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Original Article

Isolation and biological characteristics of human cervical cancer side population cells

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Abstract: Objective: By primary cultivation of clinical specimens of human cervical cancer, the main and side population cells were isolated and their biological characteristics were analyzed. Methods: Clinical specimens of human cervical tissue were cultured to get the primary cells. After staining by Hoechst33342, flow cytometry was used for sorting to get the main and side population cells. Their molecular biology, morphology and tumorigenicity were compared with that of Hela cells. Results: The primary cervical cancer and side population cells were isolated successfully, and the two cells have obvious tumor cell morphology. CK17, CK19, P63 and β1-integrin and other related markers were expressed. Tumorigenicity of side population cells was significantly higher than the main group of cells. Distribution of the cytoskeleton and expression associated tumor markers were not affected before and after tumorigenesis of the two cells. Conclusion: Side population cells which were cultured and isolated from clinical specimens of human cervical cancer have the characteristics of tumor stem cell, which can be used for further study of targeted therapy for cervical cancer.

Keywords: Cervical cancer, main population cells, side population cells

Introduction

Cervical cancer is one of serious risk factors that threaten women's health. Its morbidity and mortality rank second in the female reproductive system cancer, which has become a serious public health problem [1, 2]. Although the incidence of invasive cervical cancer has declined, the incidence of early cervical cancer increased, especially for its trend in the young [3]. In order to find the ultimate cause of cervical cancer, in basic research aspect, the molecular mechanism of onset is always a hot topic. With the cancer stem cell theory being put forward, as well as a growing number of cancer stem cells were isolated successfully in tumor tissue for cancer research, they provide a new way for cancer research. Cervical cancer stem cell research has become a hot topic for diagnosis and treatment research.

In recent years, flow cytometry found that in many tumor tissues the of the main group of cells staining (Main population cells in many tumor tissues, MP cells), which occupied the majority and can be stained by bisbenzimide (Hoechst33342) and rhodamine (Rhodanmin 123), and a very small amount, possible efflux of such DNA dyes and genotoxic chemotherapy drugs and other substances side population cells (Side population cells, SP cells) existed [4-6]. The latter has the characteristics of stem cells, which is considered to be the maintenance of tumor development for initiating cells and may play a key role in the mechanism of resistance and tumor recurrence [7, 8]. Currently, the main method of analysis of the SP tumor cells was that after DNA-specific dye or cell surface protein mark, flow cytometry was used for sorting. Dye method is the easiest for isolating cancer stem cells, in particular for the method which cannot determine a specific type of cancer stem cell molecular marker [9, 10].

The study combined Hoechst33342 staining with flow cytometry sorting method. From human clinical specimens, cervical primary MP and SP cells were isolated. Biological character-

istics and histological features were compared with Hela cells.

Materials and methods

Cell culture and sorting

Hela cells were purchased from Shanghai Chinese Academic Sciences Cell Bank. They were cultured in 1640 (Gibco) complete medium containing 1% antibiotics (Penicillin/Streptomycin, Gibco) and 10% FBS (Gibco). The temperature of the incubator was 37°C with 5% CO_2 and saturated humidity. Cervical tumor tissue was provided by our hospital. Necrotic tissue was removed under sterile conditions. Blood were washed out by PBS. Tissue blocks were cut into 1 mm^3 in size by ophthalmic scissors. They were placed in a pre-gelatin-coated dish with an appropriate distance evenly arranged, and then add an appropriate amount of 1640 complete medium into the incubator. Primary cultured cells, after adding an appropriate amount of digestion sorting buffer and adjusting the cell density to 5~8 × 106/ml, were stained by Hoechst33342 solution. They were filtered through sterile 300 mesh filter and then transformed into the stream pipe. Appropriate parameters were set. Hoechst-33342 excitation wavelength was 355 nm, wherein 460/50 band pass collect blue (Hoechst blue) and 670/30 collect red (Hoechst red). FACSVantage SE flow cytometry were used for cell sorting.

