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Retinoic acid increases the anticancer effect of paclitaxel by inducing differentiation of cancer stem cells in melanoma

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Malignant melanoma is a highly lethal disease, and advanced stages of melanoma have proven to be resistant to many chemotherapeutic drugs including temozolomide and paclitaxel. Cancer stem cells (CSCs) have been identified and isolated in different cancers including melanoma, and have been proven playing important role in the drug resistance. Retinoic acid (RA) is a promising anticancer agent, which can induce differentiation of CSCs. The main purpose of the present study was to evaluate the possible RA-induced differentiation of melanoma CSCs and sensitization of melanoma CSCs to paclitaxel. Our results show that CSCs of human melanoma A375 cells was more tolerant to paclitaxel than other non-CSCs melanoma cells. On the contrary, RA had stronger inhibitory effect on melanoma CSCs than on non-CSCs. At the same time, RA could arrest the cell cycle of CSCs and reduce the expression of Sox-2, Oct-4 in CSCs of melanoma, thereby induced the differentiation of CSCs and increased its sensitivity to paclitaxel. With this study we concluded that RA increases the anticancer effect of paclitaxel by inducing differentiation of cancer stem cells in melanoma, and the combined application of RA and paclitaxel may be more effective in the treatment of melanoma.

1. Introduction

According to the World Health Organization, malignant melanoma remains among the most aggressive human cancers, and its incidence has increased dramatically over the last decades (Nikolaou and Stratigos 2014; Garbe and Leiter 2009). Although significant progress has been made in melanoma treatment, the prognosis for melanoma remains very poor. Five-year survival rates of patients with stage IV of less than 5% (Chi et al. 2011; Cummins et al. 2006) and resistance to many chemotherapeutic drugs including temozolomide and paclitaxel is one of the primary reasons.

Multiple mechanisms contribute to the development of drug resistance in melanoma (Helmbach et al. 2001; La Porta 2007). Cancer stem cells (CSCs) in advanced stage of melanoma are one of the main reasons for the drug resistance. CSCs are a subset of cancer cells which have the ability of self-renewal and multi-lineage differentiation (stemness) (Lee et al. 2011). Higher expression of intracellular and extracellular drug transport system and stronger ability to proliferation, anti-apoptotic and repair damage to DNA of CSCs make them more tolerant to chemotherapic drugs than other cancer cells which is non-CSCs (Colak and Medema 2014; Fang et al. 2005).

Retinoic acid (RA) is a small lipophilic molecule, which is derived from vitamin A and involved in regulating many developmental processes (Marlétaz 2006). In cancer treatment, RA has been proven to induce differentiation of cancer stem cells, reduce the proliferation and migration ability of CSCs, and increase the sensitivity of CSCs to chemotherapeutic drugs (Ginestier et al. 2009; Lim et al.2012). However, RA has poor inhibitor effect on cancer cells of non CSCs (Kraemer et al. 1988).

So far, there were only few studies on inducing differentiation of melanoma CSCs to reduce their drug resistance by RA. To provide evidence for the clinical combined application of RA and paclitaxel in melanoma treatment, the CSCs of human melanoma cells were sorted, then the effect of RA and paclitaxel was assessed and the underlying molecular mechanisms were explored.

2. Investigations and results

2.1. Sorting of melanoma cancer stem cells (SP cells)

Side population cells (SP cells) are a small number of cells, which have stem cell-like properties and are capable of self-renewal and differentiation. Therefore, sorted SP cells were often used as the cancer stem cells (Li et al. 2011). The melanoma cancer stem cells were sorted from A375 cells and by the FACSDiva. The results showed that the percentage of cancer stem cells (SP cells) was 2.27±0.2 % (Fig. 1). The SP cells and Non-SP cells were sorted and used for the next experiments.

