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Indole-3-Carbinol Mediated Anti-Proliferative Regulation of Breast Cancer Stem Cells and Malignant Melanoma-Initiating Cells

By

Anna Hyo Park

A dissertation submitted in partial satisfaction of the
Requirements for the degree of

Doctor of Philosophy

In

Endocrinology

In the

Graduate Division

Of the

University of California, Berkeley

Committee in charge:

Professor Gary L. Firestone, Chair
Professor Jen-Chywan (Wally) Wang
Professor Thomas J. Carlson

Fall 2016

ABSTRACT

Indole-3-Carbinol Mediated Anti-Proliferative Regulation of Breast Cancer Stem Cells and Malignant Melanoma-Initiating Cells

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Doctor of Philosophy in Endocrinology

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Professor Gary L. Firestone, Chair

The cancer stem cell theory ascertains a subpopulation of tumorigenic cells that selectively possess the ability for tumor initiation and self-renewal capacity to differentiate into nonmalignant cancer cells, known as bulk tumor cells. Cancer stem cells were first identified in hematopoietic cancers but subsequently have been implicated in ovarian, melanoma, liver, sarcoma, head and neck, pancreatic, glioblastoma, and breast cancers since. As a result, malignant tumors can be composed of genetically and morphologically heterogeneous cell populations with varying degrees of differentiation, self-renewal ability, and metastatic capacity. Experimental pursuits have sought to isolate and analyze the cancer stem cell population in primary tumors and molecularly strategize to identify phenotypic targets. A lack of standardized cancer stem cell *in vivo* and *in vitro* models have slowed down the development of proper therapies as well as the elucidation of scientific mechanisms that dictate cancer stem cells. One method of detection has been the utilization of various stem cell markers to categorize molecular phenotypes. In breast cancer stem cells, biomarkers such as the nucleolar GTPase nucleostemin; the detoxifying enzyme, ALDH-1; and cell surface marker, CD44 aid in identification and isolation of cancer stem cells for research studies. Furthermore, exclusion of the Hoescht Dye assays for the cell's ability to efflux current therapeutic molecules extra-cellullarly, evading apoptosis and cell cycle arrest. Furthermore, melanoma cancer stem cells, also known as malignant melanoma-initiating cells (MMICs), contain subpopulations expressing CD133, CD20, and ABCG5 have defined features of unlimited self-renewal and proliferative capacities for melanoma cancers. While cancer incidence overall has decreased, melanoma continues to rise, with patients with metastatic and malignant melanoma continue to relapse from current therapeutic strategies. This thesis details the characterization of human breast cancer and melanoma cell lines of varying tumorigenic potential and their subsequent response to the phytochemical, indole-3-carbinol, and its synthetic derivative, 1-benzyl-I3C. I3C selectively targets breast cancer stem cells through different molecular mechanisms such as protein-protein interactions of the stem cell marker nucleostemin and its binding partner, murine double mutant 2 (MDM2) in the nucleolus, thereby freeing p53 to activate apoptosis. Furthermore, I3C sensitizes cells with a wild type p53, wild type PTEN phenotype or even more tumorigenic melanoma cells that possess the rare BRAF

V600D mutation and PTEN deletion. Given I3C's and its more potent derivative, 1-benzyl-I3C's, anti-proliferative modulation of the p53 pathway in breast cancer cells and the PTEN/ Wnt pathways in malignant melanoma cell lines, preclinical results can implicate I3C as a novel chemopreventative molecule that selectively targets cancer stem cells.

Acknowledgments

I would like to thank Professor Gary Firestone for your mentorship professionally and personally in my life. Our small chats about Berkeley sports to our meaningful discussions about our futures has brought much light into my career here at Berkeley. I can't speak to the volumes of engagement and thoughtfulness you've shared with me in 591 LSA. Even though my years at Berkeley end here, I know the influence you've had over my life will be infinite.

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This work is dedicated to my parents and my grandmother

Youn Suk Park and Yeun Sil Woo and Kim Keum Oak

The greatest pillars of support in my life.
Thank you for your unconditional support, unwavering truth, and unparalleled dedication to my integrity.

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Anti-Cancer Properties of Phytochemicals

Phytochemicals are naturally occurring chemicals in plants, and have been identified as promising anti-cancer, health-promoting agents. They include phenolics, such as tanins and flavonoids; alkaloids, such as caffeine; nitro-containing compounds; organosulfur compounds, such as indoles; phytosterols; and carotenoids [1].

Phytochemicals have been identified to have potential in various medicinal pathways such as hormonal regulation, oxidative damage reduction, DNA repair, genomic stability, inflammation, and cellular proliferation. Some molecular reactions phytochemicals are known to promote both *in vivo* and *in vitro* are apoptosis mechanisms such as PARP cleavage, ATP-dependent chromatin remodeling, tumor angiogenesis, differentiation in the Wnt pathway, and tyrosine kinase receptor upregulation. Epidemiological studies do suggest that consistent uptake of fruits and vegetables, and other plant foods, is linked to reduce cardiovascular and chronic diseases [2]. As a result, it has become critical to understand the underlying molecular mechanisms of phytochemicals in promoting its health benefits.

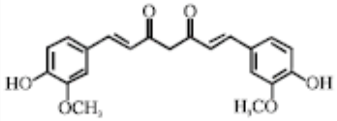
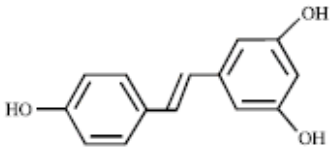
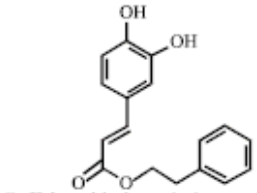
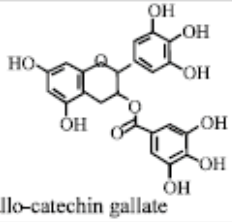
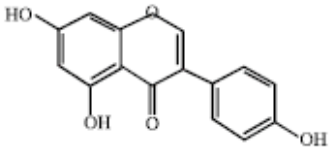
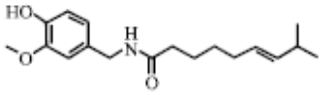
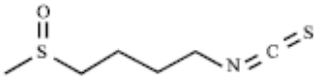
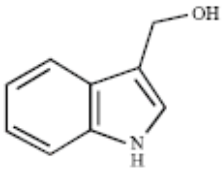
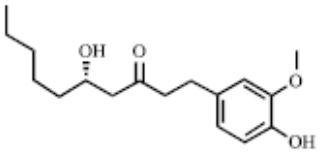
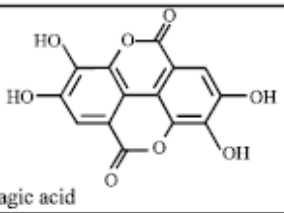
One of the toughest challenges of phytochemical usage is proving the effect of cancer prevention. Preventative epidemiological studies on a human population are difficult to prove within a reasonable timelines, as most require evidence of up to 30 years. Molecular studies in identifying progression of a tumor after dosage of phytochemicals, however, have been critical in quantifying reduction of oncogenic biomarkers and stabilization of tumor suppressors [3, 4].

One of the promising phytochemicals explored in my laboratory is, indole-3-Carbinol (I3C), which is found in *Brassica* vegetables, such as brussel sprouts, cauliflower, and napa cabbage. It's digestive derivative, is known as diindolylmethane (DIM), forms via condensation in the acidic environment of the stomach. Both have exhibited critical anti-proliferative effects in hormone responsive cancers such as prostate, ovarian, and breast cancers. Important indole derivatives, such as 1-benzyl-I3C, has been proven in our lab to be effective at a much lower dose, highlighting the potential for synthetic derivatives in battling these cancers [5, 6].

Figure 1

Cancer Preventative Phytochemicals.

Various chemopreventative agents in natural fruits, vegetables, and plant products shown to have antitumor and anti-proliferative effects in cancer, such as ovarian, breast, prostate, and melanoma.

Natural source	Chemopreventive phytochemicals	Natural source	Chemopreventive phytochemicals
Turmeric 	 Curcumin	Grapes 	 Resveratrol
Honey 	 Caffeic acid phenethyl ester	Green tea 	 Epigallo-catechin gallate
Soybean 	 Genistein	Chilli pepper 	 Capsaicin
Broccoli 	 Sulphoraphane	Cabbage 	 Indole-3-carbinol
Ginger 	 [6]-Gingerol	Strawberry 	 Ellagic acid

Indole-3-Carbinol and Breast Cancer

Indole-3-carbinol is a phytochemical naturally occurring in Brassica vegetables and have been associated with anticancer activities of cruciferous vegetables. It is produced by members of the family Cruciferae and the genus Brassica, including such vegetables as cabbage, brussel sprouts, cauliflower, radishes, and broccoli. Early studies have I3C has been a promising agent in reducing the incidence of spontaneous and carcinogen-induced tumors of certain cancers such as colon, skin, liver, cervix, and lung [7, 8].

In vitro, I3C has shown the suppress the proliferation of various tumor cells including prostate, breast, endometrial, leukemic, and colon cells by inducing G1/ S cell cycle arrest by downregulation of cyclin dependent kinases, cyclin D1, and cyclin E or upregulation of p15, p21, and p27; or activating apoptosis by downregulation of antiapoptotic gene products, such as Bcl-2, Bcl-xL, and surviving or upregulation of proapoptotic proteins like Bax [9-11].

In vivo, I3C was found to be a potent chemopreventive agent for hormonal-dependent cancers such as breast and cervical cancer by inducing apoptosis, stimulating 2-hydroxylation of estradiol, inhibiting DNA-carcinogen adduct formation, and inhibiting angiogenesis and invasion. Initial clinical trials in women show that I3c is an effective therapy against breast and cervical cancers [8].

I3C has been shown to activate or inactivate multiple nuclear receptors, decrease mitochondrial membrane potential induce endoplasmic reticulum stress, and regulate multiple cellular signaling pathways. I3C has a widespread effect on multiple cancer cells, such as breast cancer, melanoma, and prostate cancers [5], This demonstrates the I3C plays a significant role in either the cell cycle arrest or apoptosis of various cancers.

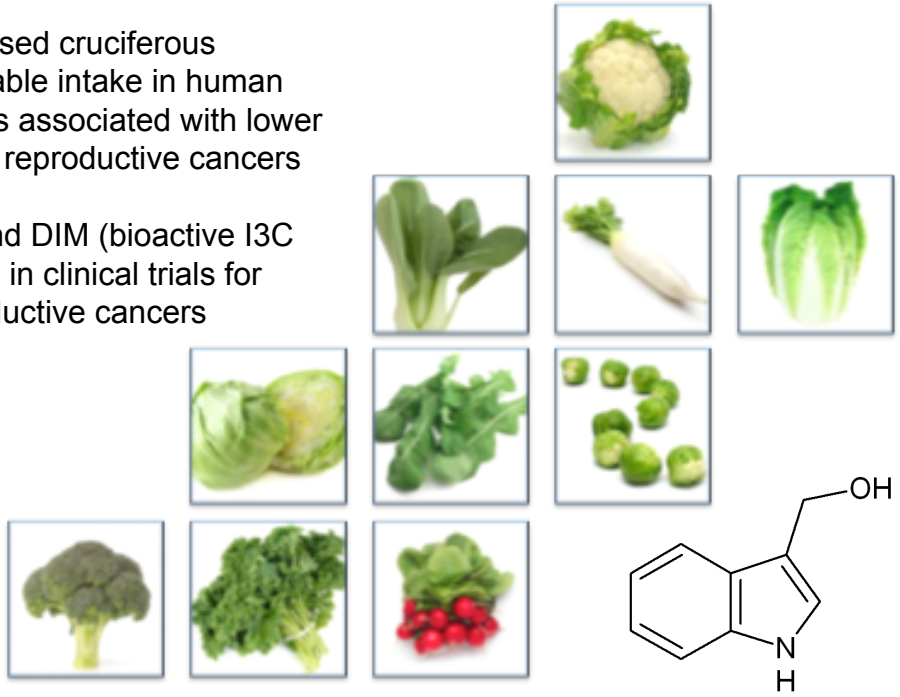
Figure 2

Indole-3-Carbinol (I3C).

I3C is produced by members of the family Cruciferae, and particularly members of the genus Brassica.

Indole-3-Carbinol (I3C)

- Increased cruciferous vegetable intake in human diets is associated with lower risk of reproductive cancers
- I3C and DIM (bioactive I3C dimer) in clinical trials for reproductive cancers



The heterogeneity of a cancer stem cell in a whole tumor

Cancer stem cells were first documented in leukemia and myeloma cancers, identifying a small side population of cancer cells as responsible for tumorigenesis and proliferation. Basic cancer research has questioned the effects of particular mutations on the proliferation, differentiation, and survival of certain cancer cells. It ha been shown only a small population of cells in a tumor are clonal, while the majority of solid cancers are comprised of cells that are phenotypically heterogeneous [1, 12, 13]. Both normal stem cells and tumorigenic cancer stem cells give rise to phenotypically heterogeneous cells that convey different degrees of differentiation. While some of the heterogeneity of a tumor can be due to prolonged mutagenesis, it is more likely that heterogeneity arises from the irregular differentiation of normal cancer cells [14-18].

The molecular signature of a malignancy is well-characterized and strategies to enrich a cancer population and re-sensitize them to treatment are being explored. However, cancer stem cells from solid tumors are much more elusive, with ambiguous phenotypic identifies and significant challenges for drug development in must tumor types [12, 19, 20]. However, based on certain characteristic properties such as tumor-initiating potential, niche dependence, detoxification/ drug resistance, activation of stem cell pathways, and clonogenicity, cancer stem cells can be identified and defined. In breast cancer cells, the presence of ALDH1, CD44, and nucleostemin as well as the lack of CD24 expression are key characteristics of a breast cancer stem cell phenotype [21-25].

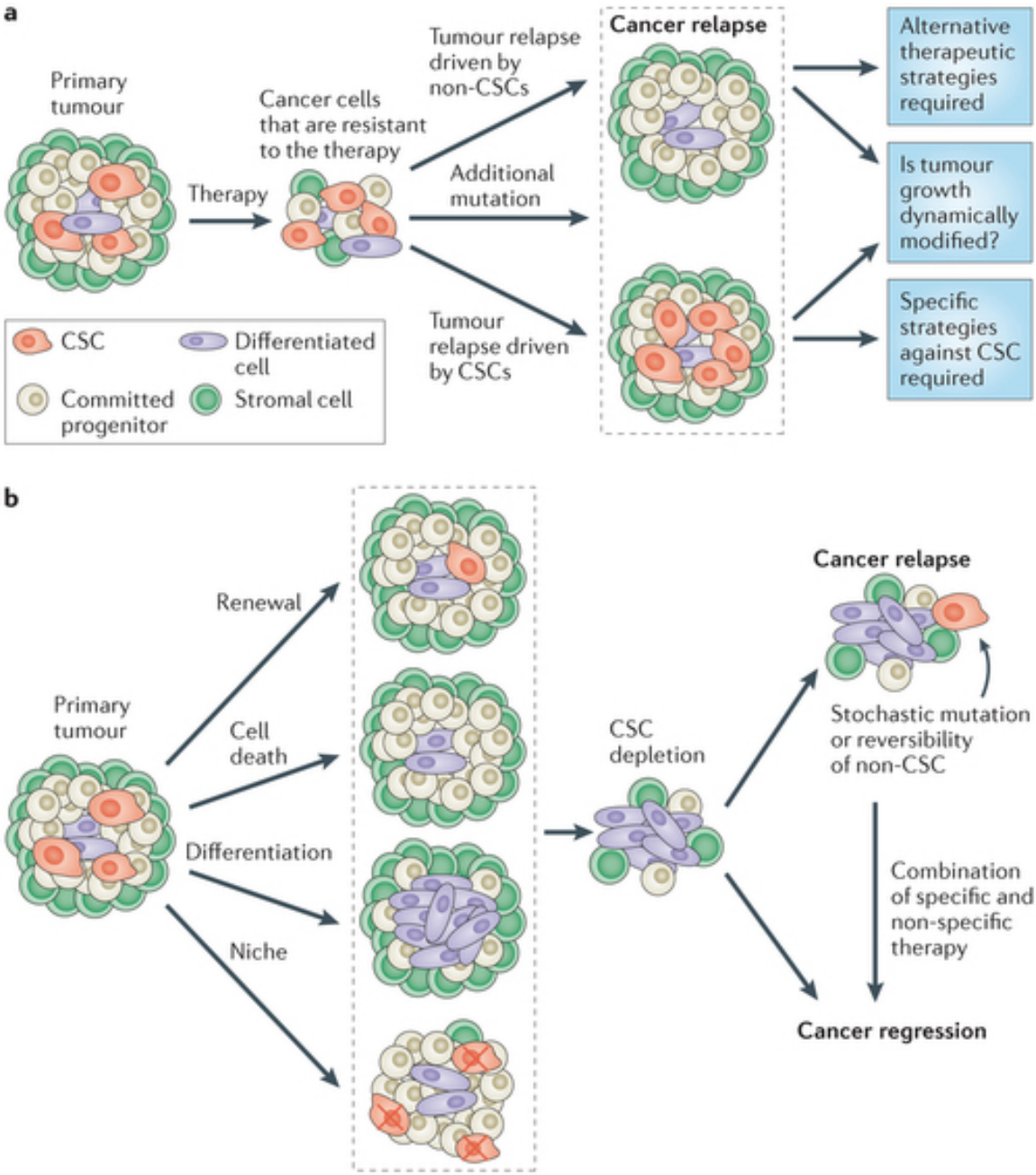
Stem cells are characterized by their ability to differentiate and self-renew, which allows them to generate all cell lineages within a given tissue. Both stem cells and cancer stem cells show activation of self-renewal associated pathways such as sonic Hedgehog, Notch, Wnt/ Beta-catenin, and PTEN [26, 27]. Through this property, tumor may originate from the transformation of normal stem cells, similar signaling pathways pay regulate self-renewal in stem cells and cancer cells, and cancer cells may include cancer stem cells.

As a result, mortality from breast cancer remains high because current therapies are limited by the emergence of therapy-resistant cancer cells [1]. Metastatic breast cancer still remains an incurable disease by current treatment strategies therefore it becomes critical to understand the molecular mutations that underlie the biology of these cells that may lead to new effective therapies [28, 29]. If a small population of cancer stem cells causes cancer growth and proliferation, novel therapies must exist that no only target and ablate solid tumor size, but also target cancer stem cells specifically.

Figure 3

Cancer Stem Cell Progression.

The heterogeneity of a tumor rises from the differentiation of cancer cells. This can lead to therapy resistance and cancer relapse, promoting poor prognosis for cancer patients. Novel molecular therapies can more effectively target the side population of cancer stem cells in a primary tumor and engage in cancer regression.


a
Primary tumour
Therapy
Cancer cells that are resistant to the therapy
Tumour relapse driven by non-CSCs
Additional mutation
Tumour relapse driven by CSCs
Cancer relapse
Alternative therapeutic strategies required
Is tumour growth dynamically modified?
Specific strategies against CSC required
CSC
Differentiated cell
Committed progenitor
Stromal cell
b
Primary tumour
Renewal
Cell death
Differentiation
Niche
CSC depletion
Cancer relapse
Stochastic mutation or reversibility of non-CSC
Combination of specific and non-specific therapy
Cancer regression

Nucleostemin, as an actor of self-renewal and cell cycle arrest

Nucleostemin is a GTP-binding protein concentrated in the nucleolus of most stem cells and now implicated in cell-cycle progression due to its p53-dependent activity [30-32]. It was discovered because of its high expression in neuroepithelial stem and progenital cells and was later discovered in other types of stem cells, tumor initiating cells (TICs), and tumor cells. In differentiated cells and tissues, however, nucleostemin is expressed at a much lower level, identifying nucleostemin as functionally critical in undifferentiated phenotypes [33-35].

Recently, downregulation as well as overexpression of nucleostemin has been identified in causing G1 cell-cycle arrest in cancer cells and stem cells. Nucleostemin upregulation will induce compartmentalization of murine double minute 2 (MDM2) in the nucleolus, stabilizing p53. Furthermore a new finding has identified depletion of nucleostemin promotes the interaction of MDM2 with ribosomal proteins L5 and L11, which also hinders p53 degradation [36].

