Investigation on how mechanical force transducer PIEZO1 controls the polarization of tumor-associated neutrophils and its relationship with colorectal cancer development

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Abstract:

Nowadays, during the polarization of neutrophils, the function of the mechanical force transducer PIEZO1 is still unclear. Our research shows that the PIEZO1 gene knockout in tumor-associated neutrophils (TANs) can suppress the polarization of TANs towards the N2 phenotype induced by TGF-β; Activating PIEZO1 by Yoda1 promotes polarization. We discover that cancer cell proliferation is enhanced by coculturing colorectal cancer cells with N2 TANs. On the contrary, when PIEZO1 is knocked out, the cancer development alleviates due to its prevention of TANs polarizing towards N2. Our research hints that mechanical force transducer PIEZO1 might be a new target spot during the treatment of colorectal cancer.

Keywords::Tumor-associated neutrophils;polarization;Piezo1;colorectal cancer;TGF-β

1. Introduction

The tumor microenvironment (TME) is the cellular environment around tumors, such as immune cells, stroma cells, and extracellular matrix (ECM). The dynamic interaction between tumor and TME plays a significant role during cancer cell survival and metastasis. TANs are a group of the most important immune cells in TME. Past research has discovered that TANs have different effects on cancer according to the cytokines and chemokines in TME. N1 TANs retards cancer growth by cytotoxicity, while N2 TANs can promote tumor growth by angiogenesis, releasing cytokines and chemokines, and immunosuppression.

Colorectal cancer is the third most popular malignant tumor with high mortality around the world. The number of patients has increased continuously and reached 1.9 million incidences in 2020. As TANs have become one of the most immune cells in TME, their function is increasingly dug in by scientific research and experimental proofs.

Some research shows that interferon INF1 can polarize TANs to anti-tumor N1 phenotype, While TGF-β in TME polarizes TANs towards the N2 type. However, the mechanism behind TGF-β-induced polarization is still unclear.

As a receptor of cellular mechanical signals, PIEZO1 transforms the signal, such as tumor hardness, into signal molecules and passes to other downstream molecules. Therefore, we predict that PIEZO1 might be involved in TAN polarization and further tumor cell development. This research will clarify the function of PIEZO1 during polarization and discuss whether it can be utilized as a possible target for pharmacological treatment, which has a profound meaning for human colorectal cancer. As shown in Fig.1-1, the whole process can be more easily illustrated. This research mainly focuses on the mechanism of the TGF-β induction of N2 neutrophils.

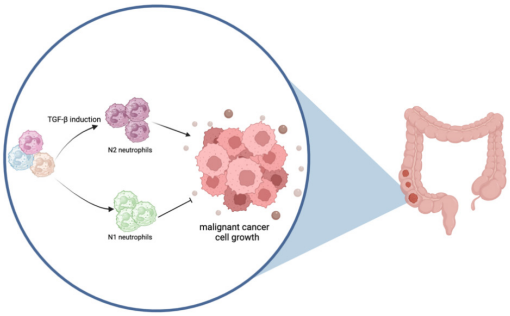

Fig.1-1 The whole process related to the cancer cell growth, promoted by N2-phenotype neutrophils

2. Materials and methods

2.1 Apparatus and Materials

Laboratory animal: C57BL/6 male mice with age of 8 weeks (from Shanghai Lingchang biotech company)
Apparatus: Low-temperature high-speed centrifuge (from Thermo Fisher Scientific), PCR thermal cycler (from ABI company), Liquid nitrogen container (from Thermo Fisher Scientific), Protein Electrophoresis Equipment (from Bioread company), Thermostat water bath (from Shanghai Sangon Biotech), and Cell counting chamber (from MARIENFELD).
Material: RPMI-1640 solution (from China Cyagen Bioscience Inc.), FBS (from GBICO company), trypsin solution (from GBICO company), TRIzol reagent (from Thermo Fisher Scientific), RT reverse transcriptase (from NEB company), Cell Counting Kit-8 solution (from MCE company), PBS solution (from Thermo Fisher Scientific), and Flow Cytometry Staining Buffer (from Shanghai Sangon Biotech).

