THE ERGOTHIONEINE TRANSPORTER

Entia Biosciences, Inc.

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Background:

SLC22A4 is a sodium-ion dependent transporter that efficiently and specifically carries L-Ergothioneine across the cell membrane. L-Ergothioneine (ERGO) is a naturally occurring amino acid and thiourea derivative of histidine that cannot be made in human cells and must be absorbed from the diet. Dr. Gründemann, the discoverer of the ERGO transporter (ETT), states that ETT is necessary for the supply of ERGO to erythrocyte (red blood cell) progenitor cells and to monocytes (white blood cells) (Gründemann, 2005). The function of ERGO and its transporter and its role in metabolism and the pathophysiology of human disease are currently unknown, but variations in SLC22A4 have been associated with susceptibility to inflammatory disorders, such as rheumatoid arthritis and Crohn's disease, and expression has been documented in a variety of human tissues, including inflammatory cell types such as macrophages and monocytes. The existence of a specific ERGO transporter suggests that ERGO is advantageous to long-term human health (Grigat, 2007). Dr. Solomon Snyder, Johns Hopkins University School of Medicine, states the following: "ERGO is as potent as glutathione. Because of its dietary origin and the toxicity associated with its depletion, ERGO may represent a new vitamin whose physiologic roles include antioxidant cytoprotection." Dr. Snyder further states that the high density of ERGO within mitochondria implies a unique role in protecting mitochondrial DNA from damage induced by free radicals and reactive oxygen species (Paul, 2009). Mitochondria are cytoplasmic organelles responsible for life and death. Evidence from animal and clinical studies suggest that mitochondria play a critical role in aging, cancer, diabetes and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Simon, 2004; Lin, 2006; Reddy, P.H., 2009).

Further supportive evidence of the potential protective role of ERGO was recently shown by the presence of elevated levels of ERGO in the red blood cells of pregnant women with the condition preeclampsia. The symptoms of preeclampsia include high blood pressure, protein in urine and fluid retention and affects almost 10% of pregnancies after 20 weeks. Left untreated, the condition can cause a range of problems such as growth restriction in babies and even fetal and maternal mortality. There is no known cause of the condition. Dr. Fisher states: "Ergothioneine is known as an antioxidant and antioxidants have been proposed to be helpful in reducing the risk of preeclampsia. It is therefore very interesting that we have found it to be in excess for women with the condition" (Turner, E., 2009).

The purpose of this study is to evaluate the expression pattern of SLC22A4 in a variety of human tissues and diseases through immunohistochemistry. We hope that the data obtained will provide needed information to explain the physiologic and potential health protective role of this master antioxidant that no human can produce.

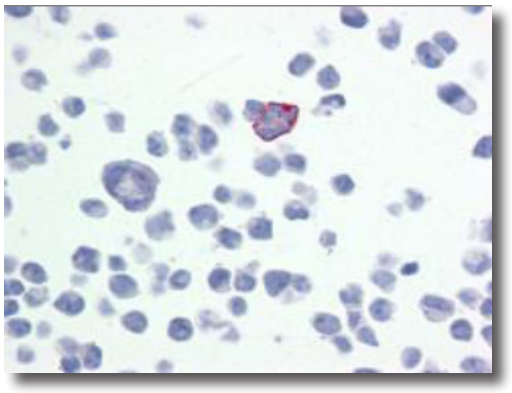
Methodology:

Five individual antibodies to SLC22A4 were evaluated on formalin-fixed, paraffin-embedded (FFPE) positive and negative control cell lines and a multi-tissue array of human normal tissues to identify the best reagents and concentrations for use in immunohistochemistry. The cell lines tested were a positive cell line TNCS1a-ETTh, which expresses SLC22A4, and a negative control cell line TNCS1a-CTTh, which expresses carnitine transporter. The human multi-tissue block included cores of the following normal tissues: adrenal, brain, breast, colon, heart, small intestine, kidney, liver, lung, skeletal muscle, pancreas, placenta, prostate, skin, spleen, testis, thymus, thyroid, tonsil, and uterus.

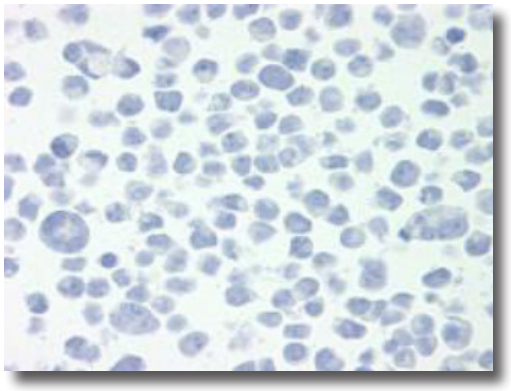
Antibody titration experiments were conducted with all 5 antibodies; formalin-fixed, paraffin-embedded human tissues were supplied by LifeSpan and control cell lines (ETTh and CTTh) supplied by Entia Biosciences, Inc. (Dr. Dirk Gründemann) prepared by LifeSpan. The 5 antibodies were initially applied to the tissues and a proprietary LifeSpan detection system was then used including a Vector Red substrate kit to produce a fuchsia-colored deposit, identifying the presence of the SLC22A4 transporter. The slides were interpreted by a pathologist and each antibody was evaluated for the presence of specific signal, level of background, and concordance with expression results reported in the literature. Staining was recorded on a 0-4 scale (0=negative, 1=blush, 2=faint, 3=moderate, 4=strong). Slides stained at the reported concentrations or dilutions were imaged with a DVC 1310C digital camera coupled to a Nikon microscope. Images were stored as TIFF files with Adobe Photoshop. The antibodies that were negative were imaged at the highest titers.

