Abstract. The aim of the present study was to establish an ovarian cancer (OC) cell line from ascites of an ovarian serous cystadenocarcinoma patient and investigate the biological characteristics of its side population (SP) cells. The OC cell line was established by isolating, purifying and subculturing primary cells from ascites of an ovarian serous cystadenocarcinoma patient (stage IIIc; grade 3). SP and non-SP (NSP) cells were isolated by fluorescence-activated cell sorting and cultured in serum-free medium and soft agar to compare the tumorigenesis and colony formation capacities. Furthermore, SP and NSP cell tumorigenesis was examined by subcutaneous and intraperitoneal injection of the cells to non-obese diabetic/severe combined immune deficiency (NOD/SCID) mice. Drug resistance to cisplatin was examined by cell counting kit-8. The OC cell line was successfully established from ascites of an ovarian serous cystadenocarcinoma patient, which exhibited properties similar to primary tumors subsequent to >50 passages and >2 years of culture. The SP cell ratio was 0.38% in the OC cell line, and a similar SP cell ratio (0.39%) was observed when sorted SP cells were cultured for 3 weeks. Compared with NSP cells, SP cells exhibited increased abilities in differentiation and tumorigenesis and colony formation, in addition to the formation of xenografted tumors and ascites and metastasis of the tumors in NOD/SCID mice, even at low cell numbers (3.0x10^3 cells). The xenografted tumors demonstrated histological features similar to primary tumors and expressed the ovarian serous cystadenocarcinoma marker CA125. In addition, SP cells demonstrated a significantly stronger drug resistance to cisplatin compared with NSP and unsorted cells, while treatment with verapamil, an inhibitor of ATP-binding cassette transporters, potently abrogated SP cell drug resistance. In conclusion, the present study verified SP cells from an established OC cell line and characterized the cells with self-renewal, differentiation, proliferation, tumorigenesis and stronger drug resistance capacities.

Introduction

Ovarian cancer (OC) is the leading cause of mortality resulting from gynecological cancers, and worldwide, ~114,000 patients succumb to OC annually (1). Although OC patients exhibit a 5-year survival rate of 90% when treated at an early stage, 80% of patients cannot be diagnosed until the advanced stages; therefore, the 5-year survival rate is 30% (2). OC recurrence occurs in 70% of patients following surgery and platinum-based chemotherapy combined treatments, which causes patients to become insensitive to chemotherapy (3). Consequently, tumor recurrence and drug resistance are two common set-backs in the treatment of OC (4,5). Cancer stem cells (CSCs) are a type of tumor cell that possess an unlimited potential for self-renewal and may differentiate into multiple tumor cell types (6). Numerous studies have demonstrated that CSCs possess various characteristics, including a silent phenotype, enhanced DNA repair capacity, expression of ATP-binding cassette (ABC) transporters and anti-apoptotic proteins, and resistance to conventional chemotherapy and radiotherapy (6-14). Conventional chemotherapy may eliminate the majority of tumor cells, but it has little impact on rare stem cell-like cells; and remaining CSCs cause tumor recurrence and metastasis. Therefore, studies concerning cancer should consider CSCs as a vital target to achieve the complete ablation of tumors, and should not solely focus on temporarily shrinking the tumor mass.

Goodell et al (15) reported that a small cell population isolated from murine bone marrow demonstrated distinct fluorescence-activated cell sorting (FACS) results compared with the main cell population, termed the side population (SP) cells. Numerous studies have demonstrated that SP cells, isolated from numerous tumors, richly contain tumor-initiating cells that possess stem cell characteristics (16-20). A
low-fluorescence staining phenotype is mediated by ABC transporters (21), which provide a functional method for isolating SP cells.

Although SP cells have been successfully isolated from certain human and mouse ovarian cell lines (22,23), the present study established an immortalized OC cell line from primary cells in ascites and identified SP cells from this cell line. Additionally, the present study investigated the biological characteristics of the SP cells, including differentiation and tumorsphere and colony formation, in addition to xenografted tumor formation and ascites, metastasis and drug resistance of the xenograft tumors.

