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# Dietary Protein Influences Paracellular Calcium Transport: Two Molecular Targets

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Dietary Protein Influences Paracellular Calcium Transport:  
Two Molecular Targets

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Dietary Protein Influences Paracellular Calcium Transport:  
Two Molecular Targets

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## Abstract

The effect of dietary protein on bone health remains controversial. We have shown that increasing dietary protein increases calcium absorption in both humans and rats with no change in bone resorption. We used a rat model in which dietary protein increases intestinal calcium absorption and whole body calcium retention, to explore the molecular basis for this effect. Female Sprague-Dawley rats were fed a low (5%) or high (40%) protein diet for 7 days. On day 7, duodenal mucosa was harvested and total RNA isolated. Microarray analyses using these RNA samples identified 208 genes whose expression was at least 1.5 fold different between groups. Of particular relevance, the calcium permissive tight junction protein, claudin-2 (Cldn-2), was found to be upregulated 2.9-fold by the high protein diet and a posited calcium intolerant tight junction protein, kallikrein-related peptidase-10 (KLK-10), was found to be downregulated 5.5-fold by the high protein diet. These results were confirmed at the mRNA level by qPCR and at the protein level by western blot analysis. To examine cellular expression of Cldn-2 and KLK-10, Caco-2 Bbe cells (14 days post-confluent) were incubated with control media (CM) or CM + 2X amino acids for 6 hrs, or 1,25(OH)<sub>2</sub>vitamin D for 24 hrs. Both Cldn-2 and KLK-10 were found to be expressed in the cytoplasm, as well as at the cell periphery, the latter consistent with tight junction expression. In at least some experiments amino acid and 1,25(OH)<sub>2</sub>vitamin D treatment increased Cldn-2 tight junction expression and its apparent colocalization with ZO-1, a known tight junction membrane protein. In preliminary experiments, amino acid treatment decreased KLK-10 tight junction expression and its apparent colocalization with ZO-1. These data suggest

that amino acid-induced changes in the expression and/or subcellular distribution of Cldn-2 and KLK-10 may be part of the molecular mechanism by which dietary protein increases paracellular calcium flux.

## Introduction

Osteoporosis affects an estimated 10 million people in the United States<sup>1</sup>. Nutrition's role in the prevention and treatment of bone loss is of increasing interest. The effect of dietary protein on intestinal calcium absorption remains a controversial area of research. However, a large number of cross-sectional, as well as longitudinal observational studies have found that high protein diets are associated with a higher bone mineral density (BMD) and slower rates of bone loss<sup>2-10</sup>. Increasing dietary protein is known to increase urinary calcium (UCa) excretion, which led to the hypothesis that the increased UCa was due to loss of skeletal calcium<sup>11, 12</sup>. Another possible source for this additional UCa observed during a high protein diet is increased intestinal calcium absorption. We have previously shown that the increased UCa excretion, which accompanies increasing dietary protein, can be quantitatively explained by an increase in calcium absorption in both humans and rats without increasing bone resorption<sup>13, 14</sup>.

Dietary calcium is absorbed by both transcellular and paracellular mechanisms. Transcellular mechanisms usually involve specific cell surface transporters or acceptor molecules that allow for selective uptake of molecules that are transported across the cell; this transport is often achieved with the assistance of carrier proteins that are either utilized by the intestinal epithelia or exported through the basolateral membrane to the systemic circulation. Transcellular calcium absorption occurs primarily in the duodenum and is the sum of three steps: apical calcium entry via TRPV5 and TRPV6, shuttling of cytosolic calcium to the basolateral membrane by calbindin, and extrusion through the basolateral

membrane via the plasma membrane Ca ATPase or the Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger<sup>15</sup>. In contrast, paracellular transport involves the movement of molecules, usually across a concentration gradient, between cells and relies for its selectivity on the specific composition of the proteins that form intercellular junctions. Passive calcium absorption occurs along the length of the small intestine and transport takes place across the tight junctions into the circulation; driven by the electrochemical gradient for calcium<sup>15</sup>. Tight junctions are composed of membrane spanning proteins, whose components traverse the paracellular space, and scaffolding proteins, that link membrane proteins directly to the actin cytoskeletal network. Membrane spanning proteins, including claudin, occludin, junction adhesion molecule (JAM), and scaffolding proteins, including the zona-occludens, collectively regulate the permeability of tight junctions<sup>16</sup>. Along with the accepted notion that the transcellular absorptive pathway is regulated, recent data suggest that the protein composition of the tight junction is also dynamically regulated<sup>17</sup>.

Claudins are essential for barrier function by virtue of their critical role in regulating selectively permeable ion channels in tight junctions. The family of claudin proteins currently consists of 24 members. Claudins can be classified as either pore forming or pore sealing. Pore forming claudins increase paracellular permeability through the formation of channels, whereas pore sealing claudins reduce paracellular permeability<sup>18</sup>. A wide variety of incompletely understood factors determine whether claudins enhance or reduce paracellular permeability. Several claudins have been reported to have functional roles in the intestine. Claudin-1, -3, -4, -5, and -8 function as pore sealing proteins whereas claudin-2



(Cldn-2) forms charge selective pores, for example, facilitating the paracellular transport of calcium<sup>19, 20</sup>. In particular, with regard to Cldn-2, Fujita et al. demonstrated that 1,25(OH)<sub>2</sub>vitamin D induced the expression of Cldn-2 in Caco-2 cells and this was accompanied by a concomitant increase in paracellular calcium transport. Suppressing Cldn-2 expression using anti-sense technology inhibited paracellular calcium transport<sup>19</sup>. Collectively, these data establish that Cldn-2 is a regulatable mediator of paracellular calcium transport. Claudin-12 has also been reported to increase paracellular calcium transport in enterocytes<sup>19</sup>.

In addition to claudins, recent studies indicate that tissue kallikrein may also be a physiologic regulator of calcium transport<sup>21</sup>. The family of kallikrein-related peptidases currently consists of 15 members. Kallikrein-related peptidases are enzymes with highly conserved trypsin- or chymotrypsin-like serine proteases. The general term kallikrein is used to describe an enzyme that releases kinins (bioactive peptides) from precursor molecules (kininogens). However, among the 15 known human kallikrein-related peptidases, only tissue kallikrein (KLK-1) has this ability to release a bioactive kinin from kininogen. In fact, the majority of kallikrein-related peptidases enzymatic activity has not yet been determined<sup>22, 23</sup>. A role for KLK-1 in mediating renal tubular calcium reabsorption was recently identified by exploring the phenotype of the KLK-1 knockout mouse. These animals demonstrated hypercalciuria due to impaired tubular calcium reclamation. Calcium transport was determined to act through a non-kinin-mediated mechanism<sup>21</sup>. However in a following study, it was suggested that KLK-1 stimulates calcium reabsorption via the bradykinin-activated PLC/DAG/PKC pathway and the subsequent stabilization of

the TRPV5 channel at the plasma membrane<sup>24</sup>. This raises the possibility that kallikrein-related peptidases may have novel, perhaps regulatory, actions in the tight junction that can affect calcium flux. Kallikrein-related peptidases-1, -10, -11, -12, and -14 are all expressed in the small intestine<sup>23</sup>, making this a tenable notion.

