REGULATION OF EXTRACELLULAR VESICLE POPULATIONS RELEASE IN GLIOBLASTOMA CELLS BY PYK2 AND MEK/ERK SIGNALING

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Abstract

Glioblastoma (GBM), the deadliest brain cancer, is typically fatal within a year despite treatment. Tumor-associated myeloid cells (TAMs) support GBM growth, treatment resistance, and immune surveillance via extracellular vesicles (EVs), which exchange biomolecules. EVs travel through the bloodstream, triggering signaling pathways in distant organs, recruiting cells, and modulating immune responses, crucially regulating the tumor immune microenvironment. We hypothesize that Pyk2/MEK/ERK signaling regulates EV release by modulating the actin cytoskeleton, impacting TAM activation in GBM. This study aims to investigate the role of Pyk2/MEK/ERK in EVs release in GBM cells, potentially offering insights into therapeutic targets. Two humans primary GBM cell lines with and without Pyk2 CRISPR/Cas9 knock-out (Pyk2KO) were used. Confocal imaging and flow cytometric analysis of EVs, purified from cell conditioned medium were employed. The study identified that knocking-out Pyk2 shifted EVs to the population of smaller diameter. Using Integrin as plasma membrane marker, we identified 84.70% Integrin+ and 15.30% Integrin- EV's, purified from medium conditioned from WT cells, compared to 89.07%/10.93% from Pyk2KO cells, respectively. Treatment with MERK/ERK inhibitor Avutometinib altered EVs populations to 79.11% Integrin+ and 20.89% Integrin-. Combining Avutometinib with Pyk2 knock-out resulted in 78.83% Integrin+ EV and 21.17% Integrin-.Pyk2 is responsible for the upregulation of EVs derived from the endoplasmic reticulum. Conversely, MEK/ERK signaling primarily enhances the release of EVs shed from the plasma membrane. Overall, these findings underscore the interplay between Pyk2, ERK/MEK signaling, and plasma membrane dynamics in regulating EV biogenesis and composition.

Introduction

GBM is the most aggressive brain cancer and usually fatal within a year after diagnosis, regardless of treatment protocol. Current treatment approaches provide only modest, few months, life extension. EVs are a heterogeneous group of cell-derived membranous structures comprising exosomes (ES) and microvesicles (MV), which originate from the endosomal system or shed from the plasma membrane, respectively. EVs are critical mechanisms of cell-to-cell communication, allowing cells to exchange proteins, lipids, RNA and genetic material (Tkach et al, 2016). Once attached to a target cell, EVs can induce signaling via receptor-ligand interaction or can be internalized by endocytosis or fused with the target cell's membrane to deliver their content into its cytosol, thereby modifying the physiological state of the recipient cell.

Our preliminary data and literature reports identified that EVs, released from GBM cells, contain cytokines and mRNA for CCL2, CCL12, CCL5, that are involved in regulation of chemoattraction and polarization of tumor infiltrating myeloid cells, thus regulating tumor immune microenvironment (Cherry et al., 2020; Popiolek-Barczyk et al., 2020; Skuljec et al., 2021). As EVs can enter circulation and distant tissues, they promote signaling and metabolic modifications of distant organs, the recruitment of bone-marrow-derived cells, and regulation of immune response. Thus, EVs represent a mechanism of distant regulation between cells of different genesis within same organ and in between of organs. It was shown that Pyk2 is involved in the release of EVs through the activation of RhoA and ERK signaling, leading to actin cytoskeleton reconstruction and vesicles release (Zhong at al., 2021; Okigaki et al. 2003). This study investigated the role of Pyk2 in regulating the biogenesis and protein and mRNA content of EVs through the MEK/ERK signaling pathway.

Purpose

This study aims to investigate the role of Pyk2/MEK/ERK in EV populations release in GBM cells, potentially offering insights into therapeutic targets.

Hypothesis

We hypothesize that Pyk2/MEK/ERK signaling regulates the release of exosomal and microvesicle populations, as well as the presence of CCL2, CCL12, CCL5, TNF, and VEGF cytokines/chemokines proteins and mRNA within EVs in GBM.

Methods

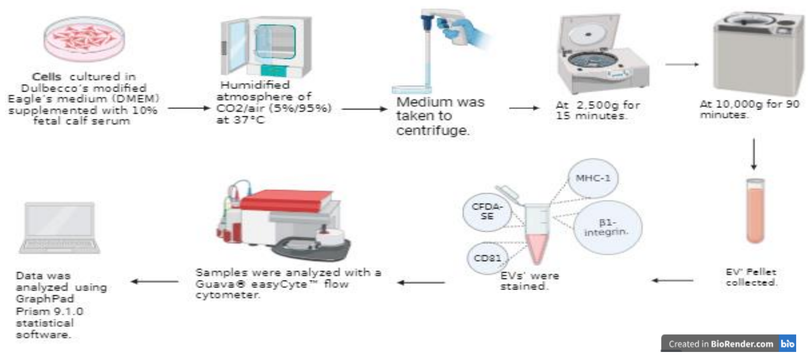

Figure 1. Extracellular vesicles (EVs) isolation and flow cytometric analysis. Cell conditioned medium underwent serial centrifugations to collect the EV-containing pellet. Flow cytometric analysis of EVs utilized staining with CFDA-SE and CD81 for total EV markers, as well as MHC-I and β1-integrin for microvesicle (MV) markers. Gating based on FSC/CFDA-SECD81was performed to identify EV populations, followed by analysis for MV and exosome (ES) populations using MHC-I and β1-integrin expression. Forward Scatter analyses was used for the EV size identification. Compensation was executed using single-fluorophore-labeled compensation beads before each experiment.