Materials and methods

Establishment of an ovarian cancer cell line. Primary cells were isolated from ascites of an ovarian serous cystadenocarcinoma patient. Briefly, primary cells were harvested by centrifugation at 300 x g for 5 min and red blood cells were removed by 1X BD lysis buffer (BD Biosciences, Franklin Lakes, NJ, USA) on ice for 1 min, followed by centrifugation at 300 x g for 3 min. Primary cells were cultured for 3 weeks in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Gibco®; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Floating cells were collected and re-cultured. Subsequent to subculturing for 15 passages, primary cells were identified by a tumor xenograft model; the tumor tissues were examined with hematoxylin and eosin staining and CA125 immunostaining.

Isolation of side population cells. The cells were trypsinized, resuspended at 1.0x10^6 cells/ml in pre-warmed DMEM containing 2% flow cytometry staining buffer (CycleTEST™ PLUS DNA Reagent kit; BD Biosciences) and incubated at 37°C for 10 min. The cells were labeled with 5 µg/ml Invitrogen™ Hoechst 33342 dye (Thermo Fisher Scientific, Inc.) at 37°C for 80 min, alone or combined with 50 mM verapamil (Sigma-Aldrich, St. Louis, MO, USA), an inhibitor of ABC transporters. The cells were counterstained with 1 µg/ml propidium iodide. In total, 100,000 cells were analyzed on a BD Influx cell sorter (BD Biosciences) and data were processed by BD FACSDiva version 6.1.1 software (BD Biosciences).

Tumorsphere formation assay. A total of 500 SP and non-SP (NSP) cells were plated onto a 24-well ultra-low attachment plate, and cultured in a DMEM/F12 serum-free medium (Gibco®; Thermo Fisher Scientific, Inc.) supplemented with 4 µg/ml insulin (Sigma-Aldrich), 10% human leukocyte antigen B27 (Gibco®; Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich), and 20 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich), for 10 days. Tumorspheres >50 mm in diameter were counted under a phase-contrast microscope (IX50; Olympus Corporation, Tokyo, Japan).

Soft agar colony formation assay. A total of 200 SP and NSP cells were resuspended in a 0.8 ml growth medium (DMEM with EGF, bFGF and B27) containing 0.3% low-melting agarose (Sigma-Aldrich) and plated 3 times onto a 24-well plate pre-coated with a base layer of 0.8 ml growth medium containing 0.6% low-melting agarose. The plates were incubated for 14-15 days until the size of colonies was large enough to count. Colonies >75 µm in diameter or colonies that possessed >50 cells were counted as positive colonies.

Xenograft tumor assay. In total, 45 female 5-week-old non-obese diabetic-severe combined immune deficiency (NOD/SCID) mice weighing 16-20 g were purchased from Vital River Laboratories, Co., Ltd. (Beijing, China). The mice were housed in a sterilized room with 12 h light/dark cycle, at a temperature of 22°C with 40-60% humidity. Food and water were provided ad libitum. Animal experiment protocols were approved by the Ethical Committee of the First Affiliated Hospital of Jilin University (Changchun, China). Subsequently, 1.0x10^6 OC cells were injected into the dorsal flank of 5-week-old female NOD/SCID mice, and tumor formation and growth were observed after 6 weeks. SP and NSP cells were sorted by FACS and resuspended in serum-free DMEM. In 0.1 ml DMEM, 3.0x10^3 or 3.0x10^4 SP and NSP cells were subcutaneously injected into the dorsal flank of 5-week-old female NOD/SCID mice, or intraperitoneally injected into 5-week-old female NOD/SCID mice (n=5). Formation of tumor and ascites were examined subsequent to 3 weeks.

