 18 | -- | -- |</p>
<table>
<thead>
<tr>
<th>Cytoskeleton</th>
<th>--</th>
<th>13.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>B</td>
<td>11.8</td>
</tr>
<tr>
<td>Involucrin</td>
<td>B</td>
<td>2.0</td>
</tr>
<tr>
<td>Integrin β8</td>
<td>B</td>
<td>1.7</td>
</tr>
<tr>
<td>Adducin3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue Development</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Collagen Type 1α1</td>
<td>B</td>
<td>10.3</td>
</tr>
<tr>
<td>Collagen Type 7α1</td>
<td>B</td>
<td>6.4</td>
</tr>
<tr>
<td>Cellular retinoic acid binding protein 2</td>
<td>B</td>
<td>8.1</td>
</tr>
<tr>
<td>Desmoplakin</td>
<td>B</td>
<td>3.0</td>
</tr>
<tr>
<td>Reproductive physiological process</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Inhibin, beta A</td>
<td>F</td>
<td>-1.8</td>
</tr>
<tr>
<td>PTHrP</td>
<td>D</td>
<td>7.8</td>
</tr>
<tr>
<td>Pappalysin 1</td>
<td>A</td>
<td>-2.3</td>
</tr>
<tr>
<td>Steroid sulfatase</td>
<td>D</td>
<td>2.3</td>
</tr>
<tr>
<td>Pregnancy specific beta-1-glycoprotein 4</td>
<td>D</td>
<td>1.6</td>
</tr>
<tr>
<td>Morphogenesis</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Wnt5A</td>
<td>F</td>
<td>-2.2</td>
</tr>
<tr>
<td>TGFB2</td>
<td>F</td>
<td>-4.1</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>D</td>
<td>25.2</td>
</tr>
<tr>
<td>Gap junction protein, alpha 1, (connexin 43)</td>
<td>D</td>
<td>114.4</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GATA-3</td>
<td>D</td>
<td>1.9</td>
</tr>
<tr>
<td>Forkhead Box Q1</td>
<td>F</td>
<td>-2.5</td>
</tr>
<tr>
<td>Forkhead box O1A</td>
<td>B</td>
<td>3.3</td>
</tr>
<tr>
<td>GABA A Receptor π</td>
<td>B</td>
<td>231.0</td>
</tr>
<tr>
<td>Crystallin, alpha B</td>
<td>D</td>
<td>27.8</td>
</tr>
<tr>
<td>CD10 (MME, CALLA)</td>
<td>B</td>
<td>4.8</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>B</td>
<td>2.3</td>
</tr>
<tr>
<td>Occludin</td>
<td>B</td>
<td>3.5</td>
</tr>
<tr>
<td>Claudin 1, 4, 7, 8</td>
<td>B</td>
<td>2.3, 3.8, 2.9, 20.5</td>
</tr>
</tbody>
</table>

The extraordinary change in expression level of GABA$_{\lambda}$ (GABRP) warranted further investigation into its function. When functional, the GABA$_{\lambda}$ receptors are heteropentameric ligand-gated Cl$^-$ channels. Thus, GABA$_{\lambda}$ is one potential subunit forming a larger receptor. Previously, it was known that GABA$_{\lambda}$ was found in the luminal epithelium of the mammary gland, but in the context as a cancer marker. In two separate studies, GABA$_{\lambda}$ expression was shown to be either upwardly or downwardly regulated compared to normal tissue, and depending
on the stage of cancer (51, 54). However, its function in normal mammary gland has not been investigated. In the lung, GABA$_{\alpha}$ receptors (assembled from various subunits, including pi), are responsible for fluid secretion that covers the lung epithelium (55). To verify the finding from the microarray, immunolocalization for GABA$_{\alpha}$ was performed on Transwell® cultures at plateau resistance (Fig 21). GABA$_{\alpha}$ was predominantly localized to the superficial, barrier-forming layer of cells. In addition it may be found with microdomains within the plasma membrane, indicated by the punctate patterning (Fig 21).

To investigate whether this mammary gland epithelium model expressed a functional GABA$_{\alpha}$ Cl$^-$ channel, I performed a preliminary electrophysiology experiment. Using the chopstick electrode, resistance and potential can be monitored and currents then calculated. GABA, picrotoxin (GABA$_{\alpha}$ antagonist), and Isoguvacine (GABA$_{\alpha}$ agonist) each at three concentrations, were applied to MCF10A Transwells® at plateau resistance, and the aforementioned measurements were recorded. Figure 22 displays the results of the preliminary experiment (n=3). The results are uninformative at this point. Practically, an Ussing chamber would be a better tool for real-time electrical measurements on an epithelium such as this. The chopstick electrode system is subject to variability in time, because of the physical movement of the electrode when switching from reading potential to reading resistance. Regardless, the MCF10A Transwell® model represents a unique opportunity to uncover the role of GABA$_{\alpha}$ receptors in the mammary epithelium.

**Discussion**

Advances in mammary gland and breast cancer biology sometimes are occasioned by
Figure 22. Electrophysiological measurements (potential & resistance measured; current calculated) of MCF10A Transwells following addition of one of the listed drugs to both chambers of media.

development or refinement of useful experimental models, such as has been the case with the widespread use of genetically-manipulated mice (56, 57). In the case of human breast biology, advances depend largely on in vitro methods, and associated xenografting techniques (8, 21, 58). There are many human cell lines derived from breast cancers, but only a few from normal (i.e., untransformed) human mammary epithelium. Among these, MCF10A cells and derivatives of this line have been the most widely used (36, 59). Here we provide evidence of multipotential epithelial differentiation of these cells at the molecular and cellular levels. These characteristics are prerequisites to study the mechanisms of normal differentiation, physiological regulation, and homeostasis in the human mammary epithelium. Salient features of this model include reliable differentiation of multipotent cultures into basal and lumenal phenotypes, formation of a high-resistance epithelial barrier, responsiveness to differentiating signals, and rational changes in the expression of genes involved in cell growth, differentiation, and reproductive function.

Before a recent paper was published from our lab (3), there were no reports demonstrating tight junction formation by human breast epithelial cells, including MCF10A. On the contrary,
an absence of tight junctions in MCF10A cells was detailed in two separate papers (27, 28). In one case, ultrastructure criteria were used with 3D mammosphere cultures, showing that MCF10A cells formed desmosome and adherens junction contacts between adjacent cells, but no tight junctions (27). In a second paper transepithelial resistance and ZO-1 immunostaining were measured in Transwell® cultures, showing a lack of tight junction development (28). Our system using MCF10A cells not only shows that this cell line can form tight junctions, but also that tight junction functionality is highly regulated (present studies and (3)) The difference between our studies and those published by other labs raises the obvious question: why? In general, the culture methods we used in either 3D mammospheres on Transwells® were similar to the others, with the single exception that our culture conditions omitted cholera toxin. Cholera toxin was identified early in our studies as being incompatible with developing tight junctions in MCF10A Transwell® cultures. Among other features of the system that were tested, the choice of substrate (in this case uncoated polyester), inclusion of EGF and hydrocortisone, and the quality of lot-tested horse serum were found to be important.

The mammary gland parenchyma is composed of two highly differentiated epithelial cell types (lumenal and myoepithelial), and less-specialized cells in the basal compartment which include one or more types of progenitor cells (29, 60-62). Lumenal cells include multiple subtypes, comprising cells that line both the ducts and the alveoli. Although both ductal and alveolar cells express milk proteins, they can be distinguished by the expression of aldolase C (34) and greater lipid secretory capacity in alveolar cells. Lumenal epithelial cells also vary in their expression of hormone receptor genes (63-67). The apparent ductal phenotype of the lumenal cells in this model is evidenced by their lack of prolactin receptor expression and downregulation of keratin 18 (unpublished results, Table 1, (68). Myoepithelial cells are specialized contractile cells found between the lumenal cells and the stroma. They express smooth muscle α-actin, as well as CD10 and are stimulated to contract by oxytocin. Within the basal compartment there are stem and/or progenitor cells, which various authors have proposed to number from <1% to nearly 10%. Many studies have attempted to characterize the mammary gland stem cells either by functional assays
(self renewal, long term label retention), or by the presence and absence of particular cell markers such as MUC1, CD10, keratins, SM α-actin and epithelial specific antigen (ESA) (19, 20, 60, 69, 70). As multifaceted and vast as this body of literature is, the nature and complexity of the apparent hierarchy of mammary stem/progenitor cells is still unresolved. Our model shows that the MCF10A cells possess properties of multipotent progenitor cells, as evidenced by differentiation into basal (CD10+, MUC1−) and lumenal (CD10−, MUC1+) phenotypes. Consequently, this model will be a useful tool to test pathways involved in proliferation and differentiation of these populations.

Broad-based gene profiling by microarray hybridization was very valuable as a means of validating the kind of changes one might expect during differentiation of MCF10A cultures. One issue that this method resolves is the nature of the multiple cell layering that occurred on the Transwell® substrate. Highly proliferative, substrate-independent cancer cells avoid “contact inhibition” and pile up in multiple layers based on unrestrained proliferation. Suppression of cell cycle genes and induction of epithelial development and differentiation genes (Table 1), along with the morphological evidence (Fig 9), demonstrates that the multiple cell layers in Transwell® cultured MCF10A represents an organized developmental process that is similar to the multiple cell layers observed in vivo, as opposed to a disorganized pathology. One source of ambiguity in gene profiling is the presence of multiple cell types. And, while this is less of a problem when using an established cell line, clearly the differentiated MCF10A cell samples that we profiled include at least two (superficial and underlying) cell types, and probably a third (suprabasal cells with cytoplasmic E-cadherin (Fig 9d arrowheads). While this limits the information that we could extract from this initial profiling study, the relative simplicity of producing differentiated MCF10A cell populations, compared with purifying single cell populations from human specimens, should make it possible to do very extensive gene profiling on well-defined cell populations that can be sorted from the MCF10A cultures based on markers we have identified here. One interesting question to address would be the relationship of the underlying population of MCF10A cells to basal cells in vivo, particularly the myoepithelium.