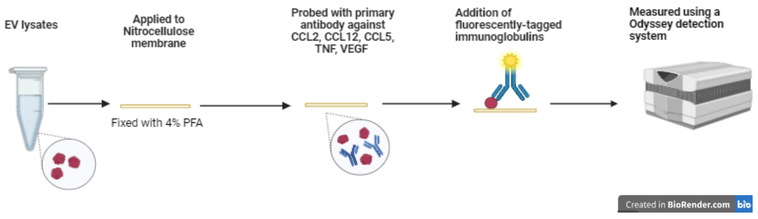

Figure 2. Dot blot analysis: Primary antibody against CCL2, CCL12, CCL5, TNF, VEGF (Cell Signaling) followed by corresponding Ig were used. The signal intensity was measured and analyzed using an Odyssey detection system (Lycor).

Results

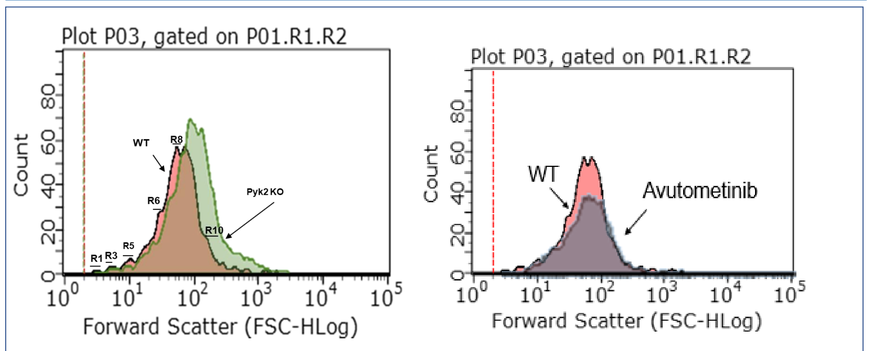

Figure 3. Pyk2 knock-out and MEK/ERK Inhibition result in reduction of Extracellular Vesicle's size in Primary GBM Cells. Flow cytometric analysis of EV's size. CFDA-SE was used as a marker of EV.

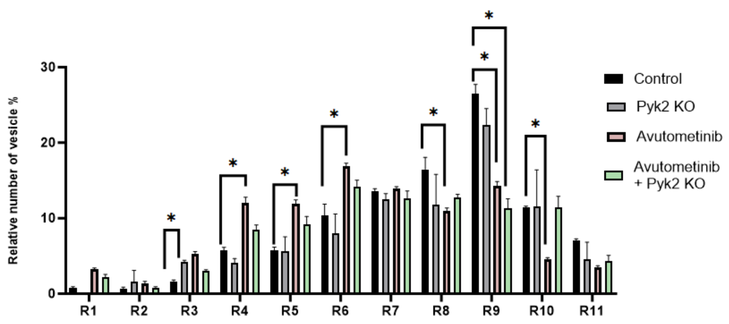

Figure 4. The effect of MEK/ERK inhibition with Avutometinib (1μM) on extracellular vesicle (EV) total size populations in primary GBM cells with and without Pyk2 knock-out. Flow cytometric analysis of EV size obtained from 965 cell line, stained with CFDA-SE, was performed. A shift to smaller sizes was detected in both Pyk2 KO EVs and upon MEK/ERK inhibition. Avutometinib treatment significantly (*, P<0.05) decreased the number of vesicles in R8-R11 populations and increased R3-R6 populations compared to the control. Slight shift toward smaller size of EV was detected in Pyk2 KO cells. Bars represent mean+/- SEM.

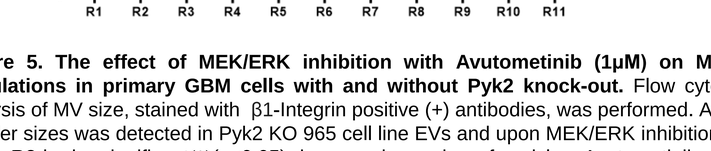

Figure 5. The effect of MEK/ERK inhibition with Avutometinib (1μM) on MV size populations in primary GBM cells with and without Pyk2 knock-out. Flow cytometric analysis of MV size, stained with β1-Integrin positive (+) antibodies, was performed. A shift to smaller sizes was detected in Pyk2 KO 965 cell line EVs and upon MEK/ERK inhibition. Pyk2 KO in R8 had a significant(*)(p<0.05) decrease in number of vesicles. Avutometinib resulted in an increase R3-R6, and reduction in R7-11 populations compared to control. Bars represent mean+/- SEM.