To evaluate the mechanism for protein-induced intestinal calcium absorption we established an animal model in which female Sprague-Dawley rats were fed a low (5%) or high (40%) protein diet<sup>25</sup>. By qPCR, we determined that the expression of the known calcium transporters TRPV5 and TRPV6 were not altered by this dietary intervention. Therefore, to look for novel calcium transporters, we undertook a whole genome microarray screen using RNA isolated from duodenal enterocytes of our animal model. Microarray analyses identified 208 genes whose expression was at least 1.5 fold different between groups. Of particular relevance, the calcium permissive tight junction protein, Cldn-2, was found to be upregulated 2.9-fold by the high protein diet and the posited calcium intolerant tight junction protein, kallikrein-related peptidase-10 (KLK-10), was found to be downregulated 5.5-fold by the high protein diet.

The primary purpose of the current study was to determine, in vitro, if amino acids directly effect Cldn-2 and KLK-10 expression, and in turn regulate intestinal calcium absorption.

## Methods

### *Animals and Diets*

For the microarray study, 18 adult female Sprague Dawley rats weighing 250-300 g were purchased from Charles River Laboratories, Inc (Wilmington, MA). Animals were housed in the Yale Animal Resource Center and cared for in accordance with institutional animal care and use policies. All experiments were approved by the Yale Institutional Animal Care and Use Committee. Upon arrival, all rats were placed on a standard diet (Harland Teklad, Inc., Madison, WI - #2018) for a minimum of two weeks to allow for acclimation. Experimental diets were also obtained from Harlan Teklad Inc. Rats were randomly assigned to receive either 5% casein protein (low, n=9) or 40% casein protein (high, n=9) *ad libitum* for 1 week with free access to tap water. These diets were used to develop our rat model as previously described<sup>25</sup>.

### *Solutions and Chemicals*

High-glucose DMEM, L-glutamine, D-glucose,  $\text{NaH}_2\text{PO}_4$  were purchased by Sigma (St. Louis, MO). Qualified FBS, L-glutamine, non-essential amino acids, essential amino acids and penicillin/streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA). KCl,  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{HPO}_4$ ,  $\text{MgCl}_2$ , glycine and NaCl were purchased from Fisher Scientific (Suwanee, GA). Sodium borate, sodium borohydride, PIPES, 16% paraformaldehyde, BSA, PBS, nocodazole, phorbol myristate acetate, and forskolin were purchased from Sigma (St. Louis, MO).

### *Isolation of Duodenal Total RNA and Microarray Analysis*

Total RNA was isolated from the duodenum of rats habituated to the 5% (n=9) or 40% (n=9) casein protein diet for one week. Rats were anesthetized, the abdominal cavity opened and the first 10 cm of the small intestine distal to the pyloric sphincter was removed. The intestine was placed on a plastic tray on ice and mucosal tissue was removed by scraping. The mucosa was snap frozen in liquid N and stored at  $-80^{\circ}\text{C}$  until analysis. For total RNA extraction, mucosa was ground in liquid N using a mortar and pestle and extracted with TRIzol (Invitrogen, Carlsbad, CA) followed by purification using the RNeasy Mini Kit (Qiagen, Valencia, CA). The quality of the RNA isolated from individual mucosa was assessed by microcapillary gel electrophoresis and determined to be suitable for microarray analysis (28S:18S = 2:1, OD 260/280 > 1.8, OD 260/230 > 1.8). Four micrograms of RNA from three rats in each group were pooled together such that there were three pooled RNA samples from each experimental diet for microarray analysis resulting in three microarray chips for each group. The pooled RNA samples were reversed transcribed to cDNA and hybridized to the Affymetrix GeneChip Rat Expression Set 230 2.0 per company protocol. We imported raw signal intensity values into GeneSpring® X (Agilent Technologies, Santa Clara, CA) and applied RMA normalization. The normalized signals were then filtered by probesets with a signal >100 in at least one sample per condition and then analyzed by student's t-test ( $p < 0.05$ ). The resulting list of significantly regulated genes was filtered by fold change of at least 1.5.

### *Quantitative Real-Time PCR*

Aliquots of the same RNA used for the microarray analysis were used for qPCR confirmation of Cldn-2. RNA was isolated from a separate set of animals adjusted to a 5% or 40% protein diet for qPCR confirmation of KLK-10. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), and the RT reaction was performed on MyCycler thermal cycler (Bio-Rad, Hercules, CA) using the following protocol: 25°C x 5 min, 42°C x 30 min, 85°C x 5 min, 4° hold. QPCR was performed using TaqMan® Gene Expression Assays for the following targets: Cldn-2 (Rn02063575\_s1) and KLK-10 (Rn01475770\_g1) (Applied Biosystems, Foster City, CA) and QRT-PCR Master Mix (Stratagene, La Jolla, CA). As an endogenous reference,  $\beta$ -actin was used. (Rn00667869\_m1, Applied Biosystems, Foster City, CA).

Caco-2 Bbe cells were kindly provided by Dr. Mark Mooseker (Dept of Molecular, Cellular & Developmental Biology, Yale University). Caco-2 Bbe cells were grown in T-75 flasks and routinely passaged using Trypsin/0.5% EDTA when 80-90% confluent. Cells were cultured in growth media containing high glucose DMEM, 10% qualified FBS, 1% L-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin and media was changed every 2-3 days. Caco-2 Bbe cells were seeded into 6-well plates at a density of  $1.0 \times 10^6$  cells / well and studied 18-22 days post confluent. To examine the direct effect of amino acids on the expression of genes identified in the microarray screen as differentially regulated by protein, cells were incubated with control media (CM) or CM + 2X amino acids for 6 hours, or CM + 1,25(OH)<sub>2</sub>vitamin D (100 nM) for 24 hours. Control media contains only the

minimum amount of substrates to maintain cell viability. 2X amino acids represents twice the amount of amino acids found in DMEM (the normal growth media for Caco-2 Bbe cells). Total RNA was isolated from Caco-2 Bbe cells using TRIzol reagent and the Qiagen RNeasy kit. TaqMan® Gene Expression Assays used were: Cldn-2 (Hs01549234\_m1), KLK-10 (Hs00173611\_m1) and GAPDH (Hs99999905\_m1) was used as an endogenous reference.

The PCR reactions were run on an iCycler iQ5 PCR Thermal Cycler (Bio-Rad, Hercules, CA) using the following protocol: 95°C x 10 min, followed by 40 cycles at 95°C x 20 sec and 60°C x 1 min. Relative quantification of target genes was calculated using the comparative C<sub>T</sub> method.

#### *Western Blot Analysis*

Cell lysates were prepared from Caco-2 Bbe cells seeded into 10cm dishes at a density of  $1.0 \times 10^6$  cells / well and grown 21 days post confluent. The cells were treated with ice-cold RIPA buffer (1% Triton-X 100, 1% Na deoxycholate, 0.1% SDS, 158 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.2) containing protease inhibitors. Cells were scraped and then homogenized with an 18G needle followed by a 22G needle, and incubated on ice for 15 min. Following homogenization, samples were centrifuged at 10,000 RPM for 10 min at 4° C. Supernatant was frozen at -20° C until analysis. Total protein concentration was determined using a BCATM protein assay and the manufacturer's recommended protocol (Thermo Scientific, Rockford, IL). Subsequently, 50 µg of duodenal mucosal protein in sample buffer was heated at 100° C for 5 min followed by separation on a 10% SDS-polyacrylamide gel. Proteins

were then transferred to nitrocellulose and the membrane was cut horizontally across 37 kDa. The membrane below 37 kDa was incubated at 4° C overnight with either rabbit anti-human Cldn-2 (1:330) (Invitrogen, Carlsbad, CA) or rabbit anti-human KLK10 (1:500) (Bioss, Woburn, MA) in TBST with 5% nonfat milk. The membrane above 37 kDa was incubated at 4° C overnight with mouse anti-human  $\beta$ -actin (1:2,000) (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST with 5% nonfat milk for an endogenous control. After washing both sections of the membrane with 0.1% TBST, the lower membrane was incubated with an HRP-conjugated anti-rabbit antibody (1:10,000) and the upper membrane was incubated with an HRP-conjugated anti-mouse antibody (1:10,000) for 60 min. After washing with 0.1% TBST, the blots were developed using ECL chemiluminescence.