Among the gene expression changes in the Transwell® differentiation process, particular
interesting genes were identified, in addition to the general suppression of the cell cycle and shifts in expression of cytoskeleton and extracellular matrix genes. For example, PTHrP expression was induced approximately 10-fold during differentiation. This hormone is a master signal for development and maintenance of the mammary epithelial phenotype (71, 72). PTHrP receptors also were expressed in the MCF10A cultures, but the receptor levels did not change under the various conditions. The presence of the receptors along with differential expression of the ligand leads one to speculate that PTHrP may function as an autocrine/paracrine factor in the MCF10A cultures. Among the small number of genes that fell into Cluster “F” (declined significantly after plating on Transwells®, then reinduced as TEER increased), IGFBP3, TGF-β and FOXQ1 are associated in a growth inhibitory pathway downstream of Akt/protein kinase B (73, 74). The induction of these genes implies that a second, Akt-dependent growth inhibitory pathway is induced during final differentiation of the MCF10A cultures.

In summary, we have demonstrated multipotent differentiation of human mammary epithelial cells that predictably assemble tight junctions and thus develop a low conductance barrier. Tight junction integrity is dependent on the composition of the bottom chamber medium and retinoic acid accelerated and amplified the TEER of MCF10A membranes. Overall, the MCF10A Transwell® model recapitulates several important characteristics of the in vivo environment of the mammary gland (barrier formation, lumenal versus basal cell phenotypes with appropriate expression of markers for each). This model is very useful for studying endocrine and autocrine-paracrine developmental regulation and physiological changes of human mammary epithelium. Further refinements of this system are likely to be possible (see Chapter 5).

References


gamma-aminobutyric acid receptors involving alveolar fluid homeostasis. *J. Biol. Chem.* **281**:36012-36020.


CHAPTER III

EFFECTS AND POSSIBLE ALTERNATE LIGANDS OF 5-HT7 SIGNALING IN THE MAMMARY EPITHELIUM

Aaron M. Marshall

Systems Biology and Physiology Program
Department of Molecular and Cellular Physiology
University of Cincinnati, Cincinnati, Ohio 45267-0576
**Introduction**

Milk synthesis is regulated within the alveolar units of the breast so as to control the degree of alveolar distension in the short term, and to adjust milk secretion to the demands of the offspring in the longer term. Correspondingly, each functional unit within the gland is regulated by local signals. Local or regional blockage within the gland, as during mastitis, results in suppression of secretion within the affected regions, although secretion from the remainder of the gland(s) continues normally. In the normal lactogenic cycle the milk stasis-associated feedback is essential between bouts of nursing, or artificial milking in dairy animals. Studies, primarily done in goats, showed that physical distension of the gland was insufficient, by itself, to cause inhibition of milk secretion. The implication of this line of studies was that some chemical signal, in addition to gland distention, was necessary for feedback control of milk secretion (1-3). However, neither the identity of a chemical signal, nor a mechanism of action has yet been proven.

Our lab has proposed that the local feedback on milk synthesis is mediated by discharge of 5-HT into the luminal interstitium, which implies that 5-HT is either secreted across the basolateral membranes into the interstitium, or that apically-secreted 5-HT leaks out of the milk space and is detected by 5-HT receptors (Fig 23). While these are not mutually-exclusive mechanisms, they are functionally different and should be testable as alternatives. We showed previously that TPH1 is induced during milk accumulation, and now have shown that an increase of TPH1 would lead to elevated 5-HT biosynthesis. Consequently, it is possible that an increase of 5-HT synthesis alone, coupled with basolateral secretion into the interstitium, could explain the observed feedback effects of 5-HT. Then again, we showed previously that 5-HT is present in milk (4), implying that it is secreted apically, and therefore would enter the interstitium by leaking through the tight junction barrier.

As shown in figure 23, the 5-HT receptor identified as being responsible for the negative feedback to milk synthesis was 5-HT$_7$ (HTR7). The HTR7 transcript is processed by alternative splicing to produce three variants (HTR7a, b, d) that encode proteins with apparently identical signaling properties (5). The mechanism of action hypothesized to lead to the feedback is
5HT₁-dependent disruption of tight junction formation (Fig 23). Two signaling pathways have subsequently been identified downstream of 5HT₁; cAMP-dependent PKA pathway and cAMP-dependent p38MAPK pathway. The latter leads to the disruption of barrier function in the long term, while the former transiently increases the impedance of the tight junctions (6). This biphasic effect downstream of 5-HT₁ may play a role in the temporal control of barrier breakdown and subsequent glandular involution.

Unlike some other serotonergic receptors, 5-HT₁ has a high level of constitutive activity, which results in ligand-independent homologous desensitization of 5-HT₁ (7, 8). Antagonists that inhibit the ligand-independent receptor activity are referred to as “reverse agonists”, and in the case of 5-HT₁, these include methysergide, metergoline and SB266790. One effect of these reverse agonists is re-sensitization of the receptors. In MCF10A cells we observed by immunostaining that receptors accumulate in the area of the basolateral membrane after reverse agonist treatment, suggesting that the drug induces re-sensitization by cycling receptors to the membrane. Consistent with this, pretreatment with MG overnight caused a leftward shift in the concentration-response
curve for 5-HT (6)(9).

One of the physiological events that is essential for lactation is the establishment and maintenance of epithelial tight junctions (10, 11). The full functional integrity of epithelial tight junctions is established shortly after parturition, and they remain closed throughout lactation (10, 12-14). Changes in breast fluid composition attributable to tight junction closure (i.e., altered sodium, potassium and serum protein concentrations) occur after parturition in women, indicating that the same general process of tight junction closure occurs in the human breast (15). Conversely, the opening of tight junctions has been suggested to be one of the early events in mammary involution. The clearest evidence of the homeostatic role of mammary tight junctions comes from studies of dairy animals (goats and cows). Lactose, which is secreted only in the mammary glands, is present at a low level in the plasma of lactating cows or goats, but when milking is suspended a spike of lactose is observed in the blood after several hours (10, 14, 16). These results indicate that leakage increases because of widespread weakening of the tight junctions when milking is suspended. Since leakage of milk constituents is known to accelerate involution, and serotonin concentrations in milk are relatively low, I hypothesize that serotonin may exist in the milk space covalently attached to a milk protein.

The process of serotonin being used as a covalent modification onto proteins was shown by Diego Walther in 2003 in platelets, and was termed serotonylation (17). Serotonylation occurs in platelets because of the action of the enzyme transglutaminase 2 (TG2). TG2 serotonylates small GTPases such as Rho, Rac, and Rab in a Ca++ dependent process, thereby rendering the small GTPases constitutively active, and resulting in exocytosis of α-granules containing growth factors and clotting proteins. Furthermore, another group described a subpopulation of platelets, termed COAT platelets, which use serotonylation by TG2 to augment retention of procoagulant proteins on their cell surface (18). These data not only demonstrate TG2’s ability to use serotonin as a substrate to modify peptides, but also demonstrates physiological actions of these serotonylated proteins. Tissue transglutaminase (TG2) is also expressed in mammary epithelial cells (Figure 6). Transglutaminase catalyzes the formation of an iso-peptide bond between specific glutamine
residues (acyl donor) and either a lysine or monoamine (amine-donor) such as serotonin, histamine, dopamine, spermidine etc. (19, 20). The transcription of TG2 is induced by several factors including retinoic acid, prostaglandin E2, interleukin-6, and TGFβ (21-24). Furthermore, early in vitro transamidation studies used beta-casein, a milk protein, as the source the acyl donor (25).

Methods

Cell Culture

An immortalized human mammary epithelial cell line, MCF10A was used for the present studies. The normal growth media for MCF10As was DMEM:F12 (1:1, Cellgro) with 2mM glutamine, containing 5% horse serum, insulin (10 μg ml⁻¹) (Gibco), hydrocortisone (0.5 μg ml⁻¹) (Sigma), EGF (20 ng ml⁻¹) (Upstate) and 1 I.U. ml⁻¹ penicillin, 0.1 μg ml⁻¹ streptomycin, 0.25 μg ml⁻¹ amphotericin B (Cellgro). Cells were grown in monolayer to 90-95% confluency, trypsinized and counted for seeding onto permeable supports (Transwell®, 0.4 μm pores, polyester) in normal growth medium. MCF10A cells were seeded on 12-well Transwells® (Corning) at 10⁵ cells/cm². Both chambers of media were changed strictly on a 24-hour schedule, unless otherwise noted. Where indicated, interleukin-1 beta (IL-1β, Peprotech) was administered in both chambers. Transepithelial electrical resistance (TEER) was measured daily with Epithelial Volt-Ohm Meter (EVOM; World Precision Instruments), prior to media change.

Matrigel® Culture.

MCF10A cells were cultured in Matrigel® (BD Bioscience) as previously described with some modifications.(26) The medium supplied during differentiation in Matrigel® contained 2% horse serum, insulin (10 μg ml⁻¹) (Gibco), hydrocortisone (0.5 μg ml⁻¹) (Sigma), EGF (5 ng ml⁻¹) (Upstate) and 1 I.U. ml⁻¹ penicillin, 0.1 μg ml⁻¹ streptomycin, 0.25 μg ml⁻¹ amphotericin B (Cellgro), but did not contain cholera toxin. Metergoline (Tocris) was added at the second medium change (day 7) at 10 uM.

Transfection of 5-HT₇α

The plasmid containing the human serotonin receptor 7a was purchased from www.cdna.org, located at the University of Missouri. The gene was cloned into the pcDNA3.1(+) vector
(Invitrogen). The vector contains the following elements; CMV promoter and bovine growth hormone polyadenylation sites surrounding the 5-HT7a gene, and the SV40 promoter and SV40 polyadenylation sites surrounding the neomycin resistance gene. For stable transfectants the plasmid contained a neomycin resistance gene. Lipofectamine 2000 (Invitrogen) was the transfection agent, and the protocol was performed according to the manufacturer’s instructions, with varying lipofectamine:plasmid ratios to optimize transfection efficiency.