2.2 Extraction of mice primary neutrophils

As shown in Figure 1-1, to obtain the femur and tibia, C57BL/6 laboratory mice are sterilized, and muscles are eliminated. It is placed in a sterile Petri plate with RPMI-1640 solution. Separate them at the knee joint and cut the ends of the bones. Bone marrow is rinsed through RPMI solution into a centrifuge tube, and by centrifugation for 7 min (4°C, 427 × g), we can collect bone marrow cells. Resuspend cells in NaCl solution(20 mL, 0.2%), and add the same volume of relatively concentrated NaCl solution(1.6%)for lysis of erythrocytes. After adding the RPMI-1640 solution, repeat the centrifugation process. Then, bone marrow cells are obtained.
Add Enrichment Cocktail to the bone marrow cells. Mix evenly and incubate (2 - 8°C for 15 min). Wash the cells by topping the sample tube with RPMI-1640 solution and centrifuge. Then, discard the supernatant and resuspend cells with the medium. Add Biotin Selection Cocktail, mix and incubate(2 - 8°C for 15 min). We obtain the isolated neutrophils using the automated RoboSep™ Protocol with the mode of Mouse Neutrophil Negative Selection 19762.

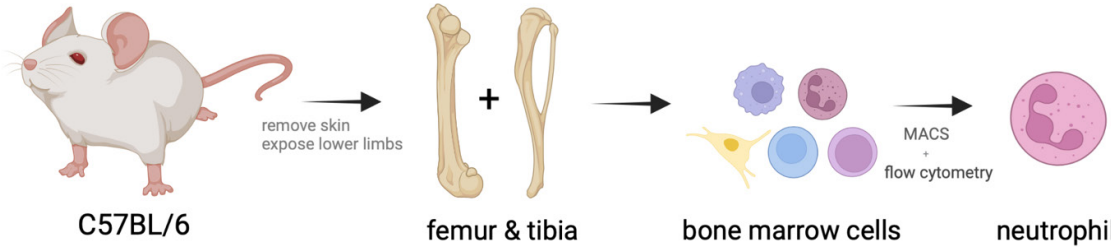

Figure 2-1 illustration of the process of extracting and purifying mouse neutrophils

2.3 Flow cytometry

Prepare neutrophil suspension. Add Flow Cytometric Staining Buffer (FCSB) containing Flow Primary Antibody to the Neutrophil Sample Cell Suspension. Vortex gently and incubate (2 - 8°C for 30 min). Wash cells with PBS solution, then centrifuge (400-600 x g for 5 minutes, RT). Discard the supernatant. Add fluorescent secondary antibodies to PBS and incubate (2 - 8°C for 30 min). Repeat the washing process and discard the supernatant. Resuspend the cells with FCSB. Up-sample into a flow cytometer for analysis.

2.4 RNA extraction and reverse transcription of cDNA

Add 1 mL TRIzol reagent per 5~10^6 cells and incubate (RT, 5 min) to fully lyse cells. Afterwards, add 0.2 mL of chloroform to each 1 mL of TRIzol. Incubate (RT, 3 min) and centrifuge (for 15 min, four °C) to separate the organic and RNA-containing aqueous phases. Move approximately 400 mL of RNA-containing solution into another EP tube. Add 400 μl isopropanol, and centrifuge (10 min, 4°C) to precipitate mRNA. Discard the supernatant. Dry naturally at RT for 5 – 10 min.
Then, reverse the mRNA into cDNA by a two-step method. Build the first step system containing 2 μg RNA, 1 μl dNTP, 1 μl oligo-dT, and 13 μl DEPC-treated water. Place in PCR instrument and react for 5 min. Then, construct the second step system, containing 2 ml DTT(0.1 M), 4 μl reverse transcriptase buffer, and 1 μl RT reverse transcriptase. Synthesize cDNA, which is used as a template for further PCR. Subsequently, we use a qPCR system to detect the expression of relevant genes.

2.5 Real-time PCR technology

Primer sequence: through the PrimerBank design website, design the primer sequence, synthesized by Shanghai Sangon Biotech)
The real-time qPCR system and its reaction conditions are shown below.