Drug resistance assay. SP, NSP and unsorted cells were seeded in 96-well plates at 3,000 cells/well. Subsequent to 24-h culture, cells were treated with 0.25, 0.50, 1.00, 2.00, 4.00, 8.00 and 16.00 µg/ml cisplatin (Sigma-Aldrich) for 72 h. All treatments were run in triplicates. Cells without cisplatin treatment were used as negative controls. Following treatment, all cells were treated with 10 ml cell counting kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Kumamoto, Japan) in 100 ml complete high-glucose DMEM (Gibco®; Thermo Fisher Scientific, Inc.) and cultured in normal culture conditions (DMEM with 10% FBS, 5% CO_2 at 37°C) for 2.5 h. In total, 3 wells without cells were used as blank controls. Absorbance at 490 nm was examined by a Model 550 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was determined by the following formula: Cell viability (%) = [optical density (OD) values of drug-treated cells – OD values of blank controls] / (OD values of negative cells – OD values of blank control) x 100. Cisplatin doses of 50% growth inhibition (IC_{50}) in SP, NSP and unsorted cells were calculated using GraphPad software, version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA).

To additionally verify SP cell drug resistance, unsorted cells were treated with the IC_{50} dose of cisplatin for 3 h and cultured in a drug-free complete medium for 72 h. The SP cell ratio was examined by FACS. To investigate the association between drug resistance and ABC transporters, SP cells were treated with IC_{50} dose of cisplatin or cisplatin with verapamil for 3 h, and were then cultured in a drug-free complete medium for 72 h. Cell viability was examined using the CCK-8 kit.

Statistical analysis. Statistical significance of differences between two groups was analyzed using two-tailed unpaired Student’s t-test. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significantly difference.
Results

Establishment of human ovarian cancer cell line from ascites. Primary OC cells were purified from ascites of an ovarian serous cystadenocarcinoma patient (stage IIIc; grade 3) and cultured for >50 passages over 2 years. Subcutaneously, 1.0x10^6 OC cells were injected into the dorsal subcutaneous thigh of female non-obese diabetic/severe combined immune deficiency mice (n=3) and the tumor was observed 8 weeks later. Histology of a primary OC tumor and xenografted OC tumor from an established OC cell line (hematoxylin and eosin staining; scale bar=100 mm). (C) Expression of ovarian serous cystadenocarcinoma marker CA125 in xenografted tumors by immunohistochemistry. Scale bar=100 mm. OC, ovarian cancer.

Isolation of SP cells from established OC cell line. SP cells possess low-fluorescence staining properties (21). FACS was used in the present study to isolate SP cells using Hoechst 33342 staining. Verapamil is an ABC transporter protein inhibitor that may effectively reduce the SP cell ratio (25). The present study observed weak Hoechst staining in certain cells, indicating that these are SP cells (Fig. 2A). The SP cell ratio in the OC cell line was 0.38% (n=5), while the SP cell ratio was significantly reduced in the presence of verapamil (P=0.001; Fig. 2B and C).

Biological characteristics of SP cells: Differentiation potential of SP and NSP cells. To evaluate the differentiation potential of isolated SP cells, 1.0x10^3 SP and NSP cells were cultured for 3 weeks and the SP cell ratios were compared between the two groups. The average SP ratio was 0.39% following SP cell culture for 3 weeks in vitro, which was a similar ratio to unsorted OC cells. The average SP ratio was 0.06% in NSP cells subsequent to 3 weeks of culture, which was significantly lower compared with SP and unsorted OC cells (P=0.004; Fig. 2D and E). These results indicate that SP cells exhibited competent self-renewal and differentiation capacities in vitro, but NSP cells did not.

In vitro tumorsphere formation of SP and NSP cells. In total, 500 single SP and NSP cells were cultured in a serum-free medium supplemented with growth factor for 10 days, and...
tumorsphere formation was calculated. The present study demonstrated that the average number of tumorspheres formed in SP and NSP cells were 42.7 and 6.3, respectively (P=0.009; Fig. 3A and B). These data indicate that SP cells possess improved self-renewal capacities compared with NSP cells.