**Luciferase assay**

Since 5-HT₇ is a Gs-coupled protein receptor, luciferase driven by the CRE promoter was used as the endpoint to determine if there is activity at the receptor in the transiently transfected CHO K1 cells. Stimulation of the cAMP cascade downstream of adenylyl cyclase will cause transcription and translation of the luciferase protein. After the indicated incubation, treated cells were lysed and protein isolated. A luciferase substrate was added to a sample of protein isolate causing emission of light which was quantified on a luminometer. Luciferase activity was corrected for transfection efficiency by cotransfection of the lacZ gene driven by the CMV promoter. Each protein isolate’s β-gal concentration was quantified by adding OPNG to each sample and measuring color intensity at 450 nm on a microplate reader.

**Results**

**Immunolocalization of 5-HT₇**

Pharmacological evidence previously published by our laboratory identified 5-HT₇ as the mediator of 5-HT dependent effects on tight junction disruption (27). Since a barrier-forming MCF10A model had been established (see Chapter II), the pharmacologic effects of serotonergic drugs was demonstrated to be basolaterally mediated. To confirm this finding, I performed immunostaining for 5-HT₇ on MCF10A Transwell cultures. Serotonin receptor 7 protein was localized to the basolateral aspect of the superficial cells (Fig 24). Although cross reactivity among serotonin receptor antibodies has been observed, the combination of pharmacologic and immunohistological
evidence strongly supports the supposition that 5-HT, mediates mammary epithelial tight junction disruption.

**Downstream effector of 5-HT, dependent tight junction disruption**

Since tight junction disruption is known to be a part of the involution process (see Chapter I), we sought to identify signaling and/or paracrine molecules downstream of 5-HT. As part of the aforementioned microarray study (see Chapter II), we treated MCF10A Transwell cultures with a concentration of serotonin known to cause TEER decline and tight junction disruption. Messenger RNA was isolated at two time points during TEER decline, at ~40-50% decline (5-HT mid) and 80-90% decline (5-HT bottom) respectively. One of the genes that was induced by 5-HT, and has been shown to increase its expression during involution, but without a well-defined role (28), was interleukin-1 beta (IL-1β). When added to MCF10A Transwells IL-1β, similar to 5-HT, causes a significant decline in TEER over 48 hours (Fig 25). However, the maximum response was less than that for 5-HT.

**Stable expression of 5-HT, in MCF10A cells**

To corroborate the evidence that 5-HT, is responsible for the changes in barrier function observed in our Transwell model, MCF10A cells were stably transfected with 5-HT. There are three splice variants of 5-HT, (a,b,d) and all are apparently functionally equivalent (5). We created 5-HT, and 5-HT, stable cell lines. Only results from 5-HT, are shown here, however similar results were observed in 5-
HT7b clones as well. To verify and visualize protein expression of 5-HT7 and distinguish it from endogenous receptor GFP was coexpressed along with the receptor. Figure 26 shows a photograph of clone 1 from the 5-HT7a stables verifying expression of exogenous receptor. Having verified the expression of plasmid derived 5-HT7a, two clones (1 and 3) were plated onto Transwells. Both stable cell clones reached a comparable plateau TEER as compared to parental MCF10A cells (~3500 Ω x cm²). Upon treatment with three doses of 5-HT (100 μM, 400 μM, 1 mM) the clones elicited different responses. Clone 1, as anticipated, was more sensitive to 5-HT than the parental cell line (Fig 27). However, clone 3 did not respond to 5-HT, except at the highest dose given. The parental MCF10A cells responded to the low doses with an increase in TEER and to the high dose with a crash in TEER. This biphasic response has been observed and published previously (6). Overall, Clone 1 was more sensitive to 5-HT, and Clone 3 did not retain the increase in TEER normally seen with low doses of 5-HT.

5-HTr antagonist effects on MCF10A cells

In the mammary gland, 5-HT is induced by milk stasis, disrupts tight junctions and affects milk protein synthesis (4, 27). In other organs 5-HT is induced by stasis and causes fluid reabsorption (see Chapter I). Therefore, it is possible that 5-HT receptor antagonists could increase milk
accumulation. To test this hypothesis, MCF10A cells were cultured as hollow mammospheres in Matrigel and given the 5-HT receptor antagonist, metergoline. Metergoline can antagonize 5-HT\textsubscript{1,2,6,7} with highest potency for 5-HT\textsubscript{7}. Mammospheres cultured with control medium were translucent. However, in the presence of metergoline, a dark material occupied the center of the mammosphere (Fig 28). An extension of this observation is that metergoline antagonized basolateral 5-HT\textsubscript{7} receptors, and enhanced fluid secretion leading to an opaque mammosphere (Fig 28).

**Development of a system to test for alternate 5-HT\textsubscript{7} ligands**

The summation of the following evidence hinted that free 5-HT may not be the ligand for the 5-HT\textsubscript{7} receptor or that 5-HT is stored in an alternate form, that is later released into a free form. First, quantification of free 5-HT in mouse milk is low, presumably due to expression of the serotonin transporter in the apical membrane (see Chapter IV). Second, the increase of fluid secretion following receptor antagonism is clearly apical secretion (Fig. 28). Third, the recent identification of a serotonylation pathway, whereby serotonin is covalently attached to amino acid residues via a transamidation. Fourth and finally, much of the prior research on transamidation utilized beta-casein, a milk protein, as a target. Moreover, because of the apical expression of the serotonin transporter in mammary epithelial cells, it was presumed that serotonin was secreted apically as well. Operating with the hypothesis that serotonin is secreted into the milk space in an alternate form, I began to develop a system to test for 5-HT\textsubscript{7} signaling. To do this, CHOK1 cells, which do not express 5-HT\textsubscript{7}, were transiently transfected with two plasmids: one coding for human 5-HT\textsubscript{7} (HTR7) promoted by
CMV, and the second coding for the luciferase gene promoted by the cAMP response element (CRE). Since 5-HT, signals through cAMP, any ligand activating 5-HT, would lead to an increase in luciferase protein, which can be assayed. When 5-HT, was transfected into CHOK1 cells, they were unable to respond to exogenous 5-HT or forskolin (Fig. 29). Interestingly, the levels of luciferase detected in any of the 5-HT, transfected groups were similar and less than the vector control with only CRE-Luc transfected.

**Discussion**

Given the importance of tight junction integrity for lactation (10, 11), previous studies focused on physiological characterization of 5-HT signaling on the regulation of epithelial tight junctions (27). As a mediator of milk stasis-induced involution, 5-HT has effects on a variety of aspects of lactating mammary epithelial cells, including milk protein synthesis, fat globule synthesis, fluid and solute secretion, and cell survival (4). Many of these effects, including the effects on tight junctions, could be indirect, and identifying the relevant signaling pathways will allow the design of experiments to sort out the hierarchy of regulatory effects on mammary cell functions. To this end, a comparative gene expression profiling was performed and IL-1 was upregulated by 5-HT. Both isoforms, alpha and beta, were upregulated and IL-1β was shown here to recapitulate the drop in TEER caused by 5-HT. Interleukin 1 is known to signal through p38MAPK, the same pathway shown to be activated in a time-dependent manner after 5-HT is administered (6). This cytokine has also been shown to be expressed during involution, and may be the link between 5-HT and the cascade of downstream events (29, 30).

One of those downstream events important for involution is fluid clearance. If 5-HT causes
fluid clearance, then an antagonist to 5-HT activity should increase milk secretion. Here we have shown that the receptor antagonist metergoline may promote fluid accumulation in MCF10A mammosphere culture. This observation has since been extended to dairy animals. In that case, methysergide another broad spectrum antagonist, caused an increase in milk yield in dairy cows (31). Interpretation of receptor activities in the bovine are complicated by the presence of multiple isoforms (Hernandez L.L. et al., in press). Further studies with receptor isoform specific antagonists would help identify which receptor is causing fluid clearance and would be viable approach to enhancing milk production in dairy animals.

The development of a system to test for alternate 5-HT7 ligands using CHOK1 cells did not succeed. One potential reason for this would be the high constitutive activity of the 5HT7 receptor leading to desensitization (7, 8). In a system where the receptor is being overexpressed, it should not be surprising that the cells have become refractory to any cAMP stimulation, including forskolin. This explanation is supported by the observation that 5-HT7 transfection suppressed luciferase expression below background level (vector only). An inducible system that can be controlled may be the best option for future pursuit of this hypothesis.

References


CHAPTER IV
SEROTONIN TRANSPORTER IN THE MAMMARY GLAND MODULATES SECRETORY ACTIVATION AND INVOLUTION

Aaron M. Marshall\textsuperscript{1,2}, Laurie A. Nommsen-Rivers\textsuperscript{3}, Laura L. Hernandez\textsuperscript{1}

\textsuperscript{1}Systems Biology and Physiology Program
\textsuperscript{2}Department of Molecular and Cellular Physiology
University of Cincinnati, Cincinnati, Ohio 45267-0576
\textsuperscript{3}Division of Neonatology
Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229
Introduction

It is currently recommended that infants be exclusively breastfed for the first 6 months of life, and continue to be breastfed with addition of appropriate complementary foods for the first 1 to 2 years of life (1, 2). Breastfeeding benefits both the recipient infant and the lactating mother (3-6). For the infant, the benefits of receiving human milk include decreased risk of acute otitis, lower respiratory tract disease, gastrointestinal infections, sudden infant death syndrome, and the future development of type 1 and type 2 diabetes (6). In the case of maternal benefits, it has recently been reported that mothers with a total lifetime history of at least 12 months of lactation are at significantly lower risk of developing hypertension, diabetes, hyperlipidemia and cardiovascular diseases after menopause (3). In addition, having lactated decreases the risk of developing breast and ovarian cancers (6). Despite these important health benefits, only 11% of mothers in the United States breastfeed exclusively for the recommended duration of 6 months (7).

For mothers who initiate breastfeeding, the early postpartum period sets the stage for sustaining exclusive breastfeeding for as long as intended. Women who supplement with infant formula because of breastfeeding difficulties in the early postpartum period are likely to continue to supplement, and/or wean prematurely(8-11). One early breastfeeding difficulty that is particularly common among women in the United States is delayed onset of lactogenesis (12, 13). Incomplete expulsion of the placenta leading to elevated serum progesterone levels prevents tight junction (TJ) closure and can cause delayed onset of lactogenesis (14, 15). Other biologic mechanisms contributing to delayed onset of lactogenesis remain poorly defined, although primiparity is known to be a strong risk factor (13).

