

# Understanding the molecular link between SPOP gene expression and breast cancer stem cell

W. Wang, R. Chen, Y. Zhang, K.J. Zou, M.L. Ye, X.H. He, F. Zhang, J. Han, H.Y. Dong

Department of General Surgery, Hainan General Hospital, Haikou (China)

## Summary

**Purpose:** Breast cancer stem cells are responsible for tumor initiation, abnormal cellular proliferation and metastasis. The activation of breast cancer stem cells is regulated by many genes and the expression of tumor suppressors has a control over it. Speckle-type POZ protein (SPOP) is similar to tumor suppressor that their intact presence inhibits tumor development and any mutation in this gene is responsible for many cancer types. However the exact mechanism of SPOP in controlling cancer stem cells still has not been revealed. **Materials and Methods:** NOD SCID mouse was used for initiating breast tumor by injecting MDA-MB-231 cell type. The expression of ALDH1 and SPOP are analyzed using Immunohistochemistry and Western blotting. **Results:** The mice injected with tumor cells develop a primary tumor at four weeks and it develops advanced stage of breast cancer at nine weeks. Tumor formation and associated cellular complexity are observed through histological evidence. Also, the expression of ALDH1 shows overexpression pattern as tumor progress to the next level. Interestingly, the authors observed the expression of SPOP shows an abundant expression pattern in the initial stage of breast cancer and noticed its downregulated expression as tumor adverse. **Conclusion:** The results demonstrate that the highest expression of SPOP had a control over cancer stem cells in the initial stages of breast cancer.

**Key words:** SPOP; ALDH1; MDA-MB-231 cells; Cancer stem cells; Primary tumor.

## Introduction

Even after the improvement in early screening strategy and development of an advanced treatment procedure, breast cancer remains a major disease in the female population. The major reasons behind that are treatment resistant, recurrence, and metastasis behavior [1]. The highest incidence of breast cancer is due to lifestyle changes such as alcohol consumption, weight gain, exposure to radiation, and carcinogens [2, 3]. Women until they attain puberty and after pregnancy, the breast cells undergo higher proliferation which direct evidence the presence of breast stem cells [4]. Tumor initiating cells or breast cancer stem cells share many common biological properties with normal stem cells that are responsible for breast tumor initiation, proliferation, maintenance, and treatment resistance [1]. The enriched population of breast cancer stem cells is notably observed following chemotherapy suggest their aggressive nature [5].

Many genes and enzymes are responsible for promoting the function of breast cancer stem cells like CXCR4 [6], PRMT5, and histone modifying enzymes [1]. Targeting the cancer stem cells helps to develop effective treatment therapies for which the basic functional aspect of genes, that are governing the cancer stem cells are needed to be understand in depth. Speckle-type POZ protein (SPOP) protein guides in normal DNA repair process and mutation in this

gene leads to genomic instability with broken chromosome [7]. The SPOP gene mutation is observed in many cancer types like prostate, endometrial, gastric, colorectal and in thyroid follicular tumors [8, 9]. The physiological role of SPOP mutation in developing pathogenesis and cancer progression has remained unclear. The lack of intact SPOP increases cellular proliferation and cell invasion, suggesting their presence as a tumor suppressor [10]. In this study, using mouse model the authors analyzed the link between SPOP and breast cancer stem cell in developing breast cancer.

## Materials and Methods

Immunocompromised, two-month old female NOD SCID mice (n = 18) were purchased from Jackson Laboratory and were injected with aggressive human breast carcinoma cells (MDA-MB-231, dose range  $2 \times 10^7$  in 200  $\mu$ l) directed into the tail vein. Following injection, the tumor appearance and growth are monitored every week. Following injection, one set of mice (n = 6) are sacrificed after four weeks of observing the primary breast tumor development and another set of mice (n = 6) were allowed up to nine weeks for developing advanced stage breast tumor. Animal maintenance, injection, and handling procedure are approved by the Hainan General Hospital ethical committee specifically made for animal care.