Real-time qPCR system

Reagents	Volume
cDNA	5 μl
Forward primer (10μM)	1 μl
Downward primer(10μM)	1 μl
HieffTM qPCR SYBR Green	5 μl
ddH2O	3 μl

The whole qPCR reaction condition is that:
1. 95℃ 10 min
2. 95℃ 30 s
3. 60℃ 30 s
4. 72℃ 30 s (2-4 steps do 30 cycles)
5. 4℃ Storage

2.6 CCK8 cell activity assays

Add 100 μL of cell suspension to a 96-well plate and incubate for 24 h to make the cells adhesive to the wall. Different treatment drugs were added and incubated, and then ten μl CCK-8 solution was gently dropped (ensure no bubbles were produced). The absorbance (450 nm) was measured with an enzyme meter after four hours of incubation.

2.7 Cell culture and passage

2.7.1 cell recovery

Place the tubes in a constant temperature water bath (37℃). Mix the cell suspension with the culture solution and add it to the centrifuge tube. After centrifugation, discard the supernatant, resuspend the cells, and put them into the incubator (37℃, 5% CO2) for static incubation. If the cells are more than 95% adhesive to the wall the next day, the cell activity is well.

2.7.2 Cell culture

Preheat the medium and PBS for 15 minutes and add the cells moistened with the appropriate amount of PBS (after removing the PBS) into the medium (containing 10% FBS+double antibiotic) for culture. Observe the cells under the microscope, and after confirmation, culture again.

2.7.3 Cell freezing

Centrifuge the cells for 15 min (800 g) to obtain the cell precipitate. Then, resuspend the cells with the addition of a complete medium and slowly drop into the freezing solution (DMSO). Dispense the cell solution evenly into cell preservation tubes (1 mL). Deposit into the cryopreservation box for cooling and transfer into liquid nitrogen.

2.8 Knockdown of PIEZO1 gene using shRNA

Anneal the shRNA sequence containing the enzyme cleavage site to generate a double-stranded structure. Ligate it with the enzyme cleavage vector pLKO.1. Transform the receptor DH5α and extract the plasmid as a template for PCR amplification. Then, the correct base information was determined by agarose electrophoresis and shRNA sequencing. The shRNAs with good knockdown effect were screened (determined from transcription and protein levels). It was found that the plasmid construction was successful.
(shRNA is designed by the TRC team, with the sequence of ATCCAAAAGCGCTTGCTAG)
Neutrophils were cultured with Opti-MEM (serum-free medium) for 24 h (density ≈70%). Later, transfected into cells by the polyethyleneimine PEI method. The constructed plasmid and PEI were added to 100 mL of Opti-MEM at a ratio of 1:3. Stir it evenly, then add 900 mL of Opti-MEM. Leave it for a while. The plasmid-PEI mixture was added again with the cell (supplemented with Opti-MEM to prevent the cells from drying out), and the transfected cells were collected after 4-6 h incubation and 48 h culture.

2.9 Statistical analysis

The data collected were then analyzed using SPSS20 software. The statistical significance is also analyzed using a t-test and a one-way ANOVA. If the resulting p-value was less than 0.05, it proves the result is significant.

3. Experimental results and analysis

3.1 Successful extraction of neutrophils from lab mouse bone marrow

Extract mice bone marrow cells from 8-week-old C57BL/6 mice. Further, isolate neutrophils with the magnetic bead negative selection kit for neutrophils. Eventually, the FACS process will examine the extraction results.
The test results in Figure 3-1 showed that the purity of neutrophils reached 96.04% after sorting, which was significantly higher than 31.58% before sorting ($P<0.05$).

