Role of BMP4 and RHOA Signalling in Breast Cancer

Abstract

Breast cancer is the second leading cause of cancer-related death in women worldwide and most of these deaths are due to metastatic breast cancer. Metastatic breast cancer is not easily treatable since it is invasive, and spreads to several organs. Targeted therapy is available for some breast cancer subtypes, based on their receptor expression and tumour stage. However, triple-negative breast cancer (TNBC), which lacks estrogen, Her2, and progesterone receptors, is aggressive and does not respond to hormone therapy. Surgery and chemotherapy are still the standard treatments for TNBC, however, they are effective only in the early stages. Therefore, one of the ways to effectively combat cancer is to understand the underlying signalling pathways that drive them so that they can be targeted for therapy. Although there is a myriad of interconnected signalling pathways that get activated in the spread of cancer, we have focused mainly on the BMP, WNT, and RHOA signalling pathways.

Four model breast cancer cell lines ZR75.1 (ER^+ PR^+ $HER2^+$), MCF7 (ER^+ PR^+ $HER2^-$), SKBR3 (ER^- PR^- $Her2^+$), and MDA-MB -231 (ER^- PR^- $HER2^-$) were used in this study. As per the cell surface marker expression, ZR75.1, MCF7 and SKBR3 are $CD24^+$ and $EpCAM^+$, while MDA-MB-231 is $CD44^+$. The three-dimensional spheroid growth represents that ZR75.1, MCF7, and MDA-MB-231 form dense, compact spheroids, whereas SKBR3 failed to form spheroids. Also, the migration analysis revealed that ZR75.1 and SKBR3 are majorly non-migratory, which may be responsible for tumour growth confined to a specific area, whereas MCF7 migrated at a rate of 2-3 µm/h and MDA-MB -231 had a migration speed of 6-8 µm/h, which correlates with their metastatic potential. The differential expression of BMP4, RHOA and β-catenin in these cell lines were analysed and accordingly, SKBR3, MCF7 and MDA-MB-231 were used for studying the BMP signalling and RHOA and Wnt signalling were analysed in detail in MCF7 and MDA-MB-231 cells.

Bone morphogenetic proteins, often known as BMPs, are proteins that control the fate of developing cells and also play a role in the progression of cancer. In this study, we explored the role of BMP4 in the proliferation of breast cancer cells, as well as its function in anoikis resistance, metastatic migration, and therapy resistance. In order to gain an understanding of the functional effect that BMP4 has on breast cancer, we used breast cancer cell lines as well as clinical samples that represent different subtypes. The involvement of the BMP pathway in breast cancer cells was further studied by utilizing the small molecule inhibitor LDN193189

hydrochloride (LDN), a BMP receptor inhibitor. Activation of BMP signalling through exogenous addition of BMP4 led to an increase in the expression of stem cell genes *CD44* and *ALDH1A3*, as well as the anti-apoptotic gene *BCL2*, and boosted anoikis resistance in MDA-MB-231 cells. Additionally, LDN treatment downregulated anoikis resistance and the proliferation of anoikis-resistant breast cancer cells in an osteogenic milieu. BMP4 upregulated Notch signalling, which resulted in increased chemoresistance and accelerated self-renewal of MDA-MB-231. Conversely, BMP4 decreased anoikis resistance in MCF7 and SKBR3 cells, and downregulated proliferation, and colony-forming abilities, while LDN treatment enhanced the formation of tumour spheroids and growth. These findings suggest that BMP4 plays a context-dependent role in breast cancer cells. Furthermore, our findings with MDA-MB-231 cells, which represent triple-negative breast cancer, implying that BMP pathway inhibition may hinder the tumour's ability to colonise new sites and expand metastatically.

To better understand the signalling pathway that regulates the metastatic migration of breast cancer cells, the well-known RHO-ROCK signalling pathway associated with actin organisation was investigated. Breast cancer cells were treated with Y27632, a putative inhibitor of Rho-associated protein kinase (ROCK). Inhibition of ROCK significantly reduced 3D spheroid formation in MCF7 and SKBR3 cells but not in MDA-MB -231 cells. In MDA-MB -231, however, there was a decrease in the $CD44^{+}/24^{-}$ population, suggesting that ROCK is primarily responsible for cell proliferation. Both MDA-MB -231 and MCF7 breast cancer cells showed enhanced 2D (wound healing assay) and 3D migration (spheroid migration assay) when ROCK was inhibited. Additionally, the inhibition of ROCK resulted in a significant increase in the clonogenic potential of MCF7, while it had no impact on the proliferation of MDA-MB-231 cells. Thus, this part of the study suggests that an active RHOA pathway is required for proliferation, while downregulation of ROCK is required to promote metastasis and survival during breast cancer development.

Furthermore, both RHOA and β-catenin were silenced in order to obtain a comprehensive view of the effects of Wnt signalling on the progression of breast cancer. Multiple functional assays and gene expression analyses confirmed that silencing RHOA and β-catenin in MCF7 significantly reduced proliferation, self-renewal, migration, anoikis resistance, and shear stress tolerance. In contrast, self-renewal was inhibited in MDA-MB-231 by suppressing RHOA and β-catenin, but 3D growth and migration remained unaffected. Nevertheless, the most intriguing observation is that a higher expression of ERK under stress conditions provides a new perspective on where further research should be conducted by considering the ERK signalling in context. Doxorubicin treatment increased the ability of

MDA-MB-231-shRHOA cells to form spheroids. The RHOA-silenced MDA-MB-231 cells displayed greater tolerance to shear stress, as evidenced by a greater number of colonies, whereas β-catenin silencing significantly reduced the shear stress tolerance of MDA-MB -231.

The immunoblotting analysis further revealed that suppressing RHOA in MDA-MB-231 can upregulate β-catenin expression, indicating an increase in canonical WNT signalling. β-Catenin silencing in MDA-MB-231 decreased pERK1/2, but RHO silencing did not affect pERK1/2 levels. This finding suggests the existence of a cross-talk between the canonical and non-canonical pathways of WNT signalling in TNBCs, in which inhibition of one pathway leads to an increase in the activity of the other. The increased expression of pERK1/2 during shear stress and spheroid formation suggests that ERK signalling becomes hyper-activated when cells are subjected to stress. Gene expression analysis was performed to determine the interaction between BMP, RHOA, and WNT signalling. Concerning cross-talk, there appears to be a compensatory relationship between RHOA and β-catenin in a stage-specific manner in both cell lines. It has been observed that the Wnt/β-catenin signalling pathway modulates the BMP4 pathway, and BMP4 regulates RHOA signalling via some unknown transcription factors. Thus, there is a possibility that inhibiting Wnt signalling could serve as a potential treatment option for breast cancer. More research is needed to establish a comprehensive link between these three pathways and locate a central point at which the entire signalling cascade can be inhibited to control the progression of cancer.