After initiating breast tumor, the mice were scarified at four and nine weeks to harvest the primary and advanced breast cancer tissues. Following dissection, the tissues were kept in a fixative

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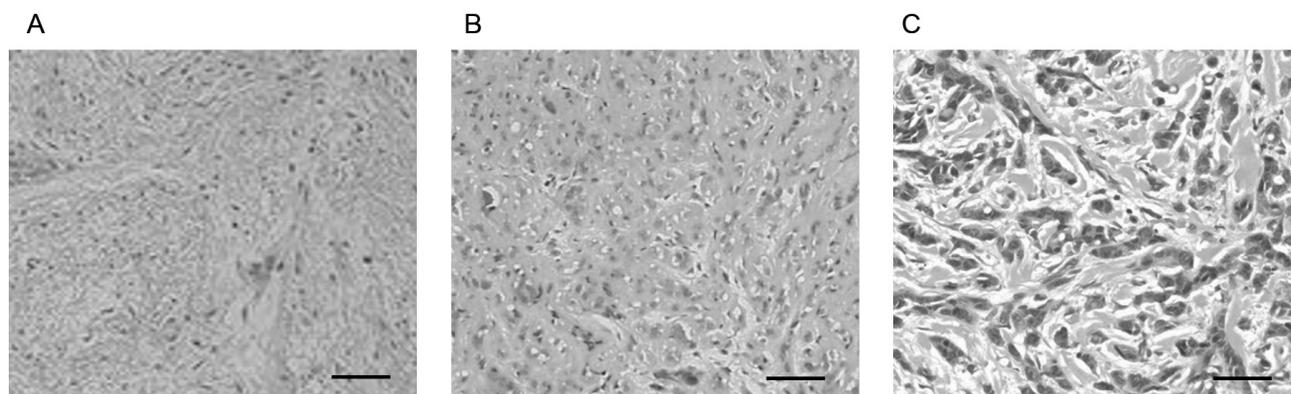


Figure 1. — Histological observation of breast cancer progression.. A) Control breast tissue of NOD SCID mice. B) Primary breast tumor with high proliferative cells. C) Advanced breast tumor tissue with multinucleated lump of cells. Scale – 50  $\mu$ m.

