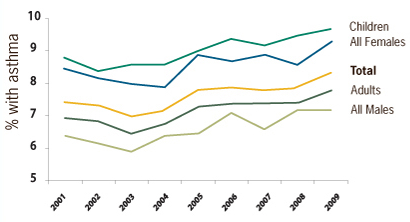
**Regulation of ORMDL3 Gene Expression through the ESK/CREB Pathway**

**I. Introduction**



**Figure 1 Asthma in the U.S. by Age and Gender from 2001-2009.** A gradual increase in the percentage of those afflicted with asthma in the population can be observed, with a large amount of the affected population being made up of children. Adapted from the NIAID (reference 6).

Asthma is a chronic inflammatory disease that is associated with airway obstruction, inflammation, and hypperreactivitiy. Recent surveys have shown that about 5-16% of the world’s population is affected by asthma; the United States remains to be one of the countries with the highest asthma rates, and the rates continue to increase each year with a significant amount of the affected population being children (Figure 1) (Martinez and Vercelli 2013). Inhaled corticosteroids is the common treatment when presenting with wheezing and other asthmatic symptoms, but community studies have shown that compliance to daily therapy is only about 20%.

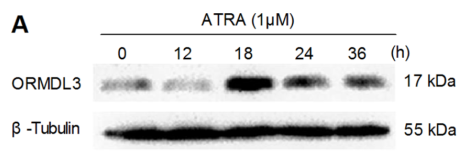
Vitamin A supplementation has been a recent treatment under consideration for asthma. It has been known to play a role in immune response and in maintaining the epithelium of the airway. Though there have been consistent findings of decreased levels of vitamin A in asthmatic patients, treatment through vitamin A supplementation remains controversial; supplementation at an early age is not associated with the decreased risk for the onset of asthma, and in other cases, supplementation at an early age cause the release of cytokines that produce the asthmatic response. Over 150 genes have been associated with producing these responses, one of which is ORMDL3, where ORMDL3 is more explicitly involved with the onset of childhood asthma (Zhuang et al. 2013a).

Orosomucoid 1-like 3 (ORMDL3) is a gene that is a part of the ORMDL family that is found in humans. While ORMDL1 and ORMDL2 are both known to be expressed throughout adult and fetal tissue, ORMDL3 is specifically expressed in bronchial epithelial cells. The gene encodes for an endoplasmic reticulum (ER) trans-membrane protein that then binds to a pump regulating the calcium ion (Ca2+) found on the sarco-endoplasmic reticulum; contact between the two then regulates the Ca2+ content in the ER (Ha et al. 2013, Zhuang et al. 2013b). Altering the pump’s activity, in turn, affects the unfolded protein response, which plays a key role in the development of inflammatory diseases like asthma (Kang et al. 2012). Earlier this year, a study found that all-trans retinoic acid (ATRA), a metabolite of vitamin A, was capable of increasing the expression of ORMDL3 (Zhuang et al. 2013a).

A number of additional signals were involved in allowing ATRA to eventually express ORMDL3. The expression of ORMDL3 is triggered when a cAMP response element (CRE) found in the promoter region of the gene binds to a cAMP response element-binding protein (CREB) (Zhuang et al. 2013a). While there are a number of kinases known to bind to CREB, Zhuang et al. (2013) found that protein kinase A (PKA) phosphorylated CREB and then allowed for ORMDL3 expression. When inducing cells with ATRA, ORMDL3 expression also increased accordingly, and they concluded that PKA must be the connecting factor between ATRA induction and ORMDL3 expression. Of the other kinases capable of activating CREB, extracellular signal-regulated kinase-1 and 2 (ERK1/2) is known to activate CREB via phosphorylation (Aggerwal et al. 2006). Canon et al. (2004) further showed that retinoic acid quickly stimulates ERK1/2 to then phosphorylate CREB in neuronal cells. However, it is not known if the ATRA-activated ERK1/2 causing the activation of CREB would then also lead to the expression of the ORMDL3 expression, specifically; previous studies have failed to link the pathway to the expression of the ORMDL3 gene. The purpose of this experiment will be to determine whether or not ERK1/2 also induces ORMDL3 to further understanding of how vitamin A supplementation may be used as a treatment to prevent the onset of childhood asthma.