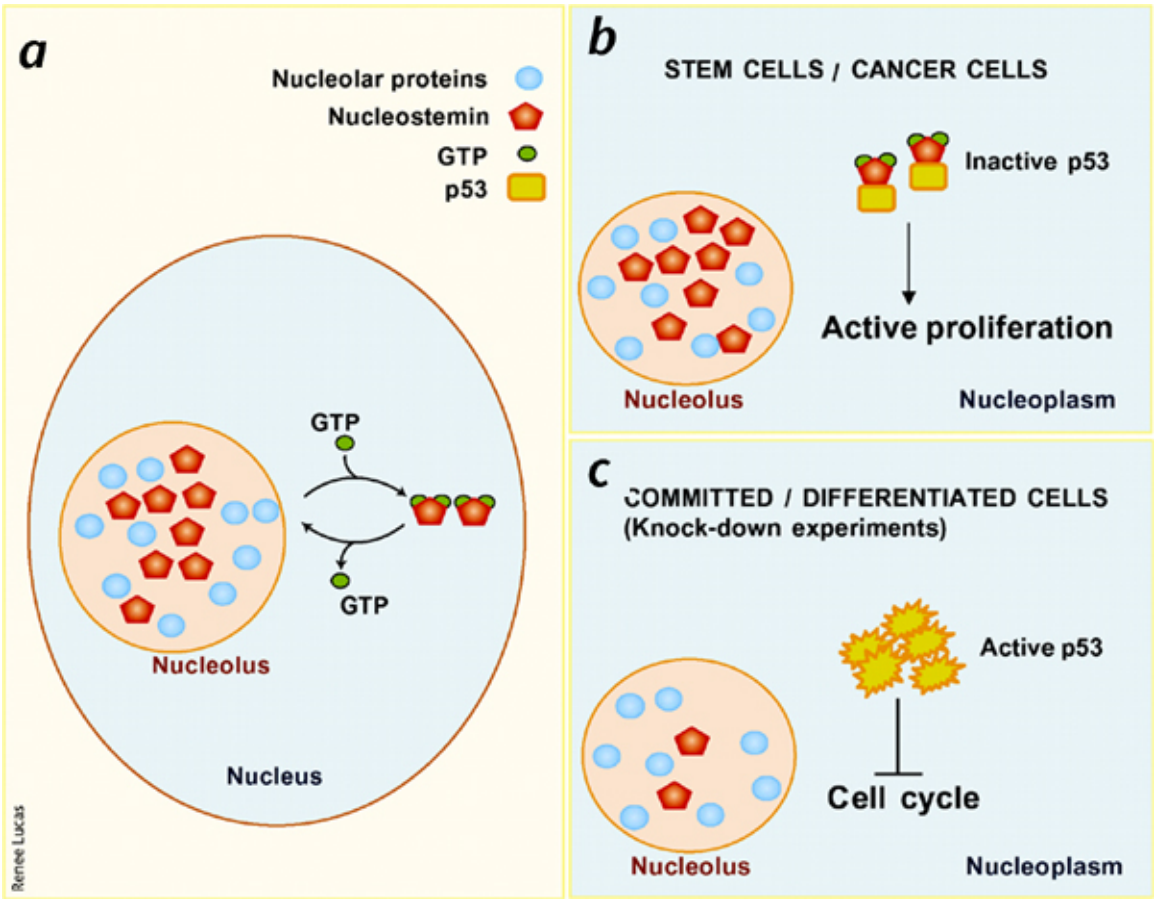
Mechanistically, the function of nucleostemin is unclear but certain studies recently have sought to explicate the genome-protective role of nucleostemin in stem cells and cancer stem cells. Several findings have implicated nucleostemin in reducing DNA damage on telomeres and non-telomeric chromosomes through modification of TRF1, also known as telomeric repeat binding factor. Nucleostemin also has the ability to recruit RAD41, a core player of the homologous recombination machinery, to protect the genomic integrity of stem or progenitor cells from replication-induced DNA damage [36].

Another mechanism critical to nucleostemin's activity is its ability to sequester MDM2, a negative regulator of p53, in the nucleolus [32, 36]. There are some studies that support p53-depdenncy of nucleostemin activity, in which cell cycle arrest (G1 or S) or apoptosis is triggered by knockdown or knockout of nucleostemin can be reversed by p53 knockdown. Furthermore, nucleostemin has been found to directly interact with p53 and MDM2 as well as p14ARF, a tumor suppressor that co-localizes MDM2 and enhances p53's apoptotic activity [30, 31, 37].

Figure 4

Localized nucleostemin regulates MDM2 binding and p53 stability.

Cancer stem cells express high levels of nucleostemin, capable of p53 and MDM2 regulation through protein binding.


a
Nucleolar proteins
Nucleostemin
GTP
p53
GTP
GTP
Nucleolus
Nucleus
Renee Lucas
b
STEM CELLS / CANCER CELLS
Inactive p53
Active proliferation
Nucleolus
Nucleoplasm
c
COMMITTED / DIFFERENTIATED CELLS
(Knock-down experiments)
Active p53
Cell cycle
Nucleolus
Nucleoplasm

Tumorigenic protein-protein interactions of ARF

P14ARF tumor suppressor is a critical player in cancer as a key sensor of signals that instruct a cell to proliferate and grow [38, 39]. It is localized in the nucleoli to limit these processes and have been implicated in releasing from the nucleolus for its most active functions. It is encoded from the human INK4A/ ARF (CDKN2A) locus which translates into both the cyclin-dependent kinase inhibitor p16INK4A as well as the tumor suppressor, p14ARF. ARF is translated in an alternative reading frame, and creates distinct ARF and INK4A proteins after translation [40-42].

In human cancers, one of the most recurrent cytogenic events is the homozygous loss of the Ink4A locus as mutation at this locus is only second to the p53 locus. Arf -/- mice commonly are susceptible to sarcomas, lymphoid malignancies, carcinomas, and tumors in the nervous system [43]. Furthermore, they are commonly more sensitive to carcinogen-related tumor formation, such as when exposed to 7,12-dimethylbenz-α-anthracene (DMBA). Arf loss also enhances aggressive phenotypes from other mutations, such as those observed in Bcr-Abl induced acute lymphoblastic leukemia. Mice disrupted for only exon 1β develop tumors as early as eight weeks. After one year, 80% of the mice die from spontaneous tumor development, with a mean survival latency of 38 weeks [38, 44].

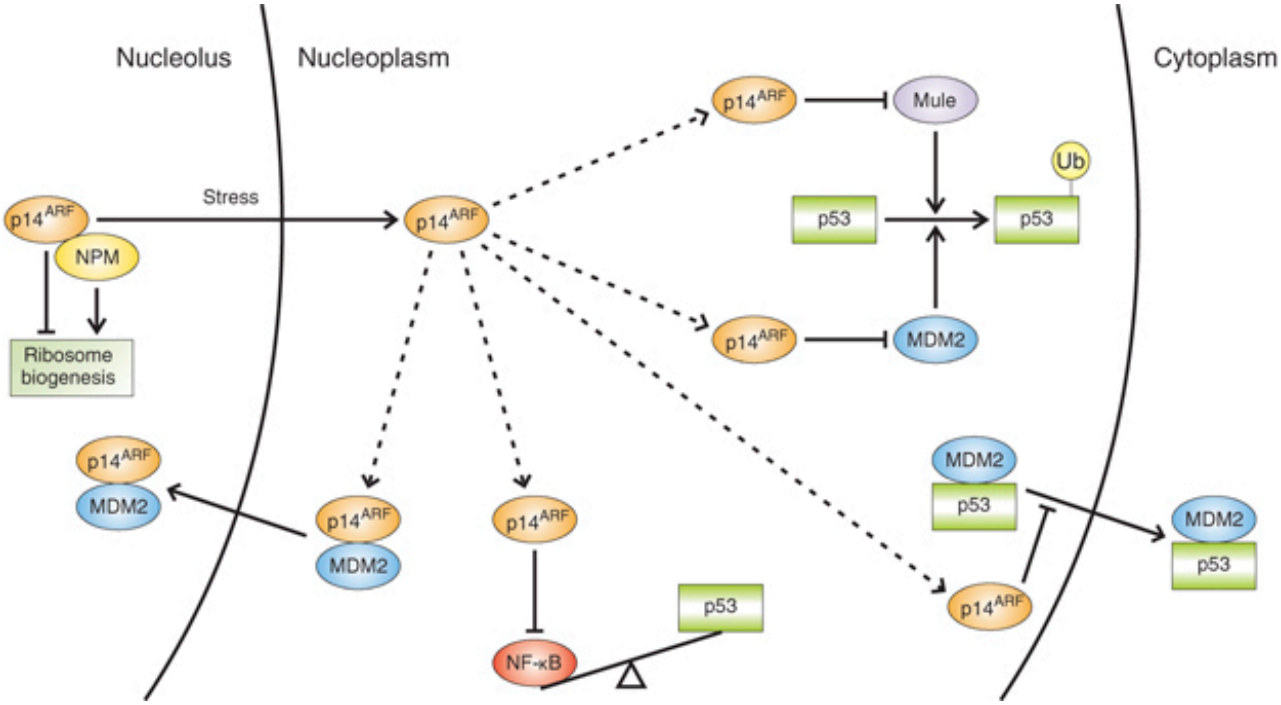
P14ARF has many oncogenic and tumor suppressive binding proteins due to its basic chemical nature conferred from its high concentration of arginine residues, It is relatively small and has a half-life of about 6 hours, ablated by ubiquitin-mediated proteosomal degradation in the N-terminus. As a result, ARF has been shown to interact with 30 other proteins, such as transcriptional activators, transcriptional repressors, posttranslational modifying enzymes, DNA modifying enzymes, and nuclear/ nucleolar proteins. ARF is typically localized in the nucleoli highly bound in high molecular weight complexes, such as with nucleophosmin, and unleashed during times of instantaneous oncogenic stress. Due to nucleophosmin interaction with ARF in the nucleolus, basal ARF might be correlated with maintaining nucleolar structure and limiting protein synthesis as well [45, 46].

P14ARF is classically involved with the p53 axis, by sequestering MDM2, an E3 ligase and potentiator of ubiquitination, away from p53. When activated by oncogenic signals, ARF's N-terminus associates with the central region of MDM2, which localizes MDM2 into the nucleolus away from MDM2 and near where ARF typically resides. The sequestration of MDM2 by ARF prevents the binding of MDM2 to p53 and the ability of MDM2 to shuttle between the nucleus and cytoplasm, thereby impeding its ability to transport p53 to the cytoplasm for degradation [47-51].

Figure 5

Cellular Localization of the tumor suppressor, p14ARF.

The tumor suppressor, p14ARF, is known to associate with multiple binding partners in the nucleolus and the nucleoplasm in order to promote p53-dependent apoptosis.


Nucleolus
Nucleoplasm
Cytoplasm
p14ARF
NPM
Stress
Ribosome biogenesis
p14ARF
Mule
Ub
p53
p53
p14ARF
MDM2
p14ARF
MDM2
p14ARF
MDM2
p14ARF
MDM2
p53
MDM2
p53
p53
NF-κB

Transcriptional Regulation of Nucleophosmin, Myc, and NFkB in Cancer

Nucleophosmin, also known as B23, is an acidic nucleolar protein with many binding partners that exhibit critical roles in proliferation and cell growth. It has been identified in a wide range of developmental pathways such as cellular proliferation, ribosomal genesis, DNA repair response, genomic stability, and most importantly, in cancer, apoptosis [52, 53].

As an acidic protein, multiple proteins interact with nucleophosmin in the nucleolus, such as the tumor suppressor responsible for p53 activation, p14ARF. The relationship between p14ARF and nucleophosmin is complex. For example, p14ARF binds to nucleophosmin in the nucleolus to promote degradation of the protein and induce cell death. There is increasing evidence of nucleophosmin's ability to retain p14ARF in the nucleolus and hinder its ability to bind to MDM2 and activate p53 [54]. However, other studies have found that nucleophosmin is also able to bind to MDM2 directly, and protect p53 from MDM2's role in ubiquitin degradation. Furthermore, nucleophosmin is enhanced by its binding to Akt1, an oncogene important in cellular proliferation and canonically known to phosphorylate and activate MDM2. Akt1, as a result of growth factor activation, protects nucleophosmin from proteolytic degradation from caspase-3 and stimulates cellular survival. By evidence of binding to p14ARF, MDM2, p53, and Akt1 directly, these studies suggest that nucleophosmin has a key regulatory role in mediating apoptosis and the p53-axis [47, 49-51].

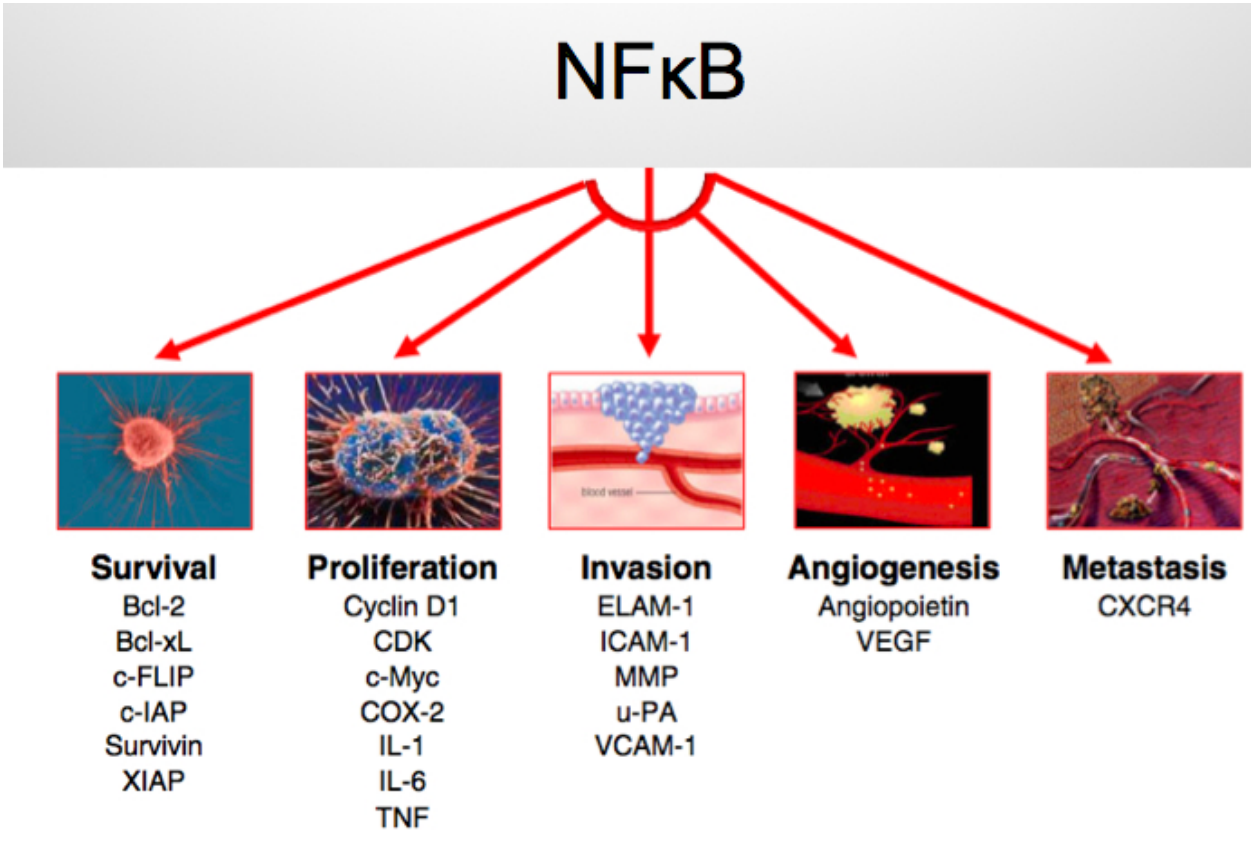
One known transcription factor of nucleophosmin is myc, a transcription factor responsible for unregulated expression of many genes in cancer, such as cellular proliferation, growth control, differentiation and apoptosis. The most common mutation of myc results in gene amplification and overactivation of chromosomal replication. Because it is overexpressed in a large majority of human cancers, it is a prime anti-cancer therapeutic target and has been implicated in colon, breast, stomach, lung, and cervical cancers. Functionally, myc's binding to the canonical enhancer box sequences, also known as E-boxes, and recruitment of histone acetyltrasnferases (HATs) in inducing transcription of downstream target genes, such as NF-kB [55-58]. The target genes of myc regulate cellular survival, protein synthesis, cell cycle, cell adhesion and metabolism.

The nuclear factor-kB family of proteins acts as dimeric transcription factors that regulate the expression of genes regarding inflammation stress response, cellular proliferation, and innate and adaptive immunity. Active NF-κB/Rel complexes are further activated by post-translational modifications (phosphorylation, acetylation, glycosylation) and translocate to the nucleus where, either alone or in combination with other transcription factors including AP-1, Ets, and Stat, they induce target gene expression [57, 58].

Figure 6

NFkB Signaling is involved in canonical and non-canonical transcriptional regulation.

The NFkB transcription factor is involved in many cancer-promoting pathways such as survival, proliferation, invasion, angiogenesis, and metastasis.


NFκB
Survival
Bcl-2
Bcl-xL
c-FLIP
c-IAP
Survivin
XIAP
Proliferation
Cyclin D1
CDK
c-Myc
COX-2
IL-1
IL-6
TNF
Invasion
ELAM-1
ICAM-1
MMP
u-PA
VCAM-1
Angiogenesis
Angiopoietin
VEGF
Metastasis
CXCR4

Melanoma stem cells, or malignant-melanoma-initiating cells (MMICs)

Malignant melanoma is highly metastatic cancer and increasingly resistant to conventional therapies due to increased molecular heterogeneity and expression of metastatic markers. Heterogeneity of a melanoma stem cell can be attributed to the cancer stem cell model. This evidence is supported by isolating cancer cells that express various levels of stem and progenitor cell markers or chemo-resistance markers, and comparing their tumorigenic capability [59]. Several biomarkers can be identified as melanoma cancer stem cells such as cell surface markers, CD133 and CD271; the ATP binding cassette transporters, ABCG2, and ABCG5; and the intracellular detoxifying enzyme expressed in many stem cells, Aldehyde Dehydrogenase (ALDH-1) [60-65]. Through these studies of cancer or progenitor stem cell markers, a distinct lineage of melanoma stem cells was isolated, positing marker-positive cells have greater tumor initiating capability in melanoma cells versus marker-negative cells which do not have the potential to recapitulate the phenotypic heterogeneity of a primary parental tumor [66-68].

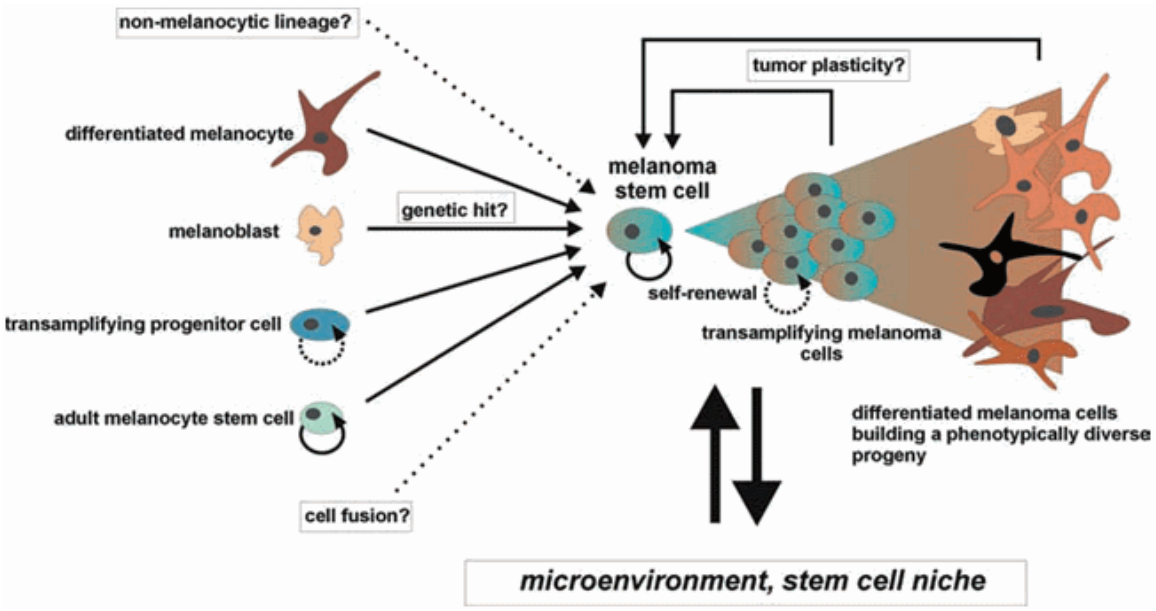
A hallmark of melanoma stem cells is initiation of tumor growth and melanoma initiation. They are a minor subpopulation, capable of self-renewal and differentiation, enriched for human malignant-melanoma-initiating cells (MMIC). A unique strategy in cancer therapeutics is to utilize molecular targets to ablate proliferation of MMICs. Gene profiling of a marker-positive population can result in the identification of more markers to elucidate the mechanism and signaling pathways relevant for potential diagnostics and prognosis. Therefore, melanoma stem cell-directed therapeutic approaches represent promising novel strategies to improve therapy.