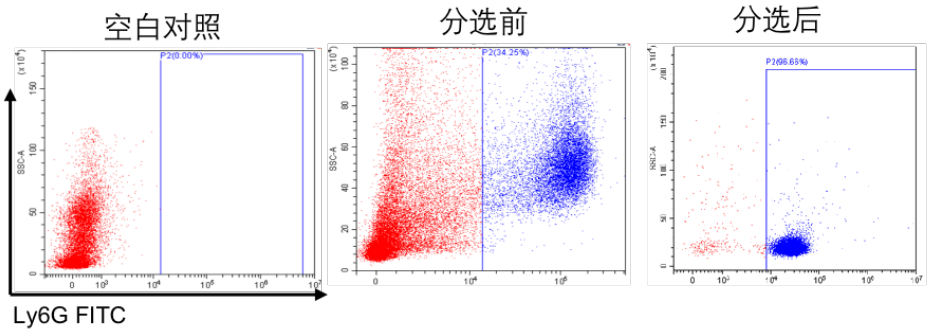

Fig.3-1 Ly6G Antibody flow cytometry test results

(From left to right, the FACS result before sorting and after sorting, respectively.)

Fig.3-1: Flow cytometry examines the neutrophil purity results after extraction. A blank control differentiates the results from the background and serves as a reference for voltage regulation, thereby circling the location of the correct negative cell population. Then, construct a scatter plot graph of FITC-A green fluorescence/SSC-A. The x-coordinate indicates the expression of Ly6G antibody conjugated to FITC tracked by FACS, representing the intensity of light; the stronger the immunofluorescence intensity, the more antibody contained, while the y-coordinate represents the internal structure and granularity of the cell.

The round-shaped neutrophils were seen by photographing with an optical microscope (Fig. 3-3), which allowed the next step of the experiment to be carried out.

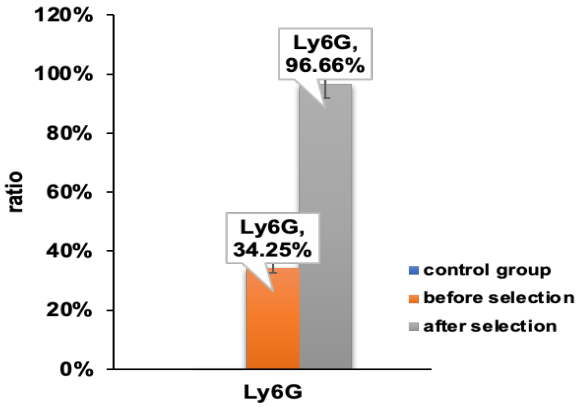

Fig. 3-2 Difference between pre- and post-sorting neutrophil magnetic beads with $p<0.05$.

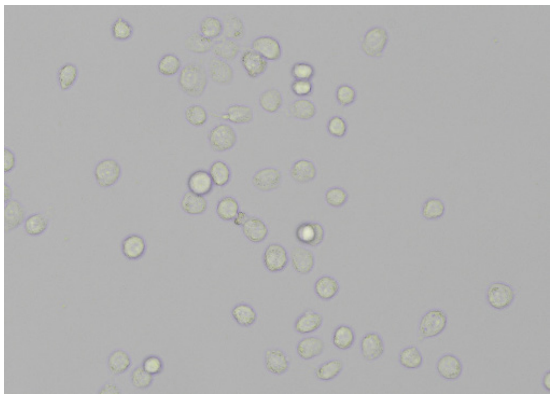

Figure 3-3 Mouse neutrophils observed by optical microscopy

3.2 The polarization of neutrophils towards N2 by TGF-β

To evaluate the phenotype of TANs, we measured the mRNA expression of anti-inflammatory markers by RT-PCR. Ym1 in the experiment was regarded as one of the markers of TAN polarization towards N2. By research,

anti-inflammatory markers YM1 were increased in N2 neutrophils instead of in N1 cells. Also, TGF-β, which can stimulate a protumor N2 neutrophil phenotype, is used as one marker. Tumor cells can also induce CCL2 expression in TANs Which is used as the biomarker in our experiment. We also test the expression of ARG2 to determine the phenotype of TANs. That gene expression (CCL2, ARG2, TGF-β, and YM1) can all promote cancer development.

Because TGF-β within the tumor microenvironment shifts a pro-tumor phenotype of neutrophils, while TGF-β blockade induces neutrophils to N1 phenotype, indicating differential neutrophil polarization, we use 40 ng/ml TGF-β to induce TANs towards N2 type for 12 hours.

