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Investigating the possible effects of AKT and FAK signaling pathways' inactivation on TNBC cell proliferation and TNBC patients' recurrence free survival via BAG 3 regulation

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Abstract. Currently, a lethal breast cancer called TNBC (triple-negative breast cancer) has been researched for decades. Related research has shown the mechanism of this cancer and the ways to recover it. According to the research, two signal pathways called AKT and FAK, existing downstream of BAG 3 (BCL2- associated athanogene3), were found as two triggers to positively regulate BAG 3 thus causing TBNC cell proliferation. Therefore, the question is: what is the effect of BAG 3 when AKT and FAK are inactivated? Will BAG 3 still be transcribed to cause TNBC cell proliferation? This paper lists all possible results of TNBC numbers (in petri dish) and tumor volumes (in mice as xenograft) changing by inactivating AKT and FAK, respectively, inactivating both or not inactivating, and lists possible reasons that cause these results.

Keywords: TNBC (triple-negative breast cancer), BAG3, AKT, FAK, recurrence-free survival.

1. Introduction

TNBC, the abbreviation of triple-negative breast cancer, is a usually uncurable cancer that has a lack of amplification of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The patient of TNBC has a poor prognosis and high recurrence-free survival [1]. The further study reported in October 2010 at the European Society for Medical Oncology meeting it suggests EGFR is a significant target in TNBC. Also, by further research, BAG3, which is the abbreviation of BCL2- associated athanogene3, can positively regulate EGFR signal transduction pathways, thus promoting tumor cell proliferation and reducing the expression of BAG3 by silencing results a small EGFR expression [2,3].

The research paper published on March 20, 2018, showed that the higher the expression of BAG3 RNA in the TNBC cell line, the poorer disease-free survival TNBC patients have [2]. This research showed the idea that expression of BAG3 correlates to high TNBC recurrence free survival, and through controlling BAG3 expression, TNBC patients may have low TNBC recurrence free survival, thus creating significant therapeutic value in TNBC.

Moving on previous research has shown that the reason BAG3 positively regulates the proliferation of TNBC cell lines is because it positively regulates TNBC via two signaling pathways called AKT and FAK. According to previous research, through silencing the expression of BAG3, the activation of signal pathways AKT and FAK will decrease [2,3]. Through a hypothetical model of potential regulation of signaling pathways that are downstream of EGFR, the activation of FAK will result in the proliferation,

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migration, and invasion of TNBC cells. In addition, the activation of AKT will cause the activation of mTOR signaling pathway or expression of IKK (IkappaB kinase) [2], thus contributing to the proliferation, migration, and invasion of TNBC cells. Therefore, the above studies state that the expression of BAG3 promotes the activation of the signal channels AKT and FAK, which makes TNBC cells proliferate and even invade, leading to TNBC patients having low recurrence-free survival.

Nevertheless, although it is shown in the hypothetical model that BAG3 expression causes TNBC cell proliferation by activating AKT and FAK signaling pathway, and there are many experimental data to confirm the above experimental results, no experiments so far have verified whether BAG3 also affects the activation of other signaling pathway or the expression of some proteins that further results in the proliferation of TNBC cells. Therefore, as for this point, the hypothesis is that BAG3 will positively regulate TNBC cell proliferation and recurrence of TNBC disease not only through FAK and AKT signaling pathways in BT549 cells, but also other pathways in vitro and BT549 xenograft. This work will treat BT549 WT, which is the one has BAG3 with increasing amounts of AKT inhibitor LY2780301 or FAK inhibitor PF-573228 for various durations with EGF ligands treatment and measure proliferation of cells with MTT assay or cell counting, and tumor weight and size in the xenografts comparing with the BT549 cells without FAK and AKT inhibitor treatment. Measure FAK and AKT activating phosphorylation by western blot to make sure the inhibitors are functioning properly. Through this hypothesis, this work will deactivate the two signaling pathways of AKT and FAK, and ensure the expression of BAG3, so as to study my research question that is what the effects of BAG3 in TNBC cell proliferation is when AKT and FAK signaling pathways is inactivated, thus understanding the effects of BAG3 on TNBC in addition to activating the two signaling pathways of AKT and FAK and know the other role of BAG3 in proliferation of TNBC beside AKT and FAK activation. This question is really important, because figuring out the influence of BAG3 in TNBC completely could be a significantly therapeutic value in preventing cancer recurrence.