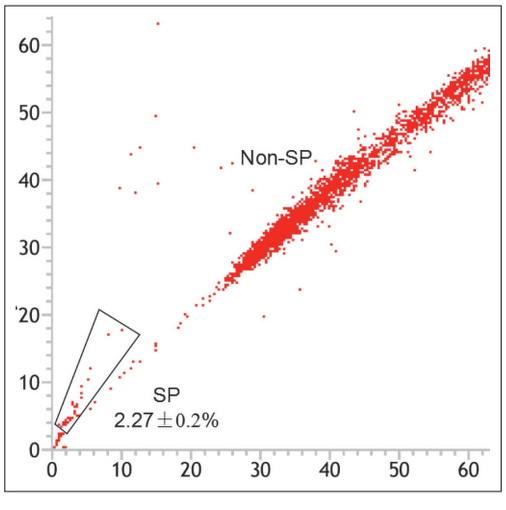

Fig. 1: Sorting of the cancer stem cells (side population cells, shown by SP cells) in human melanoma cancer cell lines. Cells were stained with Hoechst 33342 dye, and SP cells are shown in the lower left gated area. The percentage of SP cells was 1■27±0.2%.

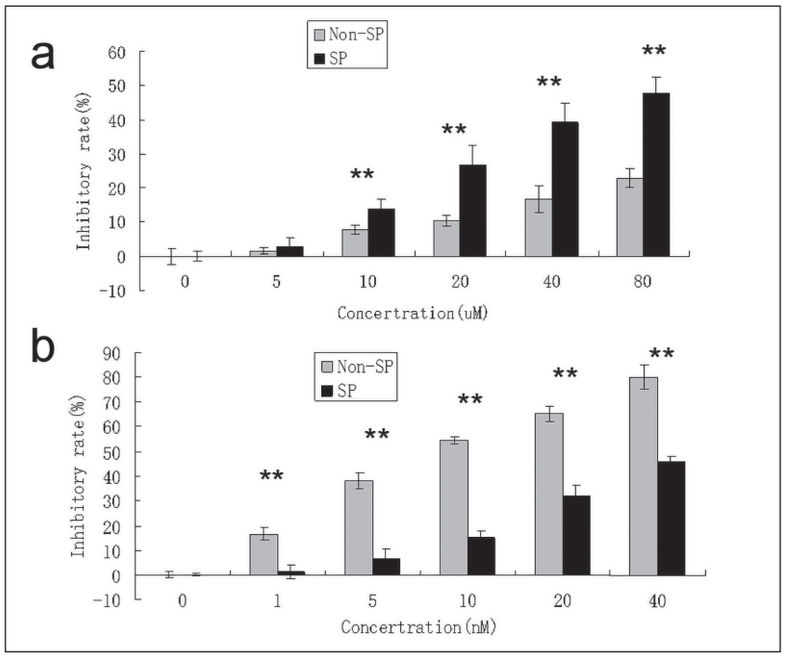

Fig. 2: Effects of RA and Paclitaxel on the A375 SP cells and the Non-SP cells. The SP and Non-SP cells were sorted from A375 cells. Serial dilutions of RA and paclitaxel were added, the MTT assay was performed at the end of incubation. a) RA produced stronger inhibitory effects in the SP cells than in the Non-SP cells. b) The SP cells were more tolerant to Paclitaxel than Non-SP cells. **P<0.01.

2.2. *Inhibitory effect on melanoma CSCs and Non-CSCs*

For evaluating the different inhibitory effect of RA and paclitaxel on melanoma CSCs and non-CSCs, the two kinds of cells were sorted and treated with various concentrations of RA and paclitaxel. The results show that RA produced stronger inhibitory effects in the SP cells than in the non-SP cells (Fig. 2a). In contrast, paclitaxel was more capable of inhibiting Non-SP cells than SP cells (Fig. 2b). The results indicated that the CSCs were more tolerant to paclitaxel than non-CSCs.

2.3. *Inhibitory effect on cell cycle of melanoma CSCs*

To analyze the effect of RA on melanoma CSCs cell cycle, the SP cells were treated with RA (20 μM) for 24 h, Non-SP cells were used as positive control, and then the cell cycle distributions of SP cells, non-SP cells , and SP cells treated with RA were analyzed by FCM. As shown in Fig. 3, compared with Non-SP cells, fewer cells of SP cells were in the G0/G1 phase, while more cells were in the G2 phase. This indicated that the SP cells were more active than non-SP cells in division and proliferation. After treated by RA, the G2 phase ratio of SP cells were decreased, while the G0/G1 phase ratio were increased, which was closed to non-SP cells. The result suggested that the SP cells were arrested at G0/G1 phases, the division and proliferation of which was inhibited.