Four types of melanoma cancer cells have been established: CD20(+), CD133(+), label-retaining or slow-cycling cells, and side-population cells with high efflux activities [13, 69, 70]. These properties are most likely overlapping for these subpopulations within a malignant lesion. Ongoing studies have been seeking to purify and characterize capabilities that determine prolonged self-renewal capability, immune evasion, vasculogenic differentiation, and microtumor niche development [12, 19, 20].

Figure 7

Melanoma Stem Cell Hypothesis.

A subpopulation of cells within melanoma with stem cell properties such as indefinite self-renewal and differentiation capacity can arise from a variety of cells with various lineages, such as a differentiated melanocyte, melanoblast, transamplifying progenitor cell, or an adult melanocyte stem cell.


non-melanocytic lineage?
differentiated melanocyte
melanoblast
genetic hit?
transamplifying progenitor cell
adult melanocyte stem cell
cell fusion?
tumor plasticity?
melanoma stem cell
self-renewal
transamplifying melanoma cells
differentiated melanoma cells building a phenotypically diverse progeny
microenvironment, stem cell niche

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Chapter I

Protein-protein interactions of the stem cell marker nucleostemin and MDM2 in breast cancer stem cells

Abstract

The cancer stem cell theory arises to explicate the heterogeneity of human breast cancer cells that possess the ability for multi-lineage differentiation and creates the survival of therapy-resistant tumor populations. In order to address the efficacy of therapies in cancer stem cells, it becomes critical to isolate and propagate cancer stem cells from primary breast tumors, which only represent 1-5% of the cells. Therefore, in order to study the affect of anti-cancer agents that can inhibit cancer stem cells to prevent apoptosis evading cellular mechanisms, an experimental capability to analyze cancer stem cells was necessary for this study. By expressing exogenous HER2 in a preneoplastic mammary epithelial cell lines, the newly created MCF-10AT-Her2 cell line expresses high levels of cancer stem/progenitor markers such as nucleostemin and ALDH1 as well as possesses increased tumorsphere forming efficiency than other cancer cell lines. Her2, or human epidermal growth factor receptor-2), is a member of the orphan epidermal growth factor (EGF) receptor gene family and is associated with poor prognosis in cancer patients and implicated with aggressive and malignant forms of breast cancer. This cell line was compared against other breast cancer cell lines of distinct lineages and of varying tumorigenic potential. Importantly, I3C induces a p53-dependent apoptosis by reducing the affinity of p53 to its negative regulators, MDM2. I3C enhances binding of nucleostemin with MDM2 (murine double mutant 2), a canonical E3 ligase of the tumor suppressor, p53. By induction of p53-dependent apoptosis through decreased interactions of MDM2 and p53, I3C initiates a cellular anti-proliferative response, inhibiting cellular growth and self-renewal capabilities. Our studies indicate the effectiveness of I3C over other established cancer cell lines of varying tumorigenecity and highlight the selective molecular response in cells with high self-renewal capacity, increased enrichment of cell population subset enriched in cancer stem cell marker profile expression, and marked notoriety to other current therapies. This new paradigm highlights the ineffectiveness of current therapies at targeting cancer stem cells over non-cancer stem cells and defines the necessity for novel treatment strategies that change today's current therapeutic standards.

Introduction

A critical issue in cancer treatment is the accurate identification, isolation and treatment of malignant and cancer stem cells, which exhibit various properties of both a cancer cell and a stem cell. One distinction is their ability to self renew in which their cell fate is segregated symmetrically or asymmetrically to daughter cells, or otherwise other tumor stem cells or bulk, non-tumorigenic cells [1, 2]. Cancer stem cells also exhibit unique markers such as high levels of the progenitor marker, nucleostemin [3, 4], and functional aldehyde dehydrogenase isoform 1 (ALDH-1)—both predictors of poor prognosis. Very little is known about the signaling pathways involved with these proteins and their abilities to influence phenotypes of cancer stem cell populations, but certain expression of stem cell markers, such as CD44 [5], nucleostemin, ALDH1, and lack of CD24 has allowed for isolation, but not propagation, of cancer stem cells [6-8]. These biomarkers have been attributed to breast cancer stem cells as well as stem cells for proteins, such as ALDH-1, which prevent mutational agents from entering the cellular membrane. For example, patients with CD44+/CD24- phenotype have been associated with poor prognosis through the Hedgehog signaling pathway [9]. Cancer stem cells are notoriously resistant to conventional therapies, such as chemotherapy or radiotherapy and no effective therapeutic strategy has yet been identified [10, 11]. Recent studies have shown that chemo- and radio-resistance of cancer stem cells in solid tumors explicates patient relapse and increased incidence of metastasis in many cancers [7]. Current therapies will attempt to ablate tumor size by targeting cancer cells in a wide fashion, rather than seeking to kill cancer stem cells, the cause of tumor burden. As a result, efficacious therapies are needed in targeting this small elusive population of cells that prevent the ablation of the entire breast tumor after various rounds of therapeutic treatments [12, 13]. However, in order to first study cancer stem cells a effective model system must be established as a model for cancer stem cells. To culture and propagate cancer stem cells *in vitro*, a stable and consistent population of cells enriched in a cancer stem cell phenotype was created by our laboratory.

By expressing HER2, a member of the orphan epidermal growth factor (EGF) receptor in the parental preneoplastic human mammary epithelial cell line, MCF-10AT [14], we engineered a breast cancer cell line in which the population is enriched with a stable stem cell-like character. As a control the 10AT cell line was also transfected with the neomycin control vector, and labeled as the 10AT-Neo control cell line from hereon. HER2 expression in breast cancer has been associated with poor prognosis in approximately 30% of breast cancers and implicated in metastasis [15-17]. Analysis of the generated MCF-10AT-Her2 cell line shows potent expression of cancer stem cell markers such as cell surface marker, CD44, the nuclear GTPase nucleostemin, and ALDH-1, a detoxifying enzyme associated with poor prognosis. We utilized this model system to elucidate the molecular mechanism of such stem cell markers through *in vitro* mammosphere assays and *in vivo* tumor xenograft capabilities in nude athymic mice. One such promising anti-cancer therapy is the chemopreventative phytochemical indole-3-carbinol [18-22], which previous research studies identified directly targets the neutrophil elastase. Binding of indole-3-carbinol to elastase stimulates interaction of nucleostemin to Murine Double Mutant 2 (MDM2), an E3 ligase for the tumor suppressor, p53 [23-25]. As a result, I3C-mediated interaction of MDM2-Nucleostemin binding in the nucleolus frees p53 to activate apoptosis in both HER2

expressing breast cancer stem cells, while the luminal cell line, MCF-7, and the triple negative cell line, both with null p14ARF phenotype, are I3C insensitive and p53 suppressive [26]. Protein levels of p14ARF, MDM2, p53, and the stem cell/ progenitor marker, nucleostemin, however, do not change, signifying the enhancement of I3C-mediated protein-protein interactions of the p53-depedent apoptotic pathway. Our study has uncovered new mechanistic insights into how the cancer stem/progenitor cell-associated component nucleostemin is directly involved in an anti-proliferative cellular signaling pathway triggered by I3C, a natural anti-cancer molecule.

Materials and Methods

Generating the Mammary Epithelial Cancer Stem Cell Line

Preneoplastic MCF-10AT human mammary epithelial cells (obtained from the Barbara Ann Karmanos Cancer Institute, Detroit, MI) were stably transfected with either the human pCMV-HER2 expression vector, which also contains the neomycin resistance gene, or with the pCMV-Neo control vector forming 10AT-Her2 cells and 10AT-Neo cells, respectively. Cells were stably selected with G418 sulfate (Cellgro, Manassas, VA) for 2 months.

Cell Culture

The MCF-10AT parent cell line and the 10AT-Her2 and 10AT-Neo cell lines were cultured in DMEM/F-12, 10% fetal bovine serum, 50 U/mL penicillin, 50 U/mL streptomycin (all media components purchased from Lonza, Allendale, NJ, and cell culture plates purchased from NUNC-Fischer, Pittsburgh, PA), 0.02 µg/mL epidermal growth factor (purchased from Promega, Madison, Wisconsin, USA), 0.05 µg/mL hydrocortisone, 10 µg/mL insulin, and 0.1 µg/mL cholera toxin (obtained from Sigma-Aldrich). Breast adenocarcinoma cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin/streptomycin, and 2 mM L-glutamine (Cambrex Bio Science, Walkersville, MD). MCF-7 human breast cancer cell lines were grown in Dulbeccos modified Eagles medium supplemented with 10% fetal bovine serum, 1.25 ml 20,000 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 10 µg/ml insulin. SKBR3 cell line was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in McCoy's 5A medium supplemented with 10% (v/v) FSC, 100 mg/l of streptomycin and 100,000 U/l of penicillin G at 37 ◦C in 5% CO2 incubator. Cells were grown to subconfluency in a humidified chamber at 37°C containing 5% CO2. For drug treatments, a 200 mmol/l stock solution of I3C, 10 mmol/l stock solution of 1-benzyl I3C, 50 mmol/l stock solution of Diindolymethane (DIM), 200 mmo/l stock solution of Tryptophol, and 300 mmol/l stock solution of Artemisinin (purchased from Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and then diluted in the ratio 1:1000 in media before culture plate application. Before each drug treatment, cells are washed in ice cold phosphate-buffered saline (PBS) (obtained from Lonza).

The 10AT-Her2 cell line, the parental MCF-10AT cells and the SKBR3 breast cancer cell line were validated by short tandem repeat (STR) DNA fingerprinting using DDC Medical Cell Line Authentication Lab Services (Fairfield, OH, USA). The STR profiles were compared to known ATCC fingerprints [83] and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 [84]. The STR profile of the 10AT-Her2 cell line matched that of its parental MCF-10AT cell line, whereas the SKBR3 cells matched the known DNA fingerprints to that of itself. Therefore, the cell population of the 10AT-Her2 cell line, which displays significant stemness-like character, is not contaminated with any other cell line, such as SKBR3 cells that also display some stemness-like character in the corresponding cell population.

Treatment With Indole-3-Carbinol

I3C was purchased from Sigma–Aldrich (St. Louis, MO), To study the effect of I3C, cells were treated with or without 200 μM I3C every 24 h for 72 h and harvested at 48 hours for western blot and flow cytometric analysis. I3C was dissolved in 99.9% HPLC grade DMSO (Sigma–Aldrich, Milwaukee, WI) and the final dilution was performed in the media aliquots used for treatment.

ALDEFLUOR Assay

ALDEFLUOR assay was performed following manufacture's recommended guidelines and protocol (Stem Cell Technologies). Live single cells were gated for analysis using Epics XL-MCL flow cytometer (Beckman Coulter) with single 488 nm blue laser filtered at 525 BP/slot 1.

Tumorsphere Efficiency Assay

Single-cell suspensions of 10AT-Her2, 10AT-Neo, MCF-7, or SKBR3 cells were plated on ultra-low attachment plates (Corning, Costar) in MammoCult Human Medium (Stem Cell Technologies, Vancouver) and cultured at 37°C, 5% CO_2. Tumorsphere formation was assessed visually by phase microscopy and quantified by counting the number of spheres formed in culture.

Western Blots

After the indicated treatments, cells were harvested in radioimmune precipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% NoNidet-p40 (Nonidet P-40, Flulta Biochemitra, Switzerland), 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 g/ml phenylmethylsulfonyl fluoride, 10 g/mL aprotinin, 5 g/mL leupeptin, 0.1 g/mL NaF, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 0.1 mM glycerol phosphate). Equal amounts of total cellular protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 ml of 14.4M βmercaptoethanol, 10% bromphenol blue, 3.13% 0.5M Tris-HCl, and 0.4% SDS (pH 6.8)) and fractionated on 10% polyacrylamide/0.1% SDS resolving gels by electrophoresis. Rainbow marker (Amersham Biosciences) was used as the molecular weight standard. Proteins were electrically transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA) and blocked for 1 hour with Western wash buffer5% NFDM (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20, 5% nonfat dry milk). Protein blots were subsequently incubated for overnight at 4°C in primary antibodies. The antibodies used were as follows, Rabbit anti-Nucleostemin, (sc-67012), Goat anti-ALDH-1 (sc-22588), were purchased from Santa Cruz Biotechnology and diluted in the ratio 1:1000 in TBST. Rabbit anti-actin (AANO1; Cytoskeleton, Denver CO) was diluted 1:1000 in TBST and used as a gel-loading control. Rabbit anti-HER2 (2165) was obtained from Cell Signaling. The working concentration for all antibodies was 1 μg/mL in Western wash buffer. Immunoreactive proteins were detected after incubation with horseradish peroxidase conjugated secondary antibody diluted to 3 x 10^4 in Western wash buffer (goat anti-rabbit IgG, rabbit anti-goat IgG, and rabbit anti-mouse IgG (Bio-

Rad)). Blots were treated with enhanced chemiluminescence reagents (PerkinElmer Life Sciences), and all proteins were detected by autoradiography. Equal protein loading was confirmed by Ponceau S staining of blotted membranes.

Results

10AT-Her2 and SKBR3 cells, both HER2+ breast cancer cell lines, reveal the highest cancer stem cell expression

To assess the varying tumorigenic capability of established human breast cancer cell lines, protein levels of established and critical breast cancer stem cell markers were analyzed in 10AT-Neo, 10AT-Her2, MCF-7, MDA-MB-231 cells, and SKBR3 cells. MCF-7 cells represent a luminal subtype with low expression of HER2, while SKBR3 cells express comparable levels of HER2 to the 10AT-Her2 cell line. MDA-MB-231 cells are triple negative basal subtype cell with slight expression of HER2. Western blot analysis shows that 10AT-Her2 cells and SKBR3 cells possess equivalent levels of HER2, Nucleostemin, CD44, and the detoxifying enzyme, ALDH-1 protein. Furthermore, both MCF-7 and MDA-MB-231 cells express much lower levels of nucleostemin compared to the more tumorigenic 10AT-Her2 and SKBR3 cell line. Through the quantification of established stem cell markers such as nucleostemin, a stem/ progenitor expression biomarker, and ALDH-1, a detoxifying enzyme implicated in therapy resistance, the characteristic of enhanced and enriched "stemness" can be analyzed per breast cancer cell line.

To quantify the population of cells within the various breast cancer cell populations that express functional levels of ALDH-1, an ALDEFLUOR assay determined the percentage of ALDH-1+ cells within each cancer cell line. The assay revealed greater than 88% of the ALDH-1 in 10AT-Her2 cell population, 44% in SKBR3, and less than 1% in MCF-7, 10AT-Neo, and MDA-MB-231 cells are enzymatically active. Upon 200 μM I3C treatment, there was no change in functional ALDH-1 subpopulations within normal and cancer human mammary epithelial cells indicating total protein levels of functional ALDH1 might not be associated with malignancy.

Figure 8

Stem Cell-Like Characteristics of Various Cell Lines.

10AT-Neo, 10AT-Her2, MCF-7, MDA-MB-231 and SKBR3 cells were harvested, total cell extracts electrophoretically fractionated and the levels of expressed HER2, CD44, CD24, ALDH-1, nucleostemin (NS) and actin protein determined by Western blots.

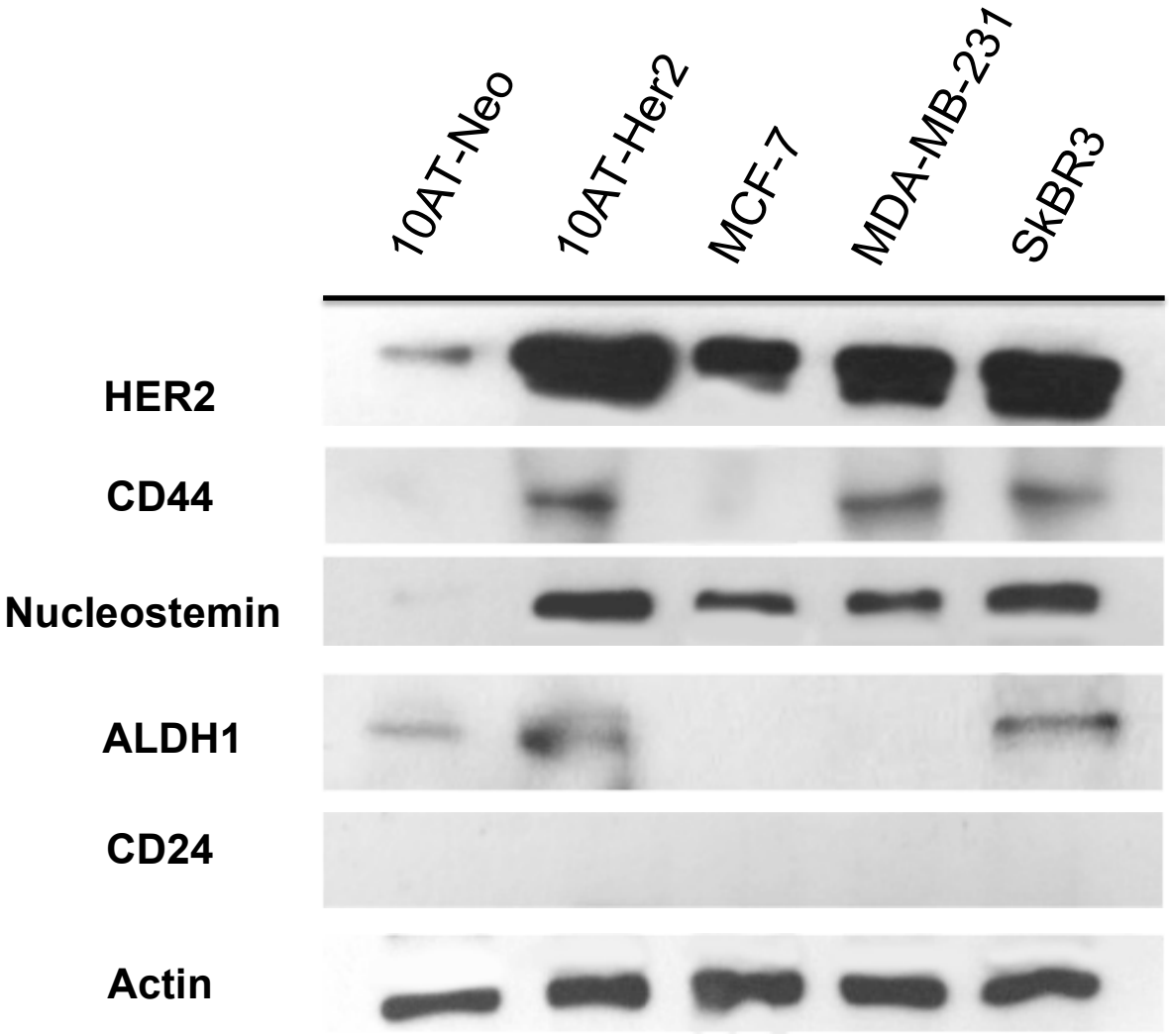
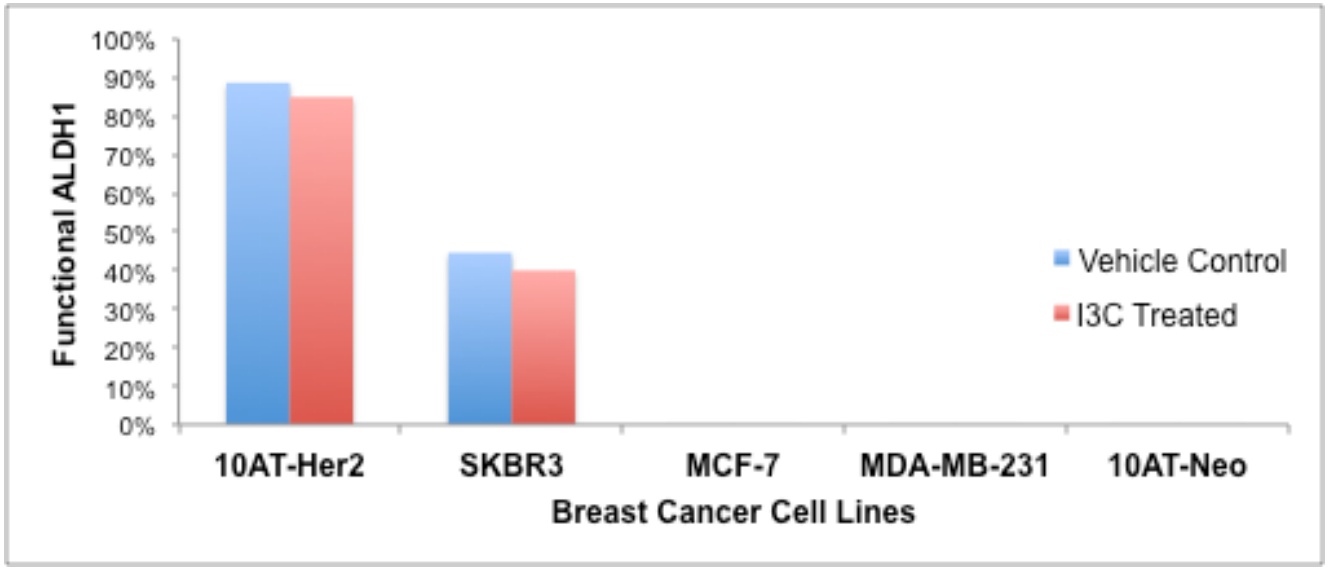

10AT-Neo
10AT-Her2
MCF-7
MDA-MB-231
SkBR3
HER2
CD44
Nucleostemin
ALDH1
CD24
Actin

Figure 9

Quantification of ALDH-1 activity.

