Final Progress Report K119223

Title: The role of the plasma membrane Ca^{2+} ATPase in cell motility

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Abstract: Cancer metastasis involves the migration of cancer cells from the original site of the tumor to distant places in the body. Actin cytoskeleton remodeling is a key factor in cancer cell migration, in which abnormal calcium signaling may play a role. The expression of the plasma membrane calcium pump PMCA4b that is essentially involved in maintaining cytosolic calcium homeostasis is often reduced in cancer. Recently, we identified PMCA4b as a metastasis suppressor that inhibited the migratory and metastatic activity of BRAF mutant melanoma cells. During the past project period we explored the pathways that regulate degradation of PMCA4b in melanoma cells, and how changes in its abundance affected actin cytoskeleton dynamics and cell migration. We demonstrated that 1/ PMCA4b is degraded through the endo/lysosomal system under regulation of the p38 MAPK pathway; 2/ PMCA4b stabilizes the actin cytoskeleton and maintains cell polarity through the control of cytosolic calcium levels; 3/ proper PMCA4b localization is required for actin cytoskeleton remodeling; 4/ PMCA4b expression can be induced by drugs already available such as the histone deacetylase inhibitor valproic acid; and 5/ female patients with high PMCA4 transcript levels had a significantly longer progression-free survival providing a gender specific prognostic impact for this pump in melanoma patients. The results from these studies may lead to new prognostic markers and/or procedures for melanoma in the clinic.

Introduction and goals

Cellular Ca^{2+} homeostasis is often remodeled during tumorigenesis resulting in an imbalance of survival and cell death. We and others have suggested that the *ATP2B4* gene encoding the plasma membrane Ca^{2+} pump PMCA4 plays a key role in this process (*see our recent review: Hegedűs et al. in Adv Exp Med Biol. 2020; 1131:93-129*). With the support of a previous research grant *ANN 110922* we identified the PMCA4b Ca^{2+} pump variant as a putative metastasis suppressor in BRAF mutant melanoma cells. An increase in PMCA4b level either by BRAF inhibition or by over-expression resulted in a remarkable reduction in both the migratory and metastatic activity of these cells.

90% of cancer patients die from tumor metastasis and cell migration is a key factor in this process, therefore, during the last project period covered by the K119223 grant we explored the pathways that controlled PMCA4b level in BRAF mutant melanoma cells, and the molecular mechanisms by which PMCA4b affected actin cytoskeleton dynamics, which is a major determinant of cell migration and metastasis. *During this research we were able to 1/ find the degradation pathways*

for PMCA4b; 2/ identify the role of PMCA4b in cytoskeletal dynamics; 3/ determine that proper PMCA4b sorting was required for actin cytoskeleton remodeling; and 4/ determine how changes in PMCA4b abundance affected distribution of cytosolic Ca^{2+} concentration in migrating BRAF mutant cells.

Major new findings during the research supported by the K119223 grant

1. PMCA4b is degraded through the endo/lysosomal system. Using immunofluorescence and confocal microscopy we determined the subcellular localization of PMCA4b and found that in BRAF mutant melanoma cells a substantial amount of PMCA4b co-localized with the late endosomal marker Rab7 and the lysosomal marker Lamp1. Inhibition of lysosomal function greatly increased PMCA4b abundance in the Lamp1 positive lysosomal compartment suggesting that PMCA4b is degrading through the endo/lysosomal system. These data indicate that enhanced lysosomal degradation contributes to the low PMCA4b abundance in BRAF mutant melanoma cells, and probably in other tumor cell types, as well (*Naffa et al. Cells, 2020*).

2. p38 MAPK promotes PMCA4b internalization and degradation. We tested the role of the signaling pathways p38 MAPK, c-jun N-terminal kinase (JNK) and nuclear factor kappa B (NFkB) in controlling PMCA4b abundance in BRAF mutant melanoma cells. We found that specific inhibitors of p38 MAPK greatly enhanced PMCA4b plasma membrane levels and diminished its intracellular localization while other inhibitors had no effect. To further prove that p38 MAPK was responsible for the enhanced PMCA4b internalization we used a HEK-mcherry-MKK6-Dox cell line. We showed that doxycycline induced PMCA4b internalization that was prevented by p38 MAPK inhibitors confirming the role of p38 MAPK in PMCA4b internalization. $BRAF^{V600E}$ mutation is associated with enhanced p38 MAPK activation that is involved in both melanoma development and chemo resistance. Inhibition of p38 MAPK reduced BRAF mutant melanoma cell migration partly through PMCA4b providing support for the advantage of using drugs targeting p38 MAPK (*Naffa et al. Cells, 2020*).

