Novel Concepts Regarding Calcium Homeostasis During the Transition Period

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Introduction

The transition period (3 weeks pre-calving through 3 weeks post-calving) is a critical time period in the life of the dairy cow. At this time, animals are highly susceptible to a variety of disorders that negatively impact their health, and hence, their overall production. Of particular concern during this time is the inability of the animal to maintain adequate blood calcium concentrations due to increased demand for calcium at the onset of lactation by the mammary gland. This increase in calcium results in decreased circulating calcium concentrations and can lead to the development of periparturient hypocalcemia (milk fever). Parturient paresis is one of the most common metabolic diseases of dairy cattle, with Jersey cows being more susceptible than Holstein (Oetzel, 1988; NRC, 2001). In fact, hypocalcemia is considered a gateway metabolic disorder that leads to increased risks of other periparturient diseases (Figure 1; DeGaris and Lean, 2008). Due to inadequate blood calcium concentrations at the onset of lactation, animals experience a range of clinical symptoms, depending on the extent of the decrease in calcium concentrations (Adams et al., 1996). Clinical hypocalcemia (**CH**) is clinically defined as a total blood calcium concentration of less than 1.4 mmol/L, and subclinical hypocalcemia (**SCH**) defined as total blood calcium of 1.4 to 2.0 mmol/L (DeGaris and Lean, 2008). Approximately 25% of heifers and 50% of older cows will succumb to SCH, and between 5 to 10% of animals will develop CH in the United States (Goff, 2008). Cattle that are afflicted with periparturient hypocalcemia exhibit a 14% decrease in milk production and are more susceptible to other transition disorders, such as ketosis, retained placenta, displaced abomasum, and muscle weakness, with the average cost of incidence of milk fever being $334/animal (Oetzel, 1988). However, should an animal succumb to additional issues due to suffering from milk fever, costs increase substantially. Subclinical hypocalcemia affects about 50% of second lactation and greater dairy cattle, and costs approximately $125/animal to treat. Overall, prevalence of milk fever and SCH are more common in Jersey cattle, likely due to their higher milk production per unit body weight (Oetzel, 1988). With a U.S. dairy cow population of approximately 10 million, an estimate for total loss due to symptomatic clinical milk fever is $240 million per year, and industry losses due to SCH are 4 times higher than that of clinical milk fever (Oetzel, 2013). Typically, in order to compensate for decreased blood calcium, increased intestinal calcium absorption and/or reduced calcium excretion from the kidney must occur; however, calcium resorption from the bone is the primary mode used during this time frame. Dairy cattle, in particular, exhibit a delay in calcium resorption from bone, causing circulating calcium concentrations to fall behind the demand from the mammary gland.

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The current working hypothesis in dairy cows is that increasing the interaction of parathyroid hormone (**PTH**) with its receptor on the bone tissue during late pregnancy can improve the dairy cow's ability to mobilize bone tissue at the onset of lactation (Goff, 2008). However, in other mammalian species, it has been elegantly demonstrated that a different hormone, parathyroid hormone related-protein (**PTHrP**), produced by the mammary gland during lactation is critical for increasing bone resorption during lactation (Wysolmerski, 2010). Recently, we have demonstrated that mammary serotonin (5-hydroxytryptamine) regulates induction of PTHrP (Hernandez et al., 2012). Manipulation of serotonin-induced PTHrP synthesis near the end of the pregnancy period could be critical in preventing the onset of hypocalcemia during the early lactation period. This is important because the early symptoms of milk fever often go undetected because they are short-lived. Data indicate that prevention of milk fever, rather than treatment, would save the dairy industry approximately $140 million per year (http://www.animate-dairy.com/dcalciumd-nutrition/index.html).

