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Time- and cell-resolved dynamics of redox-sensitive Nrf2, HIF and NF-κB activities in 3D spheroids enriched for cancer stem cells

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ABSTRACT

Cancer cells have an altered redox status, with changes in intracellular signaling pathways. The knowledge of how such processes are regulated in 3D spheroids, being well-established tumor models, is limited. To approach this question we stably transfected HCT116 cells with a pTRAF reporter that enabled time- and cell-resolved activity monitoring of three redox-regulated transcription factors Nrf2, HIF and NF-κB in spheroids enriched for cancer stem cells. At the first day of spheroid formation, these transcription factors were activated and thereafter became repressed. After about a week, both HIF and Nrf2 were reactivated within the spheroid cores. Further amplifying HIF activation in spheroids by treatment with DMOG resulted in a dominant quiescent stem-cell-like phenotype, with high resistance to stress-inducing treatments. Auranofin, triggering oxidative stress and Nrf2 activation, had opposite effects with increased differentiation and proliferation. These novel high-resolution insights into spatiotemporal activation patterns demonstrate a striking coordination of redox regulated transcription factors within spheroids not occurring in conventional cell culture models.

1. Introduction

Malignant tumors consist of a heterogenic mixture of cancer cells, and only a subset of undifferentiated tumor cells have clonogenic and tumor-initiating potential [1]. These cells are commonly termed 'cancer stem cells' (CSCs) as they share many properties with normal adult and embryonic stem cells [2]. CSCs have unlimited self-renewal capacity, can differentiate asymmetrically, and are believed to drive the heterogeneous cell populations constituting a tumor. They are either slowly proliferating or fully quiescent, and are typically resistant to chemotherapy. Factors and conditions that either control maintenance of undifferentiated clonogenic CSCs or their differentiation into more mature cancer cells are incompletely defined, but redox modulation is likely to be important. Several observations have shown that cancer cells in general have higher endogenous levels of oxidative stress than normal healthy cells [3,4] and thus up-regulate their expression of antioxidant enzymes in order to achieve redox homeostasis and cell survival [5]. How the redox state of CSCs compares to more differentiated cells from the same original cancer cell clone is not known. Redox signaling pathways that are activated in response to growth factor stimulation are typically coupled to synthesis of H_2O_2 by NADPH oxidases, but also other sources of H_2O_2 might play a role together with peroxynitrite and lipid hydroperoxides. Many transcription factors are redox regulated, including NF-κB, HIF, Nrf2, Oct-4, β-catenin, Notch, and c-Myc. All of them are known to be important mediators of development and cellular differentiation, but also of cancer promotion [6–8].

NF-κB is involved in cellular responses to inflammation [6]. Under basal conditions, NF-κB is kept inactive in the cytosol by binding to IκB, the inhibitor of NF-κB. Upon activation, a phosphorylation cascade results in the degradation of IκB and nuclear translocation of NF-κB. In relation to colorectal cancer, elevated NF-κB signaling enhances Wnt activation and can support tumor growth [9,10]. Under conditions of constitutively activated Wnt signaling, Rac1-driven H_2O_2 production is also required for NF-κB activation and initiation of colon tumorigenesis [11].

Abbreviations: AUR, auranofin; CA9, carbonic anhydrase IX; CDKN1B, cyclin-dependent kinase inhibitor 1B (p27); CSC, cancer stem cell; DMOG, dimethyloxalylglycine; GSH, glutathione; HIF, hypoxia inducible factor; MUC2, mucin2; NCL, nucleolin; NFE2L2 or Nrf2, nuclear factor (erythroid-derived 2)-like 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PHD, prolyl hydroxylase domain proteins; pTRAF, plasmid for transcription factor reporter activation based on fluorescence; ROSI, rosiglitazone; SCM, stem cell medium; XCT, cystine-glutamate exchange transporter

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The HIF1 transcription factor consists of two subunits, HIF1β and HIF1α [12]. During normoxia, HIF1α is hydroxylated by prolyl hydroxylase domain proteins (PHD), allowing the recognition and ubiquitination of HIF1α by the Von Hippel-Lindau protein followed by proteasomal degradation. Upon hypoxia (O_2 below 3%), PHDs are inactivated by a shift from Fe^{3+} to Fe^{2+} in their active center. HIF1α becomes stabilized and translocates to the nucleus, where it together with HIF1β induces HIF target genes involved in e.g. the adaptation to hypoxia, angiogenesis, glucose transport, survival and invasion. HIF1α is activated in many different types of cancers, mainly caused by the hypoxic core that develops when tumors grow bigger. For colorectal cancer, it has been shown that hypoxia promotes an aggressive CSC phenotype resulting in invasion and accelerated metastatic outgrowth [13].

During cell homeostasis, Nrf2 is bound to Keap1 and constantly degraded. Upon oxidative or electrophilic stress, Keap1 is modified, whereupon Nrf2 translocates to the nucleus to activate an array of antioxidant and detoxification enzymes, including important proteins of the glutathione (GSH) and thioredoxin systems [6,14,15]. Thus, Nrf2 provides host defense systems that can protect from cancer initiation through more efficient elimination of harmful substances. However, Nrf2 activation in cancer cells can accelerate malignant cell growth [16] and Nrf2 is typically activated in many tumors [17,18]. In Drosophila intestinal stem cells, constitutive Nrf2 activation sustained quiescence by lowering the cellular redox status via up-regulation of genes such as glutamate-cysteine ligase [19].