Immunofluorescence detection

After cell digestion, adjust the cell density to 2 × 104/ml and prepare cell climbing film. After enclosed by 4% paraformaldehyde, containing 0.5% Triton X-100 transparent PBS and 6% goat serum, 1:200 dilution of F-actin antibody (RD) were used for first antibodies and incubated at 4°C overnight; appropriate diluted amount of fluorescent was used for secondary antibodies (RD) and incubated at 37°C for 1 h; the right amount of dilution of DAPI was incubated at dark for 5 min; containing anti-fluorescence quencher Fengpian liquid were mounted and the laser confocal microscope was used to observe.

Immunohistochemistry

After cell digestion, adjust the cell density of 2 × 104/ml and prepare cell climbing film. After enclosed by 4% paraformaldehyde, containing 0.5% Triton X-100 transparent PBS, 3% H_2O_2 eliminating endogenous peroxidase activity and 6% goat serum, 1:200 dilution of CK17, CK19, P63 and β1-integrin (RD) were used for first antibodies and incubated at 4°C overnight; 1:200 dilution of biotin-labeled secondary antibody (RD) was used for secondary antibodies (RD) and incubated at 37°C for 30 min; HRP-labeled streptavidin liquid was used to incubated at 37°C for 30 min; DAB substrate was used to color for 10 min in the dark. Hematoxylin was used to restained for 1 min and washed by forte blue. Gradient alcohol dehydration and Xylene transparent process were processed, neutral gum was used for mounted.

HE staining

Gradient alcohol dehydration, xylene processing, embedded in paraffin and sliced were performed on 4% paraformaldehyde fixed tissue. After HE staining, graded alcohol dehydration and xylene processing were performed, then they were mounted with neutral gum.

Tumor formation experiment in nude mice

4 to 6-week-old BALB/C nude mice, male or female, were purchased from Experimental Animal Center of Third Military Medical University. They were randomly divided into three groups: Hela cell group, side cluster cell group, primary cancer groups with 5 mice in each group. After cell digestion, they were washed with PBS for twice. Adjust the cell density to 1×10^7/ml. 1×10^6 cells were inoculated subcutaneously in armpit portion of the nude. Since the first 10 days after inoculation, short diameter and long diameter of the tumor were measured every three days. Record tumor growth curves data. When the mice appeared back arched, activity limitation and other signs before death, the dislocation was performed and then remove tumor tissue for subsequent testing.

Results

MP and SP cells cultured and sorting

After tissue culture, we successfully obtained cervical cancer MP and SP cells (**Figure 1**). By flow cytometry the two cells underwent secondary sorting. Flow cytometry showed that more

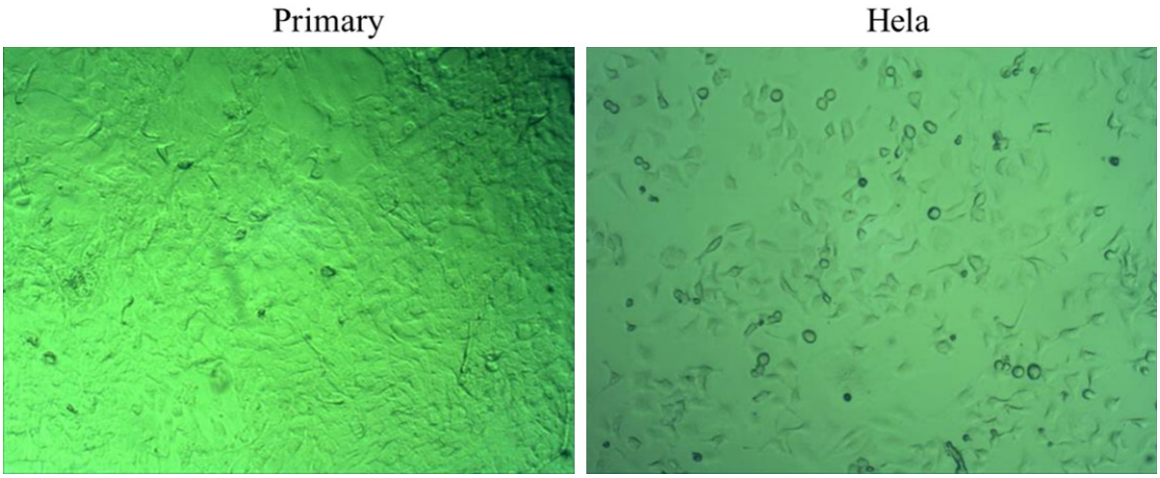

Figure 1. Culture of primary carcinoma tissue and Hela cells.