The Onset of Milk Production Drains Calcium Pools in Dairy Cows

Colostrum and milk synthesis rapidly deplete calcium from the maternal circulation, and therefore, calcium must be mobilized from maternal bone to maintain adequate circulating concentrations. Circulating calcium concentrations are tightly regulated and controlled by several hormones including: Vitamin D, calcitonin, PTH, and PTHrP (Figure 2). Liberation of calcium from bone stores can only be triggered when circulating calcium concentrations dip below the animal's minimal threshold for calcium, via a classic negative feedback loop. Dietary calcium is insufficient to maintain maternal calcium homeostasis during milk synthesis. This is demonstrated by the fact that a dairy cow will lose 9 to 13% of her bone mass during the first 30 days of lactation. Bone loss during lactation is an evolutionary strategy of mammals used to support the cow, as well as the mammary glands' demand for calcium for milk synthesis (Wysolmerski et al., 1995; Wysolmerksi, 2010; Goff, 2014).

The Mammary Gland Functions as an "Accessory Parathyroid Gland" During Lactation

The mammary gland produces the hormone PTHrP, which binds to receptors on bone to drive bone resorption and liberate calcium into the systemic circulation (Wysolmerski et al., 1995; Wysolmerski, 2010). PTHrP is only produced by the mammary gland during lactation. The calcium sensing receptor (**CaSR**) present in the mammary epithelium plays a crucial role in controlling maternal calcium concentrations during lactation. CaSR is highly expressed in the mammary gland during lactation, compared to virgin and pregnant time periods (VanHouten et al., 2003). Mammary PTHrP production is responsible for the mobilization of calcium from the bone during lactation, rather than the typical endocrine regulator of bone, PTH (Wysolmerski et al., 1995; VanHouten, 2005; Wysolmerski, 2010; Wysolmerski, 2012). Our lab made a novel discovery that serotonin is essential for the liberation of calcium from bone during lactation to sustain maternal calcium homeostasis in rodent models. Specifically, serotonin induces PTHrP synthesis by the mammary gland (Hernandez et al., 2012; Laporta et al., 2014a, 2014b). Furthermore, we demonstrated that serotonin is critical for the expression of CaSR. This finding indicates that serotonin is crucial for mammary gland sensing of systemic calcium concentrations.

Mammary Gland Coordination With the Skeletal System Liberates Calcium During Lactation

The skeletal system maintains its structural and functional roles via communication between two cell types, osteoblasts (**OB**), which are responsible for bone formation, and osteoclasts (**OC**), which are responsible for bone resorption, and thus calcium mobilization. PTH regulates this mechanism under non-lactating conditions. Research in humans and rodents has suggested that PTH action on bone is uncoupled during lactation (Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). PTHrP signals through the same G-protein coupled receptor (**PTH1R**) as PTH on the OB to decrease OB cell proliferation and up-regulate genes responsible for OC differentiation during lactation. In rodents and humans, the mammary gland is the main source of PTHrP found in the circulation (Thiede, 1994; Wysolmerski et al., 1995; Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). Mammary-derived PTHrP, not PTH, is the critical hormone responsible for induction of bone calcium mobilization during lactation (Wysolmerski et al., 1995).

Serotonin Regulates Mammary Gland Physiology During Lactation

Serotonin is synthesized in numerous tissues throughout the body and brain and is incapable of crossing the blood-brain barrier. Serotonin is synthesized from the amino acid L-tryptophan in a 2-step process. The first step is production of 5-hydroxytryptophan (**5-HTP**) via the rate-limiting enzyme, tryptophan hydroxylase (**TPH**). The second step is the conversion of 5-HTP to serotonin by aromatic amino acid decarboxylase (Wang et al., 2002). TPH1 is the rate-limiting enzyme for serotonin production in non-neuronal tissues, while TPH2 is used to produce serotonin in neuronal tissues. Our laboratory and others have shown that serotonin regulates milk protein gene expression, as well as the disassembly of tight junctions that occurs during the involution process (Matsuda et al., 2004; Stull et al., 2007; Hernandez et al., 2008; Pai and Horseman, 2008). Furthermore, we have shown that the mammary gland expresses a unique pattern of serotonin receptors in rodent, bovine, and human mammary epithelium (Hernandez et al., 2009; Pai et al., 2009). The epithelial component of the bovine mammary gland expresses at least 5 serotonin receptor isoforms (5-HT1B, 2A, 2B, 4, and 7; Hernandez et al., 2009). Our lab determined that the 5-HT2B receptor subtype modulates serotonin's regulation of PTHrP production within the mammary gland in a rodent model (Hernandez et al., 2012; Laporta et al., 2013a; Laporta et al., 2014a,b). We also confirmed that circulating serotonin concentrations postpartum are positively correlated with circulating calcium concentrations on the first day of lactation in dairy cows (Laporta et al., 2013b). Furthermore, we showed that serotonin activates expression of various calcium pumps and transporters in the mammary gland to stimulate transport of calcium from blood to milk during mouse lactation (Laporta et al., 2014a). Calcium transport into the mammary gland is thought to occur through the calcium influx channel (**ORAI1**) and subsequent pumping into the milk by the apical plasma membrane calcium ATPase (**PMCA2**; Cross et al., 2014).