#### *Immunostaining and Confocal Imaging*

Caco-2 Bbe cells were seeded at a density of  $4.6 \times 10^5$  cells / cm<sup>2</sup> into 6-well transwell plates (24mm diameter, 0.4  $\mu$ m pore size, Corning Inc., Corning, NY). Post-confluent monolayers were fixed using a pH shift method <sup>26</sup>. Specifically, media was aspirated and cell monolayers were rinsed in 37°C PIPES (pH 6.5) followed by a 5 min incubation at room temperature in 3% paraformaldehyde PIPES. The paraformaldehyde solution was aspirated and cells were incubated with 3% paraformaldehyde sodium borate for 10 min at room temperature. The paraformaldehyde was again aspirated and cells were rinsed three times in room temperature PBS (pH 8.0) followed by two successive 15 min incubations at room temperature with 0.1% sodium borohydride in PBS (pH 8.0). The fixed monolayers

were washed three times in room temperature PBS (pH 8.0) and permeabilized with 0.1% Triton in PBS (pH 8.0) at room temperature for 10 min with gentle agitation. The monolayers were washed with 1% BSA in PBS (pH 8.0) and blocked in 5% BSA in PBS (pH 8.0) for 30 min at room temperature. For each of the above fixation and wash steps, 2 ml of solution was added to the apical chamber and 3 ml to the basolateral chamber.

After fixation and washing, monolayers were incubated with rabbit polyclonal anti-Cldn-2 (2 ug/ml) (Invitrogen, Camarillo, CA) for Cldn-2 localization experiments or with rabbit polyclonal anti-KLK-10 (1:750) (Bioss, Woburn, MA) for KLK-10 localization experiments at 4°C overnight in 1% BSA. Monolayers were then washed two times with 1% BSA and three times with PBS (pH 8.0) after each incubation with an antibody. Cells were then incubated with the secondary antibody, anti-rabbit RED (1:500) (Invitrogen, Camarillo, CA) in 1% BSA at room temperature for 30 min followed by mouse monoclonal anti-ZO-1 (1:1500) (Invitrogen, Camarillo, CA) for 40 min and finally anti-mouse GREEN (1:1000) (Invitrogen, Camarillo, CA) for 30 min. After staining, adherent transwell filters were cut out of the transwell and mounted onto standard coverslides using Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized 24 - 72 hours later using a Zeiss LSM510 Meta scanning laser confocal microscope. Laser settings were kept consistent at 21% green laser output and 31% red laser output.



### *Microtubule Inhibition and PKC/PKA Stimulation*

Caco-2 Bbe cells were seeded at a density of  $4.6 \times 10^5$  cells / cm<sup>2</sup> into 6-well transwell plates (24mm diameter, 0.4 um pore size, Corning Inc., Corning, NY) and grown 14 days post confluent. For microtubule inhibition cells were treated with CM + nocodazole (10 µM) (Sigma, St. Louis, MO) for 60 min. For protein kinase C (PKC) stimulation cells were treated with CM + phorbol myristate acetate (PMA) (200 nM) (Sigma, St. Louis, MO) for 6 hours. For protein kinase A (PKA) stimulation cells were treated with CM + forskolin (10 µM) (Sigma, St. Louis, MO) for 6 hours.

### *Statistical Analysis*

Statistical analyses were performed using GraphPad Prism© version 5.0c (GraphPad Software Inc., La Jolla, CA). A Student's T-test was used to analyze experiments conducted on two groups. For all statistical analyses, differences with a *p* value <0.05 were considered significant.

## **Results**

### *Genes Differentially Regulated by Dietary Protein*

A total of 208 genes were found to be differentially expressed in the 40% casein group versus the 5% casein group. Of these differentially expressed genes, 63 were upregulated in the 40% casein group versus the 5% casein group and 145 were downregulated. None of the proteins known to be involved in transcellular calcium transport, in particular TRPV5 nor TRPV6 were found to be differentially

regulated by altering dietary protein. However, two genes, *Cldn-2* and *KLK-10* were differentially expressed and have been previously reported to be involved in epithelial calcium transport<sup>19-21</sup>. In the high protein group, *Cldn-2* was upregulated 2.9-fold vis á vis the low protein diet while *KLK-10* was downregulated 5.5-fold (Table 1). As noted above, both *Cldn-2* and *KLK-10* have posited roles in epithelial calcium transport. Therefore, these proteins were the focus of subsequent studies.

#### *Cldn-2 and KLK-10 mRNA Expression*

Microarray results were confirmed by qPCR analysis. *Cldn-2* template cDNA was generated from the same RNA samples used for gene profiling and *KLK-10* template cDNA was generated from RNA isolated from a separate set of animals adjusted to a 5% or 40% protein diet. As summarized in Figure 1, *Cldn-2* was upregulated 2.5 fold and *KLK-10* was downregulated 3.1 fold in the animals adjusted to the high protein diet compared to the low protein diet. These changes mirror those observed in the microarray screen.

Quantitative PCR analysis was also performed using cDNA prepared from RNA isolated from 21-day post confluent Caco-2 Bbe cells incubated with CM or CM + 2X amino acids for 6 hours, or 1,25(OH)<sub>2</sub>vitamin D for 24 hours. Consistent with previous reports by others<sup>19</sup>, 1,25(OH)<sub>2</sub>vitamin D increased mRNA expression of *Cldn-2* in Caco-2 Bbe cells (Fig.2). Incubating Caco-2 Bbe cells with 2X amino acids resulted in a 48% increase in *Cldn-2* expression (Fig. 2). The mRNA expression of alkaline phosphatase was not increased in Caco-2 Bbe cells exposed to amino acids (data not shown). The observation that alkaline phosphatase expression was not

increased in Caco-2 Bbe cells incubated with amino acids suggests a specific effect of amino acids on Cldn-2 expression rather than a global increase in gene expression due to increases in energy substrates.

Incubating Caco-2 Bbe cells with 1,25(OH)<sub>2</sub>vitamin D increased mRNA expression of KLK-10 via unknown molecular pathways. Incubating Caco-2 Bbe cells with 2X amino acids resulted in an undetectable level of KLK-10 expression (Fig. 3). The mRNA expression of GAPDH was not decreased in Caco-2 Bbe cells exposed to amino acids (data not shown). The observation that GAPDH expression was not suppressed in Caco-2 Bbe cells incubated with amino acids suggests a specific effect of amino acids on KLK-10 expression rather than a more generalized change in gene expression resulting from altered nutrient exposure.