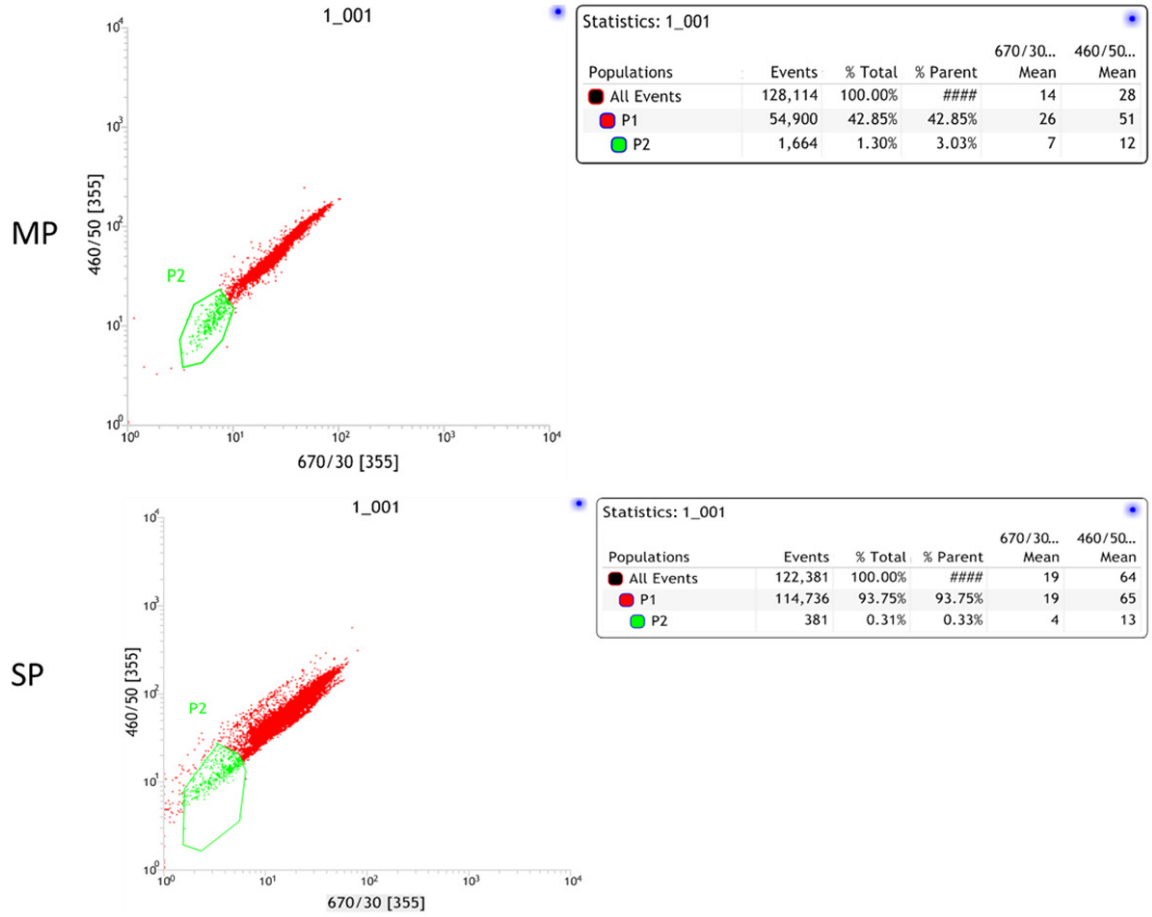

Figure 2. Sorting of MP and SP cervical carcinoma cells.