Images:

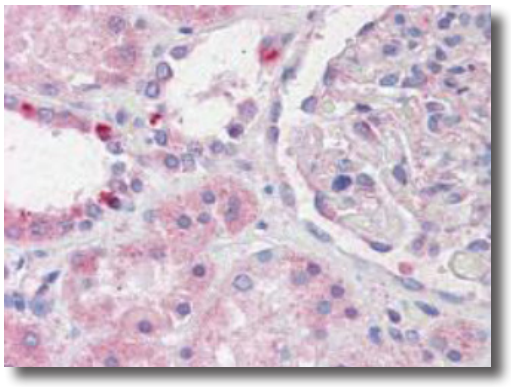
Positive Control: Cell Line, expressing SLC22A4



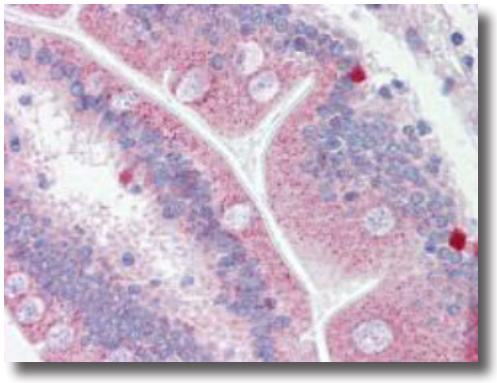
Negative Control: Cell Line, expressing Carnitine



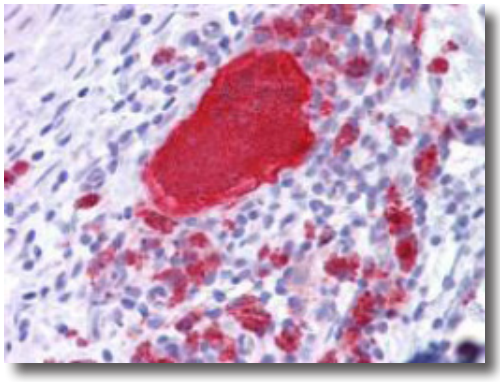
Kidney: Positive Control. Normal kidney from 68-year-old male. Proximal convoluted tubules (PCT) show moderate staining. Validates Gründemanns data that ERGO is absorbed back into the body by the PCT.



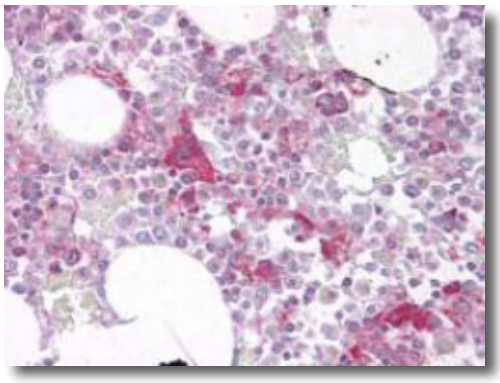
Small Intestine: Positive Control; 58 year-old male with small bowel obstruction. The antibody showed strong staining of neuroendocrine cells in the small intestine with moderate staining of epithelium and ganglion cells. Intestine has rapidly dividing tissues and would be expected to have higher amounts of the Transporter within the mitochondria of adult somatic stem cells.



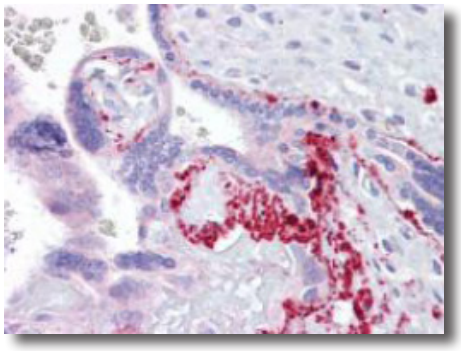
Foreign Body Granuloma: Epididymis from a 61 year-old male. Macrophages are strongly positive.