Current research in humans and rodents implicates PTHrP in the regulation of maternal calcium homeostasis during lactation. Our laboratory has demonstrated the necessity of serotonin for regulation of calcium transport in the mammary gland during lactation. Furthermore, we have demonstrated that serotonin is necessary for the production of mammary PTHrP during lactation. Mammary PTHrP is critical to the mobilization of calcium

from bone tissue to support lactation. Therefore, delineation of the mechanisms regulating the mammary gland serotonin-PTHrP axis in the dairy cow could lead to development of novel therapeutic interventions to reduce the incidence of SCH and CH in the U.S. dairy cow population.

Can We Use Serotonin to Improve Calcium Homeostasis During Lactation?

Our laboratory recently demonstrated that serotonin is necessary for mammary PTHrP synthesis in lactating rodents and mammary epithelial cells grown in lactogenic culture (Hernandez et al., 2012; Laporta et al., 2013a; Horseman and Hernandez, 2014). We also demonstrated that supplementation of a serotonin precursor, 5-HTP, to rats during the transition from pregnancy to lactation increased postpartum circulating serotonin, PTHrP, and calcium concentrations, and also increased total calcium content in milk (Laporta et al., 2013a). Furthermore, we observed increased osteocyte numbers in the femurs collected from rats supplemented with 5-HTP, indicating this response was due to bone calcium mobilization. These findings led us to perform several experiments in dairy cows in order to evaluate the utility of these findings in rodents to dairy cows.

In order to evaluate the utility of the mammary serotonin-PTHrP axis in Holstein dairy cows, we performed several observational studies. We have observed that serotonin concentrations are dynamic over the course of a given lactation and decrease around the time of calving (day 0 to 2 of lactation), rebounding by approximately 10 days into lactation (Moore et al., 2015). The overall average serotonin concentration in dairy cows is approximately 1700 ng/ml. However, it should be noted that the concentrations fluctuate depending on stage of lactation. These results combined with our rodent data support our hypothesis that serotonin and PTHrP are critical players in the regulation of calcium homeostasis in Holstein dairy cows.

Intravenous (Iv) Infusion Of 5-Htp in Late Lactation, Non-Pregnant, Multiparous Holstein Dairy Cows Increases Circulating Serotonin Concentrations and Alters Calcium Dynamics

In order to demonstrate the role of serotonin in calcium homeostasis in dairy cows, we performed a preliminary experiment in which we infused 5-HTP intravenously for one hour daily for 4 days in late-lactation dairy cows at varying doses (0, 0.5, 1.0, or 1.5 mg/kg) to determine an optimum dose of 5-HTP necessary to produce significant changes in calcium. All 3 doses of 5-HTP increased circulating serotonin concentrations (Laporta et al., 2015) to a similar extent in the two hours after dosing, with concentrations returning to baseline concentrations observed in the saline controls by two hours after infusion. In addition to serotonin concentrations, we measured circulating total calcium concentrations following the same time course post-infusion. While initially counter-intuitive, our data demonstrated that total calcium concentrations decreased in immediate response to 5-HTP treatments (Laporta et al., 2015). In order to determine where the circulating calcium was going after 5-HTP infusion, we measured urine calcium concentrations prior to the start of infusion and 2 hours after the end of the infusion. Our results indicate that there was a decrease in urine calcium output with higher doses of 5-HTP treatment.This suggests that calcium is not being lost into the urine. Therefore, we measured total calcium concentrations in the milk during the infusion periods and observed that the highest dose of 5-HTP increased total milk calcium concentrations. This supports the hypothesis that 5-HTP infusion causes transient hypocalcemia by increased calcium transport

into the mammary gland and subsequently into milk. Increased calcium transport into the mammary gland during lactation is critical for the stimulation of calcium mobilization from bone by PTHrP.