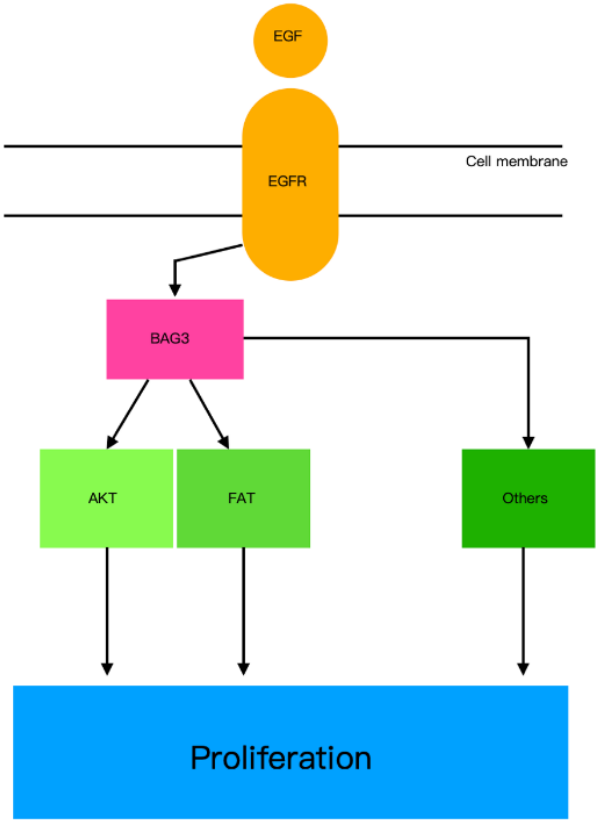

Figure 1. The interaction between BAG3, AKT, FAK in my suspected way.

DOI: 10.54254/2753-8818/45/20240784

The process of activation of EGFR and some other downstream signaling pathways will cause TNBC cell proliferation in the end, as shown in Figure 1.

2. Materials and methods

2.1. Cells, Chemicals, Animals, and Methods

2.1.1. Cells. BT549 cell line will be used in this experiment. BT549 is a cell taken from a 72-year-old white breast cancer patient. It contains various receptors and signal pathways that cause breast cancer. Therefore, it is often used for breast cancer research. The BAG3 WT will be used as the way to set the BT549 cells that contains BAG3 proteins, and I am going to use FAK and AKT inhibitor to treat the cell to see whether it proliferate or not compared with BAG WT without inhibitors treatment [4].

2.1.2. Chemicals. EGF is called epidermal growth factor. In this experiment, it should be used on the BT549 cell line. This is because the activation of AKT and FAK signaling pathways of EGFR is controlled by BAG3, but no matter what happens to the downstream signaling pathway of EGFR, it can be carried out only when EGF ligands are combined with it. Additionally, AKT inhibitor LY2780301 and FAK inhibitor PF-573228 need to be provided in the BAG3 WT. By doing this, I can understand whether the proliferation of the TNBC is related to BAG3's effects on AKT and FAK. The amount of these two prohibitions needs to be exactly the same in each group of BAG WT, and I am going to create 3 groups of BAG WT with LY2780301, 3 groups of BAG WT with PF-573228, 3 groups of BAG WT with LY2780301 and PF-573228. In this experiment, a sufficient amount of EGF ligands should be prepared to bind to EGFR on cells. The ligand must bind to the receptors of the 4 sets of groups of BT549 cells, respectively, resulting in the control of the amount of change.

2.1.3. Animals. 100 female nine-week-old adult mice were used as xenograft for research. The use of mice must strictly comply with AAAALAC guidelines. Once the experiment is completed or any discomfort besides the experiment is found, the mice will be euthanized immediately.

2.1.4. Western blot. Western blot will be used to test whether the FAK and AKT in the cell are functioning properly. Western blots were performed on all 3 groups of BAG3 WT under different inhibitor treatments, respectively. This process will use a total of 12 cell cultures.

2.1.5. MTT assay. In order to know the proliferation effect of BAG3 WT with the treatment of AKT, FAK, non, or both, MTT assay will be used. All 3 groups from all the sets of BAG3 WT with different inhibitor treatments should go through the process of MTT, respectively. This process will use a total of 12 cell cultures.