Tight junction formation in the mammary gland results in a high resistance barrier between epithelial cells. This action initiates the onset of copious milk secretion that is the hallmark of the secretory activation stage of lactation (stage II lactogenesis) (15). In women, delayed onset of lactogenesis is typically defined as maternal perception of onset of copious milk secretion after 72 hours post partum (9, 13). The initial consequence of delayed onset of lactogenesis, absent any intervention, is undernourishment of the infant (12). Mothers who experience delayed onset
of lactogenesis are at significantly greater risk for early cessation of breastfeeding (9, 16, 17). Therefore it is important to identify risk factors for delayed onset of lactogenesis so that women with these risk factors receive appropriate breastfeeding support.

Mammary epithelial TJ are dynamically regulated not only during the secretory activation phase of lactation, but also during milk stasis. Whether caused by local ductal occlusion or weaning, sustained milk stasis eventually leads to glandular involution (18, 19). Involution occurs via three sets of processes: 1) milk stasis-induced cessation of secretion combined with TJ disruption, 2) collapse of alveoli, with apoptosis of milk secreting cells and 3) remodeling of the epithelium and adipose tissue (20). Each functional unit of the mammary gland (alveolar unit) autoregulates, independent of endocrine influences (21). Of the plethora of molecules identified as being characteristic of involution (20, 22-26), most are involved in the events listed under processes 2 and 3, above. The identification of factor(s) responsible for the autocrine/paracrine negative feedback on milk secretion and barrier function (#1) have remained elusive. Recently, our lab identified the monoamine serotonin (5-hydroxytryptamine, 5-HT) as being one of these molecules (27, 28).

In the mammary glands, milk stasis induces 5-HT synthesis in the epithelium, where it acts as a negative feedback inhibitor of milk synthesis and secretion (27-29). One mechanism by which sustained 5-HT signaling contributes to this negative feedback is the disruption of epithelial barrier function (28, 30). Although these previous studies established the effects and mechanism of exogenous 5-HT on TJ, the ramifications of altering intrinsic 5-HT turnover remained unexplored.

Serotonin is a mediator of several physiological functions, and has subsidiary roles in many more. In addition to its well-recognized roles in the central nervous system, 5-HT affects the physiology of the gut, prostate, liver, lung, pancreas, heart, and vasculature. The diversity of 5-HT roles can be attributed to the seven families and more than fifteen isoforms of 5-HT receptors expressed in mammalian cells (31). Serotonin action is controlled in two ways. Intracellularly, the amount of 5-HT packaged into vesicles is controlled by a balance between synthesis (tryptophan
hydroxylase activity) and degradation (monoamine oxidase, MAO). Extracellularly, the availability of 5-HT is controlled by a recycling mechanism facilitated by the 5-HT transporter (SERT, 5-HTT). The 5-HT transporter is the target of several classes of pharmaceuticals, most particularly selective serotonin reuptake inhibitors (SSRI). This class of drugs is commonly used to treat generalized and post-partum depression. We recently demonstrated that SERT is expressed in primary human mammary epithelial cells and an immortalized mammary epithelial cell line (MCF10A) (28, 32).

In the present report we show that drugs affecting 5-HT turnover, including SSRI and MAO inhibitors, alter mammary epithelial functions, and that these actions are consistent with previous experiments that relied on extrinsic 5-HT drugs (agonists and antagonists). We also show that these effects are similar in human, mouse, and bovine systems. In addition, we present epidemiological evidence that women taking SSRI mediation are at risk for delayed onset of lactogenesis.

Methods

Animal Experiments
All mice were wild-type from the C57BL/6J strain and were between 10-16 weeks of age. Animals were housed in a barrier facility and all procedures were approved under IACUC protocol (05-01-11-01). Pregnancy day 1 is defined by the observation of a vaginal plug. Lactation day 0 is defined as the day of parturition, and involution day 1 is defined as 24 hours after forced weaning. Bovine cell preparations were a generous gift from RJ Collier at the University of Arizona. Primary human tissue was obtained under IRB approval (#) and was a generous gift from Eric Hugo.

Cell Culture and Medium Formulae
pHMECs were obtained from breast reduction mammoplasty tissue using a modification of previously described protocol for primary mouse mammary cells (33). The only modification was the collagenase incubation was 6-12 hours versus 3 hours. MCF10A Transwells® (Corning) were cultured as previously described (32). pHMEC Transwells® were similarly cultured with the following modifications. Cells were plated on collagen coated polyester membranes at 2.5x10^5 cells per cm^2 and were fed every 12 hours until plateau resistance was achieved. Tissue dissociation and isolation of bovine epithelial cells was performed as previously described (34). Thawed
pBMEC were plated on collagen coated plates. pBMEC were then plated onto Transwells® for experimental use. Drugs added to all Transwell® experiments were added to both top and bottom chambers.

MCF10A medium consists of the following: DMEM-F12 50:50 (Invitrogen) supplemented with 5% horse serum, L-glutamine (2mM), insulin (10 µg/ml Sigma), EGF (20ng/ml, Upstate) hydrocortisone (0.5 µg/ml) and 1 I.U. ml-1 penicillin, 0.1 µg ml-1 streptomycin, 0.25 µg ml-1 amphotericin B (Cellgro) (Ab/Am). The medium used to grow MCF10A cells in flasks was identical to medium supplied in Transwell® format. pHMEC medium for growth in T75 flasks contained DMEM-F12 50:50, 5% FBS (Hyclone), insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), EGF (5 ng/ml), cholera toxin (1 ng/ml) (Sigma) and Ab/Am. pHMEC medium used in Transwell® format consisted of DMEM-F12 50:50, 1% FBS, EGF (5 ng/ml), insulin (10 µg/ml) and Ab/Am. pBMEC medium consisted of DMEM-F12 50:50, 10% FBS, L-glutamine (2mM), EGF (25 ng/ml), insulin (10 µg/ml), hydrocortisone (1 µg/ml) and 10 mM sodium acetate.

**Immunocyto(-histo)chemistry and Western Blot**

Transwell® cultures used for immunostaining were fixed by a brief incubation in 4% paraformaldehyde, then rinsed and stored in sterile PBS. Sections of Transwells® were permeabilized in 0.1% Triton X-100, incubated in borate buffer pH=8.5 (80 mM boric acid, 20 mM sodium borate) overnight at 75°C for antigen retrieval. The sections were then blocked with serum, incubated in primary antibody overnight at 4°C, followed by secondary antibody incubation at room temperature for 2 hours. Mammary tissues from a lactating (day 10 post partum) mouse were excised and fixed in 4% PFA for 2 hours at room temperature then frozen in tissue freezing medium. Sections were cut at 20 µm and rehydrated with PBS and permeabilized in 0.5% Triton-X-100. The sections were then incubated in goat anti-mouse F(ab) unlabeled antibody followed by 10% normal serum, both as blocking steps. Sections were incubated in primary antibody for 30 min at room temperature, followed by secondary antibody incubation at room temperature for 20 minutes.

For Western blot, total cell lysates were obtained by RIPA buffer method from C57/B6 mouse mammary glands representing the identified stages of development. Proteins were visualized
after transfer to nitrocellulose membrane by Alexa Fluor 680 conjugated secondary antibodies and Li-Cor Odyssey 9201 machine.

Antibodies used were mouse monoclonal anti-SERT antibody (Advanced Targeting Systems, San Diego, CA), rabbit anti-occludin (Zymed), goat anti-mouse Alexa Fluor 488 (Molecular Probes, Carlsbad, CA), goat anti-rabbit Alexa Fluor 546 (Molecular Probes, Carlsbad, CA), goat anti-mouse Alexa Fluor 680 (Molecular Probes, Carlsbad, CA) and TOPRO-3 iodide (Molecular Probes, Carlsbad, CA) as a nuclear stain.

Elvax® Pellet Preparation and Implantation
Pellets were synthesized according to previously published protocols (35). Briefly, a predetermined amount of fluoxetine (FLX) (dissolved in 100% ethanol) was added to 20% Elvax® (ethylene vinyl acetate) polymer dissolved in dichloromethane. The viscous mixture was stored at -20°C in an open container to allow evaporation of the solvent. The resulting stock pellet was further dried in vacuum desiccator, and weighed. For all experiments here the pellet contained 24 μg FLX per mg of pellet weight.

For implantation, 1.0-1.5 mg pellets were trimmed off the stock pellet under a stereoscope. Wild-type lactating mice were anesthetized with isoflurane at day 8-9 postpartum. Pellets containing FLX were implanted in the #3 and #4 glands on one side, while the contralateral glands received vehicle control pellets of similar weight. India ink marked the implantation site and was later used to identify the pellet location. This was necessary due to the pellet being dissolved by xylene during the histological processing. After recovery mothers were returned to their litters until the time of sacrifice when the mammary glands were removed, fixed and processed for H & E staining.

Epidemiology data collection and analysis
All expectant primiparous women receiving prenatal care at a University of California Davis Medical Center clinic between January 2006 and December 2007 were screened for study eligibility. Selection criteria were: expecting first live-born infant, between 32-40 weeks gestation at time of interview (mean=35.9 weeks SD = 1.6 weeks), single fetus, speaks either English or Spanish, and
ZIP code in catchment area (8-mile radius of the University of California Davis Medical Center). Exclusion criteria were: referred to the University of California Davis Medical Center due to medical condition, known absolute contraindication to breastfeeding, or < 19 years old and not able to obtain parental consent. The study protocol and consent form were approved by the University of California Davis Institutional Review Board. After obtaining written informed consent, study subjects were interviewed at a prenatal clinic visit regarding demographic information; health history, including current medication use; depressive symptoms; infant feeding knowledge and attitudes; and infant feeding intentions (36). Participants were re-contacted in the hospital within 24 h of giving birth to determine if they were still eligible and willing to continue their participation in the study. If so, they were visited again between 72-96 hours postpartum (“Day 3”) to assess breastfeeding experience, including infant breastfeeding behavior (37) and the timing of the onset of stage II lactogenesis. The latter, which is characterized by the onset of copious milk production, was assessed as described previously (13), which is based on the maternal report of when her breasts felt “noticeably fuller” on a 1 to 5 scale, (where 1=no change since giving birth, 3=noticeably fuller, 5=uncomfortably full). For participants who had not experienced “noticeable fullness” by the time of the Day 3 interview, the question was repeated at the Day 7 interview. Delayed onset of lactogenesis was defined as maternal perception of onset of noticeable fullness beyond 72 hours postpartum.

