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Stem Cell: Advanced Research and Therapy

Research Article

Joun G, et al. Stem Cell Res Ther: SCRT-117.
DOI: 10.29011/SCRT-117. 000017

Cancer Cell Properties Shape Along Metabolic Activity in Human Epithelial Carcinoma

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Citation: Joun G, Fonoudi H, Asli NS (2017) Cancer Cell Properties Shape Along Metabolic Activity in Human Epithelial Carcinoma. Stem Cell Res Ther: SCRT-117. DOI: 10.29011/SCRT-117. 000017

Received Date: 20 June, 2017; **Accepted Date:** 07 July, 2017; **Published Date:** 15 June, 2017

Abstract

Intra-tumour heterogeneity is a rising concept central to differential tumour response to therapy and disease prognosis. The current study provides strong evidence for an integrated molecular regulation between different levels of heterogeneity including metabolism, cell cycle progression, EMT and cancer stem cell properties. Although the exact molecular machinery downstream of these events, and the regulatory cross-talk is far from complete, studies as such will strongly support the network view of cancer regulation and the requirement for multi-targeted drug design strategies.

Keywords: Cancer Stem Cells; EMT; Metabolic Plasticity; Quiescence; Tumour Heterogeneity

Introduction

Intra-tumour heterogeneity has been increasingly attracting interest as a dynamic core for differential tumour behaviour during tumorigenesis and in response to therapy [1]. Apart from the so-called mutational landscape, which greatly differs between clonal populations within tumours, cellular properties including states of metabolism, cell cycle and phenotypic signatures, seem to be as well differentially modulated [2,3]. The generation of a mosaic cellular landscape, with specialised functional sub-populations creates a diversified tool for tumours to dynamically morph and de-morph during progression and treatment, making them an extremely difficult clinical target for complete eradication. One major specialised subpopulation within tumours, known as tumour initiating or Cancer Stem Cells (CSC), has been suggested at the core of tumour progression, metastasis and relapse [4-7]. CSCs, have the capacity to generate the entire tumour mass through a specialised stem cell-like proliferation event, known as self-renewal. They are as well shown to express transcription factors involved in Epithelial to Mesenchymal Transcription (EMT) [8-12], have distinct cell cycle states with lower cycling propensity [13,14] and to adopt lower and/or altered metabolic profiles within tumours [15-17]. Isolation of cancer stem cells has so far been primarily dependant on the expression of cell surface antigens with little consensus in different tumours or even subtypes of the same tumour [18-21]. Moreover, the mechanistic integration of different cellular properties, i.e. metabolic profiles, cell cycle states and differential response to treatments with regards to cancer stem cell properties is yet to be fully delineated. In this study, we have used COLO316 ovarian carcinoma cells as a model of advanced-stage epithelial carcinoma and have taken the first step towards profiling cells of various metabolic levels with regards to cancer cell properties. Our findings demonstrate an integrated regulation between states of metabolism, cell cycle, EMT and cancer stem-ness.

Materials and Methods

General Tissue Culture

COLO316 ovarian carcinoma cells were cultured in standard culture medium (RPMI1640, Sigma) containing 10% fetal bovine serum (Gibco), 20mM HEPES (Gibco) and 2mM Glutamax (Gibco). The cells were incubated at 37°C in 5% CO_2.