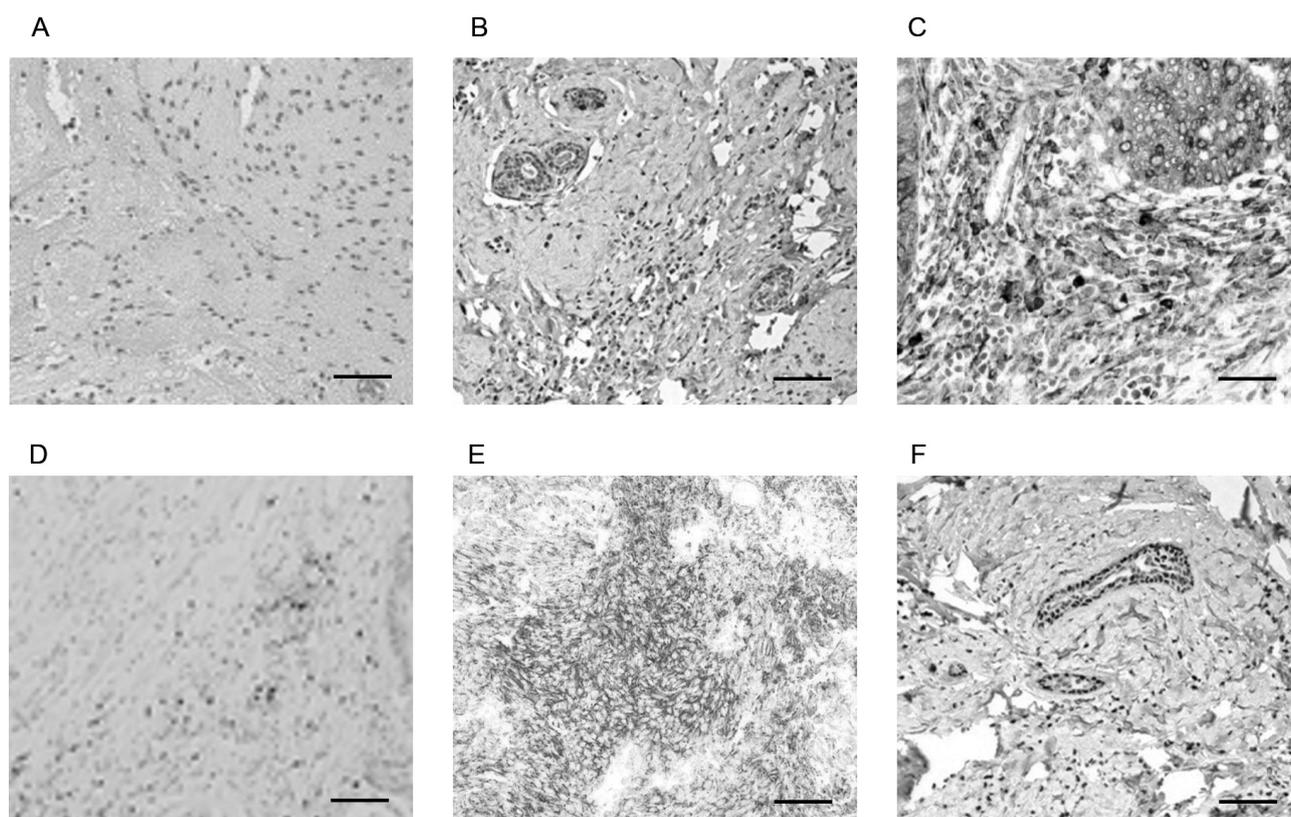


Figure 2. — Expression of ALDH1 and SPOP in different stages of breast cancer tissue. A) ALDH1 expression in control breast tissue. B) Increasing pattern of ALDH1 in primary breast tumor. C) Stronger expression of ALDH1 in advanced stage breast cancer. D) SPOP protein expression in control breast tissue. E) Overexpression of SPOP in primary breast tumor. F) Downregulated expression of SPOP in advanced breast cancer tissue. Scale – 50  $\mu$ m.

(10% paraformaldehyde) for two days at 37°C to attain adequate fixation. After the sections were washed thoroughly in distilled water and dehydrated with isopropyl alcohol in gradient step-wise manner. Lastly the tissues were impregnated with paraffin and molded into a blocks for sectioning with a microtome. The thin sections of tissue of 6  $\mu$ m size were processed in a slide and stained with Hematoxylin and Eosin for visualization.

The processed tissue sections were embedded in paraffin and mounted on a microtome for fine sectioning (4  $\mu$ m size). The sec-

tions were placed on a poly-L-lysine pre-coated slide and de-waxed using xylene. For antigen retrieval step the slides are incubated in pre-heated citrate buffer solution for 30 minutes. Following that the slides were treated with hydrogen peroxide solution (4% in methanol) for 30 minutes at room temperature. After washing, the slides were incubated with blocking solution (4% BSA in TBST buffer) for one hour at room temperature and immediately overlaid with primary antibody (anti-SPOP or anti-ALDH1) at 4°C for eight hours. Unbound and non-specifically



Figure 3. — Western blotting analysis. Left most lane – protein lysate of control breast tissue. Middle lane – protein lysate of primary breast cancer tissue. Right most lane – cell lysate with protein from advanced breast cancer.  $\beta$ -actin acts as a loading control.

attached antibody was washed and then incubated with horseradish peroxidase conjugated secondary antibody. The binding signals are detected using 3'-diaminobenzidine as a substrate which helped to amplify and visualize the signals.

After sacrificing the mice, the breast samples were immediately dissected and washed in ice cold PBS. For protein extraction, the tissue was homogenized in RIPA lysis buffer and then estimated using Bradford assay. The protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking, the targeted protein in the nitrocellulose membrane were probed by using a suitable primary antibody (anti-SPOP or anti-ALDH1). Following overnight incubation, the slides were washed twice with 1X PBS and incubated with corresponding secondary antibody for one hour at room temperature. The quantity of signals was amplified by chemiluminescence kit and documented using imaging software.

