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EZH2 is regulated by ERK/AKT and targets integrin alpha2 gene to control Epithelial–Mesenchymal Transition and anoikis in colon cancer cells

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ABSTRACT

Epithelial–Mesenchymal Transition is a good example of cell plasticity. In tumorigenesis, this process has been associated with metastasis. Overexpression of EZH2 has been detected in most malignant human tumors, including colorectal carcinomas. Herein, we provide evidence supporting the idea that oncogenic Epithelial–Mesenchymal Transition in colon cancer cell models is partially controlled by epigenetic factors such as the transcription regulator EZH2. Evaluation of EZH2 mRNA and protein levels revealed overexpression in cell lines with metastatic traits. Analysis of EZH2 mRNA expression was expanded in clinical samples of colon cancer, and high level of EZH2 correlates with appearance of metastasis. Furthermore, inhibition of ERK and AKT pathways in metastatic colon cancer cell lines attenuates EZH2 overexpression. EZH2 promoter analysis illustrates presence of putative AP-1 binding sites and occupancy of transcription factors such as FRA-1 and C-JUN is demonstrated here on EZH2 promoter. Abrogation of EZH2 expression impairs the ability of colon cancer cells to move associated with anoikis in three-dimensional environment. Integrin alpha2 was identified to be a novel EZH2 target by chromatin immunoprecipitation and short hairpin RNA analysis. This study proposes that activation of ERK/AKT pathways and FRA1/C-JUN induce EZH2 overexpression, which results in Integrin alpha2 silencing. Our results show how deregulation of epigenetic factors and mechanisms can affect cancer cell aggressiveness and propose EZH2 as a potential metastasis marker and/or therapeutic target for colorectal cancer treatment.

1. Introduction

Colorectal cancer (CRC) cells are epithelial in origin (Markowitz and Bertagnolli, 2009). During tumor progression loss of epithelial markers and gain of mesenchymal markers is observed at the leading edge of solid tumors. This process defined as Epithelial–Mesenchymal Transition (EMT) is thought to be an early step of metastasis and is reversible through Mesenchymal-Epithelial Transition (MET) (Thiery, 2002). EMT is characterized by, down regulation of the epithelial marker E-cadherin (Cano et al., 2000) and overexpression of the mesenchymal marker Vimentin (Willipinski-Stapelfeld et al., 2005).

During local invasion cancer cells colonize the surrounding tissue and later other organs. Tumor cells lose apico-basal polarity, escape the anchorage-dependent surveillance system, such as anoikis, detach and migrate through normal tissue. Because of the reversible nature of EMT and invasive/migratory capacity, unlike other cancer cell properties, they are not always associated to permanent genetic alterations, implying the existence of dynamic components like epigenetic events.

The histone methyltransferase EZH2 is the catalytic sub-unit of a complex referred Polycomb Repressive Complex 2 (PRC2) (Cao et al., 2002). PRC2 is formed by EZH2, SUZ12, EED and other components, binds to target gene promoters resulting in epigenetic repression via tri-methylation of lysine 27 at histone H3 (H3K27me3). EZH2 overexpression has been associated with poor prognosis in many aggressive cancers (Kleer et al., 2003; Varambally et al., 2002; Yu et al., 2007), yet the mechanisms underlying this are largely unknown. EZH2 down regulation can reduce growth of invasive breast carcinoma (Gonzalez et al., 2009) and tumor angiogenesis (Lu et al., 2010). A number of EZH2 target genes implicated in cancer cell migration have been revealed, like E-cadherin (Cao et al., 2008), Wnt antagonistic genes (Cheng et al., 2011), as well as metastasis suppressor RKIP (Ren et al., 2012), and yet more have to be identified.

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In CRC, crypt-derived tumor cells can invade muscular mucosa and sub-mucosa and eventually metastasize (Markowitz and Bertagnolli, 2009). While E-cadherin plays a role in cell-cell adherent junctions, cell-surface receptors formed by heterodimers of proteins belonging to the integrin (ITG) family compose focal adhesion among basement membrane of extracellular matrix (ECM) and cytoskeleton. The mammalian genomes encode 18α- and 8β-integrin subunits that in combination produce 24 different integrin (α–β-) heterodimers (Hynes, 2002). Their functions are not redundant and range from extracellular collagen receptors to signal transduction mediators (Hynes, 2002). Whether integrins act only as tumor enhancers or might contribute to tumor suppression is still debated. For example, integrin α6β4 plays a role in the regulation of cell cycle progression from G1 into S phase enhancing cell proliferation (Mainiero et al., 1997) and promotes carcinoma invasion (Shaw et al., 1997). ITGα2 forms heterodimers with ITGβ1 – α2β1 – and interacts with ECM's collagen fibers (Hynes, 2002). It has been reported that integrin α2β1 is able to block cell proliferation (Zutter et al., 1995; Ivaska et al., 1999; Ellinger-Ziegelbauer et al., 1999) and to suppress metastasis in mouse and human cancer (Ramirez et al., 2011). In prostate cancer, ITGα2 downregulation is proposed as potential tumor marker (Shaikhibrahim et al., 2011).