SP cells gathered in the middle and lower side position MP cells, indicating that SP cells had the ability to efflux Hoechst33342 stained; Fluorescence intensity of the two cells were

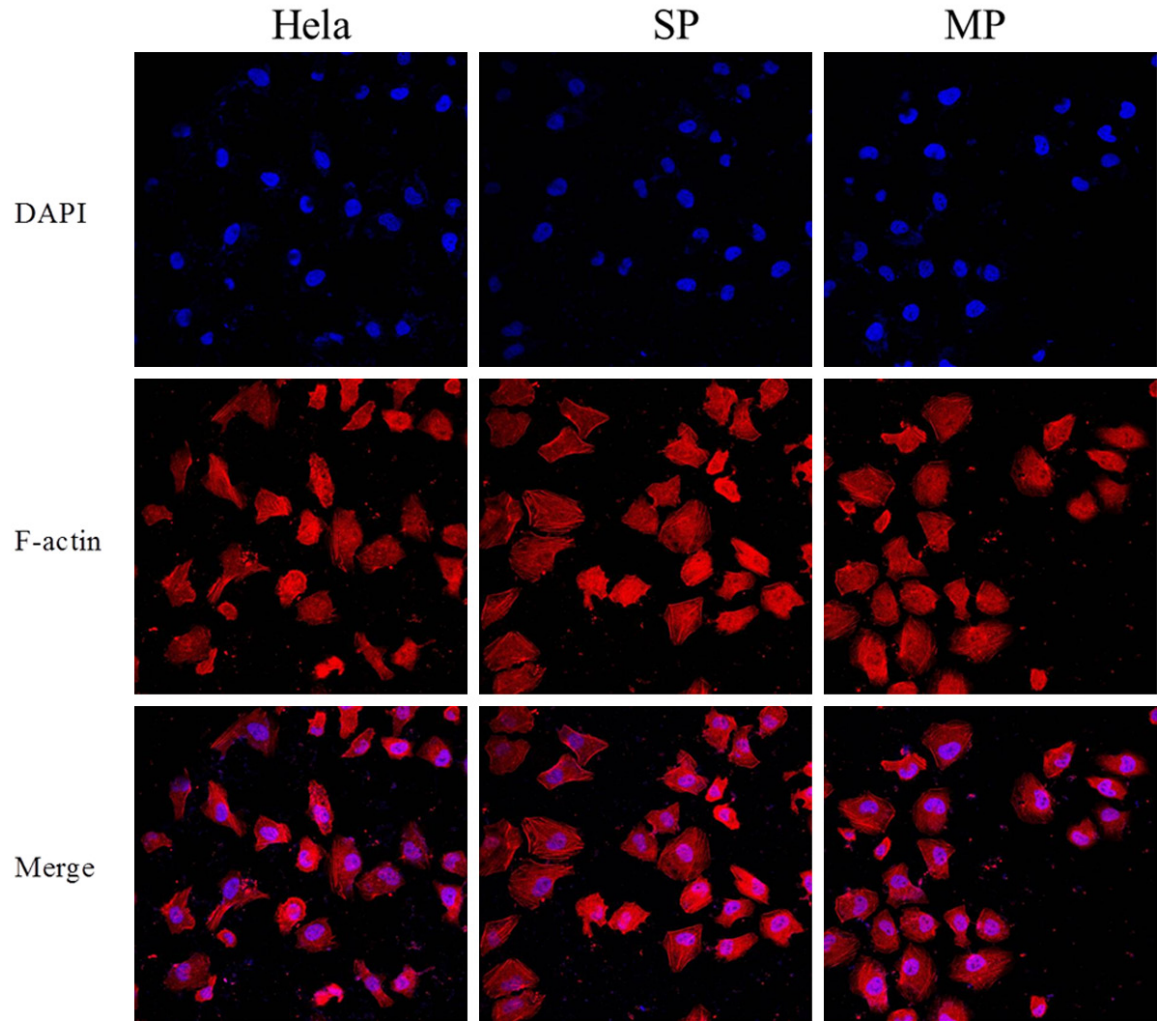

Figure 3. Skeleton detection of cervical cancer MP and SP cells.