**SP and NSP cell colony formation.** To further verify the present findings, 500 SP and NSP cells were seeded in soft agar medium and the cells were cultured for 14-15 days to observe the colony formation ratio. The present study observed that the colony formation ratio in SP cells (41.33%) was significantly higher compared with NSP cells (8.93%) (P=0.008; Fig. 3C and D). These data suggest that SP cells have greater colony formation capacities than NSP cells.

**Tumorigenesis potential of SP and NSP cells.** To evaluate the tumorigenesis potential of SP and NSP cells, 3.0x10^4 and 3.0x10^3 SP and NSP cells were subcutaneously injected into the dorsal flank of the NOD/SCID mice. Xenografted tumors were observed in all 5 mice at 52 (3.0x10^4 cells) and 63 (3.0x10^3 cells) days subsequent to the injection of SP cells. None of the mice formed tumors 70 days following the injection of 3.0x10^3 NSP cells, and only 1 xenografted tumor was observed in 1 mouse at 54 days subsequent to injection of 3.0x10^4 NSP cells (Fig. 4A). Furthermore, the present study also evaluated the intraperitoneal tumorigenesis of SP and NSP cells. Following intraperitoneal injection of 3.0x10^4 cells into NOD/SCID mice, tumors were observed in the abdominal cavities of all 5 mice by 45 days post-injection, however, only 1 mouse demonstrated ascites and metastasis after 56 days (Fig. 4B and C). At 56 days post subcutaneous injection of 3.0x10^4 cells, the xenografted tumor size from SP cells was clearly larger than the size of the tumor from NSP cells (Fig. 4C). Similarly, the present study observed blood ascites and clear metastasis in the abdominal cavity of all 5 mice following the intraperitoneal injection of 3.0x10^4 SP cells for 50 days, but this was not observed in any of the mice following the injection of the same amount of NSP cells for 56 days (Fig. 4B and D). These data indicate that the tumorigenesis potential of SP cells was significantly greater compared with NSP cells.

**Histological and cytological characteristics.** In addition, the present study investigated the histological and cytological differences between the primary and xenografted tumors. Similar to the primary tumors, xenografted tumors exhibited poorly differentiated serous cystadenocarcinoma histological and cytological features following subcutaneous and intraperitoneal injection (Fig. 5A). Furthermore, CA125 expression was observed in two types of xenografted tumors (Fig. 5B). These results verify that SP cells possess competent self-renewal and differentiation capacities in vivo.

**Drug resistance of SP cells.** To investigate the drug resistance properties of SP cells, the present study treated SP, NSP and unsorted cells with 0.25, 0.50, 1.00, 2.00, 4.00, 8.00 and 16.00 g/ml of cisplatin for 72 h and examined the cell viability using a CCK-8 kit. It was observed that the cell viability of SP cells was significantly higher compared with NSP and unsorted cells when treated with 1.0 - 4.0 g/ml of cisplatin (P=0.038 vs. NSP cells; P=0.042 vs. unsorted cells; Fig. 6A). However, there was no marked difference in cell viability following cisplatin treatment between NSP and unsorted cells (Fig. 6A). IC_{50} values for unsorted, SP and NSP cells were 1.75, 2.38 and 1.63 and µg/ml, respectively, and the IC_{50} value of SP cells was significantly higher than those of the NSP and unsorted cells (P=0.038 vs. NSP cells; P=0.042 vs. unsorted cells; Fig. 6B). These results indicate that SP cells exhibit...
greater drug resistance capacities compared with NSP and unsorted cells. Furthermore, the SP cell ratio to unsorted cells was significantly elevated following cisplatin treatment, from 0.38 to 10.18% (P=0.007; Fig. 6C). These data support the theory that SP cells are more resistant to cisplatin treatment compared with NSP and unsorted cells.