3. Epigenetic and hormonal control of PMCA4b expression in melanoma and breast cancer cells. Metastasis suppressors are usually downregulated in tumor progression and increasing their abundance is a great challenge in cancer therapy. We found that the expression of the putative metastasis suppressor PMCA4b was under epigenetic control, and treatment of both melanoma and breast cancer cells with histone deacetylase inhibitors (HDACi) resulted in a substantial increase in its expression. The increased PMCA4b abundance in the HDAC inhibitor treated cells caused strong inhibition in both random and directional movements of BRAF mutant melanoma cells. Since about 70% of breast cancers expresses ER-α, and inhibition of the ER-α receptor is an important tool in the treatment of breast cancers, we studied the role of ER-α-mediated signaling pathways in the regulation of PMCA4b expression. We found that 17β-estradiol E2 treatment increased PMCA4 expression, and fulvestrant (the specific ER-α inhibitor also used in cancer therapy) inhibited this effect. Valproate – which is an FDA-approved HDACi already used in clinical trials against breast cancer – further augmented PMCA4b upregulation.

Altogether, our results suggest that drugs targeting ER-α and epigenetic pathways may prevent PMCA4b downregulation in breast cancer and melanoma patients (*Hegedűs et al. Frontiers in Oncology, 2017; Varga et al. BMC Cancer, 2018*).

4. The role of PMCA4b in controlling the shape and F-actin dynamics of melanoma cells. Over-expression of PMCA4b in BRAF mutant melanoma cells induced a switch from a fast random moving to a persistent slow moving cell phenotype. Introducing PMCA4b in these cells resulted in a dramatic rearrangement of the actin cytoskeleton: a complete loss of protrusions, enhanced stress fiber formation and a typical mesenchymal-type front-to-rear polarity. Fluorescence recovery after photobleaching (FRAP) experiments showed no significant changes in the recovery rate and mobile fraction of F-actin suggesting that actin polymerization was not affected by PMCA4b. In contrast, PMCA4b expression resulted in cofilin rearrangement and increased F-actin stability suggesting that a reduced depolymerization rate could play a role. We showed that the typical morphology changes induced by PMCA4b expression was not restricted to melanoma cells as similar changes in morphology and actin cytoskeleton rearrangement was seen in MCF-7 breast cancer cells suggesting that PMCA4b plays a general role in the regulation of actin dynamics (*Naffa et al. Cancers 2021*).

5. PMCA4b stabilizes F-actin by reducing near-actin Ca^{2+} concentration. We demonstrated that PMCA4b induced a front-to-rear increasing cytosolic Ca^{2+} concentration gradient in BRAF mutant melanoma cells, a characteristic pattern of migratory mesenchymal cells. While a large increase in cytosolic Ca^{2+} concentration induced a dramatic loss of cell protrusions, intense membrane blebbing and cell shrinkage in the control cells with low PMCA4b abundance or in the cells expressing a non-functional mutant PMCA4b pump, the cells over-expressing the wild-type PMCA4b pump were able to retain their original shape and F-actin bundles indicating that PMCA4b function was essential in stabilizing the actin cytoskeleton even at high Ca^{2+} concentration levels (*Naffa et al. Cancers 2021*).

6. Proper PMCA4b trafficking is needed for cell polarity and reduced cell motility. We analyzed if endocytosis/recycling played a role in the anti-migratory function of PMCA4b. For these studies we used the PMCA4b-LA mutant, in which a di-leucine internalization signal was mutated that impaired endocytosis while it did not affect pump activity. Over-expression of this trafficking mutant did not affect F-actin arrangement, did not induce a Ca^{2+} concentration gradient, and the cells retained their high migratory potential suggesting that proper PMCA4b trafficking/sorting was essential for the preferred slow migratory phenotype switch (*Naffa et al. Cancers 2021*).

7. Prognostic relevance. In a more recent study, we showed that PMCA4 mRNA levels have a gender specific prognostic impact in stage I–III melanoma so that female patients with high transcript levels had a significantly longer progression-free survival. This is an interesting finding since PMCA4b expression is controlled by estradiol in breast cancer cells (*Varga et al. BMC Cancer, 2018*). Moreover, high PMCA4 transcript levels derived from RNA-seq of cutaneous melanoma are associated with significantly longer overall survival after PD-1 blockade (*Hegedus*

et al. IJMS 2022).

Impact

7 scientific papers (cumulative IF: 42,21) and a book chapter published in international journals were supported fully or partially by the present grant. In addition, the project resulted in 2 oral and 11 poster presentations at international and national conferences. Five students completed their master thesis, and three students their PhD dissertations using the results of the past research grant support. Dr. Enyedi is invited to a Gordon conference this year to present the data of the present grant on PMCA and cancer metastasis (*https://www.grc.org/calcium-signaling-conference/2022/*).

Unexpected events during the project

Because of the COVID pandemic and moving the laboratory to another department a 1.5-year extension of the grant period was needed. The original research plan could be fully completed and the data published during this time. Along with the extension all financial and personnel changes have been approved by the NRDI office.

References:

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Cumulative impact: 42,21