Use of 5-Htp Before Calving to Prevent Hypocalcemia: Is it Possible and are Breed Differences Present?

In order to determine if elevating serotonin concentrations in pre-fresh dairy cows would result in increased post-calving calcium concentrations, we treated multiparous Holstein cows with daily IV infusions of 1.0 mg/kg of 5-HTP beginning 7 days before the estimated calving date until calving. Our data demonstrates that intravenous infusions of 5-HTP pre-calving increased post-calving total calcium concentrations compared to saline treated controls (Weaver et al., 2016). Furthermore, we measured deoxypyridinoline (**DPD**), a marker of OC activity and therefore bone resorption, in the urine. These data demonstrate that cows receiving 5-HTP before calving have increased bone resorption at calving. In other words, 5-HTP treatment pre-calving may improve post-calving calcium concentrations by increasing bone calcium resorption.We performed a similar study, using multiparous Holstein cows only, with our collaborator Dr. Rupert Bruckmaier in Switzerland using the common Swiss system for raising dairy cows, and the effects of 5-HTP on total calcium concentrations post-calving were similar to those seen in our Holstein cows (Hernandez-Castellano et al., 2017). Unpublished results from the study in Switzerland have also revealed that PTH is unaffected by 5-HTP during the transition period, which supports our working hypothesis that serotonin and PTHrP are responsible for coordinating bone mobilization during lactation (Hernandez-Castellano et al., unpublished results).

In order to examine if the serotonin-PTHrP-calcium was conserved across breeds, we tested the same hypothesis in multiparous Jersey cows in the same experiment on our research farm in order to be able to make breed comparisons. Interestingly, Jersey cows responded to 5-HTP differently than the Holstein cows. Jersey cows infused with 5-HTP had significantly decreased calcium concentrations prepartum, and then began to increase calcium concentrations at calving. This was in contrast to the control Jersey cows who did not reach their total calcium concentration nadir until 1 day postpartum (Weaver et al., 2016). Furthermore, Jersey cows treated with 5-HTP had higher concentrations of calcium in their milk compared to the saline treated cows, which was opposite to what was seen in the Holstein cows. These data indicate that serotonin positively impacts calcium homeostasis in both Holstein and Jersey cows, but the underlying mechanisms appear to be different and should be further investigated.

Interrelationship of a Negative Dietary Cation-Anion Difference (DCAD) Diet and serotonin

Given that 5-HTP treatment pre-calving was capable of increasing post-calving calcium concentrations in Holstein cows, we wanted to determine if a common preventative treatment for SCH and CH, negative DCAD, controls calcium homeostasis via a serotonergic mechanism. To this end, we fed Holstein dairy cows a positive DCAD (+130 mEq/kg) or negative DCAD (-130 mEq/kg) diet for 21 days pre-calving. Upon analysis of circulating serotonin concentrations from 9 days pre-calving through 6 days post-calving, we determined that a negative DCAD diet increased circulating serotonin concentrations pre-calving, resulting in an improvement in post-calving calcium concentrations. Preliminary results from a study testing the hypothesis that 5-HTP and negative

DCAD diets have a synergistic effect on post-calving calcium concentrations indicate that the combination of 5-HTP treatment with a negative DCAD diet results in a large increase in post-calving ionized calcium concentrations.

Serotonin and Calcium: Which is the Cart or the Horse? Or are They in Their Own Feedback Loop?

