Reducing Protein Expression in the pMT Vector

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Introduction

Stem cell protein polarity is a fundamental and dynamic process that guides cellular differentiation and proliferation during development. Protein polarity can be observed in cell types ranging from zygotes to highly specialized epithelial cells. For decades an entire field of cancer research has been dedicated to understanding the mechanisms behind protein polarity, mechanisms that are dictated by an array of protein-protein interactions. One popular and highly genetically characterized model organism that is used to study protein polarity is *drosophila melanogaster*, or the fruit fly.

In the fruit fly, neuroblasts (neuronal stem cells) set up protein polarity to divide asymmetrically. While neuroblasts supply the central nervous system with neural cells of specific fate and function, neuroblasts also self renew after division in order to repeat this process. Renewal occurs because of the separation of polarity proteins to the apical and basal domains of the cytoplasm (Figure 1A). The polarity proteins that are localized to the

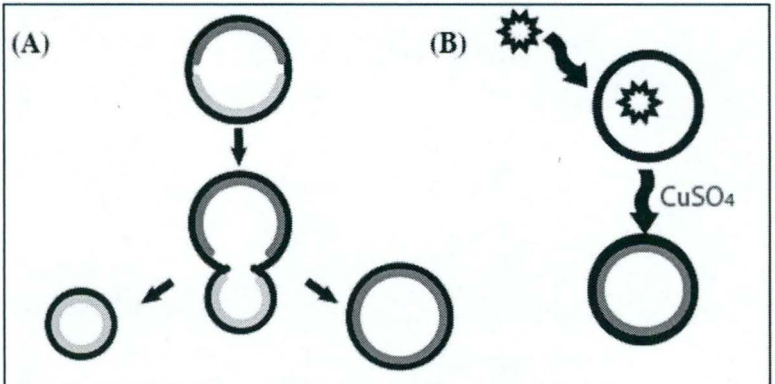

Figure 1. (A) Protein polarity in neuroblasts is critical for neural stem cell differentiation. aPKC is shown in dark gray. Miranda is shown in light gray. (B) Atypical protein kinase C (aPKC) cortical localization in Schneider 2 (S2) cells via transfection of pMT vector.

apical domain of the cell cortex act as self-renewal factors that will yield a neuroblast after division. On the other hand, separate polarity proteins on the basal cortex of the cytoplasm will yield a precursor cell of a neuron after division.

Many polarity proteins have not been well characterized, including the kinase atypical protein kinase C (aPKC), which is important to polarity because it aids in the physical restriction of basal polarity proteins. At the metaphase plate during mitosis, aPKC restricts the basal fate determinate, Miranda, to the basal cortex through phosphorylation, a process by which a protein kinase attaches a phosphate group to another protein in order to change the behavior of it. Phosphorylated by aPKC, Miranda dissociates from the apical membrane and is restricted basally. Although this provides clear evidence about the maintenance of basal polarity, this information fails to explain how aPKC is able to be anchored to the apical domain of the dividing cell. It is clear that aPKC is fundamental for maintaining protein polarity. Yet important questions remain about how aPKC does

this, what parts of it are crucial for localizing to the cell cortex, and how we tune the correct dose of aPKC in S2 cells.

To investigate these questions, constructs of aPKC were made that lacked the particular pieces of aPKC predicted to be responsible for its cortical localization. Through the method of DNA transfection, the pMT vector carried the gene of interest into host S2 cells. After transfection, expression of the gene of interest is stimulated with the addition of copper ions in the form of copper sulfate[1,2] (figure 1B). Initial imaging for the amount of protein expression revealed an overabundance of aPKC from the pMT vector. The overabundance is indicated by the fact that aPKC was present in the cytoplasm of the cell and is no longer restricted to the cell cortex. To eliminate this cytoplasmic concentration of aPKC, the promoter sequence of the pMT vector was altered to reduce aPKC expression. Developing a more efficient and rapid technique of studying polarity protein localization by creating the pMT-EboxMut and reducing the amount of aPKC, we will be able to more quickly advance our understanding of the molecular nature of cancer. Using S2 cells rather than fly embryo transfection to look at the behavior of polarity proteins can save months of time.