The characteristics of women in the SSRI versus non-SSRI groups were compared using Fisher’s exact test for dichotomous variables and Student’s t-test for continuous variables. The survival distribution functions for timing of onset of stage II lactogenesis were compared between groups using Kaplan-Meier estimator methods (SAS Proc Lifetest). The proportion in each group with delayed onset of lactogenesis was first compared using the Fisher’s exact test. Further examination after adjustment for potentially confounding baseline characteristics was done using the Mantel-Haenszel statistic (a weighted average analysis).

Results

Contribution of SERT and MAO to Intrinsic 5-HT Activity in MCF10A cells
We confirmed the apical localization of the SERT in MCF10A cells, which were cultured on Transwell® permeable membranes (28, 32). We show here with confocal microscopy that the distribution of SERT is highly restricted to the apical membrane of the superficial cell layer, which is bounded by occludin staining (Fig 30a).

Previous studies demonstrated that MCF10A cells synthesize 5-HT, and treatment with exogenous 5-HT affected TJ in a concentration-dependent manner (28). Those results and others implied that autocrine secretion of 5-HT could regulate the epithelium, but there was no direct proof that endogenous 5-HT has the same effect as that which was added exogenously. To confirm that endogenous 5-HT is sufficient to regulate epithelial TJ, we tested the consequence of blocking

Figure 30. Barrier disruption of MCF10A Transwell® cultures. (a) Immunostaining for SERT (green) and occludin (red) in MCF10A Transwell® cultures demonstrating apical distribution of SERT and well organized occludin at tight junctions. (b) Concentration-dependent TEER responses of MCF10A Transwell® cultures to FLX (1 μM – 100 μM) plotted as mean percent differences. Untransformed TEER measurements were analyzed by two-way ANOVA followed by Bonferroni tests comparing each concentration versus control (* p<0.05, @ p<0.01, # p<0.001) (n=4). (c) Response of MCF10A Transwell® cultures to serotonergic drugs 48 hours after addition of drugs; FLX (10 μM), PHLZ (10 μM), and FEN (100 μM). Results were analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test (* p<0.05, @ p<0.01). Data points represent means ± SEM (n=6). (d) Immunostaining for ZO-1 in MCF10A Transwell® cultures on Day 3 in Control (di) and FLX (dii, 30 μM) treated cultures; notice the disruption of lateral localization of ZO-1 by FLX.
5-HT reuptake in MCF10A cells with fluoxetine (FLX), a well-characterized SSRI. Tight junction permeability was monitored by measuring transepithelial electrical resistance (TEER), which is inversely related to the permeability of TJ. The data are reported here as percent difference versus untreated controls on each treatment day. Fluoxetine elicited a biphasic response in MCF10A cells (Fig 30b). At low concentrations and/or earlier time points, FLX caused a potentiation of TEER, and at later time points and at higher concentrations decreased TEER was observed. Specifically, at a concentration of 10 μM FLX, TEER was increased ∼45% and remained elevated for three days (Fig 30b – ▲). At 30 μM FLX, TEER increased on day 1, and subsequently declined on days 2-3 ( ▼). At a higher concentration (100 μM) TEER declined by 24 hours ( – ▲). Overall cell viability was not affected at any FLX dose. This biphasic pattern in TEER is similar to that observed in MCF10A Transwell® cultures treated with varying times and concentrations of exogenous 5-HT (30).

Serotonin is labile in biological environments, in part because of its biochemical conversion to 5-hydroxyindole acetic acid by MAO. Because of its role in determining 5-HT bioactivity, the MAO system, like SERT, has been used as a therapeutic target for antidepressant medications (38). Since MAO could be involved regulating endogenous 5-HT turnover in the mammary epithelium, we tested the consequences of giving the MAO inhibitor PHLZ alone or in combination with FLX. Fluoxetine was used at a concentration (10 μM) that resulted in increased TEER at 48 hours (Fig 30c). At this time point, PHLZ alone caused a small, but insignificant, increase in TEER (p>0.05). In combination with FLX there was a highly significant decrease in TEER, reversing the effects of either of the drugs alone (p<0.01, Fig 30c).

Dexfenfluramine (FEN) is an nonamphetamine anorectic drug that acts via 5-HT. Its combined use with 5-HT enhancing drugs, such as SSRIs, can precipitate episodes of “serotonin syndrome”, a potentially life-threatening reaction to excess 5-HT (39). Dexfenfluramine causes the release of monoamines from stored vesicles, and in this context, acts to increase the extracellular concentration of 5-HT. These properties allowed us to use FEN to test independently the hypothesis that endogenous 5-HT release regulates mammary epithelial TJ. When administered to MCF10A
cells, FEN caused a decrease in TEER similar to that seen with the combination of PHLZ and FLX (Fig 30c).

To confirm that a decrease in TEER represents TJ disruption we immunostained for the TJ scaffolding protein ZO-1 in FLX-treated (30 uM) cultures, in which the resistance was decreased by ~60%. ZO-1 has previously been shown to be redistributed during the disassembly of the mammary epithelial TJ complexes (30). In a high resistance barrier culture, ZO-1 staining distinctly circumscribed the cells, and was predominantly membrane associated (Fig 30di). Following FLX treatment and corresponding to TEER decline, ZO-1 staining became intracellular, and observable gaps in the circumscribing ZO-1 immunostaining could be seen (Fig 30dii).

Conservation of Mechanisms in Human Breast and Bovine Mammary Cells

To corroborate the effects of serotonergic drugs on intrinsic 5-HT activity, primary human mammary epithelial cells (pHMEC) were obtained from reduction mammoplasty and cultured on Transwell® permeable membranes. The plateau TEER achieved with these cells varied more than that of MCF10A (1200 – 2200 Ω-cm2), but they reliably polarized and formed TJ. We first confirmed the expression and localization of

![Figure 31. Conservation of 5-HT metabolic regulatory mechanisms in human and bovine mammary epithelial cells. Mean (± SEM) percent difference in TEER versus controls in response to serotonergic drugs: (a) pHMEC Transwells®; 36 hours after addition of drugs (n=4). (b) pBMEC Transwell® cultures 48 hours after addition of drugs (n=12). Drugs used were FLX (10 μM), PHLZ (10 μM), and FEN (100 μM). All results were analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test (* p<0.05, @ p<0.01, # p<0.001).
SERT in pHMEC by confocal microscopy following immunostaining. In pHMECs, like MCF10A cells, SERT expression was in the apical membrane (data not shown). The responses to serotonergic drugs (FLX, PHLZ, FEN) in pHMEC membranes was comparable to that in MCF10A (Fig 31a). While FLX alone did not change TEER, PHLZ alone caused a decrease (p<0.01). As with MCF10A cells, the combination of the two drugs amplified the decrease in TEER at 36 hours (Fig 31a). Primary HMEC cultures also responded to FEN with a 50% reduction in TEER (Fig 31a).

To extend these observations, we studied the effects of the same drug doses and combinations on primary bovine mammary epithelial cells (pBMEC). SERT in pBMEC was confirmed by immunostaining to be in the apical membrane (data not shown). pBMEC cells responded to either FLX or PHLZ alone with a modest decline in TEER (Fig 31b), suggesting that the bovine cells were more sensitive to each than either MCF10A or pHMEC. Similar to both MCF10A cells and pHMECs, the response of pBMECs to the FLX, PHLZ combination was complementary leading to a sharp decline in TEER (Fig 31b).

Expression and Localization of SERT in vivo

Having shown the contribution of 5-HT turnover mechanisms to the maintenance of epithelial barrier in human and bovine mammary epithelial cells through in vitro studies, we were interested in demonstrating the effects of SERT antagonism in the mammary gland in vivo. To establish the basis for these studies, we wanted to document the pattern of SERT expression in the rodent mammary gland, both temporally and spatially.

SERT protein levels were analyzed by western blotting in extracts of mammary gland from mice at different developmental stages (Fig 32a). Serotonin transporter protein was present in mammary gland extracts at all stages of postpubertal development. It appeared that SERT levels increased modestly with the expansion of the mammary epithelium in pregnancy, and that the elevated SERT protein content was sustained throughout lactation (Fig 32b).

To determine the pattern of cellular distribution of SERT in vivo, we performed immunostaining on lactating mouse mammary tissue (day 10 postpartum). The mouse mammary gland epithelium expressed SERT on the apical face of the epithelial cells (Fig 32c, arrow),
consistent with the distribution observed in mammary epithelial cells in Transwell® cultures.

Additional SERT immunostaining was observed in the mammary stroma (not shown), both in vascular and non-vascular elements.