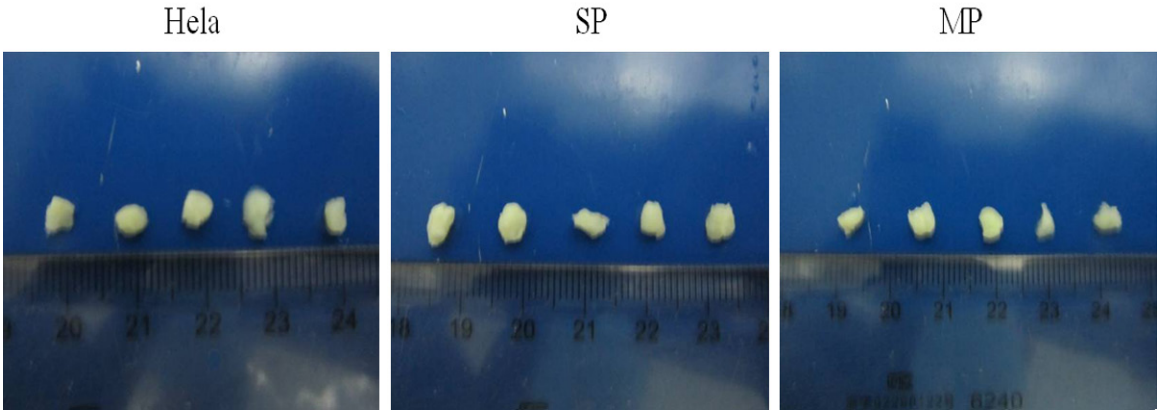

Figure 4. Size comparison of tumor formation among cervical cancer MP, SP cells and Hela cells in mice.

basically the same, indicating the size of the two cells were basically the same. After flow sorting for twice, the purity of SP cells was further increased (**Figure 2**).

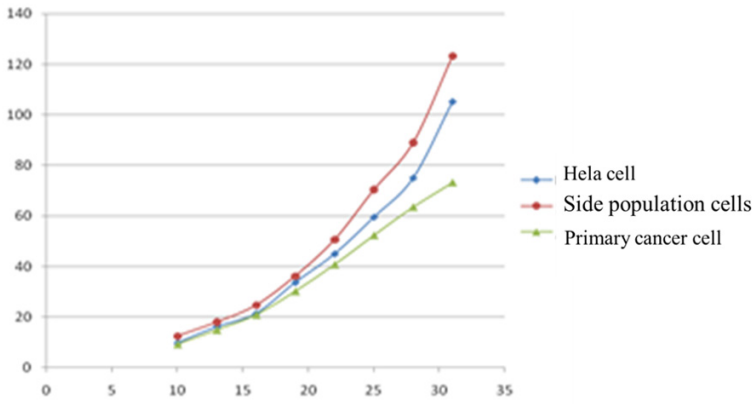

Figure 5. Comparison of tumor formation curve among cervical cancer MP, SP cells and Hela cells in mice.

MP and SP cells character identification

Immunofluorescence combined laser confocal microscope found that the nucleus size showed no significant difference between MP, SP cells and Hela cells which were isolated from human cervical carcinoma cells. The cell shape of MP and SP cells deformed into polarized lamellipodia and focal adhesion which rich in F-actin structure. It meant that migration ability of the both cells was higher than in vitro cultured Hela cells (**Figure 3**).