Recent efforts in our laboratory have focused on determining if serotonin is responsible for shuttling calcium into the mammary gland and other tissues during early lactation, or if decreased blood calcium concentrations are responsible for increasing serotonin concentrations to help restore calcium homeostasis in the circulation. We performed an experiment in dry, non-lactating dairy cows that were all fed a negative DCAD diet, but they were receiving 3 different levels of calcium in their diet (0.45%, 1.13%, and 2.02%) for 21 days. After the feeding periods were completed, all cows were subjected to a 5% ethylene glycol tetraacetic acid (**EGTA**) challenge. Our objective was to determine how cows responded to induction of a simulated hypocalcemia, and how quickly they recovered from the insult. Ionized calcium and serotonin concentrations were measured every 15 minutes until cows reached 60% of their initial ionized calcium (**Ca2+**) concentrations and at 0, 2.5, 5, 10, 15, 30, and every 30 minutes thereafter until 90% of initial Ca2+ was achieved. Our preliminary data analysis indicates that cows on the 2.02% calcium diet were more resistant to the hypocalcemic challenge, took longer to achieve the 60% target value, and recovered at the same rate as the cows on the 0.45% diet. Interestingly, the cows consuming 1.13% calcium reached 60% the fastest and took the longest to recover to 90%. Cows on the 0.45% diet reached the 60% induction at the same rate as the cows on the 1.13% calcium diet. Upon initial analysis of the serotonin concentrations during the challenge period in these cows, we observed that cows fed 1.13% calcium had the highest concentrations of serotonin compared to the other 2 treatment groups. The cows on the lowest level of calcium had the lowest serotonin concentrations, and the cows in the high group were intermediate between the other 2 during the challenge. Additionally, all serotonin concentrations in these animals were elevated compared to those in the study by Moore et al. (2015). This is in line with the unpublished studies that feeding negative DCAD diets increase serotonin concentrations as well; however, these cows are also dry and non-pregnant. Finally, these data suggest that serotonin and calcium are potentially acting in a negative feedback loop to regulate blood calcium homeostasis, rather than one or the other driving the system. We have further evidence that this may be the case in vitro in a bovine mammary epithelial cell model where we have observed that PTHrP mRNA expression is increased by both serotonin and EGTA, but the combination results in the highest level of expression. Further research will be aimed at elucidating these mechanisms of action.

Conclusion

In conclusion, we have demonstrated that serotonin plays a critical role in regulation of maternal calcium transport, maternal calcium homeostasis, and mammary PTHrP production in the rodent. Furthermore, our rodent models indicate that the mammary gland is a significant source of serotonin during lactation. Our observational data in Holstein cows suggest that serotonin, PTHrP, and calcium are interrelated during the early days postpartum. Furthermore, our initial experiment exploring the effects of 5-HTP on maternal calcium homeostasis in late-lactation dairy cows supports the hypothesis that serotonin induces transient hypocalcemia by shuttling calcium into the mammary gland

in order to stimulate mammary production of PTHrP, and the elevated PTHrP is critical to stimulate bone calcium resorption. Treating prepartum Holstein dairy cows with 5-HTP resulted in improvement of post-partum calcium concentrations both on our research farm, as well as at the University of Bern in Switzerland, suggesting that the manipulation of the serotonergic axis is conserved across management styles. It also appears that Jersey cows respond differently to 5-HTP treatment, and further research should be directed to understanding their physiology as compared to Holstein cows. Using a current therapeutic intervention for prevention of SCH and CH in the dairy industry, feeding of a negative DCAD diet prepartum, resulted in increased circulating serotonin concentrations. Our preliminary data examining the interaction of 5-HTP and negative DCAD suggests that the 2 treatments together have a synergistic effect on increasing post-calving ionized calcium concentrations. Finally, our most recent data suggest the possibility that serotonin and calcium may be acting in a classic negative feedback loop to maintain blood calcium homeostasis. Together, these findings support the possibility that serotonin is a key player in the search for prevention and treatment of periparturient hypocalcemia in the transition dairy cow.