2.4. *Effect on differentiation of melanoma CSCs*

Oct4 and *Sox2* are involved in the regulation of differentiation and proliferation of stem cells (Tay et al. 2008; Ben-Porath et al. 2008). When the two are overexpressed, stem cells are overgrown and poorly differentiated. Downregulation of their expression can

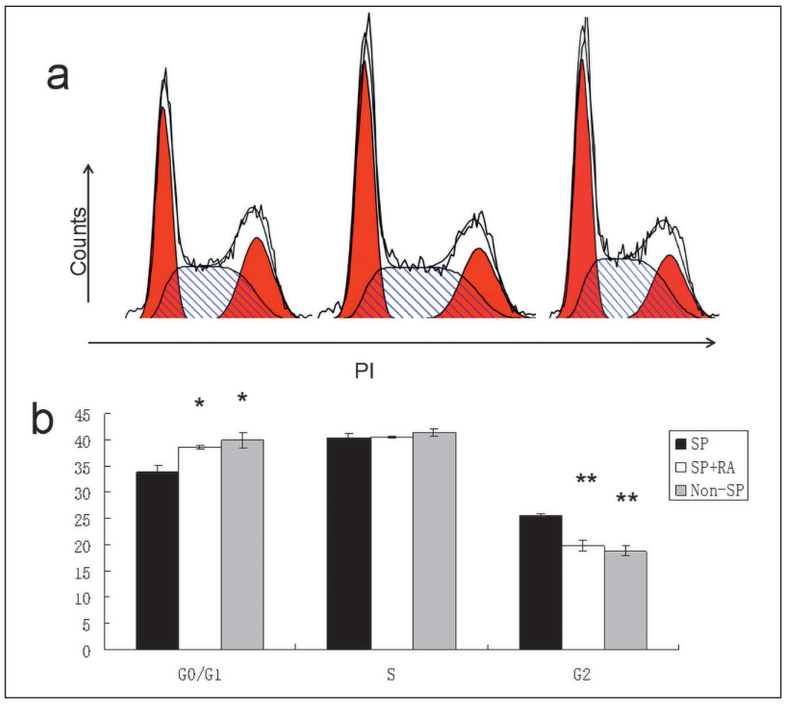

Fig. 3: Inhibitory effect on cell cycle of melanoma CSCs. More cells of SP cells were in G2 phase than Non-SP cells, while lesser cells were in G0/G1 phase. RA induced differentiation of SP cells, arrested the SP cells at G0/G1 phase, and inhibited its division and proliferation. *P<0.05;**P<0.01.

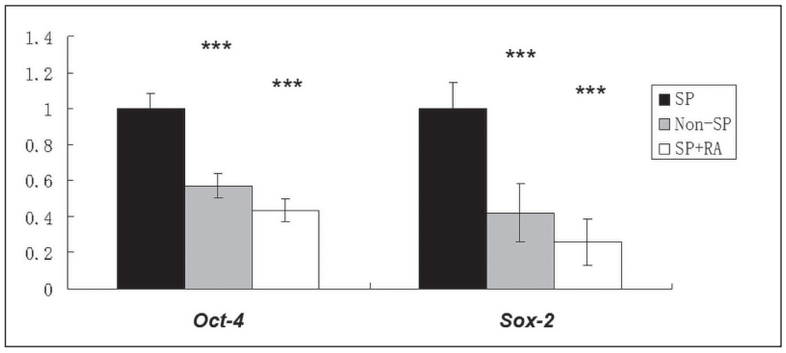

Fig. 4: Effect of RA on differentiation of melanoma CSCs. The SP cells had higher expression level of Oct-4 and Sox-2 than that of Non-SP cells. After treated by RA, the expression of the two genes of SP cells was significantly decreased, which indicated that SP cells were induced differentiation. ***P<0.001.