## Results

For investing the expression of SPOP in relation with cancer stem cell aggression, it was important to set up the experimental background. For that the authors used NOD SCID mice, which were injected with MDA-MB-231, aggressive human breast carcinoma cells. To enumerate tumor formation, the mice were sacrificed at four and nine weeks and were analyzed using histology appearance. The cellular morphology of control mice appeared as normal breast cells with extended cellular gap (Figure 1A). The mice injected with breast carcinoma cells developed a primary tumor after four weeks, which was confirmed with high proliferative and invasive nature of breast cells (Figure 1B). Following nine weeks of time after injection, the tumor promoted to advanced stage with multinucleated lump of cells (Figure 1C).

ALDH1 is an enzyme type that is enhanced in many cancers stem cells and it acts as a marker for breast cancer cell stem cells [11]. To evaluate the cancer progression, the tissue is subjected to immunohistochemistry using anti-ALDH1 antibody. In the control breast tissue, the expression of ALDH1 was almost inexistent (Figure 2A) but in the fourth week of MDA-MB-231 cells injected tis-

sue the expression of ALDH1 showed a remarkable increased in expression pattern (Figure 2B). The mice after nine weeks developed advanced stage of breast cancer, with overexpression of ALDH1 in all clustered and abnormal cells (Figure 2C). Next, the authors attempted to understand the link between SPOP and ALDH1 expression. In this investigation, they observed that in control breast tissue the expression of SPOP showed optimal expression (Figure 2D). As the tumor progressed to its initial level, the expression of SPOP showed an abundant overexpression pattern (Figure 2E), but as the tumor advanced to critical stage, the expression of SPOP was highly downregulated (Figure 2F).

Another set of experiments was carried out to assess the exact picture of ALDH1 and SPOP expression in primary and advanced stage of breast cancer. Western blotting analysis showed overlapping results with immunohistochemistry which concluded that the expression of ALDH1 gradually increased as the tumor progressed (Figure 3). SPOP responded with an overexpression in initial tumor stages and showed downregulated expression as a tumor developed to an advanced stage (Figure 3).

## Discussion

The aim of the research was to identify the SPOP expression in association with breast cancer stem cells. To initiate the research in this aspect the authors required an effective animal model which mimics primary and advanced breast tumor. They showed that they effectively induced primary and advanced breast tumor in the NOD SCID mice using MDA-MB-231 cells, which acted as an effective method of tumor initiation [12]. The severely immunocompromised mice lacked had compromised immunity and it was simple to initiate primary breast tumor in a four-week interval and proceeded to advanced stage tumor after nine weeks [13]. The histological complications helped to sort out the initial and advanced stage breast cancer, which showed tissue abnormalities as the tumor progressed (Figure 1A-C).

The research with SPOP implies that it acts as a tumor suppressor and its mutation leads to the development of different types of cancer [14, 15]. However the pathogenesis associated with tumorigenesis are still not understood in detail [16]. In the present research, the authors observed the expression pattern of SPOP which showed an overexpression pattern in the primary stage of the tumor and significantly downregulated as the tumor developed to an advanced stage (Figure 2D-F). The results imply that the injection of MDA-MB-231 cells did not cause any mutation and the intensity of intact SPOP showed a higher expression in the initial stages of tumor. The higher the expression of SPOP in primary tumor and its downregulated expression in advanced stage gave the conclusion that in the initial stage of tumor, SPOP aided to control the tumor progression. The correlation between ALDH1 and SPOP expression suggested the initial restricted signals for ALDH1 was

due to the upregulated expression of SPOP (Figures 2 and 3). However upon SPOP suppression, ALDH1 showed overexpression, which concluded the SPOP role in controlling the cancer stem cells.

In summary, the present results showed that the overexpression of SPOP had control over breast cancer stem cell expansion in the initial stages of breast tumor. However, as the SPOP downregulated it disrupted control over the breast cancer stem cells with overexpression of ALDH1.

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Corresponding Author:

H. Y. DONG, M.D.

Department of General Surgery, Hainan General Hospital

No.19 Xiu Hua Road, Xiuying District

Haikou 570311 (China)

e- mail: DerekxMartinsw@yahoo.com