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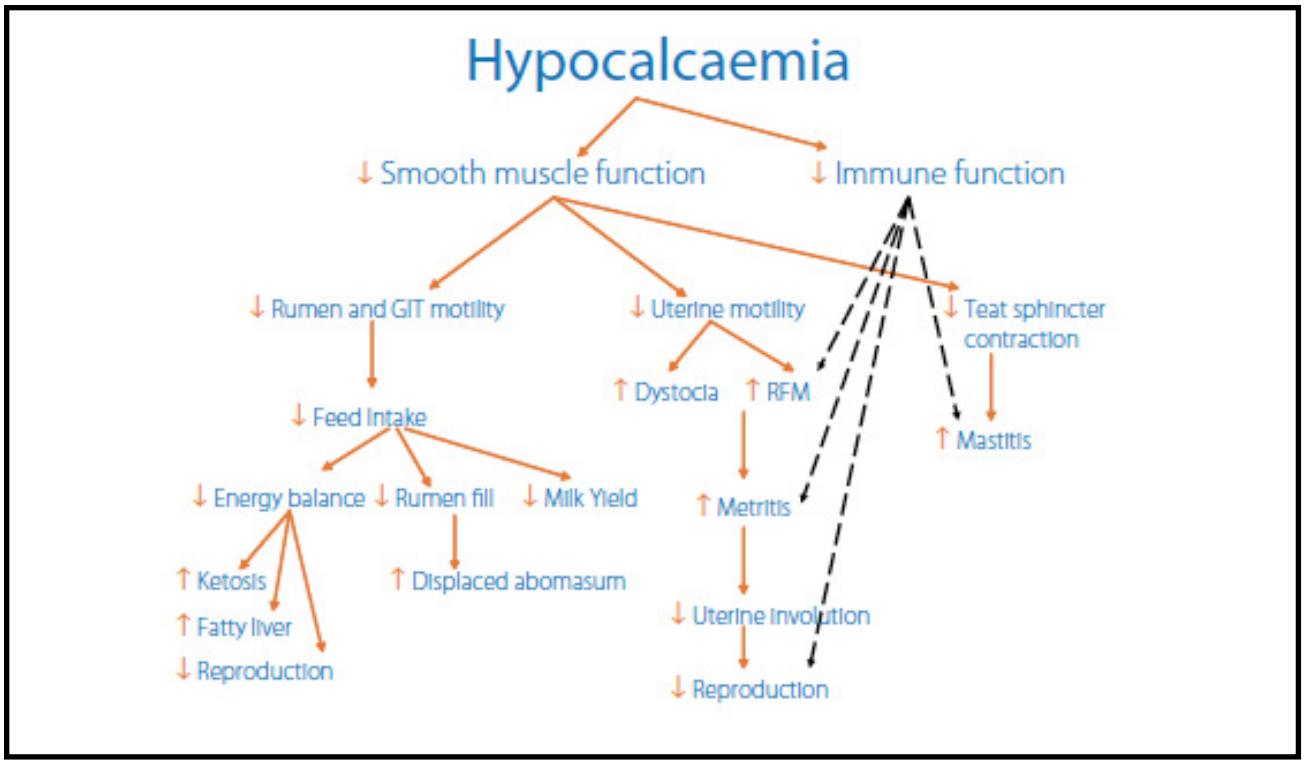

Figure 1. Hypocalcemia is a 'gateway' disease that leads to increased risks of other periparturient diseases (DeGaris and Lean, 2008).