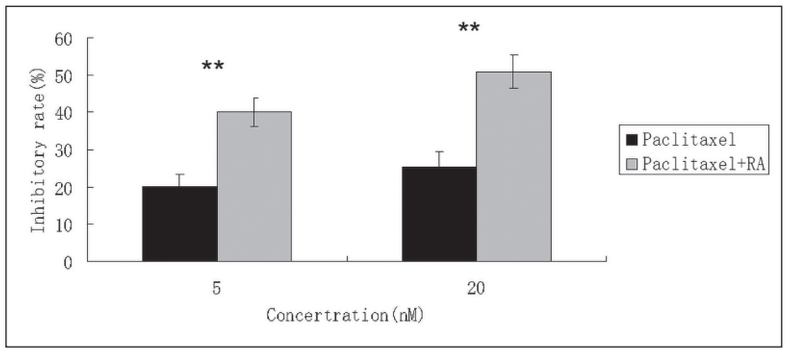

Fig. 5: Effect on sensitivity of CSCs to paclitaxel. The SP cells pretreated with RA were more sensitive to paclitaxel than that of not pretreated with RA. **P<0.01.

inhibit stem cell proliferation and induce stem cell differentiation. The results showed that the expression of *Oct-4* and *Sox-2* were higher than that of non-SP cells. After treated by RA, the expression of *Oct-4* and *Sox-2* of SP cells was significantly decreased (Fig. 4). This indicated that RA induced differentiation of the SP cells.

2.5. Effect on sensitivity of CSCs to paclitaxel

To analyze the effect of RA on the sensitivity of CSCs to paclitaxel, the SP cells were pretreated with RA (5 μM) for 48 h, then paclitaxel (5, 10 nM) was added and cells were cultured for another 24 h. The results showed that the SP cells pretreated with RA were more sensitive to paclitaxel than that not pretreated with RA (Fig. 5). This indicated that RA could increase the sensitivity of melanoma CSCs to paclitaxel.

3. Discussion

Cancer stem cells (CSCs) are a subpopulation of cancer cells with stem cell characteristics of self-renewal and multiple differentiation, which govern cancer progression, angiogenesis and metastasis, and might be the main cause of cancer progression (Bonnet and Dick 1997). Malignant melanoma is a highly aggressive and drug resistant cancer (Chin et al. 2006). Although several groups have shown the presence and involvement of CSCs in melanoma and the concept of CSCs is well accepted for many cancers (Civenni et al. 2011; Luo et al. 2012; Frank et al. 2011), the isolation and identification of CSCs in melanoma are still controversial due to the uncertainty of melanoma CSCs markers. However, an increasing number of studies proved that side population (SP) cells isolated from cancer cell lines were capable of mimicking the cancer stem cell model (Lopez de Cicco et al. 2005). The SP cells express high levels of various members of ATP-binding cassette (ABC) transporters which are responsible for chemotherapeutic resistance, and exclude Hoechst 33342 dye, thus being able to be used for sorting cancer stem cells via flow cytometry (Hirschmann-Jax et al. 2004). In our study, SP cells were sorted from melanoma A375cell line based on the same rationale. Cancer stem cells are closely related to chemotherapy tolerance of cancers (Todaro et al. 2007; Bao et al. 2006). Conventional anticancer treatments eradicate the bulk of tumor mass but are ineffective for CSCs and hence, this could be a reason for tumor reoccurrence and progression. In our study, the results also confirmed that the CSCs (SP cells) of melanoma were more capable of proliferation and resistant to paclitaxel than non-CSCs (non-SP cells).