Mitotracker staining, Cell Cycle Assay, Flow Cytometry

To assess the mitochondrial mass, COLO316 were harvested into a single cell suspension and stained with MitoTracker™ Green FM (Thermo Fisher Scientific) at a final concentration of 200nM for 15min at 37°C. The stained cell population was used for Fluorescence Activated Cell Sorting (FACS), on BD FACS Aria. Cells residing at the lowest and highest 20% margins were sorted into the standard medium and used for downstream analysis. Cell cycle assay was performed as described before [22] will minor modifications as follows: harvested/sorted cells were fixed in 80% ice-cold ethanol and stored for at least 24hrs at -20˚C. To stain for total RNA (PyroninY) and DNA (7AAD), cells were permeabilized with Cytoperm plus (BD) buffer for 5min on ice, and then washed with 1x Cytoperm/fix wash buffer (BD) for 5min at 300xg (4˚C). The cell pellet was then re-suspended in 100µl of 1x7AAD (BD) in PBS, and incubated at room temperature for 30min away from light. 150µl of PBS containing 1µl of 1mg/ml PyroninY stock (Polysciences) was immediately added to the cell suspension, and the cells were incubated for an additional 5min on ice. At the end, the cells were washed with PBS for 5min at 300xg (4˚C) and processed for signal analysis by flow cytometry. Analysis was performed on BD FACS CantoII. The G0 box was determined on a low RNA control sample treated with RNase A at 37˚C for 1hr.

For analysis of CD44 expression in COLO316 cells, cells were incubated with APC-conjugated CD44 antibody (BD) at 1:100 dilution in staining buffer (2% FBS in PBS) for 30min on ice and washed twice with the same buffer before the signal was analysed on BD FACS CantoII. The positive CD44 signal was determined using a control APC-conjugated isotype. Flow cytometry was performed at the Westmead imaging and flow cytometry facility. All flow cytometry analysis was performed using the FlowJo data analysis software package (TreeStar, USA).

Real-time RT-PCR, Gene-specific Primer Sequences

Total RNA was extracted from $Mito^{low}$ and $Mito^{high}$ fractions of COLO316 cells using Qiagen RNeasy Micro Kit. cDNA synthesis was carried out for each fraction using Superscript III reverse transcription kit (Thermo Fisher Scientific) following manufacturer's instructions. Real-time PCR was carried out using Light Cycler 480 SYBR Green I Master (Roche) following manufacturer's instructions and reactions were run and analysed on Roche Light Cycler 480. The primer sequences used were as follow (5' to 3'):

Snail: (F: ACCACTATGCCGCGCTCTT; R: GGTCGTAGGGCTGCTGGAA)

Slug: (F: TGTTGCAGTGAGGGCAAGAA; R: GACCCTGGTTGCTTCAAGGA)

Twist: (F: GGACAAGCTGAGCAAGATTCAGA; R: TCTGGAGGACCTGGTAGAGGAA)

Zeb1: (F: GCACCTGAAGAGGACCAGAG; R: TGCATCTGGTGTTCCATTTT)

E-Cadherin: (F: GCTGAGCTGGACAGGGAGGA; R: ATGGGGGCGTTGTCATTCAC

GusB: (F: CCACCAGGGACCATCCAAT; R: AGTCAAAATATGTGTTCTGGACAAAGTAA)

Data analysis was performed using the standard ΔCt/Cp methods. Final fold change was calculated relative to normalised ΔCt levels in human induced pluripotent stem cell cDNA as control epithelial tissue.

Statistical Analysis

Statistical significance was determined using the standard student T-test (Microsoft Excel) based on the average signal between biological triplicates (P-value < 0.05: *; P-value < 0.01: **; P-value < 0.001: ***). All P-values above 0.05 were regarded as Non-Significant (NS).

Results

Different Levels of Metabolism Translate to Distinct Cell Cycle Profiles

We have previously demonstrated a wide range of metabolic heterogeneity in COLO316 ovarian carcinoma cells [2]. Variations in metabolism has been associated with cell cycle activity [23] with strained metabolism having instructive roles for induction of cell cycle quiescence [24]. We therefore isolated cells residing at the far 20% ends of the mitotracker-stained COLO316 cells (Figure 1A) and checked their levels of cell cycle activity using our standard PyroninY/7AAD cell cycle assay method [2, 22] (Figure 1B and 1C). In this assay, PyroninY, marks cellular RNA content as a readout for metabolic activity and 7AAD marks the DNA content: $PyroninY^{low}/7AAD^{low}$ cells are regarded as the quiescent fraction (G0), $PyroninY^{high}/7AAD^{low}$ as G1, and $PyroninY^{high}/7AAD^{high}$ as S/G2/M. Bulk COLO316 cells, showed the expected asynchronous cell cycle profile, with an average of 10% cells in G0, ~60% cells in G1 and the rest (~28%) in the fully active phases of the cell cycle (S/G2/M). The lower 20% population of mitotracker-stained cells ($Mito^{low}$) however, showed a significant enrichment of G0 cells (~20%). On the contrary, cells residing at the higher 20% end of the mitotracker signal ($Mito^{high}$), showed a marked enhancement of the active cell cycle phases (S/G2/M). Overall, this data suggests a direct link between levels of mitochondrial mass and metabolism with cell cycle activity.