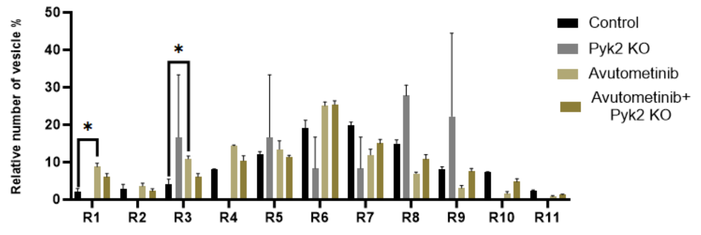

Figure 6. The effect of MEK/ERK inhibition with Avutometinib (1μM) on ES size populations in primary GBM cells with and without Pyk2 knock-out. Flow cytometric analysis of size of β1-Integrin negative(-) ES, was performed. Total reduction of ES, released from Pyk2 KO cells, comparing with WT cells, was detected. Mean ± S.E. and significant difference from R1 and R2 is shown (*,p < 0.05).

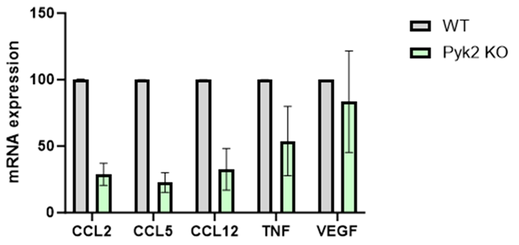

Figure 7.The Effect of Pyk2 on extracellular vesicles content of mRNA cytokines, involved in immune and inflammatory responses. RT-PCR analysis for CCL2, CCL12, CCL5, Tumor necrosis factor (TNF) and Vascular Endothelial Growth Factor (VEGF) mRNA content in EV, released from WT and Pyk2KO primary human GBM cells was performed. Reduction of mRNA for CCL2, CCL5 and CCl12 and TNF in EV, released from Pyk2KO cells compared to WT cells was observed.

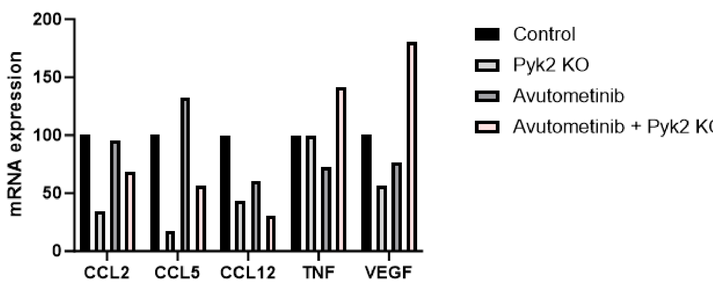

Figure 8. The effect of MEK/ERK inhibition with Avutometinib (1μM) EV content of mRNA chemokine and cytokines involved in immune and inflammatory responses. Dot Blot analysis for CCL2, CCL12, CCL5, TNF and VEGF identified reduction of CCL2, CCL5 and CCl12 in EV, released from Pyk2 KO cells, compared to WT cells.

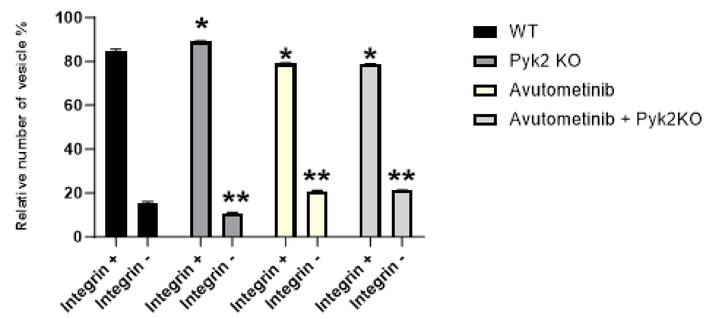

Figure 9. The effect of MEK/ERK inhibition with Avutometinib (1μM) on MV size populations with and without Pyk2 knock-out in primary GBM cells stained with Integrin(Ig). Flow cytometric analysis of EV size obtained from 965 cell line, stained with Integrin. Treatment with MEK/ERK inhibitor Avutometinib altered EVs populations to 79.11% Ig(+) a significant (*,p < 0.0001). and 20.89% Ig(-) a significant (**,p < 0.001). compared to control. Combining Avutometinib with Pyk2 knock-out resulted in 78.83% Ig(+) a significant (*,p < 0.0001). EV and 21.17% Ig(-).Pyk2 a significant (**,p < 0.001). compared to control is responsible for the upregulation of EV's derived from the endoplasmic reticulum. Bars represent mean+/- SEM.

Conclusions

- Pyk2 is responsible for the upregulation of EV's derived from the endoplasmic reticulum.
- MEK/ERK signaling primarily regulates the release of EVs shed from the plasma membrane.
- CCL12, CCL5, TNF and CCL2 protein and mRNA content is Pyk2 dependent.
- Pyk2 plays a role in regulating the release of inflammatory cytokines through the extracellular vesicle mechanism.

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