#### *Cldn-2 and KLK-10 Protein Expression*

To determine whether increased Cldn-2 mRNA expression and decreased KLK-10 mRNA expression in response to a high protein diet translates into enhanced/suppressed protein expression, we performed Western blot analysis on cell lysates prepared from 21-day post confluent Caco-2 Bbe cells incubated with CM or CM + 2X amino acids for 6 hours, or 1,25(OH)<sub>2</sub>vitamin D for 24 hours. As shown in Figure 4, Cldn-2 protein expression was increased 1.8 fold incubated with 2X amino acids and 2.2 fold incubated with 1,25(OH)<sub>2</sub>vitamin D compared to control. As shown in Figure 5, KLK-10 protein expression was decreased by 30% in the cells incubated with 2X amino acids and increased 1.3 fold incubated with

1,25(OH)<sub>2</sub>vitamin D compared to control. Thus, the changes we observed in levels of protein expression paralleled the changes in transcript expression quantified by qPCR.

#### *Cldn-2 and KLK-10 Cellular Localization*

We next explored whether incubating Caco-2 Bbe cells with 1,25(OH)<sub>2</sub>vitamin D or amino acids increased the tight junction expression of Cldn-2. Post confluent monolayers exposed to CM or CM + 2X amino acids for 6 hours, or 1,25(OH)<sub>2</sub>vitamin D for 24 hours were fixed and co-immunostained with mouse monoclonal anti-ZO-1 and rabbit polyclonal anti-Cldn-2. As shown in Figure 6, 1,25(OH)<sub>2</sub>vitamin D and amino acids caused an increase in tight junction expression of Cldn-2 compared to control treated cells.

These experiments were repeated to determine if incubating Caco-2 Bbe cells with 1,25(OH)<sub>2</sub>vitamin D increased tight junction expression of KLK-10 and/or if incubating Caco-2 Bbe cells with amino acids decreased the tight junction expression of KLK-10. Post confluent monolayers exposed to CM or CM + 2X amino acids for 6 hours, or 1,25(OH)<sub>2</sub>vitamin D for 24 hours were fixed and co-immunostained with mouse monoclonal anti-ZO-1 and rabbit polyclonal anti-KLK-10. As shown in Figure 7, 1,25(OH)<sub>2</sub>vitamin D caused an increase in tight junction expression and amino acids caused a decrease in tight junction expression of KLK-10 compared to control. Changes in tight junction expression of Cldn-2 and KLK-10 mirror the changes in levels of mRNA expression as well as the changes in levels of protein expression observed by western blot analysis.

## *The Role of Microtubules and the Cell Signaling Events that Regulate Intracellular Trafficking of Cldn-2*

Amino acids appear to both increase Cldn-2 gene expression and enhance translocation of the protein to the cell periphery, presumably the tight junction. Translocation in general is a microtubular dependent process<sup>27, 28</sup>. We therefore evaluated the role of microtubules in Cldn-2 translocation. Cellular localization experiments were conducted in the presence and absence of amino acids and the presence and absence of a pretreatment with the microtubule inhibitor, nocodazole, for 60 min. Nocodazole appeared to reduce the observed tight junction expression of Cldn-2 (data not shown). This suggests that microtubules, in part, may assist in the translocation of Cldn-2 to the tight junction after amino acid treatment.

Although microtubules may be involved in the translocation of Cldn-2, this does not reveal the signaling pathway responsible for regulating Cldn-2 expression and cellular distribution in response to dietary protein. Interestingly, it has been shown that PKC and PKA phosphorylation affect the subcellular localization of claudin-1 in melanoma cells<sup>29</sup>. In HT29 cells, claudin-1, -3, -4 and -5 and possibly Cldn-2 were redistributed to apical cell-cell contacts after PKC activation<sup>30</sup>. We therefore hypothesized that PKC and PKA activation may play a role in the subcellular localization of Cldn-2 in Caco-2 Bbe cells. Cellular localization experiments were repeated treating Caco-2 Bbe cells with CM, CM + 2X AA, CM + phorbol myristate acetate (PMA) (200 nM), CM + forskolin (10  $\mu$ M) for 6 hours. PMA is known to activate PKC and forskolin is known to activate PKA. However, preliminary data do not suggest an observed increase in tight junction expression of

Cldn-2 after PMA or forskolin treatment (data not shown). These data corroborate the report by Banan et al., that found significant correlations between PKC-activity and enhanced monolayer claudin-1 and claudin-4 integrity/stability in Caco-2 cells, but not Cldn-2<sup>31</sup>. This suggests a different signaling cascade may be responsible for regulating Cldn-2 expression and cellular distribution. Other possibilities are currently being explored.

## **Discussion**

An acute increase in dietary protein intake has been shown to augment intestinal calcium absorption in both humans<sup>32</sup> and rats<sup>25</sup>. We found that in rats, acute changes in dietary protein also affected the expression of calcium-related intestinal genes. Claudin-2 was identified as a structural protein upregulated in the high protein group. Kallikrein related-peptidase 10 was downregulated in the high protein group. Comparing Cldn-2 expression in the duodenal mucosa from rats habituated to the 5%, and 40% protein diets, revealed an increase in Cldn-2 with increasing dietary protein (Fig. 1). Comparing KLK-10 expression in the duodenal mucosa from rats habituated to the 5%, and 40% protein diets, revealed a decrease in KLK-10 with increasing dietary protein (Fig. 1). Since Cldn-2 is known to form calcium selective pores in the tight junction<sup>19</sup> and because KLK-1 knockout mice demonstrated hypercalciuria due to impaired tubular calcium reclamation<sup>21</sup>, we hypothesized that claudins and kallikrein related-peptidases may play a role in intestinal calcium regulation. We focused on Cldn-2 and KLK-10 as potential mediators of increased calcium absorption during a high protein diet.

Claudin-2 mRNA and protein was upregulated in Caco-2 Bbe cells exposed to 2X amino acids for 6 hours (Fig. 2 & 4) and increased tight junction expression of Cldn-2 was also observed in Caco-2 Bbe cells exposed to amino acids (Fig. 6). Kallikrein related-peptidase 10 mRNA and protein was undetectable or downregulated in Caco-2 Bbe cells exposed to 2X amino acids for 6 hours (Fig. 3 & 5) and decreased tight junction expression of KLK-10 was also observed in Caco-2 Bbe cells exposed to amino acids (Fig. 7). Taken together, these results support a direct effect of amino acids on paracellular calcium absorption via increased Cldn-2 expression and possibly decreased KLK-10 expression. We do not yet have direct evidence that KLK-10 is involved in paracellular calcium transport. While published data on the related protein KLK-1 suggest that this protein modulates transcellular calcium transport via enzymatic modification of TRPV5, no such data regarding mechanisms which KLK-10 may regulate calcium transport have been published.

Claudin-2 mRNA and protein was also upregulated in Caco-2 Bbe cells exposed to 1,25(OH)<sub>2</sub>vitamin D for 24 hours (Fig. 2 & 4) and increased tight junction expression of Cldn-2 was also observed in Caco-2 Bbe cells exposed to 1,25(OH)<sub>2</sub>vitamin D (Fig. 6). Our data confirm previously published data reporting that expression of Cldn-2 is upregulated in intestinal epithelial cells by 1,25(OH)<sub>2</sub>vitamin D<sup>19</sup>. Further studies will be required to determine whether the induction of Cldn-2 by amino acids and 1,25(OH)<sub>2</sub>vitamin D occur via the same or different intercellular pathways. Along with Cldn-2, KLK-10 mRNA and protein was upregulated in Caco-2 Bbe cells exposed to 1,25(OH)<sub>2</sub>vitamin D for 24 hours (Fig. 3 & 5). This corroborates with a previous microarray screen that identified KLK-5-8,

KLK-10, and KLK-13 as vitamin D-responsive genes in KerTr and NHEK cells<sup>33</sup>.