10AT-Her2, SKBR3, MCF-7, MDA-MB-231, and 10AT-Neo cells were treated with or without 200 μM I3C for 48 housr and ALDH-1 activity was quantified in 10AT-Her2 and 10AT-Neo cells by ALDEFLUOR assay, 10AT-Her2 cells and 10AT-Neo cells were quantified by flow cytometry of 500,000 cells in triplicate independent cell cultures.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Functional ALDH1
Vehicle Control
I3C Treated
10AT-Her2
SKBR3
MCF-7
MDA-MB-231
10AT-Neo
Breast Cancer Cell Lines

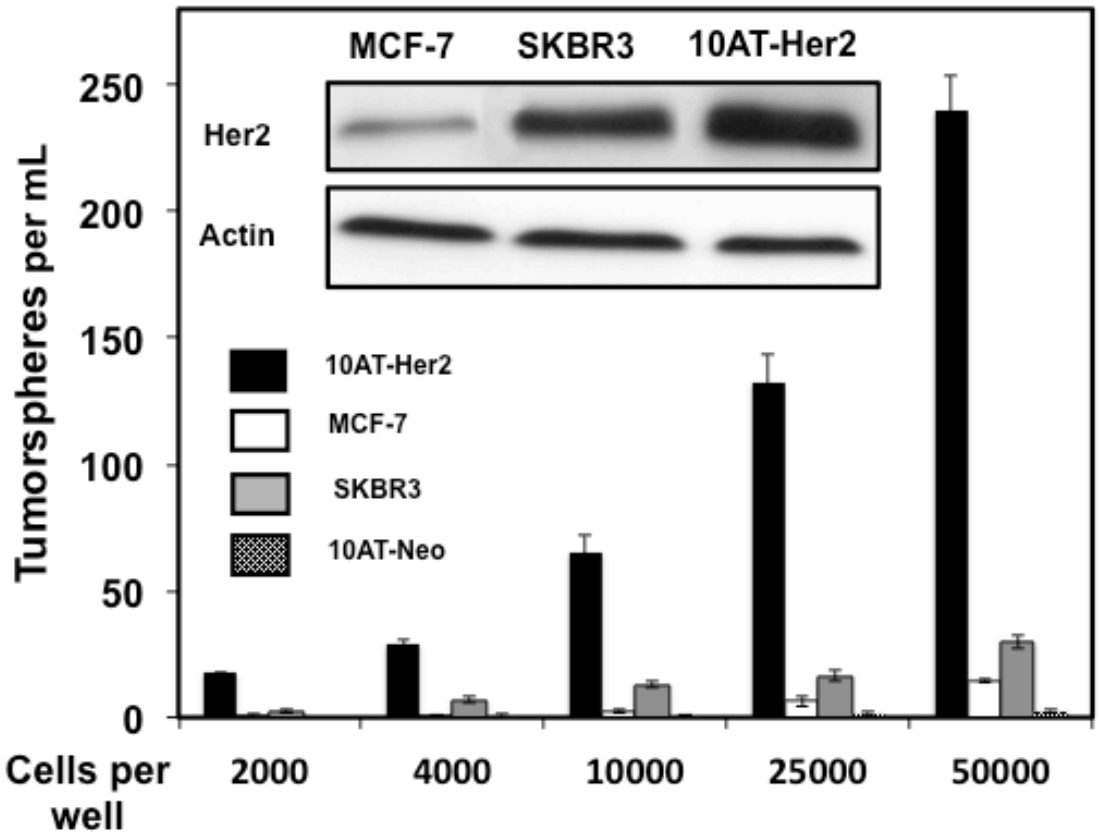
In Vitro Formation of Tumorsphere of Human Breast Cancer Cell Lines

Next, various human breast cancer cell lines were subjected to limiting dilution studies in order to understand the *in vitro* tumor-initiating capability. By assessing the formation of tumorspheres in cell suspension cultures of 10AT-Neo, 10AT-Her2, MCF-7, and MDA-MBA-231 cells, breast cancer cell lines become enriched for cancer stem cells, capable of self-renewal. 10AT-Her2 cells were the most efficient in forming tumorsphere-like formations within 2 days of culture and by 6 days the cells were identifiable as full, completed tumorspheres. However, other less metastatic cell lines such as the preneoplastic cell line, 10AT-Neo, andMCF-7 were incapable of forming tumorspheres even when 50,000 cells had been cultured. 10AT-Her2 cells displayed almost 10-fold efficiency in which cultures of 2000 cells performed similar to assays of 25,000 SKBR3 and 50,000 MCF-7 cells. Since SKBR3 and 10AT-Her2 cells express similar levels of the HER2 protein, the capacity for 10AT-Her2 cells to efficiently produce tumorspheres cannot be only ascribed to high expression levels of HER2. Therefore, tumorsphere formation, which represents a hallmark of stem cell self-renwal properties, is only attributed to the 10AT-Her2 cell line, despite its similarities in protein levels of cancer stem cell markers to SKBR3 cells.

Figure 10

Comparing 10AT-Her2 tumorsphere forming efficiency.

10AT-Her2, 10AT-Neo, SKBR3 and MCF-7 breast cancer cells were incubated at the indicated cell densities and tumorsphere formation efficiency was quantified after 6 days in culture under nonadherent conditions. The presented values are an average of three independent experiments. The gel inserts are western blots showing relative levels of HER2 protein expression and actin controls from electrophoretically fractionated total cell extracts of MCF-7, SKBR3 and 10AT-Her2 cells.


MCF-7
SKBR3
10AT-Her2
Her2
Actin
Tumorspheres per mL
250
200
150
100
50
0
10AT-Her2
MCF-7
SKBR3
10AT-Neo
Cells per well
2000
4000
10000
25000
50000

I3C enhances Nucleostemin-MDM2 binding and disrupts MDM2-p53 interactions

Co-immunoprecipitation experiments reveal the I3C-mediated protein-protein interactions of breast cancer cell lines. We examined whether cellular biomarkers that contributes towards the cancer stem/ progenitor-like phenotype might be correlated with I3C regulation of the MDM2-p53 pathway. Therefore, a critical component that is intriguingly expressed in self-renewing cancer stem/ progenitor cells is nucleostemin, a nucleolar GTPase shown to directly bind to MDM2. Binding of MDM2 and nucleostemin requires the central domain of MDM2 and the coiled-coil and acidic domains of nucleostemin (xx). While I3C had no effect on the total levels of nucleostemin and MDM2 protein in both 10AT-Her2 and SKBR3 cells, levels of serine 166-phosphorylated MDM2 decreased in cells treated with I3C. I3C treatment promoted the interactions of nucleostemin with phophrylated MDM2 as well as total MDM2 protein. This suggests that the I3C-mediated binding of nucleostemin with Ser166 phosphorylated MMD2 ablates p53 interactions with MDM2 and can be attributed for I3C's ability to activate a p53-dependent apoptotic response in 10AT-Her2 cells.

Secondly, to analyze whether the I3C regulation of MDM2 protein interactions with p53 and/or nucleostemin occurs in other indole-carbinol-sensitive breast cancer cells, three well-established cell lines, SKBR3, MCF-7 and MDA-MB-231, were treated with or without I3C for 48 hours and MDM2–p53 and nucleostemin–MDM2 co-immunoprecipitations were performed.. As shown in ----, I3C disrupted MDM2–p53 interactions and stimulated nucleostemin–MDM2 interactions in SKBR3 cells, a cell line that expresses nucleostemin and other stem/progenitor cell-like marker proteins approximately to the same levels as the 10AT-Her2 cell population (Figure 2C). Therefore, the effects of I3C on nucleostemin–MDM2 and MDM2–p53 interactions that we observed with 10AT-Her2 cells is not limited only to this newly developed breast cancer cell line. In contrast, even though MCF-7 and MDA-MB-231 cells are sensitive to the anti-proliferative effects of I3C, there were no detectable changes in MDM2–p53 or nucleostemin–p53 protein interactions after I3C treatment (Figure 5B,C). Based on expression of marker proteins, the relative stemness character of the MCF-7 and MDA-MB-231 cell populations can be considered less than that of either SKBR3 or 10AT-Her2 cells, which may be associated with the lack of any effects of I3C treatment on nucleostemin protein–protein interactions.

Figure 11

I3C induces Nucleostemin-MDM2 interactions and release p53 in cancer stem cells.

10AT-Her2 cells were treated with or without 200 μM I3C for 48 hours. Total cell extracts were immunoprecipitated with either MDM2 or nucleostemin antibodies. As a control, non-immune antibodies (of immunoglobulin G or IgG) and samples not immunoprecipitated (No IP) were used. All extracts were electrophoretically fractionated and probed by Western blot analysis using antibodies specific to p53, serine-166 phosphorylated MDM2 or total MDM2 or with antibodies specific to either serine-166 phosphorylated MDM2 or total MDM2

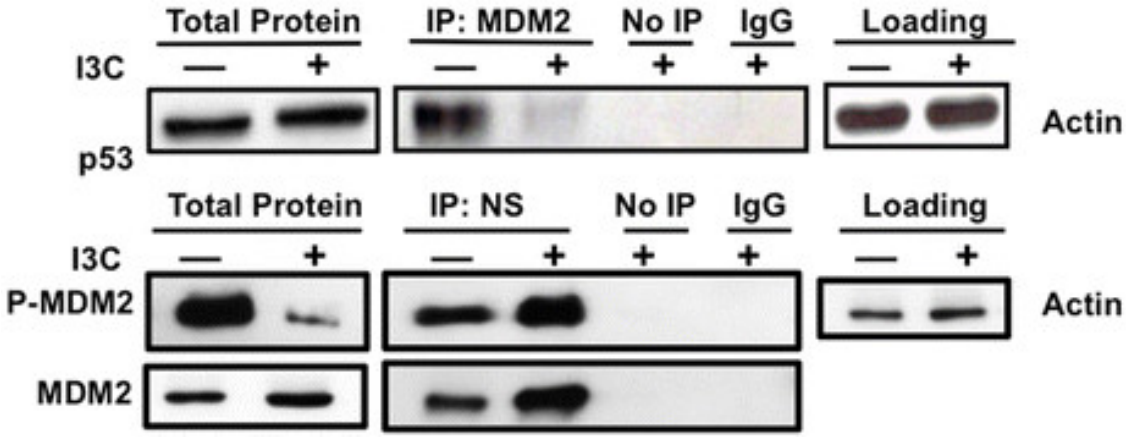

Total Protein
IP: MDM2
No IP
IgG
Loading
I3C
—
+
—
+
+
+
—
+
p53
Actin
Total Protein
IP: NS
No IP
IgG
Loading
I3C
—
+
—
+
+
+
—
+
P-MDM2
Actin
MDM2

Figure 12

I3C regulation of nucleostemin–MDM2 and p53-MDM2 protein-protein interactions in well-established human breast cancer cell lines.

SKBR3 (A), MCF-7 (B) and MDA-MB-231 (C) human breast cancer cells were treated with or without 200 μM I3C for 48 hours. Total cell extracts were immunoprecipitated with either MDM2 (top panels for each cell line) or nucleostemin (lower panels for each line) antibodies. As a control, non-immune antibodies (IgG) and samples not immunoprecipitated (No IP) were used. All extracts were electrophoretically fractionated and probed by Western blot analysis using antibodies specific to p53 (top panels) or with antibodies specific to serine-166 phosphorylated MDM2 or total MDM2 (lower panels). The levels of actin protein remaining in the cell extracts after the immunoprecipitations were used as gel-loading controls in each experiment. I3C, indole-3-carbinol; IgG, immunoglobulin G; IP, immunoprecipitated; MDM2, murine double mutant 2; NS, nucleostemin.

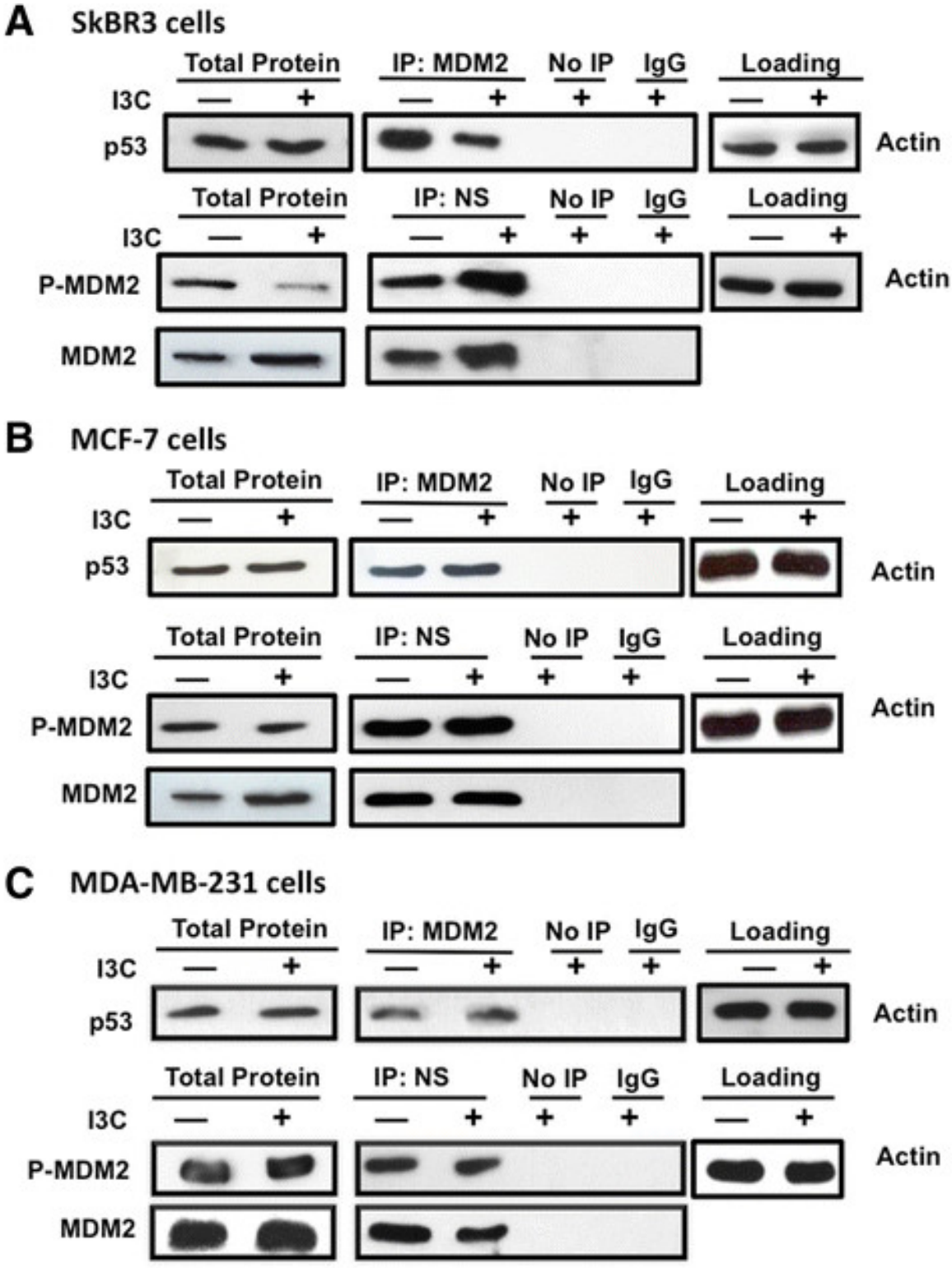

A
SkBR3 cells
Total Protein
IP: MDM2
No IP
IgG
Loading
I3C
p53
Actin
Total Protein
IP: NS
No IP
IgG
Loading
I3C
P-MDM2
Actin
MDM2
B
MCF-7 cells
Total Protein
IP: MDM2
No IP
IgG
Loading
I3C
p53
Actin
Total Protein
IP: NS
No IP
IgG
Loading
I3C
P-MDM2
Actin
MDM2
C
MDA-MB-231 cells
Total Protein
IP: MDM2
No IP
IgG
Loading
I3C
p53
Actin
Total Protein
IP: NS
No IP
IgG
Loading
I3C
P-MDM2
Actin
MDM2

Figure 13

Increased expression of Cancer Stem Cell-Like Phenotype is correlated with I3C Responsiveness.

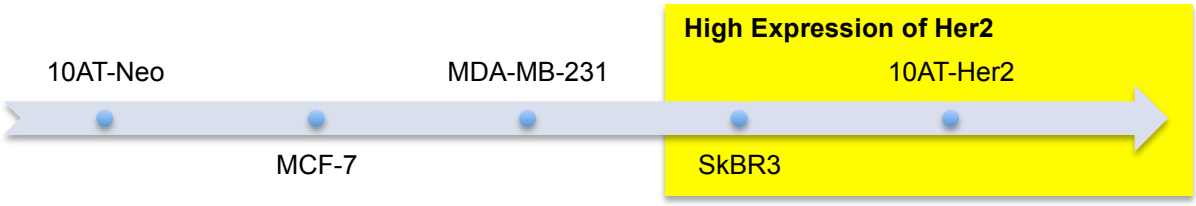
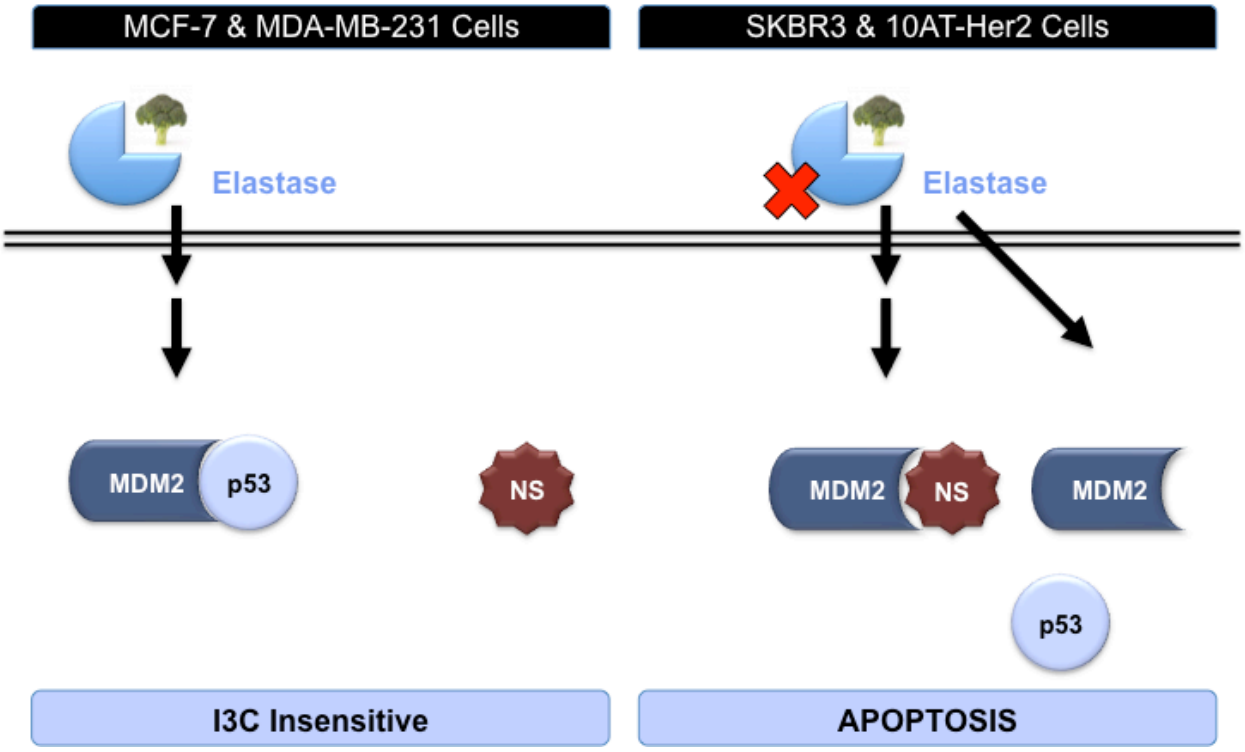

High Expression of Her2
10AT-Neo
MDA-MB-231
10AT-Her2
MCF-7
SkBR3
Increasing Stem Cell Character

Figure 14

Schematic diagram of nucleostemin-MDM2-p53 interactions.