Retinoic acid (RA) is a small lipophilic molecule, which is derived from vitamin A (or retinol) and is involved in regulating many developmental processes. Retinoic acid is a promising anticancer agent, and the anticancer activity was first discovered in the treatment of acute promyelocytic leukemia (APL), which has been confirmed in various tumor types at present (Redne et al. 1996; Haugen et al. 2004; di Masi et al. 2015). RA can induce CSCs differentiation, reduce the proliferation and migration of CSCs, and increase the sensitivity of CSCs to chemotherapeutic drugs (Ginestier et al. 2009). However, RA has a poor inhibitory effect on non-CSCs, and the capacity of inducing these cancer cells apoptosis is poor when used alone, which limits its clinical use (Kraemer et al. 1988). The results of our experiments also show that RA produced stronger inhibitory effects in the SP cells than in the non-SP cells of melanoma. Besides, RA could arrest the cell cycle of melanoma SP cells at G0/G1 phases, and downregulate the expression of Oct-4 and Sox-2. Oct4 and Sox2 transcription factors are important factors for the maintenance of stem cell characteristics, which plays an important role in the hyperproliferation and differentiation of cancer stem cells, and are considered as stem cell markers (Hochedlinger et al. 2005; Thomson et al. 2011). Overexpression of Oct-4 and Sox-2 can enhance the proliferation of CSCs, and inhibit the differentiation of CSCs, while inhibiting the expression of Oct-4 and Sox-2 can induce the differentiation of CSCs. These results indicate that after treatment with RA, the CSCs of melanoma were induced to differentiate. And when treated with RA before, the sensitivity of CSCs to paclitaxel was also increased, which suggests that a combination of RA and paclitaxel could improve efficacy of melanoma treatment.

4. Experimental

4.1. Materials

All-*trans*-retinoic acid (ATRA), Hoechst 33342, and paclitaxel were obtained from Sigma- Aldrich. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Aladdin.

4.2. Cell cultures

Human melanoma cell line A375 and was acquired from ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Newborn calf serum (GIBCO), 100 U/ml penicillin and 100 mg/ml streptomycin.

4.3. Sorting of cancer stem cells (SP cells)

Hoechst 33342 dual wavelength fluorescence analysis had been proven to be a tool in the purification and characterization of adult tissue stem cells, and the Hoechst unstained cells were described as the side population (SP) cells, namely, the stem cells (Challen and Little 2006). A375 cells were harvested and pre-warmed at 37 °C for 10 min, then were labeled with Hoechst 33342 at a concentration of 5 μg/ml in the same medium at 37 °C for 90 min (Hirschmann-Jax et al. 2004). Furthermore, the CSCs of A375 cells (SP cells) analysis and sorting were performed on a FACSDiva (Becton Dickinson, San Jose, CA, USA) by using a dual wavelength analysis (blue, 420-470 nm; red, 660-680 nm).

4.4. Proliferation inhibition assay

To evaluate the effect on the melanoma cancer stem cells of RA and paclitaxel, SP and non-SP cells were sorted from A375 cells, and seeded into 96-well culture plates at 3000 cells/well, respectively, and grown at 37 °C in the presence of 5 % CO_2 for 24 h. For SP cells DMEM medium containing 2% newborn calf serum was used, while for the non-SP cells DMEM medium containing 10% newborn calf serum was used. Then the culture medium was replaced with 150 μL of DMEM medium containing RA or paclitaxel at different doses for another 24 h. The final concentration of RA and paclitaxel was in the range of 0-80 μM and 0-40 nM, respectively. Then, 20 μL of 5 mg/ml work concentration of MTT solution was added to every cell well. The cells were further incubated for 4 h. Finally, 150 μL of DMSO was replaced for the medium of each well to dissolve formazan crystals, and then the absorbance was obtained at a test wavelength of 490 nm. The inhibitory rates were calculated by the following formula: inhibitory rate% = 100% – (absorbance of the experiment samples/absorbance of the control)×100%.

4.5. Effect on cell cycle of melanoma CSCs

The CSCs (SP cells) were sorted form A375 cells and were seeded in 6 well plates ($2*10^5$) ,using DMEM medium containing 2% newborn calf serum for 24 h. To analyze cell cycle distribution, the SP cells were treated with RA at dose of 20 μM for 24 h. Then, the cells were incubated with 0.3 mL of a solution containing 50 mg/mL propidium iodide (PI) for 30 min in the dark and analyzed by FCM (BD Biosciences).