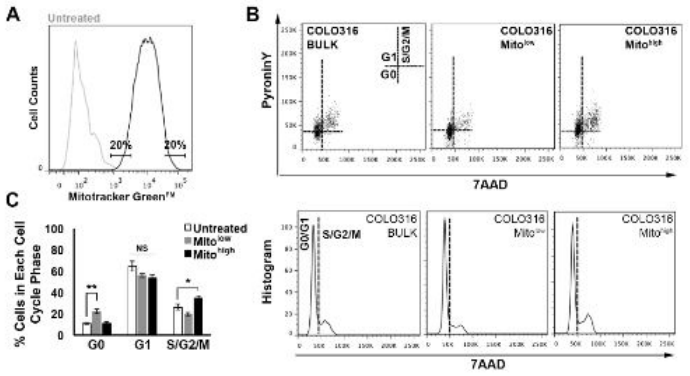

Figure 1: Cell cycle dynamics along the metabolic spectrum; **A)** Design of the Mitotracker fractionation assay. **B)** Cell cycle analysis on bulk, Mito^{low} (lowest 20%) and Mito^{high} (highest 20%) fractions. Results are demonstrated in two formats: dotplot (upper row) and histogram (lower row). Note the enriched pattern for G0 cells in Mito^{low}, and for S/G2/M cells in Mito^{high}. **C)** Quantification of cell cycle assay shown in "B", between biological triplicates.

Epithelial and Mesenchymal Signatures Differentially Associate with Levels of Metabolism

Metabolic adaptation is regarded as a mechanism for cancer cell survival reflecting both in levels and modes of metabolism [25,26]. In epithelial carcinoma, the transformation of the epithelium into loose mesenchyme known as Epithelial-Mesenchymal Transformation (EMT) also involves tight metabolic regulation with cells adopting distinct metabolic signatures throughout this process [27]. Looking at the morphologies of Mito^{low} and Mito^{high} cells in culture, we noticed a slight morphological bias between the two populations, where Mito^{low} cells seemed enriched with spindle-shaped cells with long processes (Figure 2A). We therefore looked at the expression of key EMT transcription factors in the two cell populations, anticipating a biased gene expression pattern. Among the four EMT transcription factors tested, expression of Snail and Slug were significantly enriched in Mito^{low} cells, suggesting a shift towards a more mesenchymal signature in these cells (Figure 2B). The other two EMT transcription factors, Twist and Zeb showed unchanged expression. The enrichment of selected mesenchymal markers (Snail and Slug), in metabolically-naïve cells, suggests a cross-talk between cell cycle quiescence and the induction/maintenance of the mesenchymal phenotypes.

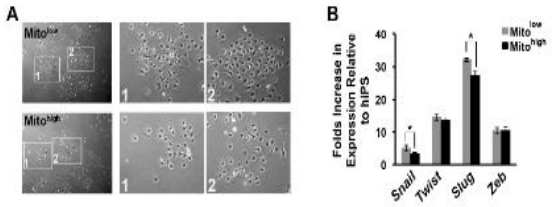

Figure 2: Analysis of EMT transcription factors in cells with different metabolic levels; **A)** Morphologies of Mito^{low} and Mito^{high} cells in culture. Note the predominance of cells with mesenchymal morphology (elongated, spindle shaped, with long processes) in Mito^{low} cultures. **B)** Expression dynamics of EMT transcription factors in Mito^{low} and Mito^{high} COLO316 cells. Fold change in expression is plotted relative to levels in control human induced Pluripotent Cells (hiPS) (*: P-value <0.05; NS: Non-Significant).