Results and Discussion

The first step focused on investigating the DNA element to which RNA Polymerase binds. RNA Polymerase binds to a DNA element known at the -10 -35 region, which is upstream from the transcription initiation site of the pMT vector.[3] This element contains a special -35 sequence known as the E-Box (consensus sequence: CANNTG). The E-Box causes high levels of mRNA expression.[4] Alterations of the E-Box from CANNTG to CTACCG decrease the enhanced green fluorescent protein (EGFP) mRNA expression in transgenic mice, as well as EGFP expression (Figure 2). [5]

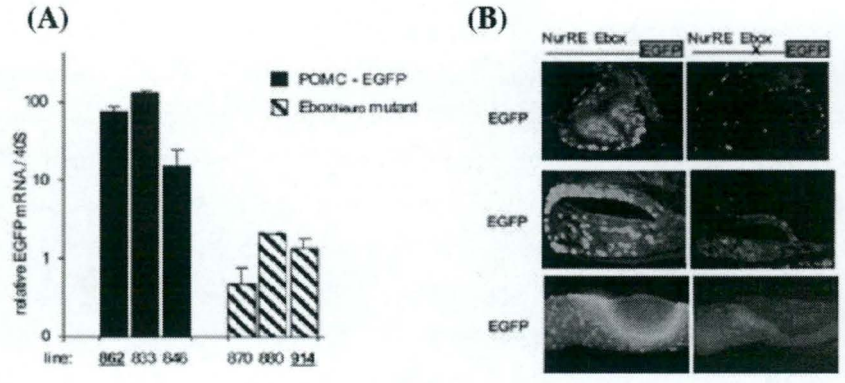

Figure 2. (A) Depiction of EGFP mRNA expression in transgenic mice lines. Striped bars indicate mRNA output by a mutated E-Box element upstream of EGFP DNA sequence. (B) Relative EGFP fluorescence expressed in mouse brain tissue with and without E-Box mutation.[5]

One of the key characteristics of the E-Box element is that it is a palendromic sequence. In other words, in the state of a single strand of DNA, these elements will fold onto themselves because they have complimentary nucleotide base pairs at either end of the DNA sequence. This palendromic structure is essential in the association of these elements to their corresponding transcription factors, including RNA polymerase.[5] The E-Box mutations designed to reduce binding to transcription factors eliminated the presence of the palendromic structure and prevented folding and association with the transcription factors. Using a qualitative comparison of protein expression levels based on relative fluorescent intensity, the pMT-EboxMut vector clearly exhibits a decrease in the amount of aPKC expressed (Figure 3).

A reduced affinity between the promoter DNA and RNA polymerase occurred because mRNA was transcribed at a lower concentration. This resulted in reduced expression of aPKC. After collecting images, quantitative analysis measured the pixel intensity emitted by the fluorescent anti-