Because KLK-10 expression is induced by 1,25(OH)<sub>2</sub>vitamin D and suppressed by amino acids, this suggests that amino acids and 1,25(OH)<sub>2</sub>vitamin D act on KLK-10 through separate signaling pathways. We are currently exploring the signaling transduction pathway(s) by which amino acids suppress KLK-10 expression.

Although our data suggest that amino acids suppress KLK-10 expression, the experiments performed in the Caco-2 Bbe cells have some limitations. First, the endogenous expression of KLK-10 in Caco-2 Bbe cells is very low. Our data show that in Caco-2 Bbe cells, KLK-10 mRNA was undetectable by qPCR after amino acid exposure and yet the protein was detectable by western blot (Fig. 3 & 5). It is therefore important for us to confirm these findings in another enterocyte cell model. Similar to Caco-2 Bbe cells, HT-29 cells are derived from colon adenocarcinomas. Both cell lines can be differentiated to a mature enterocyte phenotype with prolonged in vitro culture, and both have proved useful in studying paracellular calcium transport<sup>34</sup>. HT-29 cell express a much higher endogenous level of KLK-10<sup>35</sup>. If we can show that amino acids suppress KLK-10 expression in HT-29 cells it will lend credence to the notion that amino acids can regulate its expression.

To our knowledge, we are the first to evaluate KLK-10's role in intestinal calcium absorption. KLK-1 has long been associated with electrolyte homeostasis<sup>36</sup>. Recent studies indicate that KLK-1 may also be a physiologic regulator of calcium transport in mice<sup>21</sup> as well as humans<sup>37</sup>. Our data suggest that KLK-10 may play a



structural role in the tight junction of the enterocyte by displaying a calcium intolerant phenotype. A model for our hypothesis is depicted in Figure 8.

Importantly, we have confirmed *in vitro*, that amino acids have a direct effect on our molecular targets identified by the microarray: calcium-permissive Cldn-2 and calcium-intolerant KLK-10. The strengths of our study include 1) the microarray analysis was robust given the large number of animals analyzed per chip, which reduced the contribution of random biological variability to our findings; 2) the qPCR data were entirely consistent with the findings in the microarray screen; 3) not only was Cldn-2 transcript expression increased and KLK-10 transcript expression suppressed, but there was evidence for increased Cldn-2 protein expression and decreased KLK-10 protein expression in Caco-2 Bbe cells exposed to amino acids by western blot analysis; 4) the tight junction expression of Cldn-2 and KLK-10 in Caco-2 Bbe cells exposed to amino acids observed via confocal microscopy are supportive of the notion that these proteins could affect paracellular calcium transport. As noted, our results are currently being validated in HT-29 cells.

To directly test our hypothesis that Cldn-2 and KLK-10 mediate amino acid induced paracellular calcium transport future studies will include developing a cell model in which Cldn-2 is suppressed as well as a cell line in which KLK-10 is suppressed to determine if the observed increases in paracellular calcium flux in Caco-2 Bbe cells treated with amino acids is due to a change in tight junction Cldn-2 and/or KLK-10 expression. We will suppress Cldn-2 and KLK-10 expression using miRNA and then incubate these knock-down cells with CM or CM + 2X amino acids for 6 hours, or 1,25(OH)<sub>2</sub>vitamin D for 24 hours. The transepithelial calcium

transport study methods of Giuliano and Wood<sup>38</sup> and Fleet and Wood<sup>39</sup> will be used for determining calcium transport across the Caco-2 membrane. In preliminary studies we have been able to suppress Cldn-2 expression by as much as 80%.

It is also important to determine if a particular functional group of amino acids plays a dominant role in the redistribution of Cldn-2 and/or KLK-10. Cellular localization experiments will be repeated treating with CM or CM + aromatic or dibasic amino acids for 6 hours, or 1,25(OH)<sub>2</sub>vitamin D for 24 hours. Aromatic amino acids (phenylalanine, tryptophan, and histidine) and dibasic amino acids (arginine and lysine) are of particular interest based on our own work in gastric parietal cells and a review of the literature. The calcium sensing receptor (CaSR) is highly expressed throughout the GI tract<sup>40, 41</sup> and we have previously reported that amino acids at concentrations comparable to those seen post-prandially can activate the CaSR on gastric parietal cells both in vivo and ex vivo<sup>42</sup>. For example L-phenylalanine and L-tryptophan (both known agonists for the CaSR) stimulated parietal cell acid production, while L-leucine (which does not activate the CaSR) did not<sup>42</sup>. Finally, in the absence of extracellular calcium, phenylalanine had no agonist activity but augmented the effect of calcium at higher levels of extracellular calcium consistent with the hypothesis that amino acids are allosteric activators of the gastric parietal cell CaSR<sup>42</sup>. In keeping with these data, Dawson-Hughes et al. have reported that supplementing a low protein diet with phenylalanine and histidine increased UCa excretion<sup>43</sup>. The dibasic amino acids also appear to have effects on intestinal calcium absorption, in particular lysine, which has been previously reported to increase intestinal calcium absorption in humans<sup>44</sup>. Using these

selected amino acids will allow us to directly test the hypothesis that amino acids which activate the CaSR, are more potent at inducing translocation of Cldn-2 to the tight junction or KLK-10 away from the tight junction than the other groups of amino acids.

Lastly, the molecular mechanisms by which amino acid redistribute Cldn-2 and KLK-10 are unclear. The time course of translocation ( $\leq 6$  hrs) suggests that this is unlikely to be a nutrient effect, and our hypothesis is that this is a cell-signaling event in which amino acids are acting as cell surface or cytosolic agonists. Preliminary studies with PKA/ PKC do not suggest these pathways are involved in the translocation of Cldn-2. Because our work and that of a number of other laboratories particularly the Conigrave group, supports the idea that some of the biological effects of amino acids can be mediated by the CaSR, future experiments will include interdicting cell signaling through the CaSR to examine the impact this has on the ability of amino acids to translocate Cldn-2 and KLK-10.

Our study does have some limitations. Key to this work were the findings in the microarray study that examined the impact of dietary protein on paracellular calcium transport in rodents. Studies in rodents may not be necessarily generalizable to humans. However, increasing dietary protein improves calcium absorption in rats, as in humans, making the rat an appropriate model in which to study the mechanism for this effect. As noted, we need to validate our findings in KLK-10 in the Caco-2 cells by repeating the experiments in the HT-29 cells. Finally, we do not have direct functional evidence that KLK-10 plays a role in paracellular

calcium transport, but as noted well-established molecular tools exist to evaluate this.