**II. Experiment**

Three cell types will be used in this experiment: mouse embryonic fibroblast cells (NIH3T3), human bronchial epithelial cells (BEAS-2B), and human type-II alveolar lung epithelium cells (A549); each of the cell types are representative of the different environments in which ORMDL3 is found to be expressed. The cells were plated onto 96-well plates, where about 15,000 cells were in each well. Each of the cell types were divided into four groups: PKA, anti-PKA, ERK1/2, and anti-ERK1/2; the first two groups were to serve as a control, and anti-PKA was to represent PKA inhibition by using a PKA inhibitor, H-89 (Zhuang et al. 2013a). Similarly, ERK1/2 was inhibited in the anti-ERK1/2 group by using U0126 (Aggarwal et al. 2006). The cells were then treated with ATRA and left to incubate for 18 hours (Figure 2). Afterwards, the cell proteins were extracted using the Total Protein Extraction Kit, and the western blot was initiated.



**Figure 2 ORMDL3 Production in Presence of PKA**. Measurement of ORMDL3 expression shows that 18 hours is the optimal amount of time needed to produce significant protein. Adapted from Zhuang et al. (2013) (reference 7).

A western blot is a test commonly done to detect for the presence of a specific protein. The process will be initiated by separating the proteins using gel electrophoresis. By running the proteins through a 12% SDS-PAGE gel, the protein will become coated in the gel. This will cause the proteins have a negative charge, and migrate down the gel towards the positive end. Following this step, the separated proteins from the gel will be transferred over to a nitrocellulose gel via the electroblotting method; the gel will be placed on top of the nitrocellulose membrane, and then both will be surrounded on by filter paper and sponges on both ends. Once the two are suspended in a transfer buffer, an electric current will be run through the two. In a similar manner to gel electrophoresis, the proteins will be moved from the negative gel side onto the membrane, and they will be preserved in the order from the gel.

The membrane will be prepared by suspending it in a TBS buffer to prevent the membrane from interacting with the proteins. After this incubation, the membrane will first be incubated with ORMDL3 antibodies (E-15) overnight, allowing the ORMDL3 proteins to be marked. However, since this alone does not allow for detection, the membrane will be incubated in a secondary antibody. Because that the ORMDL3 antibody is a goat polyclonal antibody, the secondary antibody of choice will be a near-infrared (IR) anti-goat antibody. This will allow the antibodies to only bind to the ORMDL3 antibodies, retaining the marking of only one protein. Since it is a NIR antibody, luminescence will be produced when the proteins attached to the secondary are stimulated with wavelengths of 700 nm, producing the luminescence. Because that the secondary NIR antibodies emit a constant light instead of a variable light like other fluorescent dyes, the concentration of ORMDL3 protein can also be determined. The blot will be stripped and re-probed using β-tubulin as the loading control to quantify the ORMDL3 protein present. Analysis of the blot will be done using the Odyssey System with infrared absorption with a wavelength of 700 nm. The luminescence will be measured to determine the concentration of the ORMDL3 protein.

**III. Discussion**

Should the experiment be completed without issues, I expect that the ORMDL3 protein will be present in the PKA and anti-ERK1/2 lane, and not in the remaining two lanes; the bands for the loading control, β-tubulin, should be consistent across all four lanes. I expect that the concentration of ORMDL3 protein will be greater in the PKA lanes as opposed to the anti-ERK1/2 lanes due to PKA’s higher affinity for a regulator upstream, cAMP. If a treatment were to be developed solely using knowledge of these two kinases, this experiment serves to show that a vitamin A supplementation would be best given alongside an ERK1/2 inhibitor. However, because the previous study done observes ERK1/2 activating CREB with two other kinases, it is not known if only inhibiting ERK1/2 will be effective. Further studies should be done to determine if the three kinases produce a synergistic response similar to PKA. Additionally, other CREB activators, and combinations of them, should also be tested to obtain a clearer understanding of the pathway to then develop an effective treatment to stop the onset of childhood asthma using vitamin A supplementation.