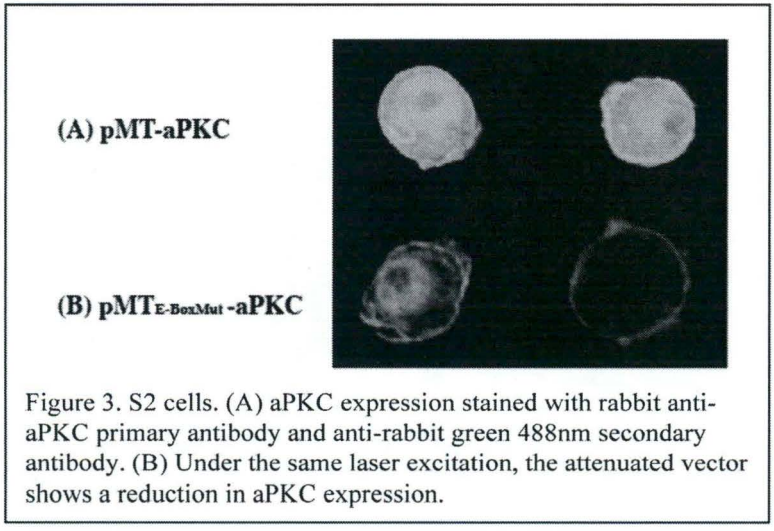

Figure 3. S2 cells. (A) aPKC expression stained with rabbit anti-aPKC primary antibody and anti-rabbit green 488nm secondary antibody. (B) Under the same laser excitation, the attenuated vector shows a reduction in aPKC expression.

bodies attached to aPKC in S2 cells. The collected data came from the same transfection session, and the analysis used the same settings on the confocal microscope. Those procedures helped to reduce variables that otherwise would interfere with the quantification. Results indicated that the attenuated pMT vector expressed a concentration of aPKC with abundance reduced by roughly 60 percent. (Figure 4). Regions measured for aPKC concentrations included the cell cortex, cytoplasm and nucleus. The areas showing the most significant reduction in aPKC concentrations were the cytoplasm and the nucleus (Figure 4). With these data, it is clear that the pMT-EboxMut controls the level of aPKC in S2 cells much as a volume knob regulates sound.

Materials and Methods

Construction of Mutant E-Box in pMT and S2 Expression

The E-box mutation was transformed from CANNTG to CTACCG using QUICKCHANGE mutagenesis. The vector and primer

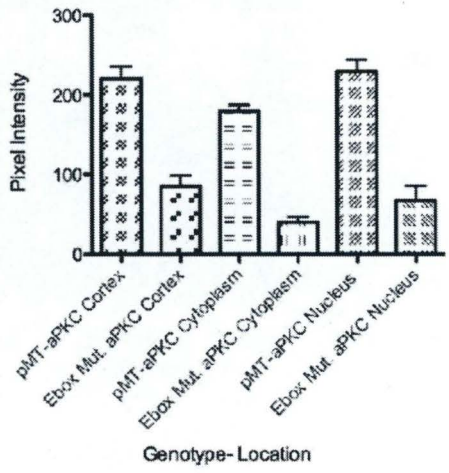

Figure 4. Relative fluorescent intensities in both regular and mutated E-Box pMT-aPKC expression based on cell location. Intensities are reduced to half or less upon expression with the attenuated vector. (pMT-aPKC Cortex, cytoplasm and nucleus n=25, EBox Mut. aPKC cortex, cytoplasm and nucleus n=27.)

samples underwent a polymerase chain reaction (PCR). The mutations were verified by DNA sequencing. The aPKC coding sequence was then inserted into the pMT and pMT E-box mutant vector at 5' BglII and 3' XhoI restriction sites. Drosophila Schneider (S2) cells were conserved in Schneider's medium with 10% fetal bovine serum (Sigma-Aldrich) at room temperature. Following procedures published by Wee et al. (2011),[6] transfection was carried out by adding approximately $3x10^6$ cells per well in a 6-well plate and transfected with 500 ng total DNA per well using the Effectene (QIAGEN) protocol. The cells were incubated for 24 hours. Gene expression was induced by adding 2.5µl of 0.5 mM CuSO4 for 24 hours.