4.6. Effect on differentiation of melanoma CSCs

The CSCs (SP cells) were sorted form A375 cells and were seeded in 6 well plates ($2*10^5$), using DMEM medium containing 2% newborn calf serum for 24 h. Then culture medium was replaced with serum-free DMEM medium containing the same dose of RA (20 μM) for another 6 h. The cells were continued to be cultured for 48 h using DMEM medium containing 2% newborn calf serum, and then the cells were harvested. The RNA was extracted according to the RNA extraction kit, and the RNA concentration was detected by NanoDrop 1000 spectrophotometer. Then RNA was transcribed to cDNA according to the PrimerScript RT Reagent Kit instructions, and Real-time PCR was used to detect the expression changes of Oct-4 , Sox2, Vimentin, and PDL-1.

4.7. Effect on sensitivity of CSCs to paclitaxel

The CSCs (SP cells) and non-CSCs (non-SP cells) were sorted and seeded in 96 well plates ($5*10^3$) cultured for 24 h in an incubator (37 °C, 5 % CO_2). For SP cells DMEM medium containing 2% newborn calf serum was sued for 24 h, and for the non-SP cells DMEM medium containing 10% newborn calf serum was used. To detect the sensitivity of SP cells and non SP cells to paclitaxel, the SP cells and the non-SP cells were continued to be cultured for 24 h with paclitaxel (5, 10 nM). Then, cell viability was determined by MTT. To detect the effect of RA on sensitivity of SP cells to paclitaxel, the SP cells were cultured with RA (5 μM) for 48 h, then paclitaxel (5, 10 nM) was added and cells were cultured for another 24 h. Then, inhibitory rates were determined by MTT.

4.8. Statistics

Results were represented as the mean±standard deviation (SD). The statistical comparisons were made by 2-tailed Student's T test. Statistically significant p values were labeled as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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Conflicts of interest: None declared.

References

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN (2006) Glioma stem cells promote radio-resistance by preferential activation of the DNA damage response. Nature 444: 756-760.

Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40: 499-507.

Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730-737.

Challen GA, Little MH (2006) A side order of stem cells: the SP phenotype. Stem Cells 24: 3-12.

Chi Z, Li S, Sheng X, Si L, Cui C, Han M, Guo J (2011) Clinical presentation, histology, and prognoses of malignant melanoma in ethnic Chinese: a study of 522 consecutive cases. BMC Cancer 11: 85.

Chin L, Garraway LA, Fisher DE (2006) Malignant melanoma: genetics and therapeutics in the genomic era. Genes Dev 20: 2149-2182.

Civenni G, Walter A, Kobert N, Mihic-Probst D, Zipser M, Belloni B, Seifert B, Moch H, Dummer R, van den Broek M, Sommer L (2011) Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth. Cancer Res 71: 3098-3109.

Colak S, Medema JP (2014) Cancer stem cells--important players in tumor therapy resistance. FEBS J 281: 4779-4791.

Cummins DL, Cummins JM, Pantle H, Silverman MA, Leonard AL, Chanmugam A (2006) Cutaneous malignant melanoma. Mayo Clin Proc 81: 500-507.

Di Masi A, Leboffe L, De Marinis E, Pagano F, Cicconi L, Rochette-Egly C, Lo-Coco F, Ascenzi P, Nervi C (2015) Retinoic acid receptors: from molecular mechanisms to cancer therapy. Mol Aspects Med 41: 1-115.

Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, Van Belle PA, Xu X, Elder DE, Herlyn M (2005) A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res 65: 9328-9337.

Frank NY, Schatton T, Kim S, Zhan Q, Wilson BJ, Ma J, Saab KR, Osherov V, Widlund HR, Gasser M, Waaga-Gasser AM, Kupper TS, Murphy GF, Frank MH (2011) VEGFR-1 expressed by malignant melanoma-initiating cells is required for tumor growth. Cancer Res 71: 1474-1485.