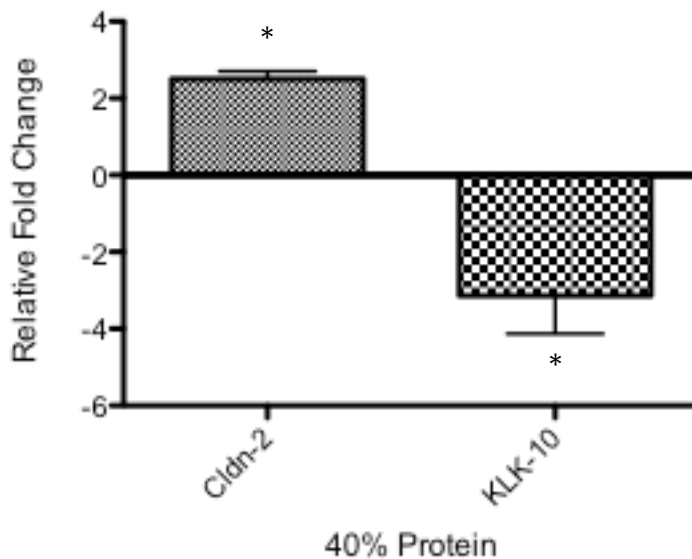
In summary, in this in vitro model, we found that: 1) Cldn-2 mRNA and protein was upregulated in Caco-2 cells Bbe exposed to amino acids; 2) tight junction expression of Cldn-2 was increased in Caco-2 Bbe cells exposed to amino acids; 3) KLK-10 mRNA and protein were downregulated in Caco-2 Bbe cells exposed to amino acids; and 4) tight junction expression of KLK-10 was decreased in Caco-2 Bbe cells exposed to amino acids. These data suggest that amino acid-induced changes in the expression and/or subcellular distribution of Cldn-2 and KLK-10 may be part of the molecular mechanism by which dietary protein increases paracellular calcium flux. Clinically, this can help to explain the findings that high protein diets are associated with a higher BMD and slower rates of bone loss<sup>2-10</sup>.

## Tables and Figures

**TABLE 1.** Calcium-related genes identified in the microarray screen

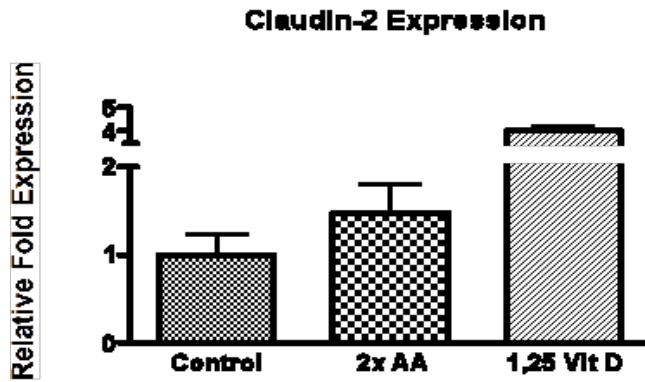
Unigene	Gene Symbol	Gene Title	Fold Change
Rn.90091	Cldn2 predicted	Claudin 2 (predicted)	2.9
Rn.21391	Klk10 /// Klk1c10	Kallikrein related-peptidase 10 /// T-kininogenase	-5.5

**FIG. 1.** qPCR confirmation of microarray hits



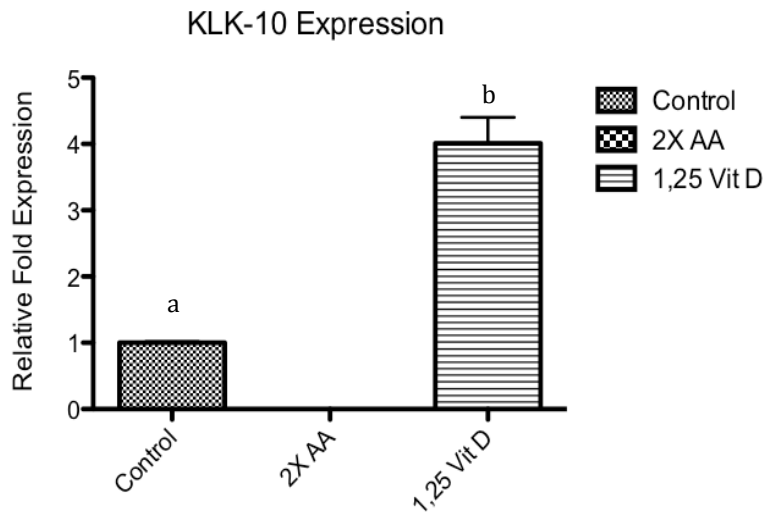
Microarray results for Cldn-2 were confirmed by qPCR using the same RNA samples employed in gene profiling. Microarray results for KLK-10 were confirmed by qPCR using template cDNA generated from RNA samples isolated from a separate set of animals consuming either 5% (n=4) or 40% (n=5) protein diets. Data are shown as means  $\pm$  SEM. (\*  $p < 0.05$ )

**FIG. 2.** Cldn-2 mRNA expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino acids



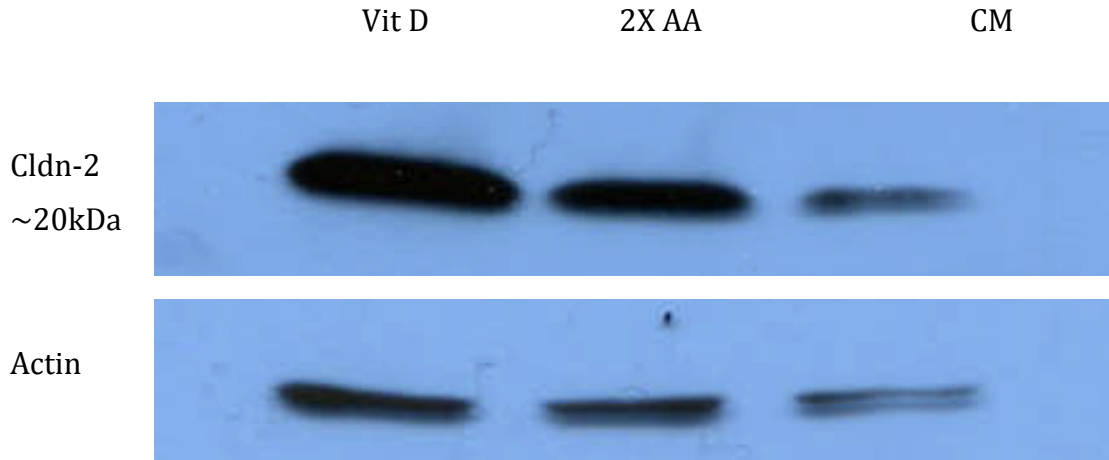
Caco-2 Bbe cells (21 days post-confluent) were treated with CM (Control) or CM + 2X amino acids (2X AA) for 6 hours, or CM + 1,25(OH)<sub>2</sub>vitamin D (100 nM) (1,25 Vit D) for 24 hours. Data are shown as means  $\pm$  SEM.

**FIG. 3.** KLK-10 mRNA expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino

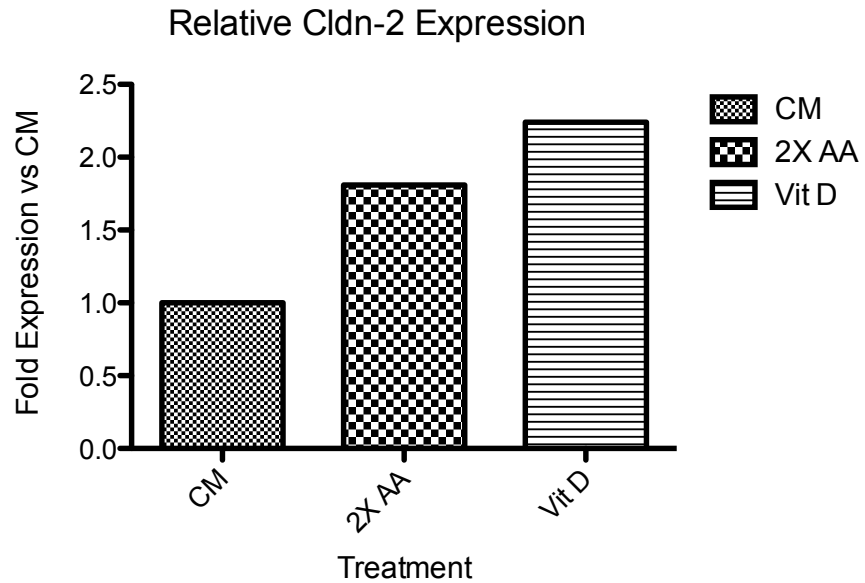


Caco-2 Bbe cells (21 days post-confluent) were treated with CM (Control) or CM + 2X amino acids (2X AA) for 6 hours, or CM + 1,25(OH)<sub>2</sub>vitamin D (100 nM) (1,25 Vit D) for 24 hours. Data are shown as means  $\pm$  SEM. Letters not shown in common indicate significant differences. ( $p < 0.05$ )