In the present study, we set out to analyze the activation patterns of Nrf2, HIF and NF-κB in relation to development of spheroids established from individual clones of human HCT116 colon cancer cells. HCT116 cells are known to have a high fraction of CSCs and a low ability to differentiate [20,21]. During the last years, 3D culture models have been increasingly used to study tumor properties and CSC functions, as such cultures are believed to mimic tumor traits better than classical adherent 2D cell cultures. For example, spheroids mirror oxygen and nutrient gradients typical for tumors, such as lower oxygen tension and nutrient supply, but higher lactate concentrations in their cores [22]. Here, HCT116 spheroids were developed under low-attachment culture conditions and with FBS-free medium, which promotes formation of CSCs [23]. To enable studies of the activation of Nrf2, HIF and NF-κB in this system, we stably transfected HCT116 cells with the pTRAF vector (plasmid for transcription factor reporter activation based on fluorescence), which allows for concomitant determination of Nrf2, HIF and NF-κB activation patterns at single-cell resolution by fluorescence detection [24]. In 2D cultures this methodology has revealed a high degree of stochastic variation in these transcription factor activities between individual cells, while here we asked if or how the activation patterns are differently coordinated during development of HCT116 spheroids.

2. Materials and methods

2.1. 2D and 3D cell culture

The human colorectal carcinoma cell line HCT116 was purchased from ATCC (CCL-247) and cultured as described [24]. The 3D spheroids were grown in serum free advanced DMEM/F12 medium (Life Technologies) supplemented as described [23]. To generate 3D cultures, adherently growing cells, derived from a single original clone, were resuspended in stem cell medium at a density of 4000 cells per 100 µl and pipetted into each well of a transparent Nunclon SpheraTM ultra-low surface round bottom 96-well plate (Thermo Fisher; 174925). Whole plates were centrifuged for 5 min at 1200 rpm. One spheroid per well developed within three days, which was monitored daily for up to 14 days.

2.2. qPCR and western blotting

Standard protocols were used to isolate RNA and perform quantitative real-time PCR (Fig. S1A for primers), SDS page, and Western blotting. Further details are described in the supplementary (see primers Fig. S1B experimental setup).

2.3. Stable transfection and pTRAF activity

HCT116 cells were stably transfected with the vector $pTRAF^{Nrf2/HIF/NFkB}$, a pGL4.32 vector containing response elements for the transcription factors Nrf2, HIF, and NF-κB in front of sequences encoding for three fluorescent proteins, mCherry, YPet, and Turquoise fluorescent protein (TFP), respectively [24,25]. Single cell clones were picked, and selected for further analyses based on their basal and inducible Nrf2, HIF, and NF-κB activity. Five different $HCT^{(pTRAFNrf2/HIF/NFkB)}$ clones were studied in parallel. To monitor transcription factor activities of 2D cultures, cells were seeded at a density of 30,000 cells per well of a black 96-well plate (Thermo Fisher, 165305). The day after, cells were exposed to 2 and 10 ng/ml tumor necrosis factor alpha in 3D and 2D cultures respectively (TNFα, Sigma Aldrich), 1 or 2 µM auranofin for qPCR and 2D/3D cultures respectively (AUR, Enzo Life science), 250 µM dimethyloxalylglycine (DMOG, Sigma Aldrich), 10 µM rosiglitazone (ROSI, Sigma Aldrich), or 10 mM lithium chloride (LiCl, Sigma Aldrich) for 20 h. Nuclei were stained with 40 ng/ml Hoechst (Life technologies). For 3D cultures, the $HCT^{(pTRAFNrf2/HIF/NFkB)}$ clones were seeded as described above and 3 days after seeding, spheroids were treated (Fig. S1B). Transcription factor activities were monitored over time in living spheroids using the Operetta HTS system, an automatized fluorescent, confocal microscope (Perkin Elmer). In confocal mode, we screened 10 confocal layers. The Columbus software was used to quantify fluorescence intensities and Image J to measure spheroid size.

2.4. Statistics

Data are shown as mean + SD. All treatments were performed at least in duplicates per plate. Accordingly, the mean was calculated out of the four individual clones in case of the pTRAF fluorescent experiments. For qPCR (measured in triplicate) and Western Blot analyses, three independent experiments were performed with one selected clone and averaged thereafter. Statistical significance was calculated by GraphPad Prism version 6 using oneway analysis of variance (ANOVA) with Tukey's multiple comparisons test or unpaired Student's *t*-test as indicated in figure legends. A p-value < 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Spheroid formation promotes cancer stem cell characteristics

We first characterized the cellular phenotypes of our $HCT(pTRAF^{Nrf2/HIF/NFkB})$-derived clones during spheroid formation (Fig. 1A). After 24 h of culturing under 3D conditions, the $HCT(pTRAF^{Nrf2/HIF/NFkB})$ cells increased their expression of well-established stemness markers such as NANOG, AXIN2, LGR5, CD44, and CD133 (Fig. 1A). CD44 and CD133 expression indicate subpopulations of cells capable to establish tumors upon implantation in immune deficient hosts [26,27]. This property was also shown for LGR5-expressing cells, initially identified as intestinal stem cells [28]. Both LGR5, the co-receptor for the WNT ligand R-spondin, and AXIN2, are target genes of β-catenin/TCF. Especially in colon, β-catenin activity is also considered as a suitable CSC marker [29]. AXIN2 showed an almost 30-fold increase in expression during the first day of spheroid formation, clearly indicating that Wnt signaling was further activated. NANOG was chosen to extend the signature to contain a marker for