**In Vivo Blockade of 5-HT Reuptake During Lactation**

Implantable pellets provide a slow and sustained release of drug or hormone and offer a unique option to deliver substance(s) locally in the mammary gland, without having systemic effects (33, 40). We constructed pellets containing FLX (24 μg/mg dry pellet weight), which were implanted into lactating mice at day 8-9 post partum. Two drug-containing pellets were implanted in each mouse (one each in the #3 and #4 glands on one side), and the contralateral glands were implanted with pellets prepared with solvent vehicle only, to serve as control treatments. Pellet locations were marked with India ink. After surgery the mothers were returned to their pups and resumed normal nursing habits, and mice were sacrificed on each of the subsequent 4 days to collect tissue specimens for histological analysis.
Figure 33. Effects of Elvax® FLX pellet implants into lactating mammary glands. (a) Displayed are representative hematoxylin and eosin stained sections of mammary glands following Elvax® pellet implantation: day 1 through day 4 of vehicle (left column) and FLX (center and right column) treated lactating mouse mammary glands. Proximal (≤0.5mm) and distal (≥5mm) are relative to the pellet implantation site. Scale bar=75 μM. (b) Mean (± SEM) lumen diameters from vehicle and FLX treated lactating mouse mammary gland pellet implants (n=3 mice x 2 mammaries/mouse/day). Shown is day 1 through 3 (x-axis) for each of relative photographed sites. Data were analyzed by one-way ANOVA followed by Dunnett’s multiple comparisons test (@ p<0.01) as compared to vehicle for that same day.
The mammary gland tissue surrounding the control pellets were histologically indistinguishable from normal lactating mouse mammary gland. These tissues were characterized by distended alveoli filled with milk, surrounded by basophilic epithelial cells containing secretory vesicles and lipid droplets. In addition, the affiliated stroma was devoid of large adipocytes (Fig 33a, “vehicle proximal”). Specimens were also analyzed from areas of the drug-treated glands that were as far as reasonable from the marked pellet sites (Fig 33a, “fluoxetine distal”). These areas were very similar in histological appearance to the control-implanted glands. In contrast, glandular morphology was starkly altered in the tissue surrounding the FLX pellet (Fig 33a, “fluoxetine proximal”). Moreover, these alterations displayed a time dependence. On the first day after implantation, alveoli proximal to FLX pellet began to collapse, and they continued to do so through day 3. By day 4, the beginning of adipose tissue re-differentiation became apparent in these regions (Fig 33a, “fluoxetine proximal”). Importantly, these changes occurred in the context of continued positive endocrine influences brought on by the suckling stimulus. Figure 33b shows quantification of the alveolar lumen diameters in the three treatment groups through day 3. Alveoli distal to the FLX pellet tended to be smaller, than those in the control glands, but the difference was not statistically significant. On the contrary, lumen diameters from alveoli proximal to FLX pellets were significantly smaller and themselves trended down over the three day period (Fig 33b). Because alveoli could not be defined structurally in the FLX-treated glands on day 4, there was no quantification of these specimens.

Assessment of SSRI as risk factor for delayed onset of lactogenesis

Based on our in vitro findings, we hypothesized that women taking SSRI medication might be at greater risk for delayed onset of stage II lactogenesis, which requires TJ closure (41, 42). Data were collected as part of a longitudinal cohort study examining barriers to early lactation success. The study group was a multi-ethnic population of primiparous (i.e., first live birth) women.

Over the 24 months of study enrollment, 768 of those screened met the eligibility criteria and 532 of these women agreed to the prenatal interview (69% of those eligible). Acceptance rates were not significantly different by education level (P = 0.22). Reasons for refusal were: too
busy, 51%; not interested, 25%; study too intrusive, 18%; doesn’t want to be interviewed about breastfeeding, 3%; and miscellaneous, 2%. Of the 532 women enrolled prenatally, 40 (7.5%) were lost to follow-up by the time of delivery and 44 became ineligible for continued follow-up (preterm birth, n=11; unable to initiate feeding at the breast within 24 hours for medical reasons, n=21; and mother chose not to initiate breastfeeding, n=12). Thus, 448 breastfeeding mother-infant pairs remained in the study for postnatal follow-up.

In the study group, 431 (96%) had yielded data on the timing of onset of lactogenesis, including 8 (1.9%) women who indicated regular use of an SSRI medication (FLX, n=3; paroxetine, sertraline, citalopram, escitalopram, duloxetine, n=1 for each).

The characteristics of the SSRI and non-SSRI mothers are shown in Table 3. Women were similar in most characteristics, except that women in the SSRI group were significantly more likely to have scored in the “at-risk” range on the CES-D depressive symptoms scale (43) (as expected),
and somewhat (but not significantly) more likely (P=0.11) to have delivered by Cesarean section.

Figure 34 shows the timing of onset of stage II lactogenesis in the SSRI group versus the non-SSRI group. Median onset of stage II lactogenesis was 85.8 hours postpartum in the SSRI-treated mothers, versus 69.1 hours in the non-SSRI mothers (p=0.004). All women in the SSRI group had experienced stage II lactogenesis by the day 7 interview. Among women in the non-SSRI group, 2.1% at day 3 interview and 1.7% at the time of the day 7 interview were censored. Delayed onset of lactogenesis occurred in 7 of 8 women in the SSRI group (87.5%), as compared to 43.5% of women in the non-SSRI group (relative risk [RR]=2.0, Fisher’s exact test, two-tailed P-value=0.02). The relationship remained significant after Mantel-Haenszel adjustment for potentially confounding factors, including maternal age, (common RR=1.9, P=0.02), maternal obesity (common RR=1.8, P=0.04), Cesarean section delivery (common RR=1.9, P=0.02) or infant gestational age (common RR=2.0, P=0.01). There was a single subject in the SSRI group that did not meet the a priori definition of delayed onset of lactogenesis (> 72 hours postpartum), and her onset occurred at 72.0 hours.

Table 3

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-SSRI group (n=423) Mean (standard deviation) or %</th>
<th>SSRI group (n=8)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>26.1 (6.0)</td>
<td>28.8 (6.6)</td>
<td>0.28</td>
</tr>
<tr>
<td>&gt; 30 years old</td>
<td>27.2%</td>
<td>35.5%</td>
<td>0.69</td>
</tr>
<tr>
<td>Attended college</td>
<td>59.8%</td>
<td>67.5%</td>
<td>1.00</td>
</tr>
<tr>
<td>Public health insurance</td>
<td>48.9%</td>
<td>50.0%</td>
<td>1.00</td>
</tr>
<tr>
<td>Body mass index (BMI), kg/m²</td>
<td>28.7 (6.1)</td>
<td>31.9 (8.4)</td>
<td>0.17</td>
</tr>
<tr>
<td>BMI in obese range (&gt; 30.0)</td>
<td>31.7%</td>
<td>57.1%</td>
<td>0.22</td>
</tr>
<tr>
<td>Depressive symptoms score &gt; 16b</td>
<td>17.5%</td>
<td>50.0%</td>
<td>0.04</td>
</tr>
<tr>
<td>Cesarean section delivery</td>
<td>30.5%</td>
<td>62.5%</td>
<td>0.11</td>
</tr>
<tr>
<td>Breastfed &gt; 8 times in first 24 hours</td>
<td>85.5%</td>
<td>100%</td>
<td>1.00</td>
</tr>
<tr>
<td>Infant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>39.6 (1.0)</td>
<td>38.9 (1.1)</td>
<td>0.05</td>
</tr>
<tr>
<td>Birth weight, kg</td>
<td>3.38 (0.43)</td>
<td>3.41 (0.40)</td>
<td>0.86</td>
</tr>
<tr>
<td>Apgar score, 1-minute</td>
<td>7.7 (1.5)</td>
<td>7.8 (0.9)</td>
<td>0.95</td>
</tr>
<tr>
<td>Apgar score, 5-minute</td>
<td>8.8 (0.5)</td>
<td>8.6 (0.5)</td>
<td>0.27</td>
</tr>
<tr>
<td>&gt; 60 ml formula, 0-48 hours postpartum</td>
<td>30.5%</td>
<td>37.5%</td>
<td>0.69</td>
</tr>
<tr>
<td>Sub-optimal breastfeeding behavior, day 0</td>
<td>62.5%</td>
<td>62.5%</td>
<td>1.00</td>
</tr>
<tr>
<td>Sub-optimal breastfeeding behavior, day 4</td>
<td>28.2%</td>
<td>62.5%</td>
<td>0.05</td>
</tr>
<tr>
<td>Sub-optimal breastfeeding behavior, day 7</td>
<td>22.8%</td>
<td>25.0%</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Student’s T-test for comparison of means; Fisher’s exact test for comparison of proportions

bCut-off for at-risk of depression based on CES-D scale (Center for Epidemiologic Study)

cAccording to Infant Breastfeeding Assessment Tool score ≤ 10 (37)

Quantification of 5-HT

99
Having shown that SSRI can affect tight junctions and modulate stage II lactogenesis and involution, we set out to prove that 5-HT is indeed elevated, as predicted, in the \textit{in vitro} model or \textit{in vivo}. To enhance the potential for differences in 5-HT concentration in the media, the combination of PHLZ and FLX were given to MCF10A Transwells at plateau resistance. Medium from the top and bottom chambers were subsequently collected at 2, 8, and 24 hours. Media samples were immediately frozen on dry ice until they could be analyzed by ELISA for 5-HT. By 24 hours, PHLZ and FLX combination did cause an increase in 5-HT concentration in the medium in both the top and bottoms chambers (Fig 35).

To corroborate this increase observed in Transwell cultures, 5-HT concentration was measured in mouse milk (see methods) after an acute injection with FLX (10mg/kg). To validate the sensitivity of the assay, we spiked mouse milk samples with 5-HT and performed either a simple defattening procedure, or a defattening plus a perchloric acid precipitation of proteins. The rationale is that milk is commonly used as a generic antibody blocker in western blotting, and therefore it was important to assess whether the assay would be able to detect 5-HT in milk. The gray bars in Figure 36 represent the concentration of 5-HT spiked. The results indicate that the assay is sensitive enough to detect 5-HT in milk when a simple defattening procedure is performed.

**Figure 35.** Quantification of 5-HT by ELISA in MCF10A Transwells. P + F = phentolamine (10 \textmu M) and fluoxetine (10 \textmu M) (n=3).

**Figure 36.** Quantification of 5-HT by RIA in mouse milk after 5-HT spike (n=3). Gray bars are the spiked concentration and blue and red bars are the concentrations detected by assay after respective extractions. Skim milk is defattened according to methods.
The perchloric acid extraction decreased the recovery of 5-HT from milk. Thus, the sample collected from mice after drug injection were defatted only. Four hours after an acute injection with FLX, mouse milk was collected, defatted, frozen and analyzed for 5-HT content by radioimmunoassay. 5-HT content was unaltered by FLX injection (Figure 37).

It is possible that the experiment setup (one injection) was not sufficient to measure a change in 5-HT concentration. However this needs to be further investigated.

Discussion

Previous work on the actions of 5-HT on mammary glands or mammary epithelial cultures has used exogenously added agonists and/or antagonists (27-30). One limitation in those studies was the difficulty of related pharmacological concentrations of exogenous drugs ro the intrinsic ligand levels. The need for high exogenous concentrations of drugs was not particularly surprising if 5-HT acts in an autocrine/paracrine fashion. The peak concentration of 5-HT in synapses following neuronal stimulation has been calculated to be in the millimolar range, which quickly dissipates to nanomolar concentrations (44). However, experiments that relied on exogenous addition of receptor-active agents (agonists or antagonists) could not answer directly the question of whether the autocrine secretory turnover of 5-HT was sufficient to explain feedback inhibition in the mammary gland epithelium.