As shown in Figure 3-4 a, the relative CCL2 mRNA expression of the TGF-β group is significantly higher than the control group($p<0.001$). Meanwhile, the expression of ARG2 (figure 3-4 b), TGF-β (figure 3-4 c), and YM1 (figure 3-4 d) increases as well, which dramatically outweighs the expression of the control group ($p<0.05$). The results showed that TGF-β successfully induced TANs towards the N2 phenotype.

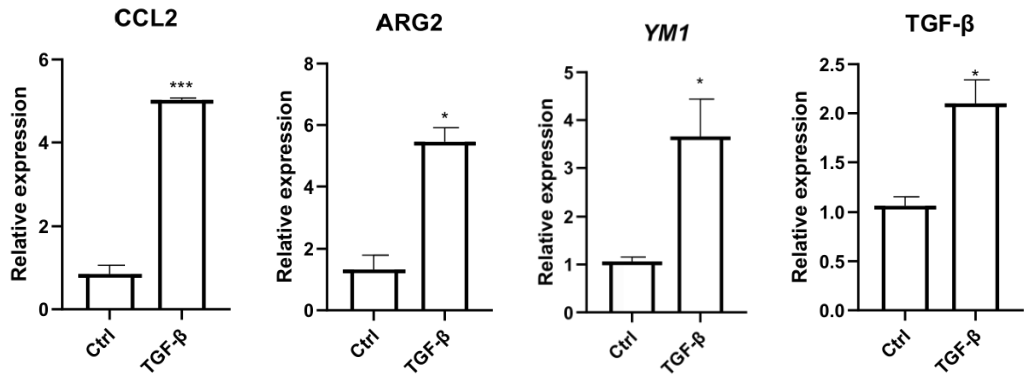

Figure 3-4 Relative expression of N2 TANs markers between the TGF-β group and Control group ($p<0.05$)

a: CCL2 gene expression between the Control group and TGF-β group

b: ARG2 gene expression between the Control group and TGF-β group

c: YM1 gene expression between the Control group and TGF-β group

d: TGF-β gene expression between the Control group and TGF-β group

3.3 N2 promotes proliferation of colorectal cancer cell line SW1116

To test whether N2 phenotype TANs can promote the SW1116 cell line development, the SW1116 cell line (to simulate tumor microenvironment) is collected and cultured in an environment with the supernatant of constructed N2 neutrophils. The cell line was cultured for 24 h/48 h/72 h/96 h for CCK-8 assay, respectively. The results showed that the proliferative capacity of N2 medium-treated SW1116 cells was significantly higher than that of the control medium ($P<0.01$), which is statistically significant. The OD value of SW1116 cells mediated by N2 phenotype neutrophils was about 0.03 higher than that of medium-treated cells (after 24 h of incubation); the OD value after 48 h of incubation was 0.12 higher; after 72 h of incubation was 0.21 higher; and after 96 h of incubation was close to 0.23. A conclusion can be drawn as a slight change in OD value reflects an intense difference in the growth of cells. The N2 group grew at an extremely high rate in the first 48 h, and then the rate was gradually similar to that of the control group, and the cell activity and growth were always higher than that of the control group. This result shows that N2 TANs can greatly promote the malignant progression of tumors.

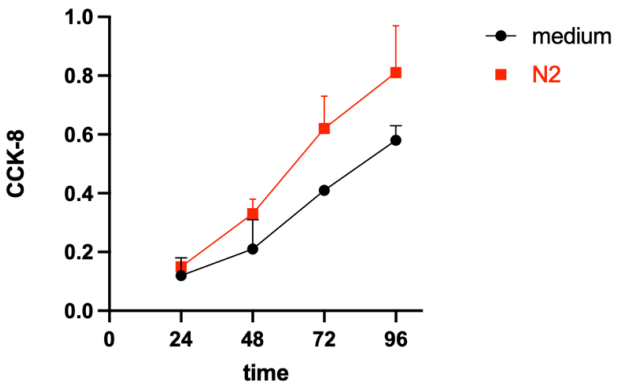

Fig. 3-5 Differences in tumor cell proliferation between control and N2 groups detected by CCK-8 assay

The graphs of CCK-8 experiment results show time (with units of h, and shown by x-coordinates) versus cell OD values (y-coordinates). The OD value of the light absorption amount of a suspension of an organic molecule is measured by a spectrometer. It's at a specific wavelength. The OD value is normally used to estimate the quantitative cell growth and metabolism. It is the most common method used in microbiology laboratories, and it is used to determine the number of cells at different stages of time and conditions by contrasting them with a blank culture medium.