I3C enhances p53 stabilization through protein-protein interactions after binding to the direct target protein elastase extracelullarly.


MCF-7 & MDA-MB-231 Cells
SKBR3 & 10AT-Her2 Cells
Elastase
Elastase
MDM2
p53
NS
MDM2
NS
MDM2
p53
I3C Insensitive
APOPTOSIS

Discussion

Indole-3-Carbinol, an antiproliferative phytochemical, has been shown to have antitumor effects in a range of cellular and animal models of cancer, and in the several well-tested systems, indole-3-carbinol and its synthetic derivatives, like 1-benzyl-I3C were shown to regulate oncogenic transcriptional and/ or cell signaling pathways [18, 20]. The MCF-10AT-Her2 model system represents an enriched population of cells with a cancer stem cell phenotype capable of tumorsphere formation and *in vivo* tumor xenograft capitulation [19]. However, tumorigenic cell lines such as MDA-MB-231 and MCF-7 cells, display cell cycle arrest in response to indole-3-carbinol treatment, but do not promote protein-protein interactions between the stem cell progenitor marker nucleostemin and MDM2 [3, 25, 27-29]. Nucleostemin is necessary for the I3C antiproliferative response in MCF-10AT-Her2 cancer stem cell line and SKBR3 cell line, which represents a HER2 positive cell line without self-renewal capability. I3C targets co-localization of MDM2 to nucleostemin in the nucleolus in which MDM2 is compartmentalized away from p53 only after I3C treatment despite presence of nucleostemin in the presence or absence of I3C treatment. As a result, the propensity for nucleostemin's activation after indole-3-carbinol treatment remains unclear. Although nucleostemin has been implicated to play a role in stem cell development and the limitless replicative potential of cancer cells, its role in cancer stem cells with MDM2 and p53 has not been fully elucidated. No protein levels of p53, MDM2, and nucleostemin were affected in any of the five breast cancer stem cells. Previous studies in our lab have identified elastase as a direct target protein in 10AT-Her2 cells, and it would be critical for future experimental studies to elucidate the direct binding of indole-3-carbinol in other tumorigenic cancer cell lines. Furthermore, since downstream targets of indole-3-carbinol have been elucidated here, it will become critical to probe the upstream Her2 regulatory signaling pathway that produces a cancer stem cell phenotype after ectopic Her2 expression.

Indole-3-Carbinol was more effective in inducing apoptosis in tumorigenic breast cancer cell lines with a cancer stem cell like phenotype, such as 10AT-Her2 and SKBR3 cells, compared to the p14ARF negative cells, MDA-MB-231 and MCF-7 cells, or nontumorigenic cell lines, MCF-10A, that was resistant to indole-3-carbinol. The observation of indole-3-carbinol's sensitivity did not depend on HER2 expression as SKBR3 cells and 10AT-Her2 cells displayed similar protein levels of HER2. However, tumorigenic cell lines, MCF-7 and MDA-MB-231 cells, which possess a p14ARF deletion, did not activate a p53 dependent apoptosis. This could potentially be explained by ARF, which is a tumor suppressor known to associate with p53 and MDM2, and their inability to form tumorspheres at the same efficiency of SKBR3 and 10AT-Her2 cells. Even though all four tumorigenic cell lines respond to I3C, only 10AT-Her2 and SKBR3 cells potentiated increased interactions between nucleostemin and MDM2. The selectivity of indole-3-carbinol has been observed in other cancer types as well. For example, indole-3-carbinol and its more potent derivative have been observed to ablate ovarian, prostate, and other reproductive cancers. This study presents indole-3-carbinol's molecular mechanism for a variety of cancers with a range of tumorigenic potential. Indole-3-carbinol and 1-benzyl-I3C represent promising antitumor preventative agents for breast cancer therapeutic molecules for a variety of human breast

cancer cell types due to their selective modulation of protein-protein interactions that affect cell cycle arrest or apoptosis.

This study presents I3C as an efficacious and novel antiproliferative phytochemical that induces apoptosis in cancer stem cell like cells by modulating the nucleostemin and p53 pathways in HER2 overexpressing cancers. These cancer cells are an opportunity to elucidate the molecular mechanisms of I3C *in vitro* in adherent and spheroid types of breast cancer cells, highlighting I3C as a potential therapeutic strategy for patients with malignant cancer tumors burdened by a cancer stem cell population.

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Chapter II

Indole-3-Carbinol regulation of the tumor suppressor p14ARF in breast cancer stem cells

Abstract

Indole-3-Carbinol is a naturally occurring compound in *Brassica* vegetables such as broccoli and brussel sprouts that can induce apoptosis in breast cancer stem cells, accompanied by selective inhibition of Akt1, an potent kinase that activates downstream oncogenes and prohibits activation of p53, the tumor suppressor. A series of coimmunoprecipitation experiments in a previously established breast cancer stem cell line, 10AT-Her2 cells, elucidate the indole-regulated changes in protein-protein interactions of the p53 axis, beginning with the direct target protein of I3C, a human neutrophil elastase. Analysis of these protein-protein complexes highlighted I3C's ability to promote anti-proliferative relationships in which tumor suppressors such as p14ARF were able to sequester the p53 E3 ligase, MDM2. Isolation of p14ARF from a stem/ progenitor stem cell marker, nucleostemin, which normally resides in the nucleolus, further revealed p14ARF's stabilization of MDM2. Furthermore, chromatin immunoprecipitation experiments revealed decreased binding of oncogenic transcription factors, nuclear factor κB and MYC DNA binding to its respective promoter sequences. In indole-treated cells, MYC and nuclear factor κB decrease in a time dependent manner transcriptionally and at the protein level, thereby reducing the presence of nucleophosmin, a nucleolar inhibitor of p14ARF. Our results demonstrate that I3C's transcriptional effects activate p14ARF, a tumor suppressor, to actively engage with activated MDM2, thereby freeing p53 to regulate apoptosis.

Introduction

ARF is a basic protein product of the INK4A locus, comprised of only 132 amino acids, and known to localize into the nucleus where it promotes its tumor suppressive functions as well as is stored in the nucleolus until activation [1-4]. Its basic nature lends itself to have many potential binding partners and as a result, is a critical component of many signaling pathways. For example, it has been implicated in sequestering mouse double minute 2 (MDM2) in the nucleus, resulting in decreased stabilization of p53 and inactivation of apoptosis in cancerous tumor cells [2]. As a critical component of tumor surveillance, ARF accumulates in response to oncogenic and genotoxic stresses and activates DNA damage pathways to halt cell-cycle division and/or eliminate cells that have sustained irreparable damage. Mutations in ARF including homozygous deletion, promoter methylation, and loss of heterozygosity, show that it is a critical component of tumor surveillance in cancers, such as lung, oral, prostate, gastric, colon, and breast. ARF-null mice have been shown to spontaneous develop tumors early in life with high proliferation rates [1, 5, 6]. Also ARF inactivity in the nucleolus is promoted by its sequestration by the nucleolar protein, nucleophosmin (NPM), which prevents ARF's interaction with MDM2 in the nucleoplasm. Little is known of the functions of p14ARF in breast cancer stem cells, but due to its structure and localization could represent a therapeutic target for cancer treatment due to its critical contribution to tumor suppressive functions [7, 8].

Previous research has identified a natural phytochemical, indole-3-carbinol, (I3C), which directly targets breast cancer stem cells by binding and inhibiting a serine protease, elastase, and ablates its enzymatic activity in the MCF-10AT-Her2 cell line [9-11]. When expressing exogenous HER2 into human preneoplastic mammary epithelial cells, the parent MCF-10AT cell line, populations are enriched with stem/progenitor cell-like character. The 10AT-Her2 cell line population expresses high levels of stem cell markers such as ALDH1, nucleostemin [12], and CD44+/CD24-; possesses high tumorsphere forming efficiency; and produces tumor xenografts *in vivo* in mice at significantly low numbers—representing an ideal model system for studying the proliferation of breast cancer via cancer stem cells [13]. These stem cell markers have been highly implicated in identifying and isolating cancer stem cells as they are associated with self-renewal properties. Furthermore, use of *in vitro* suspension assays, which plate cells in limiting dilution assays to assess for tumorsphere formation have been critical in understanding tumor-initiating capabilities [10, 14-16]. They are also highly sensitive to I3C, which is a natural plant compound, derived by hydrolysis from glycobrassicin produced in cruciferous vegetables of the *Brassica* genus such as broccoli, cabbage, and brussel sprouts. It has potent proapoptotic properties and has exhibited a wide range of anti-tumor effects in liver, colon, lung, melanomas, cervical, endometrial, prostate, and breast cancers. I3C has direct target proteins in these systems, such as elastase, which triggers distinct transcriptional and cellular signaling pathways, particularly p53-dependent apoptosis [9, 17-19]. I3C's inhibition of elastase in the breast cancer stem cell line is critical in identifying future natural and efficacious therapeutics that ablate breast cancer stem cells amongst a tumor population [10]. Cancer stem cells are notoriously resistant to conventional therapies, such as chemotherapy or radiotherapy and no effective therapeutic strategy has yet been identified. Recent studies have shown that chemo- and radio-resistance of cancer

stem cells in solid tumors explicates patient relapse and increased incidence of metastasis in many cancers [14, 15, 20, 21]. As a result, efficacious therapies are needed in targeting this small elusive population of cells that prevent the ablation of the entire breast tumor after various rounds of therapeutic treatments.

We demonstrate that I3C activates a p53-dependent apoptosis in the 10AT-Her2 breast cancer stem cell population by promoting interactions of the tumor suppressor protein p14ARF and its oncogenic substrate, MDM2 [3, 22, 23]. As an alternate reading frame protein product of the CDKN2A locus, p14ARF is responsible for inhibition of abnormal cell growth by blocking the MDM2 oncogene pathway. Inactivation via hypermethylation of the promoter region of p14ARF and other cell cycle modulators have been implicated in attenuation of p53 activity [4, 23-26]. I3C causes p14ARF's sequestration of the murine double mutant 2 (MDM2) protein into the nucleus along with its binding partner, nucleostemin, thereby allowing the p53 tumor suppressor protein to escape from the MDM2 inhibition of apoptotic activity. Furthermore, I3C's stabilization of p14ARF by depleting p14ARF's negative regulator nucleophosmin (NPM) further promotes p53 apoptotic activity. Also known as B23, this phosphoprotein is a molecular chaperone that aggregates and sequesters proteins like p14ARF into the nucleolus, preventing MDM2-p14ARF apoptotic complexes [7, 27-31]. I3C's depletion of the nucleophosmin protein is attributed to inhibited transcriptional activity upstream transcription factors, myc, and protein complexes, NFkB [27, 32-34]. Therefore, I3C's promotion of p14ARF demonstrates its critical ability to target and ablate MCF-10AT-Her2 cells, which represent an opportune model system to study nucleostemin-p14ARF-MDM2-p53 cellular pathways in breast cancer stem cells. Our study has identified novel pathways into how the cancer stem/progenitor cell-associated marker, nucleostemin, and a p53-stabilizing tumor suppressor, p14ARF, are directly involved in an anti-proliferative and pro-apoptosis cellular cascades disrupted by I3C.

Materials and Methods

Expression plasmids and transfects

Human cytomegalovirus CMV-HER2 and CMV-Akt1 expression plasmids were kind gifts from Dr Leonard Bjeldanes (Department of Nutritional Sciences and Toxicology, University of California at Berkeley). Transfection of expression vectors was performed using Superfect transfection reagent from QIAGEN (Germantown, MD, USA) per the manufacturer's recommended protocol.

Transfection of 10AT-Her2 cell line

Preneoplastic MCF-10AT human mammary epithelial cells (obtained from the Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA) were stably transfected with either the human pCMV-HER2 expression vector, forming 10AT-Her2 cells. Cells were stably selected for 2 months with G418 sulfate, purchased from Cellgro (Manassas, VA, USA). 10AT-Her2 and 10AT-Neo cell lines were cultured in DMEM/F-12, 10% fetal bovine serum, 50 U/ml penicillin/streptomycin (Lonza, Allendale, NJ, USA), 0.02 μg/ml epidermal growth factor (Promega, Madison, WI, USA), 0.05 μ g/ml hydrocortisone, 10 μg/ml insulin and 0.1 μg/ml cholera toxin (Sigma-Aldrich, St Louis, MO, USA).

The 10AT-Her2 cell line, the parental MCF-10AT cells and the SKBR3 breast cancer cell line were validated by short tandem repeat (STR) DNA fingerprinting using DDC Medical Cell Line Authentication Lab Services (Fairfield, OH, USA). The STR profiles were compared to known ATCC fingerprints and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808. The STR profile of the 10AT-Her2 cell line matched that of its parental MCF-10AT cell line, whereas the SKBR3 cells matched the known DNA fingerprints to that of itself. Therefore, the cell population of the 10AT-Her2 cell line, which displays significant stemness-like character, is not contaminated with any other cell line.

Cell Proliferation Assay

The cellular response to I3C was examined using the Cell-Counting Kit-8 (Dojindo Molecular Technologies, Inc, Santa Clara, CA, USA) based on the manufacturer's recommended protocol [53). Cells were plated at a density of 5,000 to 7,000 cells per well in 24-well plates containing 500 μl of culture medium. After the indicated treatment and incubation times at 37°C, 40 μl CCK-8 reagent was then added to each well, which were then incubated for 2 hours before reading at a wavelength of 450 nm.

Flow Cytometry

Cells were plated onto six-well tissue culture plates at 70% confluency and treated as indicated in triplicate with the medium changed every 24 hours. Following treatment, the cells were washed once with phosphate buffered saline (Lonza), also known as PBS, and subsequently harvested with 1 mL of the reagent. Cells were lysed in 300μL of DNA staining solution (0.5mg/mL propidium iodide, 0.1%% sodium citrate, and 0.05% Triton X-

100). Nuclear fluorescence of wavelength more than 585nm was measured on a Beckman-Coulter EPICS XL instrument with laser output adjusted to deliver 15 megawatts at 488nm. For each sample 10,000 nuclei were analyzed and the percentage of cells in G1, S, G2/M phase of the cell cycle was determined by analysis with Multicycle provided by Phoenix Flow Systems in the Cancer Research Laboratory, Flow Cycle Cytometry Facility of the University of California, Berkeley.

Western Blot

After indicated treatments, all cells were harvested in ice-cold PBS and lysed using a radioimmunoprecipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% NoNidet-p40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 g/ml phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, 5 g/ml leupeptin, 0.1 g/ml NaF, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate and 0.1 mM β-glycerol phosphate). After centrifugation, total protein in the lysate was estimated using the protein quantification kit (Bio-Rad, Hercules, CA, USA). Cell lysates were electrophoretically fractionated using SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk for an hour at room temperature and incubated with primary antibodies overnight at 4°C. Immunoreactive proteins were detected after a 1-hour incubation with horseradish peroxidase conjugated secondary antibodies. Blots were then treated with enhanced chemiluminescence reagents (Estman Kodak, Rochester, NY, USA) for visualization on film.

Primary antibodies, nucleostemin, (sc-67012), CD44 (sc-65412), CD-24 (sc-70598), ALDH1 (sc-22588), MDM2 (sc-5304), p53 (sc-6243), and lamin (sc-7293), MYC (sc-764), Nucleophosmin (sc-47725), and Akt1 (sc-5298) were purchased from Santa Cruz Biotechnology. Actin (AANO1) was obtained from Cytoskeleton (Denver, CO, USA). HER2 (2165), was obtained from Cell Signaling Technology, Inc (Danvers, MA, USA). All antibodies were diluted 1:1000 in TBST (0.1 M Tris, 150 mM NaCl, 0.05% Tween 20).

Reverse Transcriptase-Polymerase Chain Reactions (RT-PCR)

Cells were treated with mentioned compounds and harvested in 1 ml of Tri-reagent (Sigma, St.Louis, MO). RNA was extracted and 1 mg was subjected to RT using random hexamers, GAPDH Forward 5'-TGA AGG TCG GAG TCA ACG GAT TTG-3', GAPDH Reverse: 5'- CAT GTG GGC CAT GAG GTC CAC CAC-3'. PCR products were analyzed on 1.2% agarose gel along with 1-kb Plus DNA ladder from Invitrogen (Carlsbad, CA) and the products were visualized with GelRed from Biotium (Hayward, CA). qPCR reactions were subjected to an initial step of 10 min at 95°C to activate the AmpliTaq Gold, followed by 40 cycles consisting of 15s at 95°C, 45s at 57°C, 15s at 70°C. Fluorescence was measured at the end of each elongation step. Data was analyzed using the StepOnePlus RealTime PCR system (Applied Biosystems, Carlsbad California), and a threshold cycle value C t was calculated from the linear phase of each PCR sample.

Co-immunoprecipitation

After the indicated treatments, pre-cleared samples were then incubated with 50 μg of specific antibodies as indicated overnight at 4°C. Immunoprecipitated protein was eluted from beads by addition of gel-loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM ethylenediaminetetraacetic acid (EDTA), 0.02 mM bromophenol blue) and heating the sample at 100°C for 5 min. Samples were analyzed by Western blot. The immunoprecipitation procedure was nearly 100% efficient because the resulting supernates did not contain any of the proteins of interest. If any of the intended immunoprecipitated protein was detected in a given supernatant fraction, that experiment was not used for the study. Also, the "No IP" control in each experiment represents samples that were subjected to the usual immunoprecipitation procedure except that the beads were added without the antibody.

Chromatin Immunoprecipitation

10AT-Her2 cells were grown to ~60% confluency and treated for 48 hr with 200 uM I3C or DMSO vehicle control. DNA was cross-linked to proteins through the addition of 1% formaldehyde and incubated at room temperature for 5 minutes. The fixation reaction was quenched with glycine for a final concentration of 125 mM and incubated for 5 minutes at room temperature. Cells were then harvested and lysted with chromatin immunoprecipitations lysis buffer (50 mM NaCl, 1% Triton X-100, and 0.1% sodium deoxycholate) with protease inhibitors (50 g/mL phenylmethylsulfonyl fluoride, 10 g/mL aprotonin, 5 g/mL leupeptin, 0.1 g/mL NaF, 1mM dithiothreitol. 0.1 mM sodium orthovanadate, and 0.1 mM glycerol phosphate). Cells were sonicated and supernatants were standardized using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). 1 mg of protein was used for each IP. Protein-DNA complexses were incubated overnight with 15 μL antibody recognizing Ets-1 from Santa Cruz Biotechnology. Complexes were precipitated by adding Sepharose-G beads from GE Healthcare (Piscataway, NJ) and incubating at 4°C for 1 hour. Beads were then washed 2x with ChIP Lysis Buffer, followed by 2x with ChIP wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate and 1mM ethylendiaminetetraacetic acid). Samples were eluted with ChIP elution buffer (50 mM Tris pH 8.0, 1% sodium dodecyl sulfate, and 10 mM ethylenediaminetetraacetic acid) at 65°C for 18 hourse. PCR was carried out in a total of 50 μL. Primers for ChIP experiments were as follows: MYC-forward 5' GTAGAAGAGACGTGGAAG 3', MYC-reverse 5'TCGCCGCACGTATATATG 3' NF-κB-forward: 5'-TCTTTATCTCCCAGCCCTTTGG-3; reverse: 5'-AGTGGAAGGAGTGGCAAATAGGGT-3. Products were then visualized on a 1.6% agarose gel buffered with Tris borate-EDTA, and the products were then visualized with Ethidium Bromide.