Identification of MP and SP cells after tumor formation

Results from Isolated human cervical tissue MP, SP cells and Hela cell in nude mice showed that three kinds of cells can form solid tumor (**Figure 4**). Tumor growth curve showed three cell tumor formation rate were: SP cells > Hela cells > MP cells (**Figure 5**). HE staining of tumor sections showed three tumor cells had significant tumor tissue morphology with squamous cell differentiation. Its degree of differentiation was: Hela cell > MP cell > SP cells (**Figure 6**). Combined with the results of laser confocal immunofluorescence microscopy, it showed that after tumor formation, the nucleus size, lamellipodia and focal adhesion formation of F-actin rich in terms of structure of MP, SP cells and Hela cells in nude mice showed no significant difference. It meant that migration ability of MP, SP cells did not change significantly, while Hela cells after in vivo tumor formation, migration ability was improved (**Figure 7**). The use of immunohistochemistry was used to detect three tumor cells the keratin CK17, CK19, P63 tumor suppressor proteins and extracellular matrix β1-integrin. Result showed that after tumor formation of MP and SP cells, the expression of four related factors were consistent with Hela cells, and were not affected (**Figure 8**).

Discussion

SP cells were firstly discovered in mouse hematopoietic cells. Later it is found that SP cells do not have tissue-specificity, and it widely presents in most normal tissue and diseased tissue in humans and animals. SP cells were also presented in human and murine tumor cells, such as C6 rat glioma cell lines, human breast cancer, ovarian cancer, liver cancer, thyroid cancer, lung cancer, stomach cancer and esophageal cancer and other cell lines. The content of SP cells is 0.1% to 20% in tumor cells [11-14]. In recent years, the relationship between SP cells and tumor stem cell research became a hot spot. SP cells in tumor cells are usually able to avoid the effects of chemotherapy drugs. Studies have shown that the proportion of SP cells in primary tumor mass correlated with grading of cancer patients and prognosis [15, 16]. In addition, SP cells have self-renewal and differentiation function, which is very similar to stem cells [17]. The origin theory of stem cells has become one of mechanisms of tumor cells [18].

In this study, cervical cancer clinical samples were used for primary culture and dye method was used twice for FACS sorting. We successfully screened cervical MP and SP cells. By molecular biology and its morphological analysis, MP and SP cells were compared with Hela cells, and the cell body had lamellipodia and focal adhesion structures, indicating that primary cultured cells have higher migration capability. Cervical cancer differentiation, tumor suppressor genes and migration-related markers CK17, CK19, P63 and β1-integrin expression existed in the both group cells. The results showed that in nude mice, rate of tumor formation of SP cells were faster than that of MP. HE staining results of tumor tissue showed that

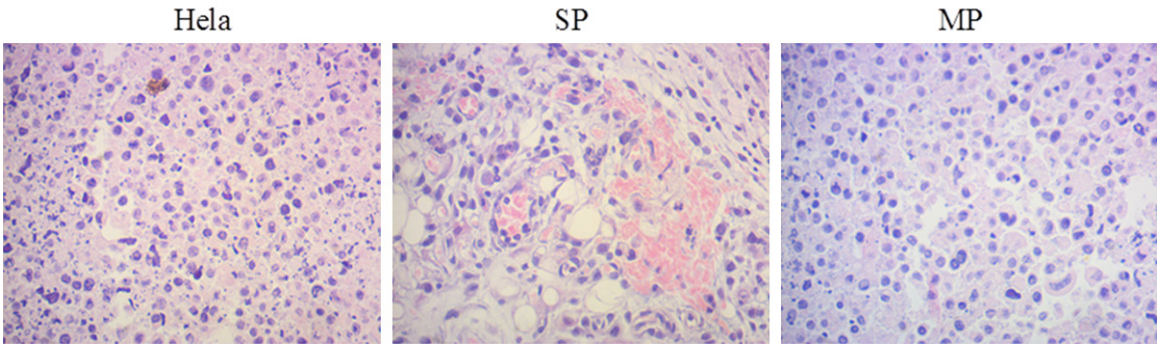

Figure 6. HE staining of Tumor cell body.