3.4 Knockout of PIEZO1 gene in N2 phenotype neutrophils inhibits the proliferation of colorectal cancer SW1116 cell line

After the knockout of the PIEZO1 gene in neutrophils, its result must be verified. As shown in Fig. 3-6a, the mRNA expression in the ShPIEZO1 group was significantly smaller than that in the negative control group ($p<0.0001$), indicating that neutrophils knocked down for PIEZO1 had been successfully constructed and could be used for the next experiments.

By CCK-8 assay, the results showed that the proliferation of SW1116 cells was significantly inhibited by coculturing SW1116 cells with N2 phenotype neutrophils, of which the PIEZO1 gene had been knocked down. After 96 h of culture, the OD value of SW1116 cells in the control group was 0.47 higher than in the Sh-Piezo1 group. The Control group shows a relatively linear growth at a constant rate. In contrast, the growth curve of SW1116 after the PIEZO1 knockdown gradually flattened and eventually stabilized to reach a plateau (Figure 3-6 b).

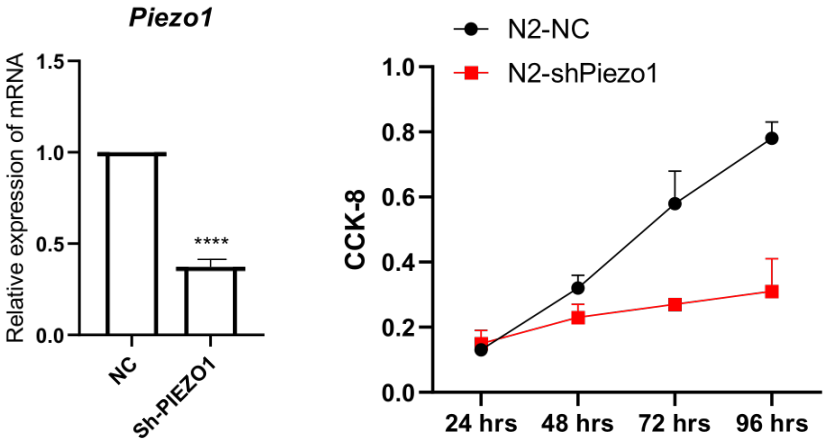

Fig. 3-6 a: Differences in Piezo1 mRNA expression between Sh-Piezo1 and control groups

Fig. 3-6 b: spectrometer detected SW1116 cells growth trend in N2-NC group and N2-ShPiezo1 group (24 h/48 h/72 h/96 h).

3.5 N2 neutrophils treated with PIEZO1 agonist Yoda1 promotes proliferation of colorectal cancer cells SW1116

Because Yoda1 activates the PIEZO1 ion channel, which is amenable to chemical activation, it is one of the key tools to learn specifically about PIEZO1 as its functions are highly activated. Similarly, N2 cells (treated with PIEZO1 or the control group) were cocultured with the SW1116 cell line for 24 h/48 h/72 h/96 h. The results of the CCK-8 assay showed that the proliferation rate of cells in the N2-Yoda1 group was much higher than that of the control group at each period (fig.3-7).

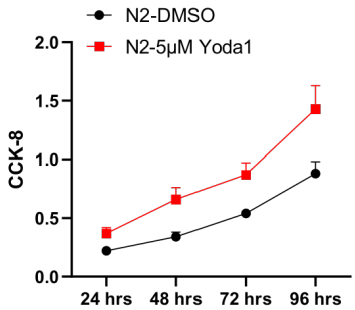

Figure 3-7 Differences in the proliferation rate of SW1116 cells between control and N2-Yoda1 groups detected by CCK-8 assay (Yoda1 agonist is in solid powder form, and its use requires the addition of dimethyl sulfoxide DMSO, and control N2-DMSO strictly adheres to the control variables.)