Results

I3C stimulates interactions of the tumor suppressor p14ARF with the apoptotic regulator MDM2 and disrupts p14ARF interactions with nucleostemin

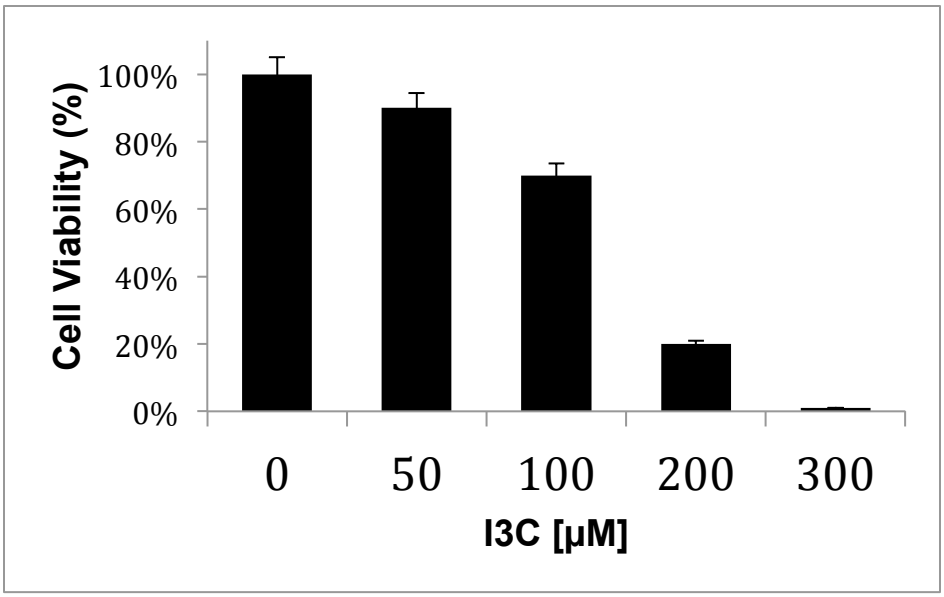
To define the proliferative context associated with the I3C regulation of p14ARF protein-protein interactions, the anti-proliferative effects of this indolecarbinol compound were initially analyzed in 10AT-Her2 breast cancer cells treated for 48 hours with a concentration range of I3C. Cells were initially cultured in adherent monolayers and total cell number was determined using cell-counting kit-8 assays. As shown in Figure 1A, similar to other human cancer cell types, 200 μM I3C induced a near maximal decrease in total cell number with a half maximal response calculated to be approximately 125 μM I3C. Flow cytometry (Fig. 1B) of nuclear DNA stained with propidium iodide revealed that 48 hours treatment with 200 μM I3C induced a significant increase in 10AT-Her cells with a sub-G1DNA content compared to vehicle-controlled cells, which is indicative of the activation of apoptosis. Therefore, 200 μM I3C was used through out this study.

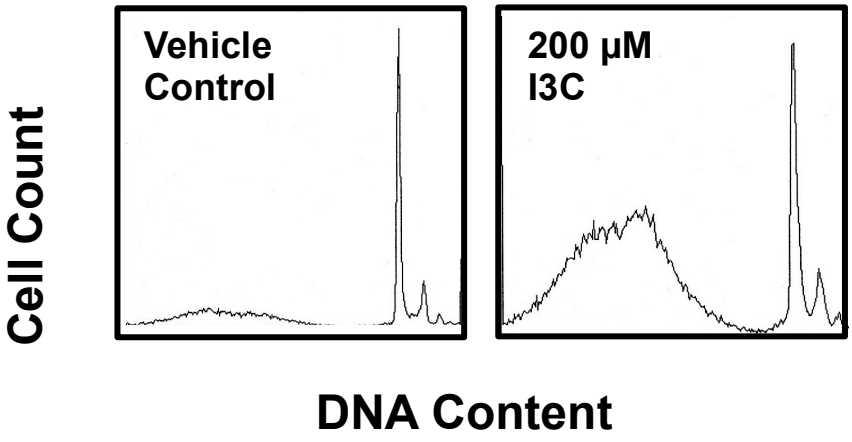
We previously demonstrated that I3C treatment disrupted the interaction of MDM2 with the p53 tumor suppressor that frees p53 to trigger its apoptotic response in 10AT-Her2 breast cancer cells as well as stimulate MDM2 interactions with the stem cell/progenitor marker protein nucleostemin ([10]). In several other cell systems, p14ARF tumor suppressor was shown to sequester activated and phosphorylated MDM2 away from p53, although little is known about p14ARF protein interactions nucleostemin in the apoptotic response. Co-immunoprecipitaitons were carried out to assess the potential I3C regulation of p14ARF protein-protein interactions in the context of the apoptotic response. 10AT-Her2 breast cancer cells were treated with or without 200 μM I3C for 48 hours and then western blots of immunoadsorbed p14ARF probed for either MDM2 or nucleostemin. As shown in Figure 1C, under conditions in which there are no changes in the total protein levels of MDM2 and nucleostemin, I3C treatment significantly increased the interaction of p14ARF with MDM2 and concomitantly decreased the interaction of p14ARF with nucleostemin. Complementary co-immunoprecipitations from cell extracts isolated from I3C treated and untreated cells showed that immunoprecipitated MDM2 pulls down significantly more p14ARF protein after I3C treatment (Fig 1D), whereas, immunoprecipitated nucleostemin pulls down significantly less p14ARF after I3C treatment (Fig 1E). I3C had no effect on the total level of p14ARF protein. Taken together, these results demonstrate that in the context of a p53-dependent apoptotic response that results from release of p53 from a p53-MDM2 protein complex [3], I3C treatment induced p14ARF-MDM2 protein-protein interactions and disrupted p14ARF-nucleostemin protein-protein interactions.

Figure 15

I3C Effects on 10AT-Her2 cells.

I3C inhibits the proliferation of cultured MCF-10AT Her2 human breast cancer cells. Cultured cells were treated for 48 hours at indicated doses and quantified by cell counting kit. 10AT-Her2 cells were treated with 200 μM I3C and the cell population contents quantified by flow cytometry.


Cell Viability (%)
100%
80%
60%
40%
20%
0%
0
50
100
200
300
I3C [μM]

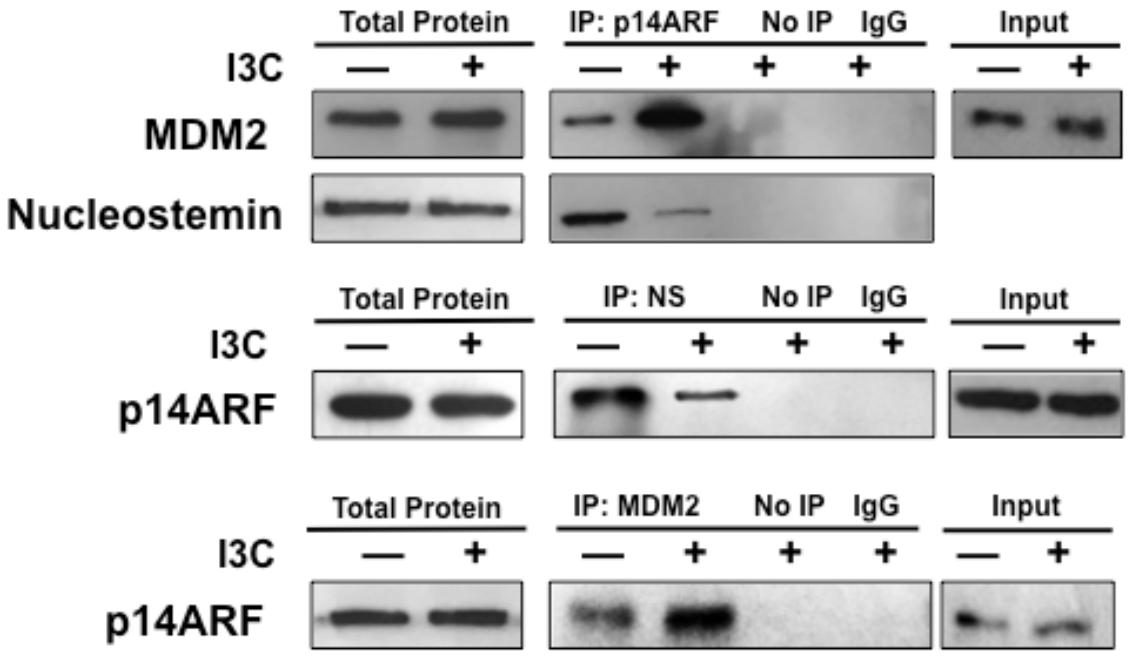

Cell Count
Vehicle Control
200 μM I3C
DNA Content

We previously demonstrated that I3C treatment disrupted the interaction of MDM2 with the p53 tumor suppressor that frees p53 to trigger its apoptotic response in 10AT-Her2 breast cancer cells as well as stimulate MDM2 interactions with the stem cell/progenitor marker protein nucleostemin [10]. In several other cell systems, p14ARF tumor suppressor was shown to sequester activated and phosphorylated MDM2 away from p53, although little is known about p14ARF protein interactions nucleostemin in the apoptotic response. Co-immunoprecipitaitons were carried out to assess the potential I3C regulation of p14ARF protein-protein interactions in the context of the apoptotic response. 10AT-Her2 breast cancer cells were treated with or without 200 μM I3C for 48 hours and then western blots of immunoadsorbed p14ARF probed for either MDM2 or nucleostemin. As shown in Figure 1C, under conditions in which there are no changes in the total protein levels of MDM2 and nucleostemin, I3C treatment significantly increased the interaction of p14ARF with MDM2 and concomitantly decreased the interaction of p14ARF with nucleostemin. Complementary co-immunoprecipitations from cell extracts isolated from I3C treated and untreated cells showed that immunoprecipitated MDM2 pulls down significantly more p14ARF protein after I3C treatment (Fig 1D), whereas, immunoprecipitated nucleostemin pulls down significantly less p14ARF after I3C treatment (Fig 1E). I3C had no effect on the total level of p14ARF protein. Taken together, these results demonstrate that in the context of a p53-dependent apoptotic response that results from release of p53 from a p53-MDM2 protein complex [4], I3C treatment induced p14ARF-MDM2 protein-protein interactions and disrupted p14ARF-nucleostemin protein-protein interactions.

Figure 16

I3C promotes p14ARF interaction with MDM2 and diminishes nucleostemin-MDM2 binding.

10AT-Her2 cells were treated with or without 200 μM I3C for 48 hours. Total cell extracts were immunoprecipitated with p14ARF, Nucleostemin, MDM2 antibodies respectively. As a control, non-immune antibodies (IgG) and samples not immunoprecipitated (No IP) were used. All extracts were electrophoretically fractionated and probed by western blot analysis. Antibodies specific to Nucleostemin, MDM2, and p14ARF were used.


Total Protein
IP: p14ARF
No IP
IgG
Input
I3C
MDM2
Nucleostemin
Total Protein
IP: NS
No IP
IgG
Input
I3C
p14ARF
Total Protein
IP: MDM2
No IP
IgG
Input
I3C
p14ARF

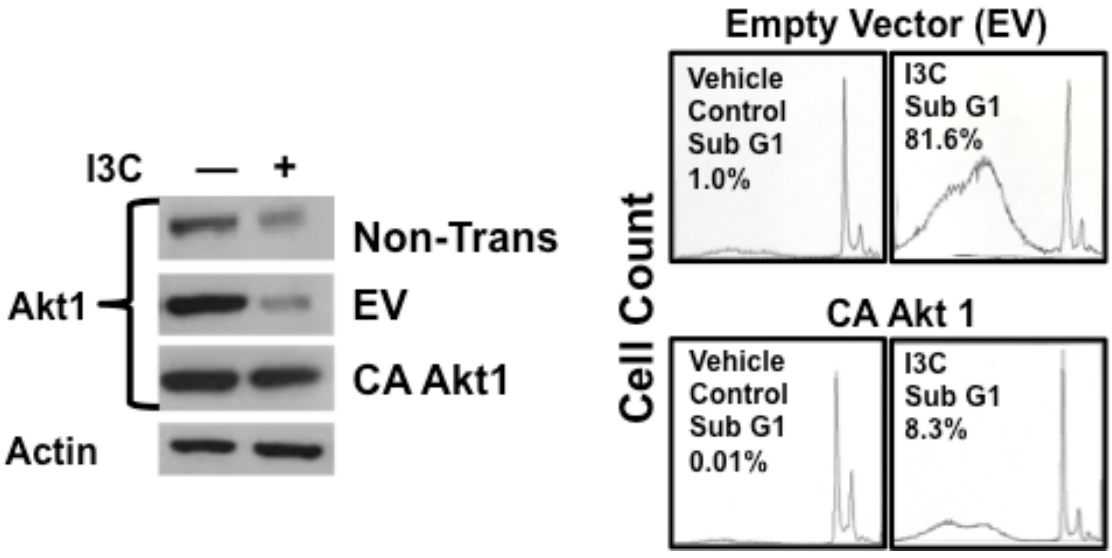
Effects of expressing a constitutively active Akt1 on the I3C apoptotic response and p14ARF and nucleostemin associated protein-protein interactions

It is well established that MDM2 function and binding with p53 is facilitated by its Akt1-mediated phosphorylation, which is mechanistically connected to the control of cellular apoptosis. Therefore, the role of Akt1 in the I3C apoptotic response and regulation of protein-protein interactions was functionally evaluated by expression stable transfection of 10AT-Her2 cells with either a constitutive active Akt1 expression vector (CA-Akt1) or an empty vector control (EV). Cells were treated for 48 hours with or without 200 μM I3C and western blots were used to evaluate the effectiveness of the transfections by probing total cell extracts for Akt1 protein using Akt-1 specific primary antibodies. As shown in Figure 2A, in non-transfected (Non-Trans) and empty vector transfected cells, treatment with I3C down regulated Akt1 protein levels, whereas in cells transfected with CA-Akt1, I3C had no effect on the levels of Akt1 immunoreactive protein in the cells (Fig 2A). Flow cytometry analysis of sub-G1 DNA content, which is indicated of apoptosis, revealed that expression of constitutively active Akt1 in 10AT-Her2 cells prevented I3C induced apoptosis, whereas, in empty vector transfected cells I3C efficiently induced an apoptotic response (Fig 2B).

Figure 17

I3C induced apoptotic response is mediated by Akt1.

10AT-Her2 cells transfected with the control vector, "Empty Vector," or with constitutively active, "CA-Akt1" were treated with or without 200 μM I3C for 48 hours. Expression of Akt1 was confirmed by western blot. DNA content was assessed by flow cytometry.

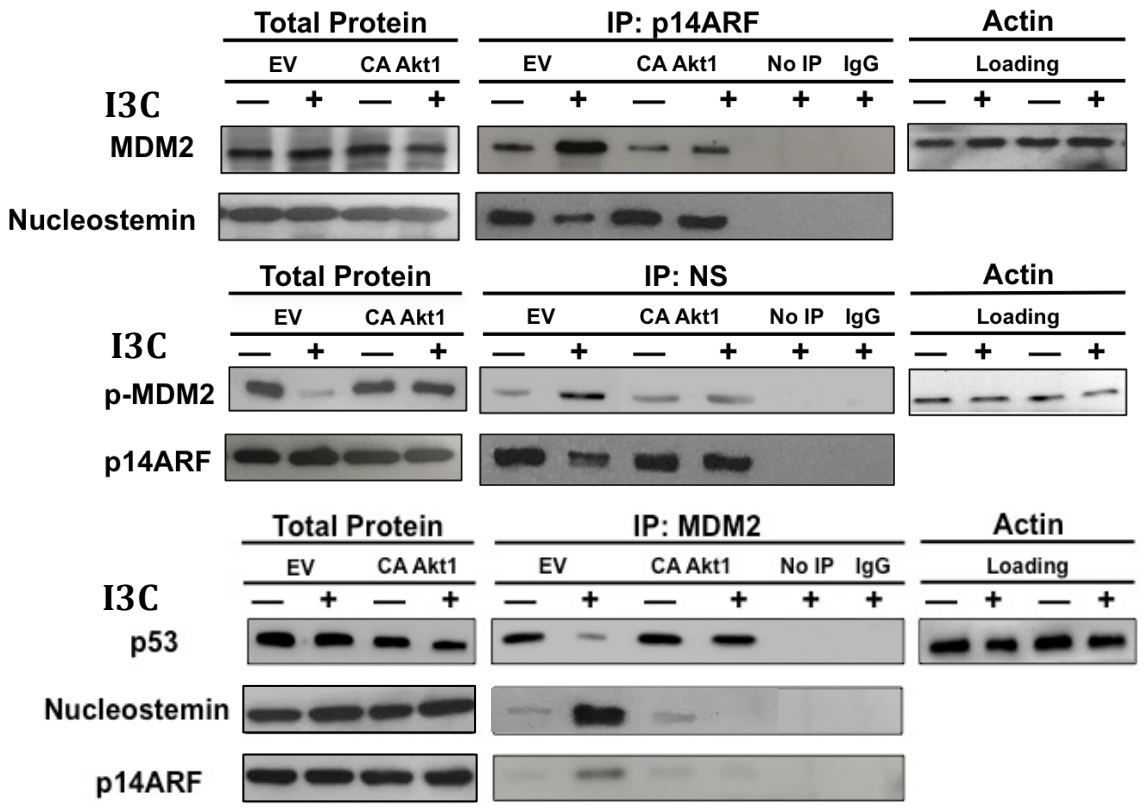

I3C
—
+
Akt1
Non-Trans
EV
CA Akt1
Actin
Empty Vector (EV)
Vehicle
Control
Sub G1
1.0%
I3C
Sub G1
81.6%
CA Akt 1
Vehicle
Control
Sub G1
0.01%
I3C
Sub G1
8.3%
Cell Count

Because of the efficient rescue from the I3C-mediated apoptotic response, we assessed the ability of expressed constitutively active Akt1 to reverse or attenuate I3C regulated protein-protein interactions involving p14ARF, nucleostemin, MDM2 and p53. The co-immunoprecipitations were carried out from cells transfected with either the constitutively active Akt1 expression vector (CA-Akt1) or the empty vector control (EV) after treatment for 48 hours with or without 200 μM I3C. Expression of constitutively active Akt-1 completely reversed the I3C stimulated protein-protein interactions involving p14ARF-MDM2 and nucleostemin-MDM2 when either p14ARF or nucleostemin were immunopreciptated (Fig 2C and 2D) or when MDM2 was initially immunoprecipitated (Fig 2E). Furthermore, expression of constitutively active Akt-1 prevented the I3C disruption of MDM2-p53 interactions and of p14ARF-nucleostemin interactions (Fig 2C, 2D and 2E), and also reversed the I3C induced loss of total phospho-MDM2 (Fig 2D). Expression of the empty vector (EV) had no effect on the I3C regulation of protein-protein interactions. Taken together, the I3C loss of Akt-1 protein levels, and its phosphorylation of its cellular targets, including MDM2, plays an important role in the control of protein-protein interactions associated with the p53 apoptotic response.

Figure 18

Constitutively active Akt1 alters I3C regulation on the molecular interactions between p14ARF, Nucleostemin, and MDM2.

10AT-Her2 cells were transfected with either constitutively active Akt1 or an empty vector control and then treated with or without 200 μM I3C for 48 hours. Total cell extracts were immunoprecipitated with p14ARF, nucleostemin, or MDM2 antibodies, electrophoretically fractionated and western blots probed with MDM2, nucleostemin, p14ARF or actin specific antibodies as indicated. Negative controls were immunoprecipitations carried out with non-immune antibodies (IgG) or samples that were not immunoprecipitated (No IP).