**FIG. 4. (A)** Cldn-2 protein expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino acids

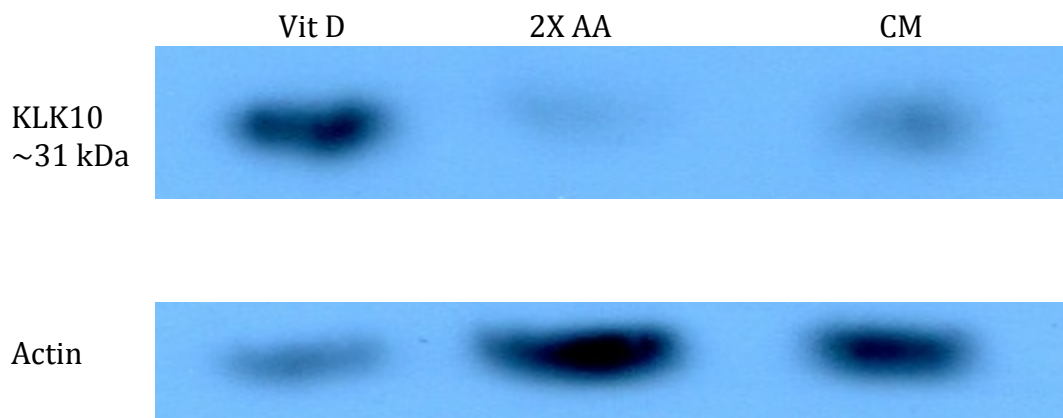


**FIG. 4. (B)** Cldn-2 protein expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino acids

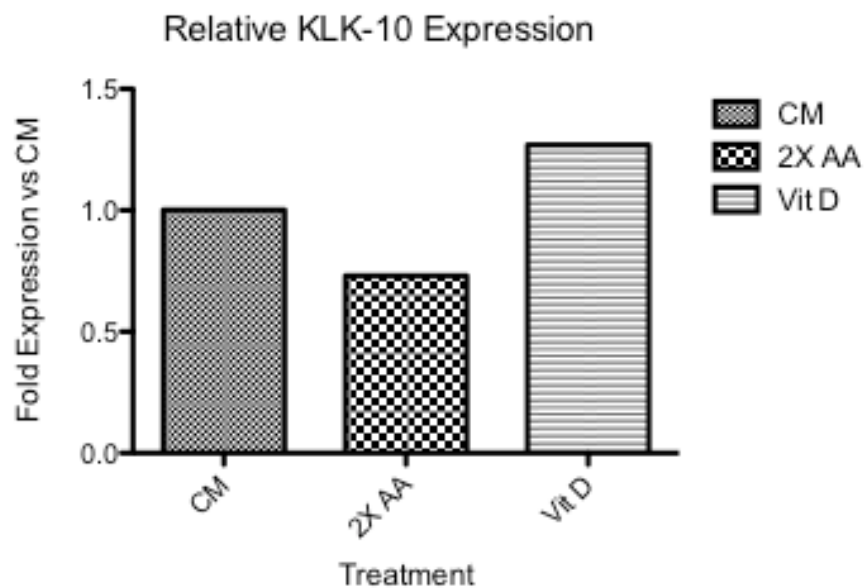


A. Cldn-2 expression by western blot in Caco-2 Bbe cells exposed to control media (CM) for 6 hours, 2X amino acids (2X AA) for 6 hours, or 100 nM 1,25(OH)<sub>2</sub>vitamin D (Vit D) for 24 hours. B. Relative expression of Cldn-2 in A., normalized to  $\beta$ -actin.

**FIG. 5. (A)** KLK-10 protein expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino acids



**FIG. 5. (B)** KLK-10 protein expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino acids

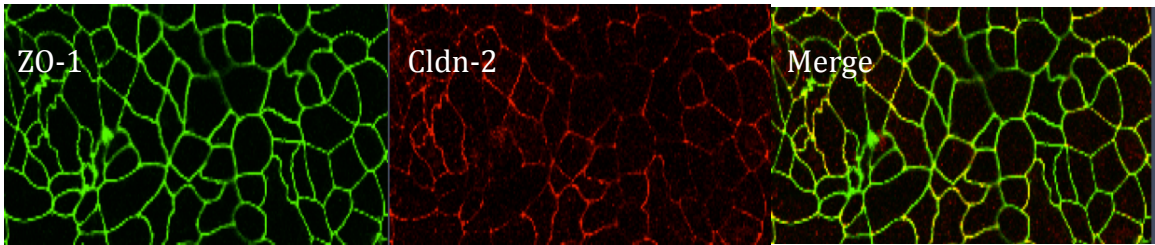


A. KLK-10 expression by western blot in Caco-2 Bbe cells exposed to control media (CM) for 6 hours, 2X amino acids (2X AA) for 6 hours, or 100 nM 1,25(OH)<sub>2</sub>vitamin D (Vit D) for 24 hours. B. Relative expression of KLK-10 in A., normalized to β-actin.

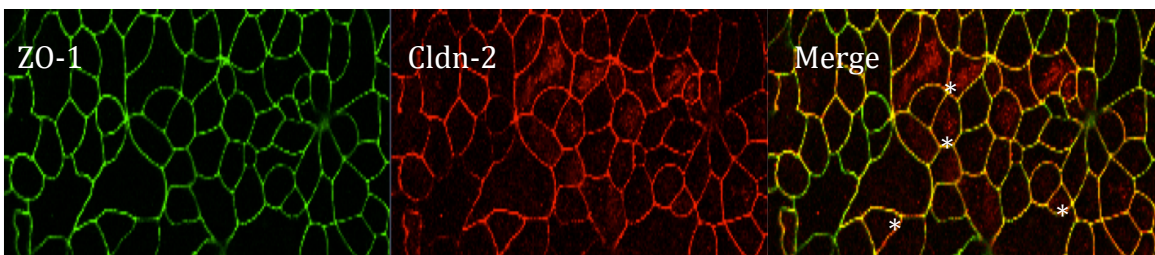


**FIG. 6.** Cldn-2 tight junction expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino acids

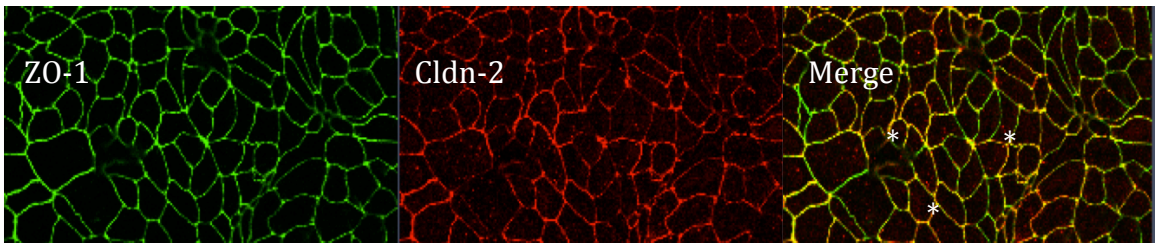
A.