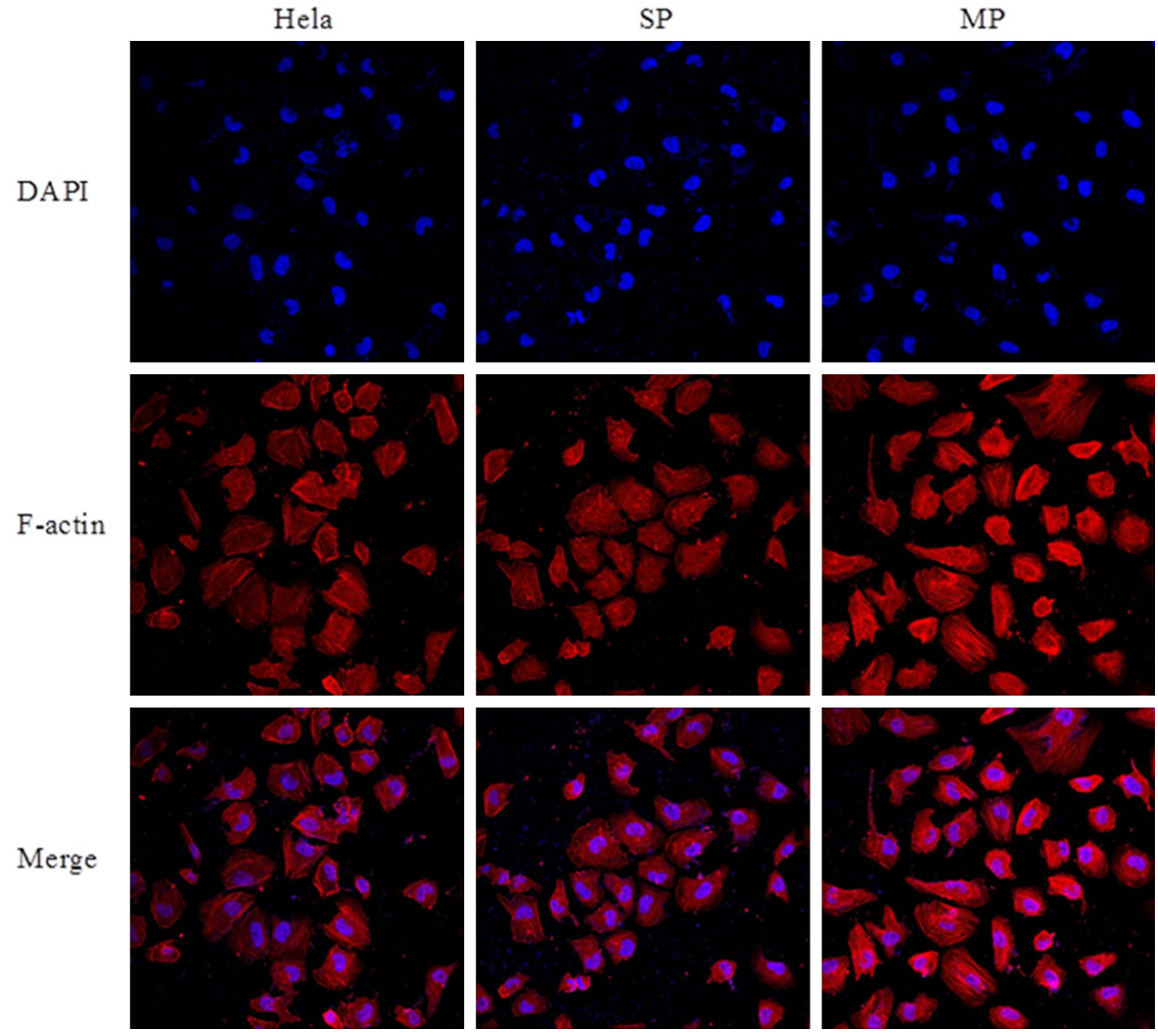

Figure 7. Cytoskeleton detection of tumor cell body.

the degree of SP tumor cell differentiation was lower than that of MP. Before and after tumor formation, the two cell skeleton distribution and expression associated markers were not affected.