To clarify the association between drug resistance of SP cells and ABC transporters, the SP cells were treated with an IC50 dose of cisplatin or cisplatin + verapamil. The results demonstrated that cell viability was 71.41% following cisplatin treatment, while it was reduced to 6.00% following treatment with cisplatin + verapamil (P=0.006; Fig. 6D). These data indicate that verapamil increases the sensitivity of SP cells to cisplatin treatment, suggesting that the drug resistance of SP cells to cisplatin depends on the drug efflux function of ABC transporters.

Discussion
Cancer cell lines are widely used in research to study the biology of cancer and examine cancer treatments. These cell lines can be cultured for long periods of time and share the tumor-associated biological characteristics of the original tumor (26). The present study successfully established an OC...
Stem cells have strong self-renewal capacities and form floating spheroids in a serum-free medium supplemented with growth factor, while differentiated tumor cells become apoptotic (6,24). The present study additionally demonstrated that ABC transporters are important in cisplatin-induced SP cell accumulation. Furthermore, it has been demonstrated that ABC transporters are important in cisplatin-induced drug resistance in OC (43,44). The present study identified an ABC transporter inhibitor (verapamil) that potently abrogated SP cell resistance to cisplatin, which is consistent with previous observations in OC cell lines (12). These data indicate that SP cell drug resistance to cisplatin depends on the efflux function of ABC transporters, suggesting a promising therapy for drug-resistant OC. In addition, previous studies demonstrated that increased chemoresistance of SP cells is associated with diverse mechanisms, including alterations in signaling pathways and enzymes, reduced apoptosis and increased DNA repair (19,45,46). However, additional study is required to verify whether these mechanisms exist in SP cells from OC.

In summary, the present study successfully isolated and established an OC cell line. Isolated SP cells from the established OC cell line possessed similar biological characteristics, including self-renewal, differentiation and high tumorigenicity. In addition, previous studies have demonstrated that certain molecular markers, including cluster of differentiation (CD)133, CD44 and CD117, may be associated with diverse mechanisms, including alterations in signaling pathways and enzymes, reduced apoptosis and increased DNA repair (19,45,46). However, additional study is required to verify whether these mechanisms exist in SP cells from OC.
characteristics as cancer stem cells, including self-renewal, differentiation, proliferation, tumorigenesis and drug resistance. This cell line and SP cells provide valuable models for studying OC tumorigenesis and drug resistance mechanisms that may aid in developing specific therapies for targeting OC SP or stem cells.

Acknowledgements

The present study was supported by the Jilin University Bethune plan B (grant no. B2012227) and Jilin Province Science and Technology Development Program (grant no. 20140520032JH).