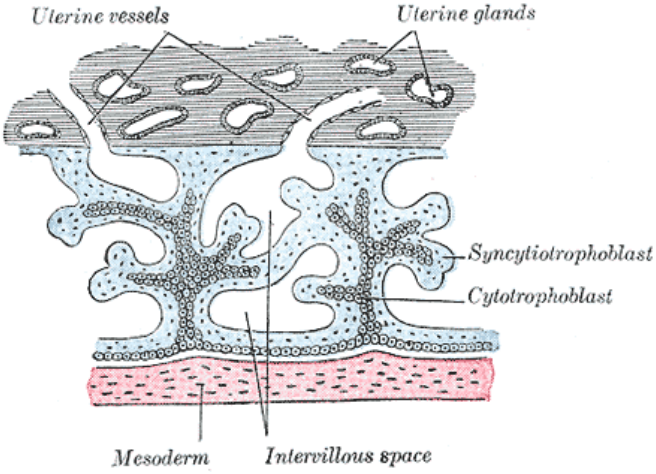
Normal Bone marrow: 74 year-old male autopsy. Moderate to strong staining of macrophages; faint to moderate staining of megakaryocytes.



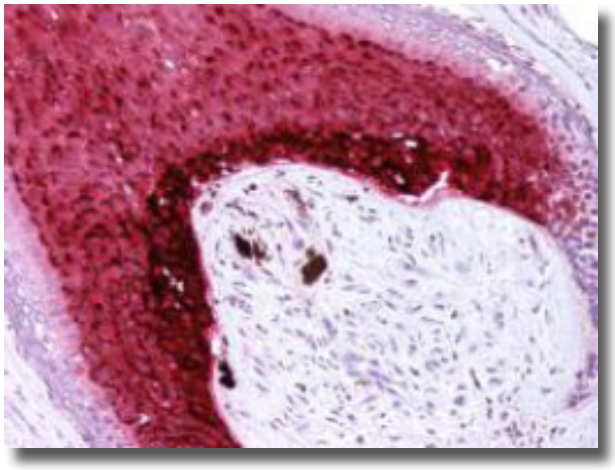
Normal Placenta: 18 year-old female. Faint to moderate staining of intermediate cytotrophoblasts; occasional strong granular staining along basal border of placental trophoblasts. Faint staining of syncytiotrophoblasts.



Primary chorionic Villi; Diagrammatic



Hair Bulb: 40 year-old who died in motor vehicle accident. Hair shaft faintly to moderate positive staining; strong staining of the hair root within the hair bulb.



Summary:

ENTIA/LifeSpan has identified the best antibody to show specific, positive membranous and granular cytoplasmic staining for the Ergothioneine Transporter (ETT). Our initial impression is that ETT is highest in concentration in the most undifferentiated and rapidly dividing tissues; findings consistent with those of Dr. Gründemann. An example of undifferentiated stem cells is the placenta image that reveals significant staining of the cytotrophoblast. The cytotrophoblast is considered to be the trophoblastic stem cell; it differentiates into the other forms of trophoblastic tissue (intermediate trophoblast and syncytiotrophoblast). The intermediate trophoblast is more highly differentiated and anchors the placenta to the maternal tissue. The syncytiotrophoblast is the epithelial covering of the placenta villous tree. These cells differentiate and secrete hormones to maintain the integrity of the uterine lining.

The intestinal lining represents cells and tissues that rapidly divide. The absorptive and protective functions of the intestines are dependent on an intact and functional epithelium. This epithelial layer undergoes continuous and rapid replacement of the differentiated cells by replication of somatic adult undifferentiated stem cells located within intestinal crypts. This process of cell renewal is based upon a limited number of long-lived multipotent intestinal stem or progenitor cells. These cells are similar to those of the hematopoietic system and also the hair follicle. These cells must have two main properties: self-renewal or the ability to maintain itself throughout long periods of time and the potential to generate all differentiated cell types present within a tissue or organ.

Mitochondria are rod-shaped organelles that can be considered the power generators of the cell, converting oxygen and nutrients into adenosine triphosphate (ATP). ATP is the chemical energy “currency” of the cell that powers the cell’s metabolic activities. These organelles are constantly exposed to free radicals and oxidative stress as part of their normal role in providing needed energy to keep cells alive. When cells are deprived of the transporter there is increased mitochondrial DNA damage, protein oxidation and lipid peroxidation (Paul, 2010).

Why are the levels of ETT higher in the mitochondria of rapidly dividing tissues? One explanation is that rapid cell division exposes the cell to free radicals and oxidative damage and the antioxidant protective role of ERGO is needed. In addition, ETT levels are directly related to different tissue requirements for mitochondrial function. For example, placenta, intestinal linings and hematopoietic tissues have high energy requirements as compared to other tissues in the body.

ENTIA/LifeSpan Phase 2 studies, currently underway, will focus on analyzing the presence/levels of SLC22A4 in a whole array of normal tissues and cancer tissues, including but not limited to breast, colon, lung, ovary, prostate, pancreas and skin. Additional studies will focus on neurodegenerative diseases, such as Parkinson’s, Alzheimer’s, Multiple sclerosis, and Lou Gehrig’s disease, Crohn’s disease, ulcerative colitis, and metabolic syndrome, especially diabetes.

It is hoped that future studies on ERGO and The SLC22A4 Transporter will provide a better platform to develop therapies to regenerate and/or treat tissues and organs that have been damaged by oxidative stress and inflammation.

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