These above results showed that the SP cells, which were isolated from cervical cancer and identified in vitro, by in vivo tumor formation and other tests, were confirmed with a low degree of differentiation, higher tumorigenicity

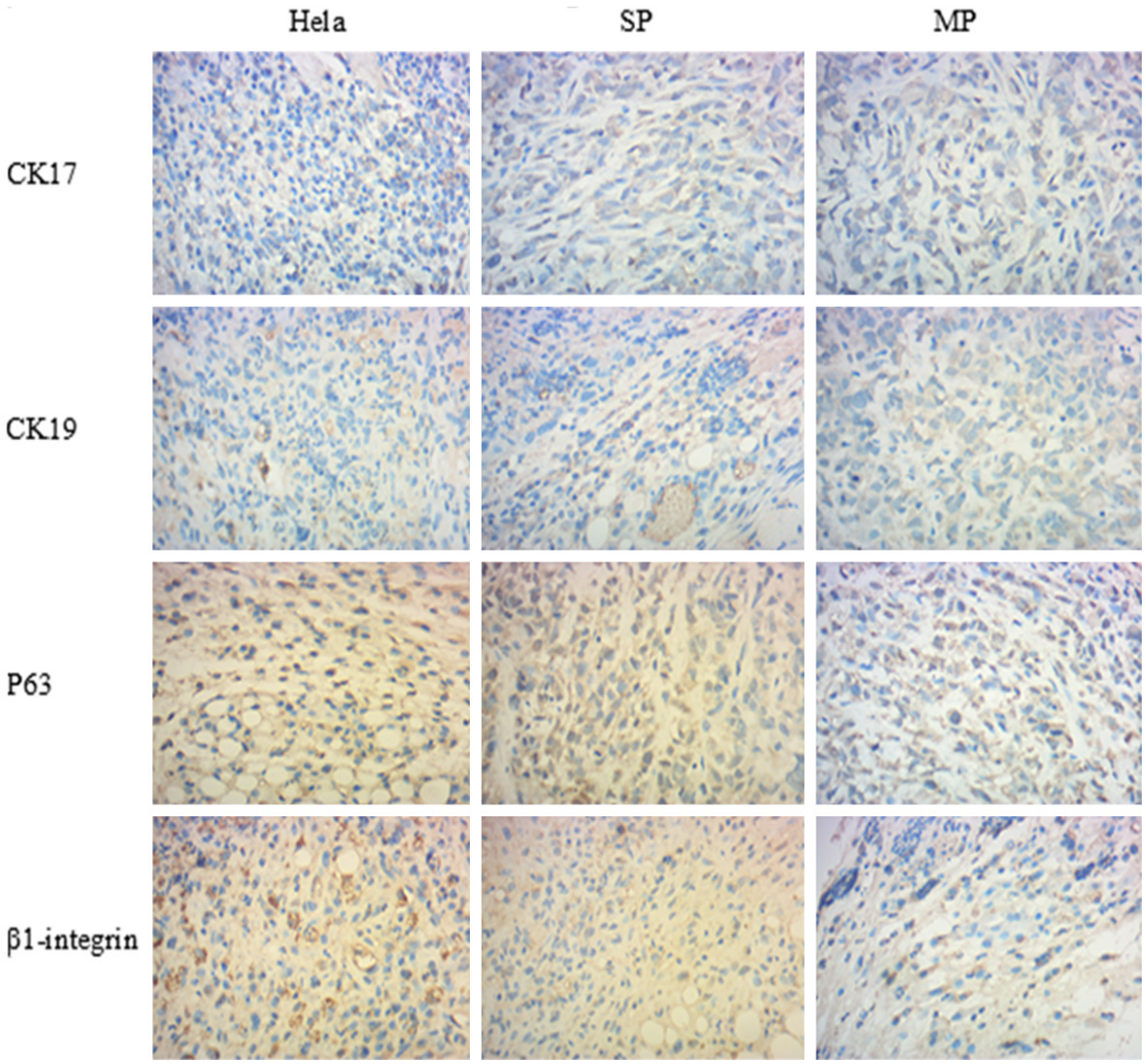

Figure 8. Related factors detection of Tumor cell body.

and characteristics of cancer stem cells. It can be used as the cut point for cancer stem cell research and provide experimental basis for cancer stem cells targeted therapy.

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Disclosure of conflict of interest

None.

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