4. Conclusion and discussion

In conclusion, the extraction method is established for primary mouse bone marrow neutrophils in this experiment. By detecting the expression of N2 markers such as CCL2, ARG2, TGF-β, and YM1, it is clarified that TGF-β could induce neutrophil polarization toward the N2 phenotype. Knockout of PIEZO1 in neutrophils using the shRNA method inhibited TGF-β-induced polarization of TANs toward N2. Coculture experiments revealed that the knockdown of PIEZO1 in N2 inhibited the proliferation of colorectal cancer cells. Conversely, we utilized that Yoda1 (an agonist of PIEZO1) could promote the proliferation of tumor cells.

In the tumor microenvironment, N2-type TANs can secrete pro-cancer inflammatory factors and cytokines and high levels of, for example, CCL2, which have been detected in the experiments. It can promote colorectal carcinogenesis by increasing the size of colorectal tumors and function of the polymorphonuclear myeloid grafted cell population, which can differentiate into mature neutrophils and be polarized into N2 TANs. ARG1 and YM1 are abundantly expressed in TANs and play an important role in the progression of colorectal cancer due to immunosuppressive effects. The present experiments have demonstrated that TGF-β cancan promote the polarization of N2 TANs, and N2 cells can also secrete TGF-β molecules, which increase tumor aggressiveness and growth due to the deteriorating tumor microenvironment. Currently, factors studied to regulate N2 polarization include molecules such as G-CSF (granulocyte colony-stimulating factor), IL-10, and TGF-β. [7] However, the mechanisms that regulate N2 polarization are unclear, except for factors secreted in the tumor microenvironment (cytokines, growth factors, inflammatory factors, etc.). And so is preliminarily clarified in this experiment.

PIEZO1/2 is a mechanical force transducer in the shape of a trimer consisting of approximately 2500 amino acids, with each subunit containing 38 transmembrane regions. The principle of PIEZO1/2 is to transmit mechanical force signals by converting mechanical force into Ca2+ permeation through a “gatekeeper-latch” mechanism. PEIZO1 is more common in TANs around the human body than PEIZO2 and is mainly investigated in this research. It acts as a Ca2+ channel that can affect calcium flow in tumor cells, and the disruption of calcium homeostasis can cause apoptosis (as well as metastasis, etc.). PIEZO1/2 can be used as a Ca2+ channel in tumor cells, which can cause apoptosis (as well as metastasis). The experiment then hypothesizes that the PIEZO1 mechanical force sensor also affects apoptosis in TANs. However, the molecular mechanisms by which PIEZO1 regulates TAN cells have not been fully investigated. At the same time, PIEZO1 can also be directly involved in regulating the proliferation and expansion of tumor cells through TAMs and within the tumor. Combined with the results of this experiment, it suggests that PIEZO1 can be used as a target for therapy, and Dooku1, an antagonist of PIEZO1, may be able to treat colorectal carcinogenesis.

This study demonstrates for the first time that PIEZO1 has a role in tumor-associated neutrophils in a tumor-promoting direction. However, it lacks animal model validation, our follow-up work. In further experiments, we will make mice subcutaneous tumors and inject the antagonist inside the tumors to determine whether the antagonist can be used for treatment, and at the same time, we will study the specific mechanism of PIEZO1 regulation of TAN cells.

The possible limitations of this experiment are that, firstly, no living animals are involved. It’s done in an in vitro environment, and PIEZO1 may have a different effect in in vivo experiments, which needs further investigation involving living mice. Another possible limitation is that the mRNA expression is used to detect the sh-PIEZO1 gene and biomarkers’ relative expression. This expression only considers the transcription process but not the translation, although it might be sometimes useful, but certainly far from perfect, in predicting protein expression levels.

5. Summary

This experiment showed that the mechanical force sensor PIEZO1 could promote colorectal carcinogenesis by regulating the polarization of tumor-associated neutrophils towards N2, and targeting the PIEZO1 channel may be a new target for treating colorectal cancer. It may be a new direction for pharmacal cure.

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