Impairment of signaling pathways in cancer cells is a frequent event as at least 50% of CRCs present deregulated RAS–RAF–MEK–ERK and RAS–PI3K–AKT pathways. Moreover, activation of proto-oncogenes lead cancer cells to escape from normal growth and differentiation control (Fang and Richardson, 2005).

In this study we focus on how MAPK and PI3K pathways are activated upon oncogenic transformation and how tumor microenvironment can regulate expression of polycomb proteins such as EZH2 in EMT cells. We show that after transient or stable reduction of EZH2 expression most malignant cancer cell properties are attenuated leading to cell death in three-dimensional culture. Finally, we demonstrate that ITGα2 is a target of EZH2 and that its de-repression is associated with reduced migratory ability and restoration of integrin-mediated surveillance systems in EMT cells.

2. Materials and methods

2.1. Two- (2D) and three-dimensional (3D) culture

Cells were cultured in a humidified atmosphere at 37 °C with 5% CO_2 and DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and non-essential amino acids (all from Invitrogen).

For 3D culture, Matrigel was used (BD, Bioscience). Polylysine pre-coated cover slips were inserted in a 24-well plate. 0.5×10^3 cells were diluted in 100 μl of cold complete DMEM and mixed with 100 μl of 30% Matrigel-DMEM. This 200 μl mix was added to the wells coated with 200 μl of 15% pre-warmed Matrigel. Plate was incubated as above for 10–12 days.

2.2. UO126, Wortmanin and 3-deazaneplanocin (DZNep) treatment

ERK phosphorylation was attenuated by MEK inhibitor UO126 at a final concentration of 40 μM for 4 h. Treatment with PI3K inhibitor Wortmanin was performed at a final concentration of 1 μM for 2 and 4 h. Cells were treated with EZH2 inhibitor DZNep at a final concentration of 5 μM for 24 and 48 h.

2.3. Migration, invasion and wound healing assays

Migration and invasion assays were performed on transwell plates (Corning Costar). Migration and invasion ability was measured as described previously (Voulgari et al., 2008). Data were obtained from two independent experiments, each repeated twice.

For wound healing assay cells were plated in 6-well plate at a 70–80% confluence. Several scratches were created in the cell monolayer by a 10 μl pipette tip. Four additional scratches were shaped orthogonally to the others in order to create a grid and pictures were taken near the corners of each scratch.

2.4. Western Blot (WB) and immunofluorescence

Antibodies used in WB and immunofluorescence assays were: EZH2, BD Biosciences; ITGA2, Tubulin, pERK, E-cadherin and H3 total, Santa-Cruz; H3K27m3, Millipore; Caspase3 and pAKT, Cell Signaling. Cells used for immunofluorescence assays were fixed with 4% paraformaldehyde or methanol/acetone solution (8:1), washed, permeabilized with 0.3% Triton-X-100, and blocked with 5% BSA prior to incubation with primary antibodies. Primary antibodies were then stained with Alexa555-conjugated secondary antibodies (Molecular Probes,). Nuclei were counterstained with Hoechst and samples were mounted in Vectashield (Vector labs).

2.5. RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions and reverse transcribed to cDNA with SuperscriptRT transcriptase II (Invitrogen). qPCR reactions were run using SYBR-Green (BioRad). The reaction was carried in 96-well plates. Melting curve analysis revealed a single product. The experiment was repeated twice for each gene and cell line. Fold change and statistical analysis for qPCR data were calculated using Bio-Rad iQ5 software. Statistical significance between high EZH2 overexpression and presence of metastasis in human CRC specimens was assessed by Mann–Whitney test. Primers used for qPCR assays are listed in Table S1.

2.6. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was essentially performed as described previously (Mazon-Peláez et al., 2010). Pre-cleared chromatin was incubated overnight by rotation with 4 μg of antibody (EZH2, BD Biosciences; H3K27m3, Millipore; C-JUN and FRA1, Santa-Cruz) or no antibody as a negative control. Inmunoprecipitates were re-suspended in 40 μl TE buffer, whereas inputs were re-suspended in 90 μl. Inputs and immunoprecipitated DNA samples were quantified by qPCR. The reaction was carried in 96-well plates and PCR reactions were run in duplicate using SYBR-Green (BioRad). Ct values of the immunoprecipitated samples were normalized with Ct input values. As an internal control GADPH locus was used. Primers used for qPCR ChIP assays are listed in Table S1.

2.7. ShEZH2 construct

To express small hairpin (sh) oligonucleotide that silences EZH2 mRNA we used the pSuper RNAi System (OligoEngine, Seattle). pSuper.neo (VEC-PBS-0004) vector was used for sh oligonucleotides cloning. The sequence of hairpin oligonucleotides was: Sh2EZH2sense 5′-GATCCCCAGCCATTTCCTCAATGTTTTTCAAGAGAAAACATTGAGGAAATGGCCTTTTTA-3′, Sh2EZH2antisense 5-AGCTTAAAAAGGCCATTTCCTCAATGTTTTCTCTTGAAAAACATTGAGGAAATGGCTGGG-3′. The cloning procedure was according to the manufacturer's instructions.