The bulk of the potential problems will arise from the reliability of the data obtained from the western blot. Because this test is extremely sensitive, it is likely that too much or too little of the antibodies could be detected, thus leading to falsehood in the data. This could be corrected by altering the incubation time after finding the optimal time or simply be doing another trial of the experiment. Transferring the gel onto a nitrocellulose membrane and then later stripping the membrane may prove to be a problem as nitrocellulose is more fragile. Should this be the case, the alternate membrane, polyvinylidene fluoride (PVDF), should be used as it is capable of handling stripping.

The main limitation of this study is the lack of kinase diversity. If this experiment were to become more expansive, the kinases coupled with ESK1/2 in Aggerwal et al.’s (2006) study, RSK and PKC, would have also been incorporated. Additionally, this mechanism is unusual in that it is appears to be independent of the retinoic acid receptor. Further testing on this key point would help define the mechanism and perhaps narrow down the kinases that are involved in the pathway. Finally, the experiment does not measure the ORMDL3 mRNA produced to determine whether or not an intermediate is being produced at all in cells that eventually did not produce any protein. Exploration of all of the aforementioned may help designing a vitamin A supplementation treatment that produces less contradicting results.

**III. References**

Aggarwal, S., Kim, S., Cheon, K., Tabassam, F. H., Yoon, J., & Koo, J. S. (2006). Nonclassical Action of Retinoic Acid on the Activation of the cAMP Response Element-binding Protein in Normal Human Bronchial Epithelial Cells. *Molecular Biology of the Cell*, *17*(February), 566–575. doi:10.1091/mbc.E05

Dijk, F. N., de Jongste, J. C., Postma, D. S., & Koppelman, G. H. (2013). Genetics of onset of asthma. *Current opinion in allergy and clinical immunology*, *13*(2), 193–202. doi:10.1097/ACI.0b013e32835eb707

Ha, S. G., Ge, X. N., Bahaie, N. S., Kang, B. N., Rao, A., Rao, S. P., & Sriramarao, P. (2013). ORMDL3 promotes eosinophil trafficking and activation via regulation of integrins and CD48. *Nature communications*, *4*, 2479. doi:10.1038/ncomms3479

Kang, M.-J., Yu, H.-S., Seo, J.-H., Kim, H.-Y., Jung, Y.-H., Kim, Y.-J., … Hong, S.-J. (2012). GSDMB/ORMDL3 variants contribute to asthma susceptibility and eosinophil-mediated bronchial hyperresponsiveness. *Human immunology*, *73*(9), 954–9. doi:10.1016/j.humimm.2012.06.009

Martinez, F. D., & Vercelli, D. (2013). Asthma. *Lancet*, *382*(9901), 1360–72. doi:10.1016/S0140-6736(13)61536-6

National Institute of Allergy and Infectious Diseases, National Institutes of Health. Asthma in the U.S. (http://www.niaid.nih.gov/topics/asthma/understanding/Pages/usAsthma.aspx; Accessed December 2013)

Zhuang, L.-L., Huang, B.-X., Feng, J., Zhu, L.-H., Jin, R., Qiu, L.-Z., & Zhou, G.-P. (2013). All-Trans Retinoic Acid Modulates ORMDL3 Expression via Transcriptional Regulation. *PloS one*, *8*(10), e77304. doi:10.1371/journal.pone.0077304

Zhuang, L.-L., Jin, R., Zhu, L.-H., Xu, H.-G., Li, Y., Gao, S., … Zhou, G.-P. (2013). Promoter characterization and role of cAMP/PKA/CREB in the basal transcription of the mouse ORMDL3 gene. *PloS one*, *8*(4), e60630. doi:10.1371/journal.pone.0060630