Total Protein
IP: p14ARF
Actin
EV
CA Akt1
EV
CA Akt1
No IP
IgG
Loading
I3C
MDM2
Nucleostemin
Total Protein
IP: NS
Actin
EV
CA Akt1
EV
CA Akt1
No IP
IgG
Loading
I3C
p-MDM2
p14ARF
Total Protein
IP: MDM2
Actin
EV
CA Akt1
EV
CA Akt1
No IP
IgG
Loading
I3C
p53
Nucleostemin
p14ARF

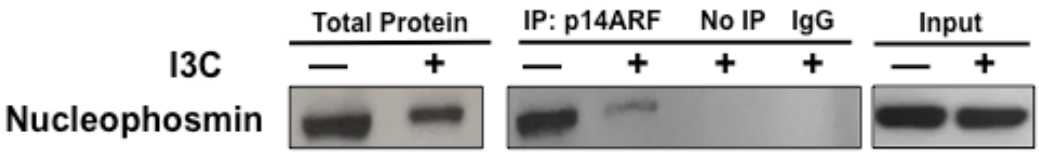
I3C down regulation of nucleophosmin gene expression accounts for the loss of the p14ARF-nucleophosmin protein complex

To further explore the mechanism underlying the I3C control of protein-protein interactions and p14ARF function, 10AT-Her2 breast cancer cells treated with or without I3C and coimmunoprecipitated p14ARF protein was probed for p14ARF binding partners. One important regulator of p14ARF is nucleophosmin, a nucleolar phosphoprotein expressed at high levels in certain highly mutated tumor types [6] that can inhibit p14ARF's tumor suppressor properties and can prevent apoptosis by sequestering p14ARF in the nucleolus [2]. Co-immunoprecipitation analysis from I3C treated and untreated cells showed that I3C strongly triggered a significant loss of p14ARF bound with nucleophosmin that was accounted for the down regulation of total nucleophosmin protein levels (Fig 3A). We propose that the loss of total nucleophosmin protein allows unbound p14ARF protein to bind to and sequester MDM2 in the nucleolus and thereby allow the p53 apoptotic response.

Figure 19

I3C releases p14ARF from nucleophosmin in cancer stem cells.

10AT-Her2 cells were treated with or without 200 μM I3C for 48 hours. Total cell extracts were immunoprecipitated with p14ARF antibodies. As a control, non-immune antibodies (IgG) and samples not immunoprecipitated (No IP) were used. All extracts were electrophoretically fractionated and probed by western blot analysis. Antibodies specific to nucleophosmin were used.

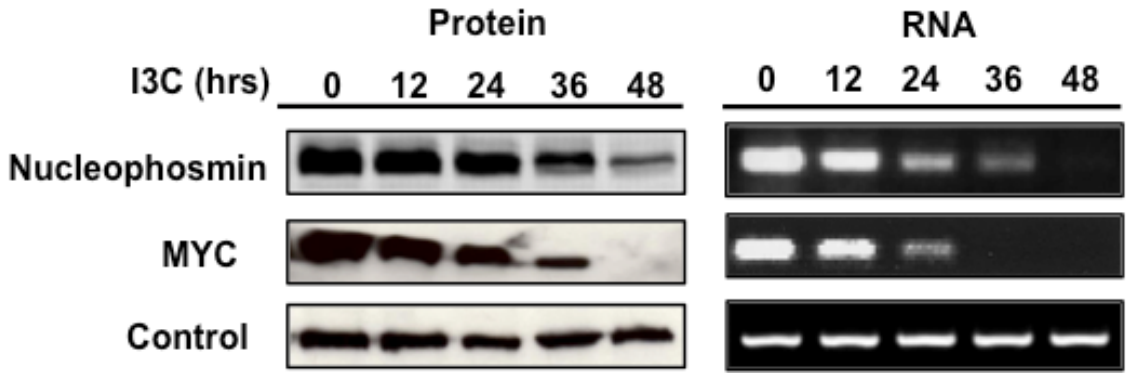

Total Protein
IP: p14ARF
No IP
IgG
Input
I3C
— + — + + + — +
Nucleophosmin

To assess the kinetics of I3C down regulation of nucleophosmin gene expression, 10AT-Her2 cells were treated with I3C over a 48 hour time course and nucleophosmin protein levels were determined by western blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) was used to monitor the levels of nucleophosmin transcripts. As shown in Figure 20, I3C strongly down regulated nucleophosmin protein levels by 48 hours and its corresponding transcript levels were down regulated within 24 hours of I3C treatment and completely ablated by 48 hours. Under these conditions, the levels of actin and GAPDH transcripts remained constant. These results suggest that the loss of nucleophosmin transcripts accounts for the loss of nucleophosmin protein. The expression of myc protein and transcript levels, a known transcriptional regulator of nucleophosmin expression, was examined over the same time course. As also shown in Figure 20, I3C more rapidly down regulated Myc gene expression compared to the observed loss of nucleophosmin expression. Myc Protein expression is almost completely abolished by 48 hours and transcript levels are diminished completely by 36 hours. These kinetics suggest that the I3C down regulation of Myc may be causal for the down regulation of nucleophosmin expression.

Figure 20

I3C downregulates protein and transcriptional levels of nucleophosmin and a transcription factor, MYC.

10AT-Her2 cells were treated with or without 200 μM I3C for the indicated durations. Total cell extracts were electrophoretically fractionated and western bltos probed for MYC and Nucleophosmin. Actin was used as a gel loading control. To assess I3C mediated regulation at the transcript level, total RNA was isolated. The transcript levels of Nucleophosmin, MYC, and GAPDH were determined by reverse transcriptase-polymerase chain reaction using specific primers and the products fractionated by electrophoresis on a 1.2% agarose gel and visualized on an ultraviolet transilluminator. GAPDH served as a gel loading control.


Protein
RNA
I3C (hrs)
0
12
24
36
48
0
12
24
36
48
Nucleophosmin
MYC
Control

I3C treatment disrupts Myc interactions with the nucleophosmin gene promoter and NFkB interactions with the Myc gene promoter.

Nucleophosmin contains a consensus Myc DNA binding site in its promoter at -730 bp upstream of the transcriptional start site, suggesting that the I3C-mediated loss of Myc protein levels should result in the loss of Myc interactions with the nucleophosmin promoter. Chromatin immunoprecipitations (ChIP) assay was used to directly test this possibility. 10AT-Her2 cells were treated with or without I3C for 48 hours, and genomic fragments that were cross-linked to protein were immunoprecipitated with anti-Myc antibodies or with an IgG control antibody. As shown in Figure 21, PCR analysis using primers specific to the Myc consensus binding site in the nucleophosmin promoter revealed that I3C significantly down regulated endogenous Myc interactions with this promoter. One percent input was used as a loading control.

The ability of constitutively active Akt to prevent the I3C regulated p14ARF protein-protein interactions (see Figure 17), suggests that a down stream component of Akt signaling may be linked to the I3C down regulation of Myc expression. One such transcriptional regulator is NFkB because the Akt mediated phosphorylation of IkB and of NFkB functions to maintain nuclear localized NFkB in an active form. A functional NFkB binding site is located at -1096 bp upstream of the Myc gene transcriptional start site. Therefore, a parallel ChIP assay was completed 10AT-Her2 cclls treated with or without I3C for 48 hours to assess endogenous interactions of NFkB with the Myc gene promoter. As shown in Figure 21, although I3C did not down regulate total NFkB protein levels, I3C strongly down regulated the ability of endogenous NFkB to interact with the Myc promoter, which we propose may mediate the I3C down regulation of Myc gene expression.

Figure 21

I3C ablates binding of MYC to the Nucleophosmin promoter and NFkB binding to the MYC promoter.

10AT-Her2 cells were treated with or without 200 μM I3C and subjected to western blot analysis and chromatin immunoprecipitation assay. Expression of MYC and NFkB were confirmed by anti-MYC and anti-NFkB antibodies through western blot. Genomic fragments that were cross-linked to protein were immunoprecipiated with anti-MYC or anti-NFkB antibodies, or with a control IgG antibody.

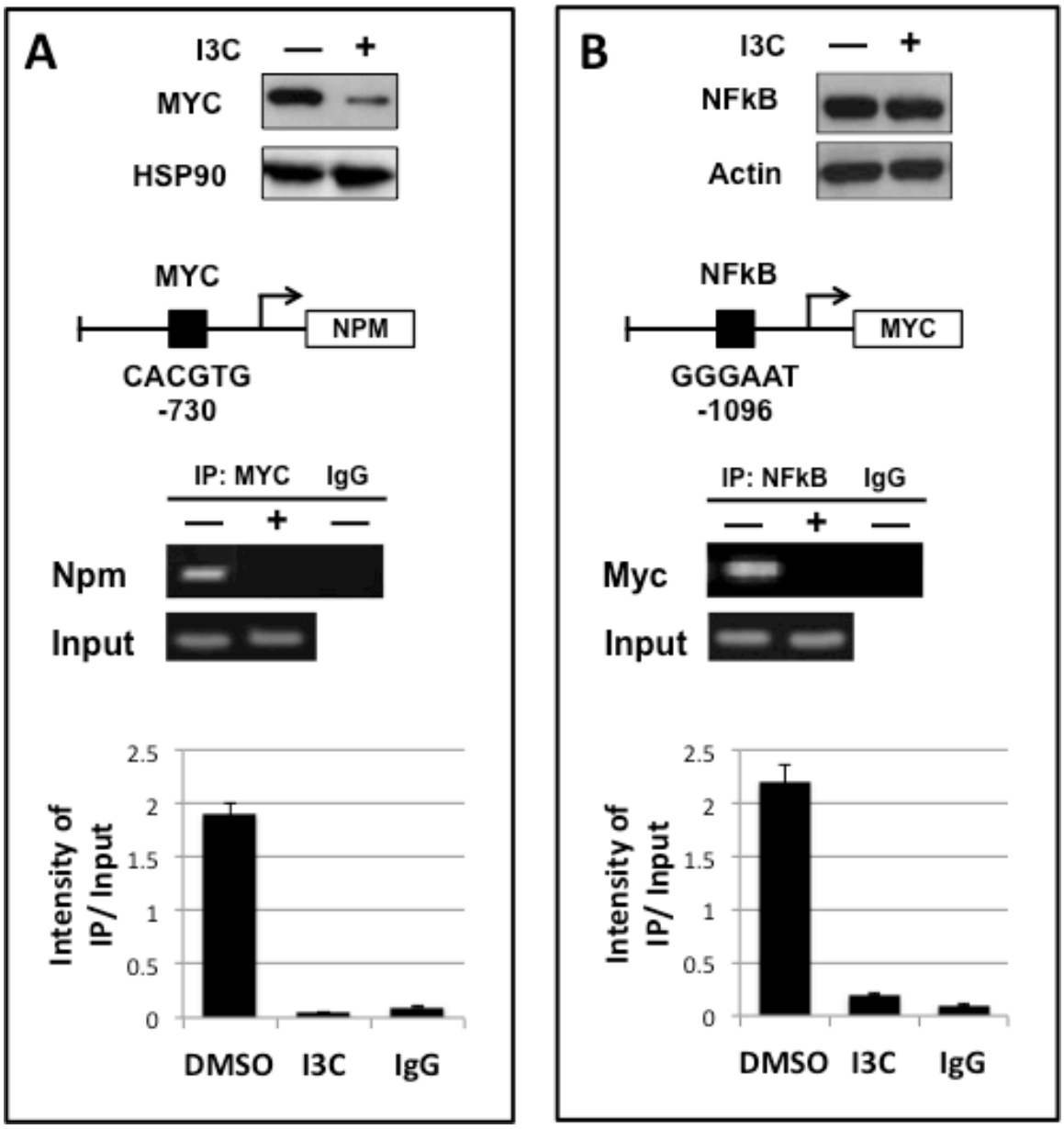
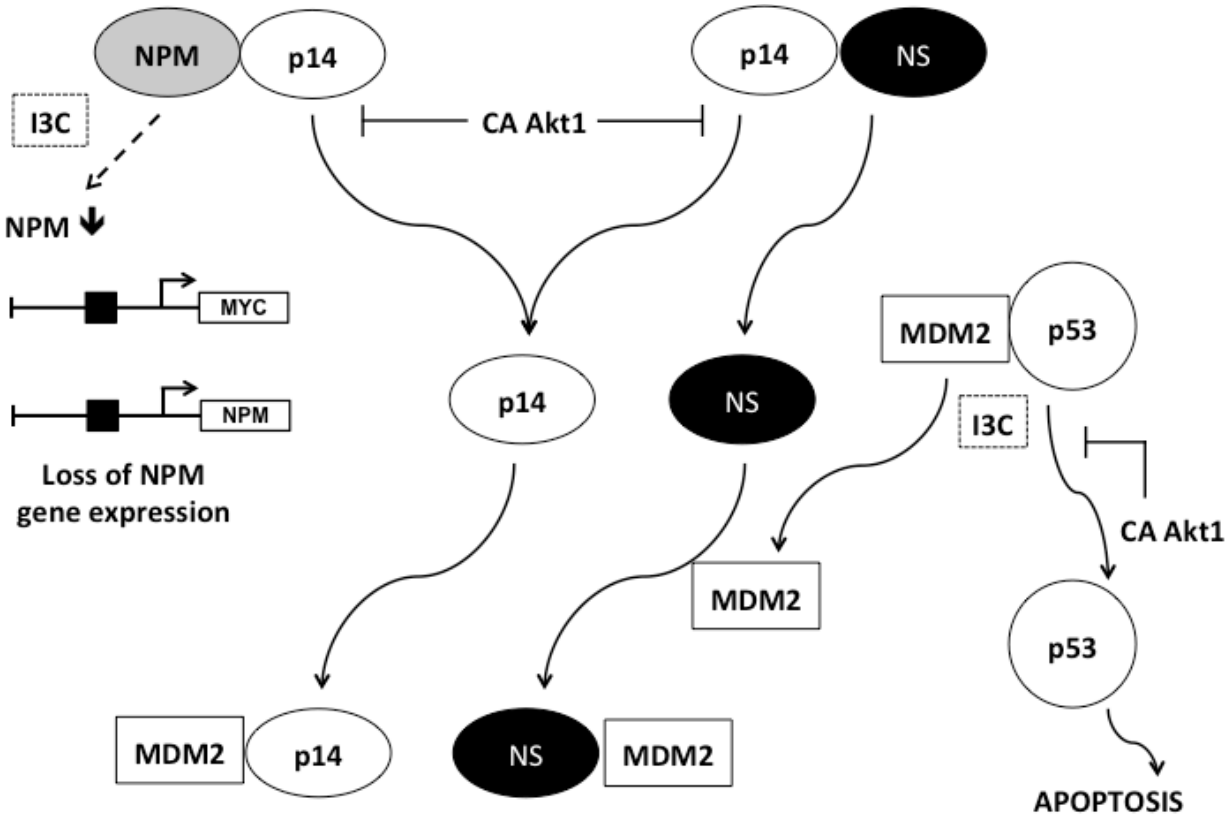

A
I3C — +
MYC
HSP90
MYC
NPM
CACGTG
-730
IP: MYC IgG
— + —
Npm
Input
Intensity of IP/ Input
2.5
2
1.5
1
0.5
0
DMSO I3C IgG
B
I3C — +
NFkB
Actin
NFkB
MYC
GGGAAT
-1096
IP: NFkB IgG
— + —
Myc
Input
Intensity of IP/ Input
2.5
2
1.5
1
0.5
0
DMSO I3C IgG

Figure 22

Summary of I3C-Mediated 14ARF regulation.


NPM
p14
I3C
CA Akt1
p14
NS
NPM
MYC
NPM
Loss of NPM
gene expression
p14
NS
MDM2
p53
I3C
CA Akt1
MDM2
p53
MDM2
p14
NS
MDM2
APOPTOSIS

Discussion

In human breast cancer cells, protein-protein interactions of the tumor suppressor p14ARF and its oncogenic substrate, MDM2, are a biologically significant relationship that activates p53-dependent apoptosis. In the p53 pathway, p14ARF maintains MDM2 localized in the nucleolus so that MDM2, its E3 ligase, cannot degrade p53, and thus promotes apoptosis in breast cancer stem cells. Previous studies in our lab have shown I3C mediated localization of MDM2 into the nucleolus in 10AT-Her2 cells to initiate a p53-dependent apoptosis. Furthermore, direct binding of indole-3-carbinol to its target protein, elastase, in breast cancer stem cells has illustrated indole-3-carbinols apoptotic regulation. I3C disrupts the interaction of elastase and CD40, preventing it from associating with a tumor necrosis factor family protein, called TRAF6. TRAF6 is known as an E3 ligase that stabilizes Akt1, and thus the impairment of Akt1's ubiquitination activates p53-dependent apoptosis [9, 10, 35]. Disruption of the p14ARF-MDM2-p53 pathways is commonly developed in many cancers, preventing the shuttling of p53 in an auto-regulatory feedback loop [22]. Our studies show that I3C inhibits MDM2-p53 interactions through increased p14ARF activation, which is promoted by ablation of protein levels of its negative regulator, nucleophosmin [28]. This suggests promising opportunities and many anti-cancer targets in patients with highly metastatic and proliferative breast cancer tumors.

Our study establishes that I3C inhibits cellular proliferation of breast cancer stem cells by regulating the protein-protein interactions of the tumor suppressor, p14ARF, and downstream transcriptional targets such as MYC and NFkB. These findings implicate the critical involvement of p14ARF signaling through its binding partners nucleophosmin and nucleostemin in attenuating cellular proliferation and inducing apoptosis in human breast cancer stem cells. The p14ARF tumor suppressor plays an important role in cancer by promoting the accumulation of the p53 tumor suppressor followed by the induction of p53-mediated cell growth arrest or cell death [24, 36-38]. Loss of either p53 or p14ARF contributes to the unrestrained growth of most cancers. Furthermore, p14ARF's regulation can be attributed specifically to its binding partner, nucleophosmin. Nucleophosmin functions to sequester p14ARF, a role that is inhibited by I3C's transcriptional regulation of nucleophosmin, as well as its transcriptional regulator, myc [39]. Based on western blot and PCR studies, I3C downregulation of nucleophosmin can be directly attributed to decreased protein and transcript levels of its transcription factor, myc as well as one of myc's own transcription factor, NFkB. NFkB is known to be a dimeric complex of transcription factors that transactivate the myc promoter in highly metastatic cancer cells. Reduced myc expression and growth arrest is accompanied by p53 activation. Furthermore, I3C was effective in inhibiting Akt activation and ablating protein expression of p14ARF's negative regulator, nucleophosmin. However, I3C did not alter protein levels of p53, nucleostemin, MDM2, and p14ARF, suggesting I3C's regulation can be on protein level as well as the protein-protein interactions. Studying the DNA binding regulatory role of I3C is a powerful approach to understanding the therapeutic value of I3C as a naturally occurring, cost-accessible plant molecule with many anti-cancer abilities.

Together with previous results from our laboratory, I3C shows to be promising therapeutic agent for ablation of breast cancer stem cells by stabilizing p53 through enhancement of

protein-protein interactions of p14ARF and MDM2, localization of MDM2 into the nucleolus, and downregulation of oncoproteins implicated in p14ARF deregulation and rampant cellular proliferation, such as MYC and NFkB [7, 27, 32, 33, 40, 41]. Overall, the identification of molecular targets in tumorigenic breast cancer cells has been important therapeutic identifications as most cancer stem cells are notoriously resistant to therapies and show poor prognosis for patients with high expression of stem cell prognosis factors, such as CD44, ALDH1, and nucleostemin. For example, tumors associated with high expression of the cell surface marker, CD44, and lack of CD24, present cells which have self-renewal capacity and ability to proliferate in suspension as mammospheres in nonadherent suspension media. Furthermore mammospheres from these breast cancer cells retain their tumor-initiating ability *in vivo* when isolated and injected into nude, athymic mice. Therefore, understand the molecular phenotype of 10AT-Her2 cells and its responsiveness to indole-3-carbinol is critical in developing more effective therapeutic strategies before the death of breast cancer patients from metastasis.[14, 21, 42] We observed that 10AT-Her2 cells are highly sensitive to the antitumor effects of I3C and triggers a molecular response that normally causes proliferation in cancer stem cells. Because 10AT-Her2 cells exhibit increased expression of nucleostemin and confers selective responsiveness to I3C, regulation of the p53's protein-protein interactions with MDM2, p14ARF, and Akt1 show consistent ablation of tumorigenic growth.