Immunohistochemistry, Staining and Imaging

To facilitate antibody staining, 200 µl of S2 cells were placed on 12-mm-diameter glass cover slips in a 24-well plate and allowed to attach to the glass for 1 hour. The cells were fixed for 20 min with 4% paraformaldehyde in PBS followed by three rinses of wash buffer (0.1% saponin in PBS) and two rinses of block buffer (0.1% saponin and 1% BSA in PBS). The primary antibody used was rabbit anti-aPKC (1:1,000; Santa Cruz Biotechnology). Cover slips were incubated overnight with primary antibodies at 4°C and rinsed three times in block buffer followed by incubation with species-specific green (488 nm) secondary antibodies (Invitrogen) diluted in 1:500 block buffer at room temperature for 2 hours. Mounting was completed by rinsing the cells three times with washing buffer and then placing each cover slip in VectaShield Hard Set (No Dapi) solution. Images of the cells were obtained by using a BioRad confocal microscope at the objective of 60X using the program LaserSharp with the following settings: 12.8-Kr, 1.6-Iris, 8.5-Gain, and 1.5-Offset. All images were taken using the same laser intensity in the green laser channel of the confocal microscope. Images were analyzed using ImageJ and graphs were created with Prism.

Conclusion

Overall, protein expression of aPKC from the pMT-EboxMut vector was reduced. These data support the findings of Lavoie et al. 2008 in that a lower expression of protein follows the elimination of the E-Box palendromic quality, a procedure followed in this study. Until now, however, these results have never been seen in S2 cells. With the reduced expression of polarity protein in S2 cells, we are now able to study polarity protein localization. Reduction in protein expression from the pMT-EboxMut vector was exemplified by measuring the reduction in protein

fluorescent pixel intensities in S2 cells, which represents the amount of aPKC expression from each pMT vector. The pMT-EboxMut vector displayed a drop of 100 pixel units or more in the nucleus, cytoplasm and cell cortex, thus providing evidence that aPKC expression is less in the pMT-EboxMut vector compared to expression levels using the unaltered pMT vector. This research will aid in the development of a cultured protein polarity model cell that can be subjected to structure-function tests of aPKC and other proteins via genetic domain deletion. The pMT-EboxMut vector will aid in these studies because it acts as a protein expression volume knob that has been turned down. With the expression knob turned down, we are able to see exactly where aPKC is localizing in the S2 cell. In all, understanding the function of polarity proteins such as aPKC will bring us much closer to understanding protein polarity in general and its relation to the behavior of stem cell division and cancer. Here we have developed a more efficient and rapid technique of studying polarity protein localization with the pMT-EboxMut vector. Now we will be able to further our studies and understandings of the molecular nature of protein polarity and cancer at a faster pace.

Future Directions

With evidence of a reduction in protein expression by the pMT-EboxMut vector, we are able to express other polarity proteins in S2 cells to replicate the dynamics and localization observed in *drosophila melanogaster* neuroblasts. We may also use various aPKC constructs with deletions in various domains in order to understand how aPKC localizes to the cell membrane.

Sources:

1. Maroni, G., E. Otto and D. Lastowski-Perry, 1986 Molecular and cytogenetic characterization of a metallothionein gene of Drosophila. *Genetics* 112, 493-504.

2. Wakiyama, M., R. Muramatsu and Y. Kaitsu, 2011 Inducible protein expression in drosophila schneider 2 cells using the lac operator-repressor system. *Biotechnol Lett.* [Epub ahead of print].

3. Yuzenkova, Y., VR. Tadigotla and K. Severinov, 2011 A new basal promoter element recognized by RNA polymerase core enzyme. *EMBO J.* [Epub ahead of print].

4. Chaudhary, J; M. Skinner, 1999 Basic Helix-Loop-Helix proteins can act at the e-box within the serum response elements of the c-fos promoter to influence hormone induced promoter activation in Sertoli cells. *Molecular Endocrinology* 12, 774-786.

5. Lavoie, PL., L. Budry, A. Balsalobre and J. Drouin, 2008. Developmental Dependance on NurRE and Ebox Neuro for expression of pituitary proopiomelanocortin. *Molecular Endocrinology.* 22(7), 1647-1657.

6. Wee, B., CA. Johnston, KE. Prehoda and CQ. Doe, 2011. Canoe binds to RanGTP to promote PinsTPR/Mud-mediated spindle orientation. *J Cell Biol* 195, 369-376.

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