Garbe C, Leiter U (2009) Melanoma epidemiology and trends. Clin Dermatol 27: 3-9.

Ginestier C, Wicinski J, Cervera N, Monville F, Finetti P, Bertucci F, Wicha MS, Birnbaum D, Charafe-Jauffret E (2009) Retinoid signaling regulates breast cancer stem cell differentiation. Cell Cycle 8: 3297-3302.

Ginestier C, Wicinski J, Cervera N, Monville F, Finetti P, Bertucci F, Wicha MS, Birnbaum D, Charafe-Jauffret E (2009) Retinoid signaling regulates breast cancer stem cell differentiation. Cell Cycle 8: 3297-3302.

Haugen BR, Larson LL, Pugazhenthi U, Hays WR, Klopper JP, Kramer CA, Sharma V (2004) Retinoic acid and retinoid X receptors are differentially expressed in thyroid cancer and thyroid carcinoma cell lines and predict response to treatment with retinoids. J Clin Endocrinol Metab 89: 272-280.

Helmbach H, Rossmann E, Kern MA, Schadendorf D (2001) Drug-resistance in human melanoma. Int J Cancer 93: 617-622.

Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, Goodell MA, Brenner MK (2004) A distinct side population of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 101: 14228-14233.

Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, Goodell MA, Brenner MK (2004) A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 101: 14228-14233.

Hochedlinger K, Yamada Y, Beard C, Jaenisch R (2005) Ectopic expression of oct-4 blocks progenitor-cell differentiation and causes dysplasia. Cell 121: 465-477.

Kraemer KH, DiGiovanna JJ, Moshell AN, Tarone RE, Peck GL (1988) Prevention of skin cancer in Xeroderma pigmentosum with the use of oral isotretinoin. N Engl J Med 318: 1633-1637.

La Porta CA (2007) Drug resistance in melanoma: new perspectives. Curr Med Chem 14: 387-391.

Lee, HE, Kim, JH, Kim, YJ, Choi SY, Kim SW, Kang E, Chung IY, Kim IA, Kim EJ, Choi Y, Ryu HS, Park SY (2011) An increase in cancer stem cell population after primary systemic therapy is a poor prognostic factor in breast cancer. Br J Cancer 104: 1730-1738.

Li RJ, Ying X, Zhang Y, Ju RJ, Wang XX, Yao HJ, Men Y, Tian W, Yu Y, Zhang L, Huang RJ, Lu WL (2011) All-trans retinoic acid stealth liposomes prevent the relapse of breast cancer arising from the cancer stem cells. J Control Release 149: 281-291.

Lim YC, Kang HJ, Kim YS, Choi EC (2012) All-trans-retinoic acid inhibits growth of head and neck cancer stem cells by suppression of Wnt/β-catenin pathway. Eur J Cancer 48: 3310-3318.

Lopez de Cicco R, Bassi DE, Zucker S, Seidah NG, Klein-Szanto AJ (2005) Human carcinoma cell growth and invasiveness is impaired by the propeptide of the ubiquitous proprotein convertase furin. Cancer Res 65: 4162-4171.

Luo Y, Dallaglio K, Chen Y, Robinson WA, Robinson SE, McCarter MD, Wang J, Gonzalez R, Thompson DC, Norris DA, Roop DR, Vasiliou V, Fujita M (2012) ALDH1A isozymes are markers of human melanoma stem cells and potential therapeutic targets. Stem Cells 30: 2100-2113.

Marlétaz F, Holland LZ, Laudet V, Schubert M (2006) Retinoic acid signaling and the evolution of chordates. Int J Biol Sci 2: 38-47.

Nikolaou V, Stratigos A (2014) Emerging trends in the epidemiology of melanoma. Br J Dermatol 170: 11-19.

Redner RL, Rush EA, Faas S, Rudert WA, Corey SJ (1996) The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. Blood 87: 882-886.

Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I (2008) MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455: 1124-1128.

Thomson M, John Liu SY, Zou LN, Smith Z, Meissner A, Ramanathan S (2011) Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell 145: 875-889.

Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP, Stassi G (2007) Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 1: 389-402.