In the current studies, we have manipulated the intrinsic 5-HT bioactivity of the mammary using agents that were predicted to alter the rates of turnover of the endogenous ligand. These included drugs that inhibit SERT or MAO (FLX and PHLZ respectively), or that activate vesicular release (FEN). Therefore, the current *in vitro* results demonstrate the importance of SERT and

![Figure 37. Quantification of 5-HT by RIA in mouse milk after one injection of FLX (10 mg/kg). Samples were collected 4 hours after injection.](image)
MAO in the dynamic turnover of 5-HT content within mammary epithelial cells. The in vivo results confirm that altering 5-HT turnover at the cellular level affects the function of the glands in the whole organism.

Understanding the physiology of lactation, and especially the factors that inhibit lactation is important in several contexts, including human breastfeeding and dairy production. In addition to the obvious goal of enhancing milk production, it is equally important to shut down the gland quickly at the end of the lactation period. The successful completion of the “dry period” in dairy cows is crucial to maximize milk production in subsequent lactations (45). Currently, it takes about 3 days for the bovine mammary gland to completely dry. During this dry-off period, dairy animals are susceptible to mastitic infections (46). Moreover, women who stop breastfeeding for medical reasons are also at increased risk for mastitis (47, 48). Understanding the physiology surrounding dry-off may provide novel approaches for ameliorating the negative consequences during this fragile period of the lactation cycle in dairy animals and women.

The discovery that the mammary gland synthesizes 5HT did not come about by histochemical evidence, as was common for other organs, but rather by a comparative gene expression approach. Messenger RNA transcripts from mammary glands between prolactin knockout mice (non-secretory) and hyperprolactinemic mice (hypersecretory) were compared using subtraction cloning. TPH1, the rate limiting enzyme in the synthesis of 5-HT was highly induced in the hyperprolactinemic mice (27). Further study, revealed that TPH1 gene expression (and 5-HT synthesis) was induced by fluid stasis. Interestingly, the mammary gland is not the only ductal organ where 5-HT has been shown to be either induced by fluid stasis or cause decreased fluid secretion. This action of 5-HT has been proposed in the developing lung, pancreas, liver, prostate, salivary gland, as well as the mammary gland (27, 49-54).

The 5-HT transporter, SERT, is the target for the most commonly-prescribed class of prescription drugs in the United States and other developed countries, SSRI-type antidepressants (55). Here we have demonstrated that SERT is expressed in mammary tissue, and specifically that SERT is present in the apical membranes of the epithelial cells in the lactating glands.
Pharmacologic antagonism of SERT in vivo affected the morphology of mammary tissue, leading locally to an involution-like state. Prior to the current study, the context of studies on SSRI drug use during pregnancy and lactation focused on: (1) whether SSRI use caused developmental defects in utero(56-58), (2) whether SSRI was passed into the milk during lactation (59, 60), and (3) the safety implications of SSRI in milk (61, 62). Our data are the first to report on another important aspect of SSRI use during the peripartum; that is, potential effects on the functioning of the mammary gland itself, including a possible role in delayed onset of lactogenesis.

The expression of SERT in the apical membranes of mammary epithelial cells may explain the relatively low concentration of 5-HT that was observed in milk (27)(Hernandez, L.L., unpublished). This localization may relate primarily to conservation of 5-HT, since 5-HT secreted into the milk space would be largely wasted (28). However, another implication is that 5-HT may be reabsorbed from milk to limit the exposure of the offspring to milk-borne 5-HT. An interesting avenue of further research would be to explore any changes in free 5-HT levels in milk as a result of SSRI use, and to determine whether there are any deleterious effects of milk-borne 5-HT on infants. At least one report of lower weight gain (average deficit of 392 g) among infants of mothers taking FLX suggests that SSRI use during lactation may either suppress infant appetite or compromise maternal milk supply(63). However this finding was not replicated in a more recent report in which the independent effect of maternal depressive symptomology on infant weight gain was taken into account (64).

Consistent with our in vitro findings, the risk of delayed onset of lactogenesis was 2-fold greater among primiparous women using an SSRI medication, as compared to women in the same cohort not using an SSRI medication. Seven of eight women in the SSRI group experienced delayed onset of lactogenesis, with the remaining woman in the SSRI group experiencing onset of stage II lactogenesis at 72.0 hours, the defined cut-off for delayed onset of lactogenesis. However, all mothers in the SSRI group did eventually experience onset of lactogenesis. Although the sub-sample of women taking an SSRI medication was small, the 2-fold greater risk of delayed onset of lactogenesis was statistically significant and remained significant after adjustment for maternal
age, obesity, delivery mode, or infant gestational age. The proportion of women in the cohort using an SSRI medication (1.9%) was less than that reported for other settings (65). The low incidence of SSRI use may relate to the study cohort (women in Northern California) or be a result of selection bias. For example, it is also possible that women on SSRI medications (potentially with some depressed symptoms) self-selected out of the study population because of “lack of interest”, a feeling that the study was “too intrusive”, or the desire not to “be interviewed about breastfeeding.” The challenge of getting full participation from mothers who might be experiencing some degree of depression is obvious. Nevertheless, further examination with a larger sub-set of women using SSRI medication would be warranted.

Although the consistency between our in vitro and in vivo results suggests a possible causal link between SSRI use and disruptions in secretory activation, there may be other contributing factors. For example, at least one study has reported an increased risk of feeding problems in newborns exposed to SSRI medication in utero (65). Newborns who are not feeding well at the breast may contribute to delayed onset of lactogenesis because of ineffective milk removal. In our study cohort, we did not observe a lower proportion of infants being rated as “sub-optimal” on breastfeeding behavior during the first 24 hours postpartum. Even though there was a significant difference in this measure at day 3, (72 to 96 hours postpartum), this is as likely to have been a consequence of delayed onset of lactogenesis (i.e. frustration or disinterest in suckling by the neonate) as to be a cause of it. There was no significant difference in breastfeeding behavior at day 7, when all of the mothers in the SSRI group had experienced onset of stage II lactogenesis.

In conclusion, we have now demonstrated the importance of the intrinsic mammary gland 5-HT system in lactation physiology. Fluoxetine alone or in combination with PHLZ resulted in changes in barrier function in the mammary epithelium. This mechanism was conserved between primary and immortalized human cells, and bovine mammary epithelial cells. We were also able to observe the intrinsic actions of 5-HT in vivo by implanting slow release pellets containing SSRI in the lactating mammary of mice. Furthermore, primiparous women taking an SSRI medication were more likely to experience delayed onset of lactogenesis, possibly through a set of 5-HT–dependent
mechanisms.

References


closure in the mouse mammary epithelium during the transition from pregnancy to lactation.
*J. Endocrinol.* **170**:347-356.

43. Roberts, R.E., and Vernon, S.W. 1983. The Center for Epidemiologic Studies Depression

rat neocortex. *Brain Res.* **111**:301-309.


47. Betzold, C.M. 2007. An update on the recognition and management of lactational breast


serotonin release from pulmonary neuroendocrine cells: implications for lung development.


51. Suzuki, A., Naruse, S., Kitagawa, M., Ishiguro, H., Yoshikawa, T., Ko, S.B., Yamamoto,
A., Hamada, H., and Hayakawa, T. 2001. 5-Hydroxytryptamine Strongly Inhibits Fluid

52. Marzioni, M., Glaser, S., Francis, H., Marucci, L., Benedetti, A., Alvaro, D., Taffetani,
growth of the biliary tree by the neuroendocrine hormone serotonin. *Gastroenterology*
**128**:121-137.

Peptide-hormone- and serotonin-immunoreactive cells in normal and hyperplastic prostate

mammalian salivary glands for 5-hydroxytryptamine receptors coupled to increased cyclic


CHAPTER V

PERSPECTIVES AND FUTURE DIRECTIONS

Aaron M. Marshall, Nelson D. Horseman

Systems Biology and Physiology Program
Department of Molecular and Cellular Physiology
University of Cincinnati, Cincinnati, Ohio 45267-0576
**Introduction**

Since our laboratory first identified 5-HT as mediator of breast function, significant progress has been made in understanding its role(s). To aid the progress, we developed a novel barrier forming epithelial model using a commonly studied cell line. Here, I speculate on the future use of this model, and potential improvements to it. This dissertation is also the first report to draw direct analogy between the 5-HT system in the breast and other 5-HT producing ductal epithelia, such as the liver, fetal lung, salivary gland, pancreas and prostate (1-4, 4, 5). Here, I will consider the origin of this system and extrapolate the commonalities into one encompassing model. In terms of understanding 5-HT function in the breast, we have gone a step further and demonstrated, through pharmacological studies, unforeseen and as of yet, unidentified contraindications of drugs targeting the serotonin system. Future studies will be needed to sort these out; however here I will hypothesize about these future outcomes.

**MCF10A Transwell® model**

The purpose of the *in vitro* model detailed in Chapter II is to provide a platform for measuring mammary epithelial tight junction regulation by monitoring trans-epithelial electrical resistance. While this in itself is not novel, several characteristics of this model make it the most tractable above all other alternatives. First and foremost, the cell line used in the model is MCF10A cells. They are immortal, untransformed and human in origin. The combination of these three characteristics have led to the most important characteristic of all; they are extremely well studied. MCF10A cells are almost always used in *in vitro* breast cancer studies as a representative of ”normal” cells, since primary breast tissue requires extra effort to acquire. These cells have also been used to validate the mammosphere method (6). Mammospheres are hollow cysts that polarize and are thought to be the best available model of an alveolus or a hollow duct. The problem with mammospheres experimentally is they are difficult to use for anything but visual data, such as immunostatining or conventional light microscopy. In contrast, the transwell model provides separate access to basolateral and apical fluid chambers. Moreover, it has been reported that mammospheres do not form tight junctions (7).
Part of the effort to characterize the MCF10A Transwell model was intended to help determine which segment of the mammary tree this polarized epithelium represents. The arborization of the mammary gland that occurs during puberty, and then again during pregnancy, creates several levels of ducts that can be defined anatomically and functionally. A single human nipple gives rise to a primary duct which then gives rise to 5-10 lactiferous ducts. Each lactiferous duct extends in a radial pattern giving rise to a triangular lobe which has a completely separate duct system from adjacent lobes. The functional unit of the human mammary gland is the terminal ductal lobular unit (TDLU). Within the TDLU, separate epithelial cells can be anatomically defined; those within the extralobular ducts, intralobular ducts, or the acini. The end of the intralobular duct flowers into several acini resulting in a grapelike shaped structure. Each acinus produces and secretes milk into the lumen during lactation. In contrast, the mouse mammary tree branches in a linear fashion, and therefore a terminal duct (analogous to intralobular duct) gives rise to a single alveolus (acinus).