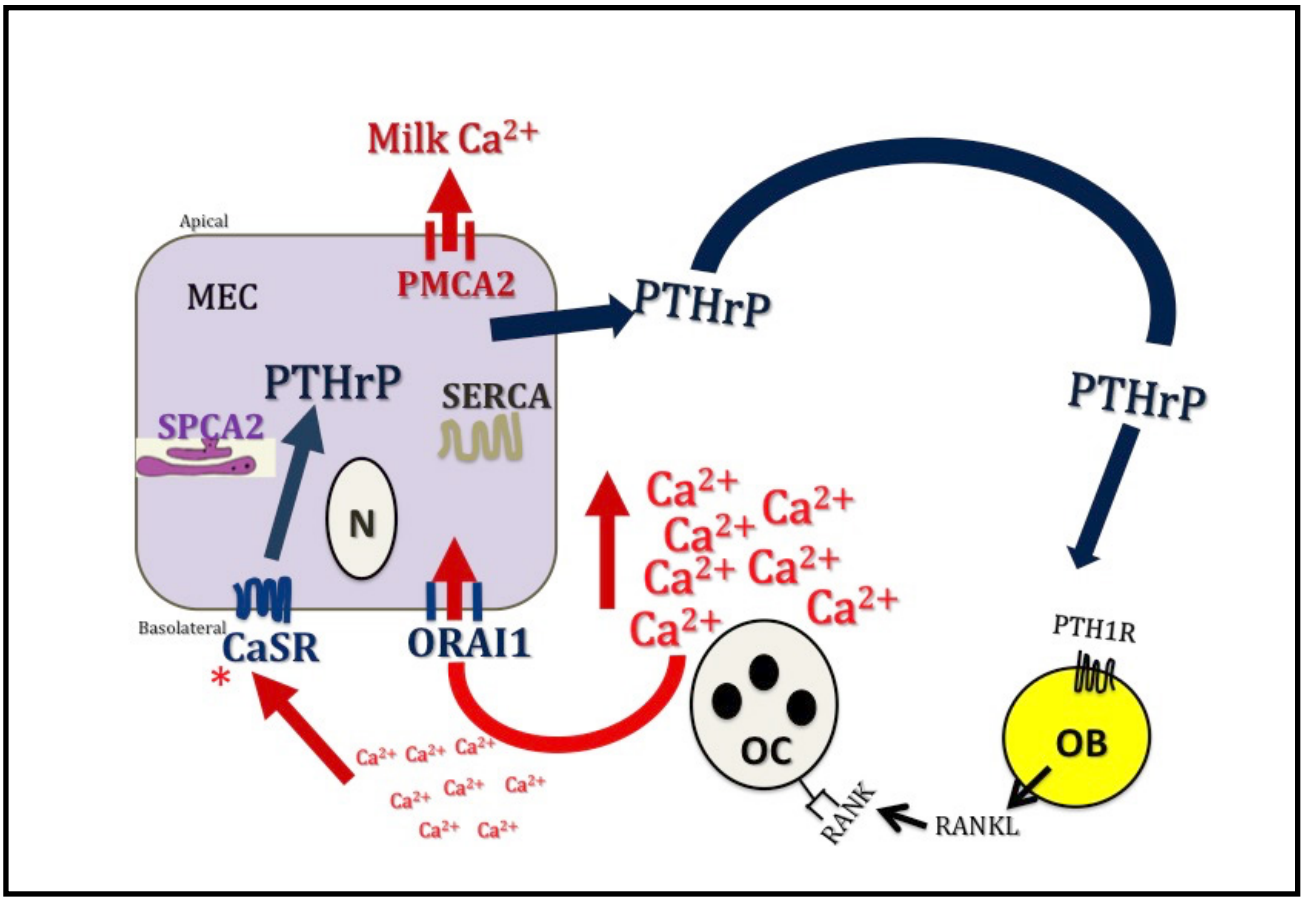

Figure 2. Maternal calcium homeostasis is regulated by the mammary gland-bone axis. During lactation, the calcium sensing receptor (**CaSR**) on the basolateral side of the mammary epithelial cell (**MEC**) during lactation detects low blood calcium concentrations due to the increased transport of calcium into the MEC by calcium release-activated calcium channel protein 1 (**ORAI1**). Calcium is either secreted into the milk through the apical plasma membrane Calcium ATPase 2 (**PMCA2**) or sequestered in the Golgi apparatus by secretory pathways Calcium ATPase 2 (**SPCA2**) or endoplasmic reticulum by the sarco(endo)plasmic reticulum Calcium ATPase (**SERCA**). Detection of systemic decreased calcium by CaSR results in parathyroid hormone related-protein (**PTHrP**) production. PTHrP is secreted into the circulation and will bind its receptor PTH1R on the osteoblast (**OB**) cell in the bone increasing production of receptor activated nuclear factor kappa B (**RANKL**), which binds its receptor (**RANK**) on the osteoclast (**OC**) cell in the bone tissue, activating calcium liberation from bone.