References

1. Pecorelli S, Favalli G, Zigliani L and Odicino F: Cancer in women. Int J Gynaecol Obstet 82: 369-379, 2003.
2. Ozols RF: Update on the management of ovarian cancer. Cancer J 8 (Suppl 1): S22-S30, 2002.
3. Ozols RF: Treatment goals in ovarian cancer. Int J Gynecol Cancer 15 (Suppl 1): 3-11, 2005.
4. Cannistra SA: Cancer of the ovary. N Engl J Med 351: 2519-2529, 2004.
5. Ozols RF, Bookman MA, Connolly DC, Daly MB, Godwin AK, Schiller RJ, Xu X and Hamilton TC: Focus on epithelial ovarian cancer. Cancer Cell 5: 19-24, 2004.
6. Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. Nature 414: 105-111, 2001.
7. Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC and Dirks PB: Tumour-initiating cells: Challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 8: 806-823, 2009.
8. Gottesman, MM: Mechanisms of cancer drug resistance. Annu Rev Med 53: 615-627, 2002.
9. Schatton T, Murphy GF, Frank NY, Yamamura K, Wuaaga-Gasser AM, Gasser M, Zhan Q, Jordan S, Duncan LM, Weishaupt C, et al: Identification of cells initiating human melanomas. Nature 451: 345-349, 2008.
10. Yu, F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J and Song E: let-7 regulates self renewal and tumorigenesis of breast cancer cells. Cell 131: 1109-1123, 2007.
11. Zhou, S., Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Laguntina I, Grosveld GC, Osawa M, Nakauchi H and Sorrentino BP: The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is amoleculardeteminant of the side-population phenotype. Nature Med 7: 1028-1034, 2001.
12. Ito, K., Hirao A, Arai F, Matsuoka S, Takubo K, Hamaguchi I, Ito, K, Hirao A, Arai F, Matsuoka S, Takubo K, Hamaguchi I, Nomiyama K, Hosokawa K, Sakurada K and Nakagata N: Regulation of reactive oxygen species levels and radioresistance in cancer stem cells. Proc Natl Acad Sci USA 103: 11154-11159, 2006.
13. Moriguchi S, Kawasaki M, Yamashita S: Characterization of side population in thyroid cancer cell line. Cell Biol Int 35: 227-234, 2011.
14. Sztoket PP, Pietrini-Vanmearcke R, Masiakos PT, Dinulde MC, Connolly D, Foster R, Dombockowski D, Preffer F, Maclaughlin DT and Donahoe PK: Ovarian cancer side population defines cells with stem-cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci USA 103: 11154-11159, 2006.
15. Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schiller JM, Yan PS, Huang TH and Nephew KP: Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 68: 4311-4320, 2008.
16. Ishikawa S, Stadler W, Adina S, Kost-Alimova M, Klippel S, Cervi D, Daley JF, Cholujova D, Kong SY, Leiba M, Blotta S, et al: Lenalidomide targets clonogenic side population in multiple myeloma: Pathophysiologic and clinical implications. Blood 117: 4409-4419, 2011.
17. Douglas DJ, Fiegler H, Rowan S, Halford S, Binkell DC, Bodmer W, Tomlinson IP and Carter NP: Array comparative genomic hybridization analysis of colorectal cancer cell lines and primary carcinomas. Cancer Res 64: 4817-4825, 2004.
18. Bonnet D and Dick JE: Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730-737, 1997.
19. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983-3988, 2003.
20. Collins AT, Berry PA, Hyde C, Stower MJ and Matlind NJ: Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65: 10946-10951, 2005.
21. O’Brien CA, Pollett A, Gallinger S and Dick JE: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445: 106-110, 2007.
22. Moriguchi S, Hawkins C, Clarke I, Squire JA, Bayani J, Hide H, Henkelman RM, Cusimano MD and Dirks PB: Identification of human brain tumour initiating cells. Nature 432: 396-401, 2004.
23. Zheng SL, Wang YS, Zhou T, Yu KW, Wei ZT and Li YL: Isolation and characterization of cervical cancer cell line. Cell Biol Int 31: 997-1002, 2004.
24. Douglas DJ, Fiegler H, Rowan S, Halford S, Binkell DC, Bodmer W, Tomlinson IP and Carter NP: Array comparative genomic hybridization analysis of colorectal cancer cell lines and primary carcinomas. Cancer Res 64: 4817-4825, 2004.
43. Moreno-Smith M, Halder JB, Meltzer PS, Gonda TA, Mangala LS, Rupaimoole R, Lu C, Nagaraja AS, Gharpure KM, Kang Y, et al: ATP11B mediates platinum resistance in ovarian cancer. J Clin Invest 123: 2119-2130, 2013.

44. Januchowski R, Zawierucha P, Andrzejewska M, Ruciński M and Zabel M: Microarray-based detection and expression analysis of ABC and SLC transporters in drug-resistant ovarian cancer cell lines. Biomed Pharmacother 67: 240-245, 2013.

45. Chen Y, Li D, Wang D, Liu X, Yin N, Song Y, Lu SH, Ju Z and Zhan Q: Quiescence and attenuated DNA damage response promote survival of esophageal cancer stem cells. J Cell Biochem 113: 3643-3652, 2012.

46. Li XX, Dong Y, Wang W, Wang HL, Chen YY, Shi GY, Yi J and Wang J: Emodin as an effective agent in targeting cancer stem-like side population cells of gallbladder carcinoma. Stem Cells Dev 22: 554-566, 2013.