2.2. The in vitro study

2.2.1. In vitro cell culture. BT548 cells were routinely cultured in RPMI-1640 medium (GIBCO) and 10% fetal bovine serum (FBS, GIBCO). They were cultured at 37 ℃ in 5% CO2 wet air, containing 21% oxygen or 1% oxygen. The total volume of the culture medium and cells should be 30ml [5].

2.2.2. Cell proliferation assay. The BAG3 WT needs to be separated into 4 groups. The first group BAG3 WT, is treated with FAK inhibitor. The second group of BAG3 WT is treated with AKT inhibitor. The third group of BAG3 WT is treated with both AKT and FAK inhibitors. The fourth group is treated by non AKT and FAK inhibitors, which is normal saline. The above steps must be completed simultaneously within one day. All these experiments need to be done at least 3 times, which creates 3 groups of BAG3 WT with AKT, 3 groups of BAG3 WT with FAK, 3 groups of BAG3 WT with AKT and FAK, 3 groups of BAG3 WT with normal saline without AKT and FAK.

DOI: 10.54254/2753-8818/45/20240784

2.2.3. Result record. After 2 days of daily treatment, take out the cell culture of the two groups of experiments, observe the cell proliferation through MTT assay, and record the test results.

2.3. The xenograft studies

2.3.1. Cells and animals' preparation. Prepare 120 female experimental mice, BAG3 WT. The cell fluid of BAG3 WT with AKT was injected into the arteries of 30 female mice; the BAG3 WT with FAK was injected into the arteries of 30 female mice; the BAG3 WT with FAK and AKT was injected into the arteries of 30 female mice; the BAG3 WT without FAK was injected into the arteries of 30 female mice.

2.3.2. Feed mice. The 100 experimental mice were raised in the same room in an adaptive cage for 2 weeks. The amount of food and drinking water provided by each mouse shall remain the same every day, and try to ensure that the mice can grow normally without accidental death due to external influence.

2.3.3. Result record. Two weeks later, observe and note down tumor values that the mice have in different groups of BAG3 WT.

3. Possible results

Table 1. Cell proliferation (growth) with inhibitor AKT, FAK, or no inhibitor in vitro through MTT assay.

Method	No inhibitor MTT	AKT inhibitor MTT	FAK inhibitor MTT	AKT and FAK inhibitor MTT
Detecting substance	Cell growth	Cell growth	Cell growth	Cell growth
Result 1 (Expected)	+	+	+	+
Result 2	+	+	+	-
Result 3	+	+	-	+
Result 4	+	-	+	+
Result 5	+	-	+	-
Result 6	+	-	-	+
Result 7	+	+	-	-
Result 8 (Completely not Expected)	+	-	-	-
Result 9	-	-	-	-
Result 10	-	-	-	+
Result 11	-	-	+	-
Result 12	-	+	-	-
Result 13	-	+	-	+
Result 14	-	-	+	+
Result 15	-	+	+	-
Result 16	-	+	+	+

Table 2. Tumor volume (growth) with inhibitor AKT, FAK, or no inhibitor in xenograft (female mice).

Method	No inhibitor mice	AKT inhibitor mice	FAK inhibitor mice	AKT and FAK inhibitor mice
Detecting substance	Tumor volume	Tumor volume	Tumor volume	Tumor volume
Result 1 (Expected)	+	+	+	+
Result 2	+	+	+	-
Result 3	+	+	-	+
Result 4	+	-	+	+

DOI: 10.54254/2753-8818/45/20240784

Table 2. (continued)

Result 5	+	-	+	-
Result 6	+	-	-	+
Result 7	+	+	-	-
Result 8 (Completely not Expected)	+	-	-	-
Result 9	-	-	-	-
Result 10	-	-	-	+
Result 11	-	-	+	-
Result 12	-	+	-	-
Result 13	-	+	-	+
Result 14	-	-	+	+
Result 15	-	+	+	-
Result 16	-	+	+	+

Table 1 shows the possible results of TNBC proliferation under several treatment in vitro. Table 2 shows the possible results of TNBC (tumor) proliferation under several treatment in xenograft (female mice).

