A perspective analysis: microRNAs, glucose metabolism, and drug resistance in colon cancer stem cells.

Sara Pagotto[1,2*], Maria Luisa Colorito[1*], Simone Di Franco[3], Annalisa Nicotra[3], Tiziana Apuzzo[3], Nicola Tinari[1,2], Feliciano Protasi[2,4], Giorgio Stassi[3] , Rosa Visone[1,2] and Angelo Veronese[2,5 ‡]

[1] Department of Medical, Oral and Biotechnological Sciences, "G. d'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy;
[2] Center for Advanced Studies and Technology (CAST), "G. d'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy;
[3] Cellular & Molecular Pathophysiology Laboratory, Department of Surgical, Oncological and Stomatological Sciences, University of Palermo, Palermo 90127, Italy;
[4] Department of Medicine and Aging Science, "G. d'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy,
[5] Department of Neuroscience, Imaging, & Clinical Sciences, "G. d'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy.

Material and Methods

Isolation and culture of Colon Cancer Stem Cells (CCSCs)

CRC specimens were obtained from patients undergoing CRC surgical resection, in accordance with the ethical standards of the institutional committee of the University of Palermo, Italy. Tumor samples were mechanically and enzymatically digested with collagenase and hyaluronidase for 1 hour at 37° C. CCSCs were isolated as previously described [1] and cultured in serum-free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Sigma-Aldrich, Saint Louis, MO, USA), and cultured in ultra-low attachment plate to promote the growth of CCSCs as spheroids (Corning, New York, USA). Cell cultures were maintained at 37°C in a 5% CO_2 humidified incubator.

Animal Procedures

All the *in vivo* experiments have been performed following the animal care committee guidelines of the University of Palermo, Italy (Italian Ministry of Health authorization n. 154/2017-PR).

The tumorigenic potential of CCSCs was assessed by harvesting $5x10^5$ dissociated tumor cells, resuspended into 100 µl thawed, cold Matrigel at a 1:1 ratio (BD Matrigel Matrix Growth Factor Reduced; BD Bioscience, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and then subcutaneously injected in NOD/SCID mice. Tumor growth was monitored and measured twice per week following cell injection using an electronic caliper. All the *in vivo* experiments were performed in 5 mice per condition. Treatments with vehicle (PBS) or with 2-DG (250 mg/kg; Sigma-Aldrich, Saint Louis, MO, USA) were performed twice a week for three weeks. Tumor volume was calculated using the formula: largest measured diameter x (smallest measured diameter)$^2 \times \pi/6$, for up to 15 weeks. At the endpoints, when the subcutaneous tumor reached 2 cm in diameter, or when mice showed the first signs of suffering, animals were sacrificed according to Directive 2010/63/EU guidelines (D.lgs 26/2016). Xenograft tumors were collected and used for paraffin-embedded tissues (FFPE), DNA, RNA, and protein extraction.

Cell viability assay

For cell viability assay, CR-CSCs were enzymatically dissociated, plated at a concentration of 500 viable cells (six replicates for experimental point) in ultra-low attachment 96-well plates (Corning, New York, USA) and then exposed to vehicle (PBS) or 2DG (5 mM) alone or in combination with 5FU (25 µg/ml) for 24 and 48 hours (Sigma-Aldrich, Saint Louis, MO, USA). The ATP levels of cells were measured using CellTiter-Glo®Luminescent Cell Viability Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and analyzed by Infinite® F500 (Tecan, Männedorf, Switzerland) to evaluate the viability percentage.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).

Total RNA was isolated from cells and homogenized mouse tissues using QIAzol Lysis Reagent (Qiagen, Hilden, Denmark) according to the manufacturer's instruction. TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to reverted total RNA (25 ng) using a specific *miR-483-3p* reverse primer designed with modifications to include the UPL #21 (Roche, Basel, Switzerland): RT_miR-483-3p: (5'GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAAGACG-3'). Reactions were incubated 30 min at 16° C, followed by pulsed RT of 60 cycles at 30° C for 30 s, 42° C for 30 s, and 50° for 1 s. qPCRs were performed on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the Universal Mastermix (Roche, Basel, Switzerland) and specific primers:

miR-483-3p_Forward: 5'-GCGGCGGTCACTCCTCTCCTC-3'; Universal Reverse: 5'-GTGCAGGGTCCGAGGT-3'. MicroRNA relative expression, normalized to the endogenous reference RNU44, was determined using the $2^{-\Delta ct}$ method (User Bulletin #2, Applied Biosystems, Thermo-Fisher Scientific, Waltham, MA, USA).

Reference

1 Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F *et al.* Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007; **1**: 389–402.