Based on the present information about the MCF10A Transwell model, I hypothesize that the model recapitulates a ductal epithelium (either extralobular or intralobular). This is based on the presence of milk proteins, but lack of prolactin receptor expression., which would be present if the cells modeled an acinus. The transition zone between the extra- and intralobular ducts is thought to contain a high concentration of stem cells that give rise to milk producing acini during menstrual cycling and pregnancy. It is possible and likely that the origin of the MCF10A cell line is stem cell, perhaps from a transition zone.

Acting on that hypothesis, one way I propose to further refine this model is to integrate the prolactin receptor gene into the genome of MCF10A cells under user control (i.e. tetracycline stimulated PRL-R expression). The reason for conditional expression is because PRL-R signaling will lead to differentiation into an acinar epithelial state, which normally do not proliferate. Thus, constitutive expression of PRL-R in these cells would be negatively selected over several passages. The goal here would be to culture the model without PRL-R induction until it has polarized and formed a high resistance barrier. Turning on PRL-R expression and stimulating with prolactin subsequently, should provide a milk producing and milk secreting in vitro epithelial cell model, of
which there is none available.

**Evolutionary conservation of 5-HT feedback system**

Bioactive 5-HT is found within virtually every multicellular animal and many plants as well. Because of the ancient “evolutionary age” of 5-HT it is not surprising that in humans it has several primary roles, and a subsidiary role in many more. Given this, I hypothesize that the mammary gland has inherited the 5-HT feedback system as a way to detect intraluminal fluid status. Indeed, some of the early studies of 5-HT on the salivary glands were performed in the blowfly (8). In addition, 5-HT has several functions in the skin glands of amphibians (9). The connection here, is that the mammary gland has evolved from skin glands of lower vertebrates.

The studies outlined in Chapter I with respect to 5-HT function in other ductal organ epithelia, were presented with different interpretations of the results. Definite commonalities between all the organs were the capacity to synthesize 5-HT in a subset of epithelial cells, secretion of 5-HT in a regulated way, and presence of 5-HT receptors on the basolateral surface. My overall hypothesis for all of these organs is that 5-HT is the chemical messenger responding to stretch initiated by fluid stasis causing increases in intraluminal pressure. I hypothesize that the resulting action of 5-HT signaling will coincide with ultimate goal of the organ. For example, in the fetal lung, stretch that is initiated by fluid and breathing results in 5-HT signaling that is crucial for clearance of lung fluid; since the ultimate goal of this epithelium is to have an interface with the atmosphere.

The mammary gland, however, needs to sense the fullness of each functional unit in order to transiently prevent leakage of milk components into the interstitium. The prevention of leakage is not to combat a potential pathology, as is the case with pancreas or liver, but rather to extend the time between nursing bouts between a mother and an infant. The evolutionary need to extend lactation without constant milk letdown is obvious. Infants do not nurse constantly. Interestingly, one mammal, the fur seal, is able to maintain lactation away from their pups for weeks, as the mother travels from shore (to nurse) to the sea (to forage) (10). It would be very interesting to investigate 5-HT function in the mammary glands of fur seals, looking for that prevent involution
from occurring.

**Serotonin’s role in the mammary involution process**

In humans and rodents however, the eventual leakage of milk components facilitates the process of involution (11). Involution is extremely important to the mother since lactation is metabolically taxing; often resulting in decreased bone density and weight loss (12). Therefore, following fluid stasis, the mammary gland wants to sustain lactation, contain the milk intraluminally for a short period of time (2-4 days), and then proceed with an ordered destruction of milk producing epithelium. The biphasic nature of the serotonin system seems to fit the bill for allowing both processes to occur. In the early transient phase tight junctions are enhanced in a coordinated effort to prevent leakage. Upon sustained 5-HT signaling, the tight junction are compromised and involution can proceed. Although the signaling pathways for each of these events has been identified: the former a cAMP dependent PKA pathway, and the latter a cAMP dependent p38MAPK pathway, it is not known whether the second delayed phase is a direct result of 5-HT signaling or indirect through another factor. Hinting at an indirect mechanism, we observed as part of our microarray study 5-HT stimulation of interleukin 1. Interleukin 1 does signal through p38MAPK, and the data reported in Chapter III, demonstrate that it can cause a crash in barrier function (13). Further studies where 5-HT is administered in IL-1 signaling is blocked in order to verify that IL-1 is responsible for 5-HT stimulated barrier disruption.

**Serotonylation of milk proteins**

Recently it was uncovered{{}} that serotonin and other monoamines are utilized in some tissues as covalent modifications onto peptides, often altering their function (14). This process is referred to as serotonylation. In Chapter III, I report the results of an attempt to develop a system for testing activity of serotonylated proteins. The motivation for this set of experiments was circumstantial evidence from published reports that indicated the negative feedback to lactation molecule was present in the milk space (15). We knew the serotonin receptor mediating feedback in the mammary was on the basolateral surface, and therefore any lumenal factor would not have access to basolateral receptors under high resistance barrier conditions (16). Therefore it seemed
unlikely that the negative feedback inhibitor (5-HT) to lactation was stored in the lumen and its receptor was basolaterally located. Nevertheless, I hypothesized a mechanism whereby serotonin was covalently attached to a milk protein.

Preliminary unpublished evidence from our lab, indicated that serotonylated proteins do exist in the mammary gland, however their identity and function are unknown. Small GTPase proteins in platelets are serotonylated and this causes a constitutive activation leading to secretion of clotting factors (14). Here, I would hypothesize that upon leakage of milk components (caused by stretch), this serotonylated protein could act as receptor ligand itself, or through a protease, the 5-HT-peptide bond is hydrolyzed, releasing free 5-HT. Indeed the presence of many proteases is necessary to facilitate involution (17), and a similar mechanism has been proposed for histamine in mast cells (18). Future studies are needed to purify and identify serotonylated proteins in the mammary gland, after which their function can be investigated.

**Lactation timing and performance effects of serotonergic drugs**

The serotonin system is comprised of the synthetic proteins (TPH and AADC), the regulatory/secretory proteins (VMAT and SERT), and the degradative protein, MAO (Fig 4). There are many drugs (natural and synthetic) that target one of these proteins. Most of them act to increase the availability or activity or 5-HT. St John’s wort (*Hypericum perforatum*), also known as Klamath weed, is known as an herbal antidepressant because of its actions to increase availability of central nervous system’s serotonin system (19). Moreover, there are many pharmaceutical drugs on the market targeting members of the serotonin system, that increase serotonin activity. Overall the potential for serotonin physiological functions to be altered is high.

Two alterations detailed in Chapter IV relate to the timing of the onset of stage II lactogenesis, and the timing of involution. I will discuss the former first. The onset of stage II lactogenesis is hallmarked by the copious milk production and is initiated by tight junction closure. To most accurately measure this, sodium concentration in the milk can be monitored. The colostrum, or first milk, contains a high concentration of sodium, similar to serum, in addition to other differences. As the tight junctions close, the sodium levels decline in milk. I speculate that late in pregnancy
and early post partum, when the mammary gland is filled with colostrum, the serotonin system is actively providing the feedback to the fullness of the gland. The removal of colostrum by suckling would then normally reduce 5-HT activity, thereby allowing the tight junctions to close. However, if a pharmaceutical (such as an SSRI) is present that increases the extracellular availability of 5-HT, then the tight junction closure is delayed. This delay in closure is what I believe is causing the delay in the onset of stage II lactogenesis.

The study reported in Chapter IV, examines women who were taking an SSRI medication during the third trimester of pregnancy. The sample size was low (n=8, 2% of total), presumably because most women are advised to stop taking medication during pregnancy. Future studies could be conducted to answer additional questions regarding 5-HT function during the peripartum. For example, many women experience some degree of post partum depression, and some women will receive an SSRI. There are two questions to examine in this case; (1) does SSRI affect overall lactation performance in terms of quantity and quality of milk and (2) are women taking an SSRI experiencing dysregulation of involution in some way. And if so, is that dysregulation contributing to tumorigenesis. It has been reported that partial involution (measured as number acini per TDLU) increases a women’s risk of breast cancer, while complete involution reduces the risk (20). To assess lactation performance, sodium concentration in milk or other involution markers can be noninvasively assessed.

So far, contraindications of serotonergic drugs have been discussed and investigated. Conversely, there are opportunities to utilize these same drugs at the appropriate time to affect a more desired outcome. Since SSRI drugs are able to cause precocious involution (see Chapter IV), they could be used to aid in complete involution. Such a therapy may reduce a women’s future risk of breast cancer, as has been reported (20). In addition to prevention of future disease, rapid involution is important for women who are HIV positive. It is thought that gradual weaning of infants, (as is normally done), increases the risk of HIV transmission to the infant, since the epithelial barrier is being breached. Therefore, HIV positive women who breastfeed, abruptly wean at 6 months; and subsequently experience discomfort and a high risk of ductal occlusion.
leading to mastitis. Serotonergic drugs could be used to aid in rapid involution in women who are HIV positive. In dairy animals the involution process (aka dry period) is known to affect future lactation cycles (21). Moreover, the risk of mastitic infection greatly increases during the dry period, and costs the industry hundreds of millions each year in lost production (22). Therefore, serotonergic drugs may be the future of dry off agents, causing more rapid involution and reducing the risk of infection.

References