3.1. In vitro

Result 1: BAG3 WT grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

Result 2: BAG3 WT grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 3: BAG3 WT grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

Result 4: BAG3 WT grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

Result 5: BAG3 WT grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 6: BAG3 WT grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

Result 7: BAG3 WT grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 8: BAG3 WT grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 9: BAG3 WT does not grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 10: BAG3 WT does not grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

Result 11: BAG3 WT does not grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 12: BAG3 WT does not grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 13: BAG3 WT does not grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT does not grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

Result 14: BAG3 WT does not grow under no inhibitor; BAG3 WT does not grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

Result 15: BAG3 WT does not grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT does not grow under two inhibitors.

Result 16: BAG3 WT does not grow under no inhibitor; BAG3 WT grow under AKT inhibitor; BAG3 WT grow under FAK inhibitor; BAG3 WT grow under two inhibitors.

DOI: 10.54254/2753-8818/45/20240784

3.2. In xenograft

Result 1: Tumor volume grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume grow under two inhibitors.

Result 2: Tumor volume grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 3: Tumor volume grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume grow under two inhibitors.

Result 4: Tumor volume grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume grow under two inhibitors.

Result 5: Tumor volume grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 6: Tumor volume grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume grow under two inhibitors.

Result 7: Tumor volume grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 8: Tumor volume grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 9: Tumor volume does not grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 10: Tumor volume does not grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume grow under two inhibitors.

Result 11: Tumor volume does not grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 12: Tumor volume does not grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 13: Tumor volume does not grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume does not grow under FAK inhibitor; Tumor volume grow under two inhibitors.

Result 14: Tumor volume does not grow under no inhibitor; Tumor volume does not grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume grow under two inhibitors.

Result 15: Tumor volume does not grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume does not grow under two inhibitors.

Result 16: Tumor volume does not grow under no inhibitor; Tumor volume grow under AKT inhibitor; Tumor volume grow under FAK inhibitor; Tumor volume grow under two inhibitors.

4. Discussion

TNBC is a very serious breast cancer, which can cause very serious injury to patients and is extremely difficult to be completely cured. In previous studies, I know that the main receptor causing TNBC cell proliferation is the EGFR receptor on TNBC cell membrane. When this receptor binds to EGF, it will cause cell proliferation. BAG3 is a protein that exists in cells, and it is closely related to EGFR. The expression of BAG3 was correlated with the expression of EGFR. Through silence BAG, the expression of EGFR also decreased slightly but significantly. At the same time, the data show that the large expression of BAG3 in TNBC patients causes TNBC to have poor disease-free survival. Thus, BAG3 has very important research significance for TNBC.

According to later research, they state that BAG3 causes cell proliferation because it leads to the activation of two very important protein pathways under EGFR, which eventually leads to a large number of cell proliferation. These two protein pathways are AKT and FAK. In order to know more about the role of BAG3, I decided to design an experiment to inactivate AKT and FAK by adding inhibitors ly2780301 and pf-573228 in BAG3 WT so as to study whether BAG3 will affect other

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pathways or affect the proliferation of TNBC through other methods. The following is my prediction of the possible results of the experiment. I divided the results of my experiments into two main categories. The first category of results is that the results I obtained in the mice match the results in the test tube, and the second category of results is that the results in vitro do not match the results in the mice.

4.1. Invitro experiment and xenograft experiment result matches.

Result 1: completely proves the experimental results, which means that BAG3 also leads to the proliferation of TNBC through stimulation to other signaling pathways. This is because BAG3 cells continue to proliferate either in the presence of FAK inhibitors or AKT inhibitors or FAK as well as AKT inhibitors. This may be caused by BAG3 alone causing cell proliferation or by BAG3 also stimulating other signaling pathways for proliferation [6].

Result 3,4: partially support my hypothesis. In the third and fourth experimental groups, the cells proliferated in the presence of FAK inhibitors and AKT inhibitors, respectively, and also in the presence of FAK and AKT together. The reason for this may be that BAG3 individually activate the FAK or AKT, but it also causes other pathways' activation. Therefore, when one of the pathways FAK or AKT is inhibited, BAG 3 can only cause other pathways to activate and create less cell proliferation.

Result 8: is completely relative to my hypothesis. This is because in this case, the cells proliferate in the presence of either the FAK inhibitor, the ATK inhibitor, or both, as opposed to the group without the inhibitor. This means that the no inactivation of both FAK and AKT causes the proliferation of BAG3 WT because BAG3 stimulates FAK and AKT these two pathways independently to cause cell proliferation [7].