The Metabolic Signature Differentially Enriches for Cancer Stem Cells

Cancer stem cells, also known as tumour initiating cells, are a subpopulation of cells within tumours that have the capacity to self-renew and form the entire tumour in solidarity [4]. Several studies have suggested a preferential settlement of cancer stem cells in tumour niches of low metabolic activity [15,16,28]. On this basis, we aimed to look at the expression of a common cancer stem cell antigen, CD44 [29] in Mito^{low} and Mito^{high} cells (Figure 3A and 3B). Our analysis, showed a significant bias in the expression of CD44 in the two cell populations, with almost double the expression of this marker in Mito^{low} cells. Enrichment of CD44 marker in Mito^{low} cells is a strong suggestion that this compartment has higher propensity for cancer stem cell properties and that CD44^{+} cancer stem cells are majorly of low metabolic activity.

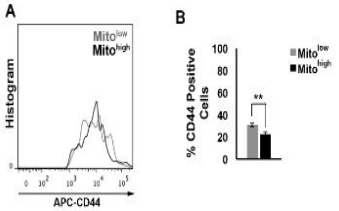

Figure 3: Expression of CD44 cancer stem cell markers in cells with different metabolic levels; A) Histogram comparing CD44 expression in Mitolow and Mitohigh cells. B) Quantification of CD44 expression in Mitolow and Mitohigh cells between biological triplicates (**: P-value <0.01).

Discussion

Metabolic plasticity is a specialized property of cells within the tumour [30,31] that can be directed as a signature by micro-environmental or positional cues. Cancer cells acquire distinct metabolic profiles based on their geographical location in the tumour mass. Factors such as access to nutrients [31,32] can have a directive role during the metabolic adaptation process. Like-wise, the hypoxic niches within the tumour mass also seem to dictate or select for specialised metabolic signatures [26,33]. The extent of heterogeneity in metabolic adaptation is further linked to tumour prognosis and treatment [34], suggesting a tight regulation between states of metabolism and other cancer cell properties. Cancer cells might as well harbour an inherent metabolic code, independent of their position or the stress cues in the tumour. In a variety of cancers, for instance, the stem cell compartment adopts a unique mode of "Aeorobic Glycolysis" that persists throughout its life cycle, as a state of low energy but high biomass output [25,35]. Although a number of studies have associated states of low metabolic activity to cancer stem-ness, there is evidence for metabolically-active cancer stem cell compartments in pancreatic cancer [30], or utilization of alternative metabolic organelles other than mitochondria [36]. In the current study, ovarian epithelial carcinoma cells manifested a broad range of metabolic activity, evident by a wide spectrum of mitochondrial mass/potential. The metabolic machinery cross-talks with a number of other cellular pathways including the cell cycle [23,24]. The association between increased cell cycle activity and states of higher metabolism is shown to be synergistically cross-regulated [37]. The metabolic machinery has also been suggested as a regulator of EMT, a process not only involved during cancer metastasis [38,39] but also a core regulator of cancer stem cell properties [8,11,40]. In our study, the cells of less metabolic activity (Mitolow), showed a higher propensity of a quiescent, mesenchymal and CSC-rich profile. A strong suggestion, that the three machineries are finely inter-tuned in ovarian carcinoma cells. The exact mechanisms of cross-regulation between these processes however is yet to be delineated. More importantly, the causal relationship between the three events is in continuous cross-talk with the tumour microenvironment [41-43], with its regulatory network undergoing major adaptive changes during the course of treatment [13,44,45]. In this respect, the plastic nature of the regulatory networks involved in these cross-talks should be closely considered in drug design and clinical treatment strategies.

Acknowledgements

The COLO316 ovarian carcinoma cells were a kind gift from Professor Anna deFazio, Westmead Institute for Medical Research.

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