B.



C.



A. Caco-2 Bbe cells (14 days post-confluent) were incubated with CM for 6 hours. For all confocal imaging, cells were grown on transwell inserts, fixed in 3% paraformaldehyde and immunostained with anti-Cldn-2 and anti-ZO-1. Cells were imaged using a Zeiss LSM510 confocal microscope.

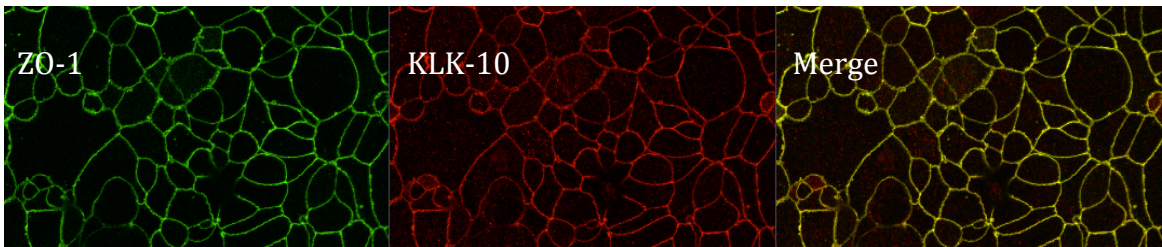
B. Caco-2 Bbe cells (14 days post-confluent) were incubated with 1,25(OH)<sub>2</sub>vitamin D for 24 hours.

C. Caco-2 Bbe cells (14 days post-confluent) were incubated with 2X AA for 6 hours.

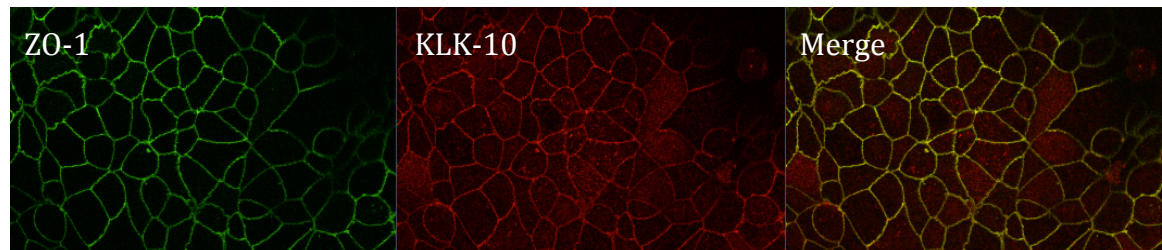
\*White stars indicate some areas of intense co-localization of Cldn-2 and ZO-1

**FIG. 7.** KLK-10 tight junction expression in response to 1,25(OH)<sub>2</sub>vitamin D and 2X amino acids

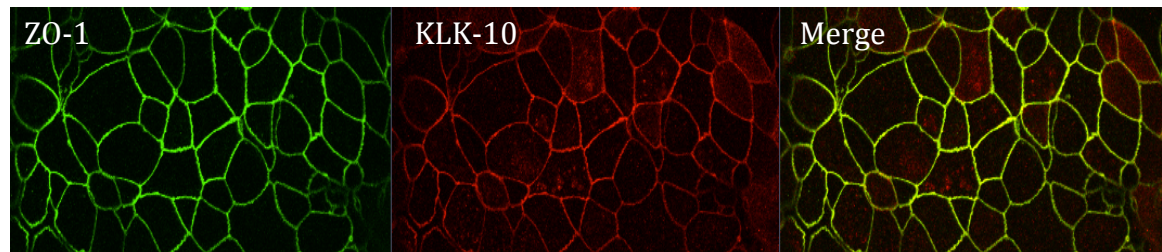
A.



B.

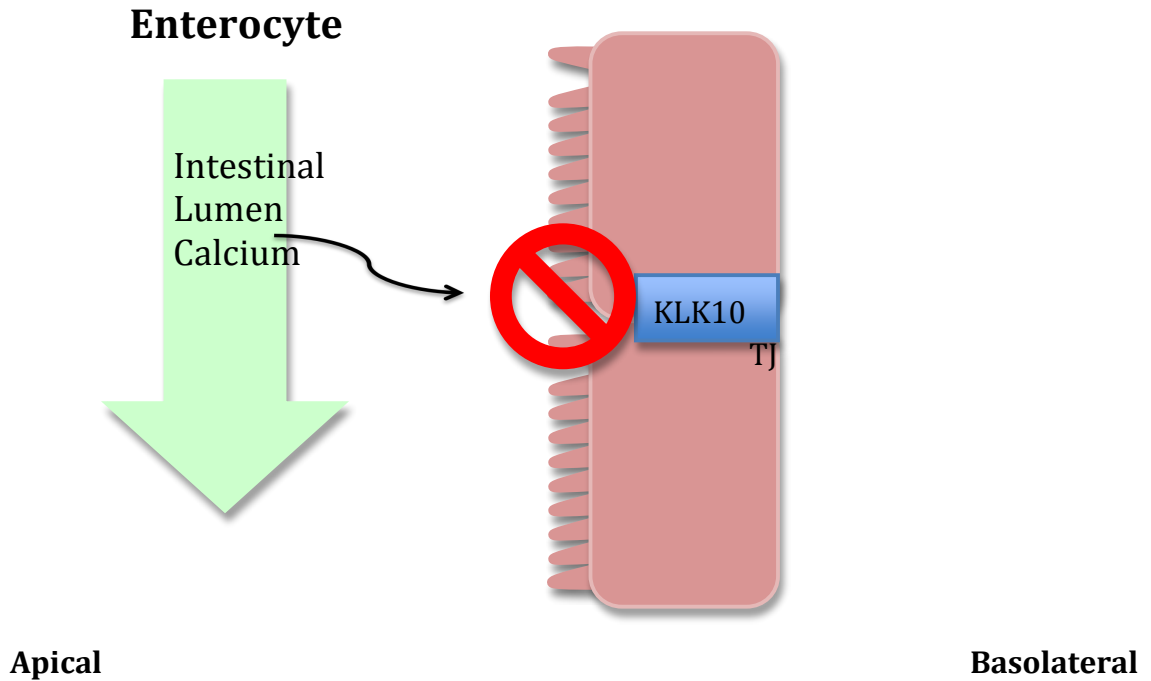


C.



- A. Caco-2 Bbe cells (14 days post-confluent) were incubated with CM for 6 hours. For all confocal imaging, cells were grown on transwell inserts, fixed in 3% paraformaldehyde and immunostained with anti-KLK-10 and anti-ZO-1. Cells were imaged using a Zeiss LSM510 confocal microscope.
- B. Caco-2 Bbe cells (14 days post-confluent) were incubated with 1,25(OH)<sub>2</sub>vitamin D for 24 hours.
- C. Caco-2 Bbe cells (14 days post-confluent) were incubated with 2X AA for 6 hours.

**FIG. 8.** KLK-10 may play a structural role in the tight junction of the enterocyte by displaying a calcium intolerant phenotype: a model for our hypothesis



We hypothesize KLK-10 is present in the tight junctions (TJ) of the enterocyte. Due to KLK-10's calcium intolerant phenotype, this structural protein acts to interfere with the flux of calcium. Decreased KLK-10 expression in the enterocyte could therefore lead to an increase in calcium absorption.

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