Result 5,7: partially does not support my hypothesis. For the 5th and 7th results, there is a significant increase in cells in the FAK inhibitor and AKT inhibitor groups, respectively. At the same time, there was a significant decrease in cells in the AKT inhibitor group and the FAK inhibitor group. This may be due to the fact that BAG3 acts alone on FAK or AKT, resulting in cell proliferation.

Result 6: For the sixth group, the cells did not proliferate in the presence of FAK and AKT inhibitors but proliferated in the presence of both FAK and AKT inhibitors. This may be due to the fact that both FAK and AKT signaling pathways act together to inhibit the activity of another signaling pathway that can cause cell proliferation.

Result 2: For the second group, the cells did proliferate in the presence of FAK and AKT inhibitors but did not proliferate in the presence of both FAK and AKT inhibitors. This may be due to the fact that both FAK and AKT signaling pathways act together to promote the activity of another signaling pathway that can cause cell proliferation.

Result 12: When the inhibitor of the FAK, such as Y15, is supplied, according to the research done by Donghang Zheng etc., it will significantly decrease cell survival and tumor apoptosis [8]. Therefore, this potential result is possible.

Result 9-11 and 13-16: For experimental results 9-16, the experimental results would not appear unless the BAG3 WT cells were taken into another kind of cells that do not contain BAG3 such as CRISPER KO BT549 by mistake during the experiment. This is because in the absence of any suppressors, FAK as well as AKT must be activated to proliferate in response to BAG3 stimulation.\

Result 1-4, 6-7, 10, 12-16: These results require the inhibitor of AKT. Recently, some investigations are putting effort to develop the inhibitors of AKT, but some challenges, such as inhibitors targeting the ATP binding site, pH domain and protein substrate binding site, and isoform selective allosteric inhibitors are still remained [9]. Hence, even though ideologically this experiment can include these potential results, it is possible that when it comes to the practical part, they will not be freely operated. Currently, the inhibitor of AKT can be API-2, which is highly selective for AKT and does not inhibit the activation of other pathways, such as phosphatidylinositol 3′-kinase, phosphoinositide-dependent kinase-1 and protein kinase C [10]. However, as mentioned above, some difficulties are still faced in creating the inhibitor of AKT, such as the protein binding sites. It is possible that although API-2 is highly selective, it might also inhibit other pathways due to the lack of specificities of its active site.

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4.2. Invitro experiment and xenograft experiment result do not matches

The tumor volume in rats should be proportional to the cell proliferation in vitro, but in fact, it is not. There are several possible reasons for this.

4.2.1. The proliferated BAG3 WT cells could not survive in mice. Although the silence of FAK and AKT caused the proliferation of BAG3 WT in vitro, that is, excessive cell proliferation caused immune or xenophobic reactions in mice, resulting in the elimination of BAG3 WT cells. Therefore, the experimental result was that cell proliferation did not occur. This can occur when the amount of BAG3 WT cells is limited, which can be suppressed by mice's self-immune system, such as lymphocytes, fibroblasts, or macrophages [7].

4.2.2. The BAG3 WT could not survive in mice. It may be due to a few CRISPR KO BT549 cells or other cells that are not BT549 injected or too strong immunity in mice, which leads to the elimination of BAG3 WT once they enter the mouse. Therefore, mice will not get breast cancer, and cell proliferation will not occur.

4.2.3. Inhibition of FAK, ATK or both in BAG3 WT does not result in the proliferation of TNBC in vitro, but it results proliferation of BT549 in xenograft. The reason for this possibility is that BAG3 WT cannot adapt to survive in the medium environment, and the breast environment in female mice may be very suitable for its proliferation.

5. Conclusion

As a conclusion, my research paper helped me design an experiment to study the effects of BAG3, AKT and FAK on the proliferation of TNBC cells. Completing this experiment can help researchers better understand the effect of BAG3 on AKT and FAK and thus on the proliferation of TNBC, which is helpful to study TNBC and treat TNBC patients and has strong medical significance. For the future experiment, since I just test whether BAG 3 independently regulate FAK and AKT to cause cell proliferation, I would like to test other pathways that are in the downstream of BAG 3 that cause cell proliferation in TNBC through BAG 3. In this case, people will further understand more about BAG 3, and create more therapeutical value.

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