Breast cancer continues to be diagnosed in one in eight American women and persists as the second leading cause of cancer death in women [15, 21, 43, 44]. Further effective treatments for breast cancer may be developed if full knowledge of mechanisms of action in various proteins involved in tumor suppressor pathways, including the p53-p14ARF pathway is revealed. Nucleostemin as an early progenitor stem cell marker may became an important prognostic marker in treatment of patients with highly metastatic and malignant Her2 positive tumors. Furthermore, previous research in our laboratory has demonstrated elastase as a direct target protein for indole-3-carbinol. I3C's anti-proliferative signaling begins by disrupting downstream targets of elastase, such as CD40. I3C disrupts the binding of CD40 to its downstream effector, tumor necrosis factor receptor activator factor-6 (TRAF6), which is also is an E3 ubiquitin ligase for Akt1. We observed that the direct interaction between elastase on the cellular membrane with I3C triggers a downstream signaling cascade involving CD40 and TRAF6 potentiates a p53 apoptotic mechanism that ablates the growth of cancer stem cells [9, 19, 35]. Through exploitation of the p53-dependent apoptotic pathway and its upstream regulator, elastase, a direct target protein of indole-3-carbinol, the phytochemical can provide ease in treatment as well as long-term prevention. The overall significance of our pre-clinical efforts can potentially be critical in furthering development of cheaper, more effective low-cost indole based molecules for targeted treatment of cancer stem/ progenitor cell populations in solid breast cancer tumors for women impacted by late stage and metastatic class cancers. Cancer stem cells possess the capacity to self-renew and to generate heterogenous lineages of cancer cells that comprise tumors, and as a result, are critical in understanding their role in tumor initiation and metastasis.

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Chapter III

Identification of malignant melanoma-initiating cells (MMICs) and varying tumorigenic potential in five phenotypically distinct melanoma cell lines and their responsiveness to indole-3-carbinol

Abstract

The cancer stem cell theory indicates that tumorigenesis is propelled by a subpopulation of tumor cells that are able to self-renew, give rise to a heterogenous progeny, and initiate the growth of new tumors. Current therapies have been ineffective in targeting cancer stem cells to reduce tumor size, cancer incidence, and mitigating poor cancer prognosis. A chemopreventative agent is indole-3-carbinol, hydrolyzed in the vegetables of the Brassica genus such as brussel sprouts and radish. I3C promotes a G1 cell cycle arrest in metastatic cancers with a wild type PTEN, wild type p53 mutation, and the most common BRAF mutation in melanomas, the V600E mutation. However, our results also indicate the molecular response of a cancer cell line enriched in malignant melanoma initiating cells, indicating a strong sensitized proliferative response in cancers with a high phenotype to cancer stem cells, a PTEN mutation deletion, and a rare BRAF mutation, the V600D mutation.

Since melanoma cancer stem cells, also known as malignant melanoma-initiating cells (MMICs) have the capability to self-renew and potentiate into tumors with poor prognosis for patients, it becomes even more critical to create molecularly targeted therapies. The origin of a cancer stem cell is poorly understood as cancer cells have properties reminiscent of both stem cells as well as cancer cells. Although not necessarily a clonal derivation of a stem or embryonic cell, cancer stem cells have presumably arisen from a malignant transformation event that deregulated the self-renewal ability of normal stem cells. Furthermore, perhaps even differentiated cells could experience a transformative mutation that led to acquiring properties of cancer stem cells. Advancements in genetic and molecular therapies to identify signaling components that promote self-renewal capacity, apoptosis evasion, and initiate carcinogenesis generate future abilities to reverse relapse in patients with rare melanoma mutations.

Our studies specify the first evidence that a natural anti-tumor agent regulates the cellular proliferative response of malignant melanoma-initiating cells, which induces a G1 cell cycle arrest and reduces the formation of increasing multiclonal tumorigencity.

Introduction

Even though overall cancer incidence has decreased, the incidence of human malignant melanoma continues to increase in the United States. As a result, melanoma is the sixth most common cancer in males and the seventh in females in the United States. Metastatic melanoma is far more dangerous than local melanoma, which can be surgically excised before it has spread. Metastatic melanoma has been associated with poor prognosis due to its drug-resistant and aggressive cancers with a median survival time of 6 months and a 5-year survival rate of less than 5% [1-3]. Since no current therapy selectively targets these malignant melanoma-initiating cells and there is no indication of the molecular signaling pathways it utilizes to evade apoptosis, there exists a significant need to develop efficacious and therapeutic drugs for metastatic melanoma.

A promising dietary anti-proliferative phytochemical is indole-3-carbinol, which has demonstrated chemopreventative properties as well as promising anti-tumor capabilities in ovarian, prostate, breast, and other reproductive cancers. Most recently, indole-3-carbinol has also been shown to sensitize melanoma cells to cell cycle arrest. Indole-3-Carbinol is derived from the hydrolysis of glycobrassicinin, a phytochemical produced from cruciferous vegetables such as broccoli, cabbage, and brussel sprouts. I3C, as well as its potent synthetic derivative, 1-benzyl-I3C, are natural agents capable of inducing G1 cell cycle arrest in a phenotypic range of cancers, such as melanoma, prostate, breast, and ovarian [4-7]. Our results demonstrate that I3C has antiproliferative effects in a selective manner of human melanoma cell lines with varying tumorigenic potential. The G1 cell cycle arrest occurs in a mutant BRAF dependent manner, targeting human melanoma cells with either a wild type PTEN and mutant V600E phenotype, or a null-PTEN and V600D phenotype, which constitutes the most cancer stem cell-like systems. While the BRAF V600E mutation is common but malignant in many melanoma patients, the V600D mutation is equally as metastatic but demonstrates worse prognosis as less treatment options exist [8]. Treatment with I3C downregulated oncogenes such as MITF-M and various stem cell markers such as CD20 and ALDH1 in melanoma cell lines that exhibited the highest cancer stem cell-like behavior. In contrast, cells with mutant PTEN or mutant p53 exhibited no I3C sensitive response.

To isolate and study the cancer stem cell, biomarkers for identification of stem cells from normal and cancerous tissues have been crucial [9]. Some biomarkers critical in acquisition of cancer stem cells for study of self-renewal capabilities are:

- **CD133**, a cell surface differentiation antigen: associated with self-renewal differentiation in brain tumors, melanoma, and ovarian cancer [10]
- **ABCG2/ ABCG5**: ATP Binding Cassette: signals pathway drug-resistance recurrence and prognosis of tumors [11, 12]
- **ALDH1**, aldehyde dehydrogenase 1: a detoxifying enzyme found in both stem cells and cancer stem cells, and linked with drug resistance capabilities [13-15]

CD133 is originally identified as a transmembrane protein for human hematopoietic stem cells and subsequently, present in progenitor and stem cells, tumors, and regenerative tissues. These functional roles for CD133 associate CD133 as a prominent stem cell marker for melanoma tissues where it is most likely involved in self-renewal, metabolism, and differentiation. Previously isolated CD133+ cells have given rise to tumorspheres and xenografts tumors in NOD/ SCID mice in multiple cancers, from colorectal cancer, ovarian cancer, glioblastoma, and gallbladder carcinomas [16, 17]. CD133 also has direct interactions with two stem cell factors, OCT4 and SOX2, two transcription factors that bind on P1 promoter region of CD133 gene loci. As a result, the role of CD133 expression in self-renewal and tumorigenesis is likely [18]. Therefore, our results demonstrate the use of indole-3-carbinol in rare BRAF V600D mutant to improve prognosis for a population of malignant melanoma patients as well as a potential regulator of transcriptional and cell cycle effects for melanoma patients with mutant BRAF and wild type p53.

One of the reasons for malignant melanoma-initiating cells is the composition of heterogeneous cancer cell subpopulations in which only the cancer stem cell will self-renew, differentiation, and initiate tumorigenesis [19, 20]. Current therapies induce great side effects by also killing bystander healthy wild type cells or mass targeting non-malignant bulk tumor cells which cause for increased clonal differentiation, and as a result, therapy resistance. Therefore, given the important function and presence of a cancer stem cell in propagating aggressive tumor subpopulations, it will be quite critical to treat MMICs. To date, there are no effective therapies that can target malignant melanoma initiating cells to improve the prognosis of patients with aggressive and drug-resistant tumors [11, 20-22]. As a result, there is a profound need for the inclusion of new therapies, such as indole-3-carbinol, to represent a promising novel target for melanoma therapy.

Materials and Methods

Cell Culture

Melanoma cell lines G-361, SK-MEL-2, SK-MEL-28, SK-MEL-30 and WM115 were purchased from American Type Culture Collection (ATCC) (Manasas, VA), and were authenticated according to the ATCC guidelines. The G361 melanoma cells were cultured in Modified McCoy's 5A cell media supplemented with 10% fetal bovine serum (Gemini Bio Products, West Sacramento, CA), 2 mM L-glutamine, and 2.5 ml of 10,000 U/ml penicillin/streptomycin mixture (Gibco, Life Technologies, Carlsbad, CA). SK-MEL-28 and SK-MEL-30 cells were cultured in DMEM with 4.5 g/L glucose supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 2.5 mL of 10,000 U/mL penicillin/streptomycin mixture in addition to 1 of MEM nonessential amino acid (Gibco, Life Technologies). WM!!% cells were cultured in "WM Media": MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% FBS, 0.2% sodium bicarbonate, 5µg/ml insulin, and 1% penicillin/streptomycin (P/S). SK-MEL-2 melanoma cells were cultured in DMEM containing 4.5 g/L glucose, 114 mg/L sodium pyruvate, and 2 mM L-glutamine, supplemented as described above. The cells were incubated in tissue culture dishes (Nalgene Nunc, Penfield, NY) at 37°C with controlled humidity and 5% CO2 air content.

Treatment With Indole-3-Carbinol

I3C was purchased from Sigma–Aldrich (St. Louis, MO), To study the effect of I3C, cells were treated with or without 200 µM I3C every 24 h for 72 h and harvested at 24, 48, and 72 h for western blot and flow cytometric analysis. I3C was dissolved in 99.9% HPLC grade DMSO (Sigma–Aldrich, Milwaukee, WI) and the final dilution was performed in the media aliquots used for treatment.

MTT Proliferation Assay

Melanoma cell lines were seeded on a 48-well plate in triplicates and upon 80–90% confluency were either treated with different concentrations of I3C or DMSO, the vehicle control, for 48 hours. Subsequently, inhibition of proliferation was assessed using the Dojindo Cell counting Kit-8 as per the protocol in the user's manual. Briefly, 50 µl of the CCK-8 solution was added to each well along with 450 µl media and incubated for 2–3 h. The absorbance was read at 450 nm and percent inhibition was calculated for each condition standardizing DMSO to zero.

Flow Cytometry

Melanoma cells were either treated with the indicated concentrations of I3C in triplicates cell cultures for 48 h, or treated with 200 µM I3C in triplicate cultures for 48 hours. The DNA content of propidium iodide stained nuclei from harvested cells were determined by flow cytometry. Briefly, cells were hypotonically lysed in 300 ml of DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, and 0.05% Triton-X 100). Emitted fluorescence from the nuclear of wavelengths more than 585 nm was measured with a

Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Ten thousand nuclei were analyzed from each sample at a rate of 300–500 nuclei/s. The percentage of cells within the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the multicycle computer program provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

Fluorescence-Activated Cell Sorting (FACS)

Fluorescence-activated cell sorting (FACS) was done on a Cytomation MoFlo cytometer (DakoCytomation, Fort Collins, CO). FITC-conjugated mAb against human CD133 was used for separation. Polyclonal antibodies were labeled by PE-conjugated goat antibodies against rabbit IgG (Sigma) and mAbs by FITC-conjugated rabbit antibodies against mouse IgG (Sigma).

Aldefluor Assay

ALDH1 activity was measured using the Aldefluor kit (Stem Cell Technologies) following the manufacturer's instructions. ALDEFLUOR assay was performed following manufacture's recommended guidelines and protocol (Stem Cell Technologies). Live single cells were gated for analysis using Epics XL-MCL flow cytometer (Beckman Coulter) with single 488 nm blue laser filtered at 525 BP/slot 1.

Tumorsphere Efficiency Assay

Single-cell suspensions of 10AT-Her2, 10AT-Neo, MCF-7, or SKBR3 cells were plated on ultra-low attachment plates (Corning, Costar) in MammoCult Human Medium (Stem Cell Technologies, Vancouver) and cultured at 37°C, 5% CO_2. Tumorsphere formation was assessed visually by phase microscopy and quantified by counting the number of spheres formed in culture.

Western Blot

After indicated treatments, all cells were harvested in ice-cold PBS and lysed using a radioimmunoprecipitation assay buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% NoNidet-p40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (50 g/ml phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, 5 g/ml leupeptin, 0.1 g/ml NaF, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate and 0.1 mM β-glycerol phosphate). After centrifugation, total protein in the lysate was estimated using the protein quantification kit (Bio-Rad, Hercules, CA, USA). Cell lysates were electrophoretically fractionated using SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk for an hour at room temperature and incubated with primary antibodies overnight at 4°C. Immunoreactive proteins were detected after a 1-hour incubation with horseradish peroxidase conjugated secondary antibodies. Blots were then treated with enhanced chemiluminescence reagents (Estman Kodak, Rochester, NY, USA) for visualization on film. Actin (AANO1) was obtained from Cytoskeleton (Denver, CO, USA).

HER2 (2165), was obtained from Cell Signaling Technology, Inc (Danvers, MA, USA). All antibodies were diluted 1:1000 in TBST (0.1 M Tris, 150 mM NaCl, 0.05% Tween 20).

Results

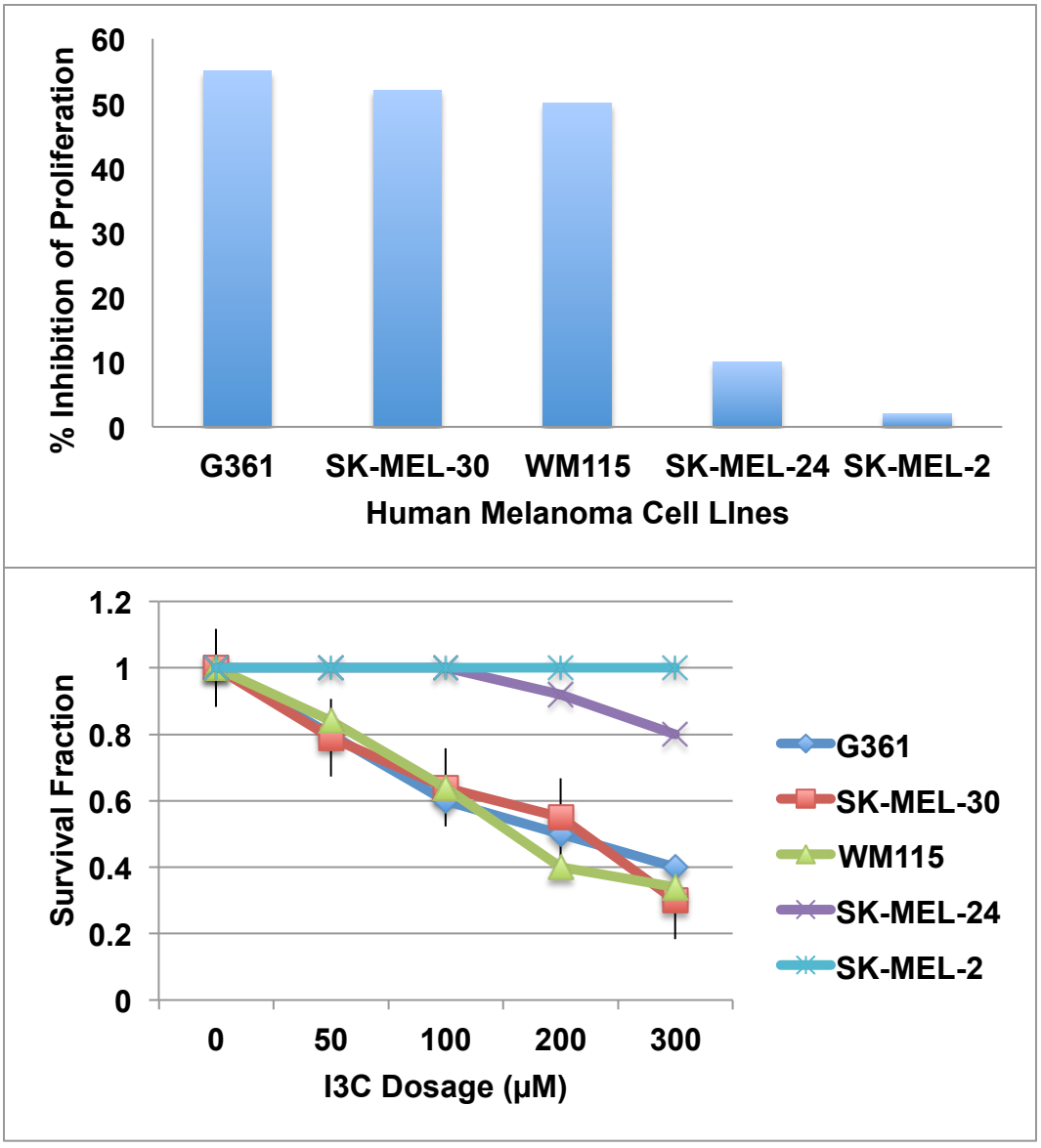
Human melanoma cells are selectively responsive to Indole-3-Carbinol

The potential effects of I3C on melanoma cellular proliferation was tested in five different human melanoma cell lines with distinct mutation profiles. Each of the cells was treated for 48 hours with or without 200 μM indole-3-carbinol, and cellular proliferation was determined using a CCK-8 proliferation assay. As shown in Figure 23, the most sensitive to the treatment were G631, SK-MEL-30 cells, and WM115 cells. The cellular proliferation of Sk-Mel-28 and Sk-Mel-2 cells, which express mutant p53, was not affected by I3C treatment.

Figure 23

I3C inhibits human melanoma cell lines of varying tumorigenic potential.

G361, Sk-Mel-30, WM115, Sk-Mel-24, and Sk-Mel-2 cells were treated with or without 200 μM I3C for 48 hours and cell number was quantified by the CCK-8 proliferation assay.


% Inhibition of Proliferation
60
50
40
30
20
10
0
G361
SK-MEL-30
WM115
SK-MEL-24
SK-MEL-2
Human Melanoma Cell LInes
Survival Fraction
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
200
300
I3C Dosage (μM)
G361
SK-MEL-30
WM115
SK-MEL-24
SK-MEL-2

I3C-Mediated G1 cell cycle arrest of G361 and WM115 cells

Our studies indicate that the antiproliferative effect of the antitumor agent indole-3-carbinol is cell type specific between cells from one tissue through regulation of cell signaling pathways and induction of apoptosis. Human melanoma cells were treated with or without 200 μM/ L indole-3-carbinol for 48 hours, and potential cell-cycle effects were analyzed by flow cytometry of propidium iodide-stained nuclear DNA. This concentration of I3C has been proven to be optimally effective in other human cancer cell lines as the functional intracellular concentration of this indole carbinol entering cells is less than 0.3% of the final concentration in the cell culture media. As shown in Figure 24, a G1 cell cycle arrest was observed in G361 and SK-MEL-30 cells, which are phenotypically wild type p53, wild type PTEN, and V600E B-raf mutation. However, in cells that display A499G-mutant PTEN or mutant p53, such as the Sk-Mel-28 cells, and Sk-Mel-2 cells, respectively, there was no indication of a cell cycle arrest. In a dose response, Sk-Mel-28 cells, showed slight sensitivity--an arrest after 300 μM of treatment or past 48 hours (data not shown). Sk-Mel-2 cells, which express a wild type BRAF, are mostly insensitive to the effects of indole-3-carbinol. Interestingly, WM115 cells, which are considered metastatic and malignant melanoma cells, were also responsive to I3C despite their null PTEN, wild type p53, and V600D-mutant B-RAF genetic molecular profile. This suggests that I3C might function in two different signaling mechanisms that is not only specific to a wild-type PTEN, wild-type p53, and V600E-mutant B-RAF genotype.

Figure 24

I3C induces strong G1 cell cycle arrest in G361 and WM115 cells.

Human melanoma cells were treated with or without 200 μM I3C for 48 hours, and the cell population DNA contents quantified by flow cytometry after staining with propidium iodide. The results are an average of triplicate results. The average error was approximately -/+ 1% for each of the results.

		Vehicle Control			Indole-3-Carbinol		
Cell Line	Phenotype	G1	S	G2/M	G1	S	G2/M
G361	Wild Type PTEN Wild Type p53 B-Raf V600E Mutation	36.3%	38.6%	25.2%	70.4%	16.8%	12.8%
Sk-Mel-28	A499G PTEN Mutant p53 B-Raf V600E Mutation	60%	28%	12	63%	24%	13%
Sk-Mel-30	Wild Type PTEN Wild Type p53 B-Raf V600E Mutation	48%	36%	14%	77%	16%	6%
Sk-Mel-2	Wild Type PTEN Mutant p53 Wild Type B-Raf	59%	28%	13%	60%	27%	13%
WM115	PTEN Deletion Wild Type p53 B-Raf V600D mutation	40%	41%	19%	73%	18%	9%

I3C ablates spheroid formation in cell lines, WM115 cells and G361 cells

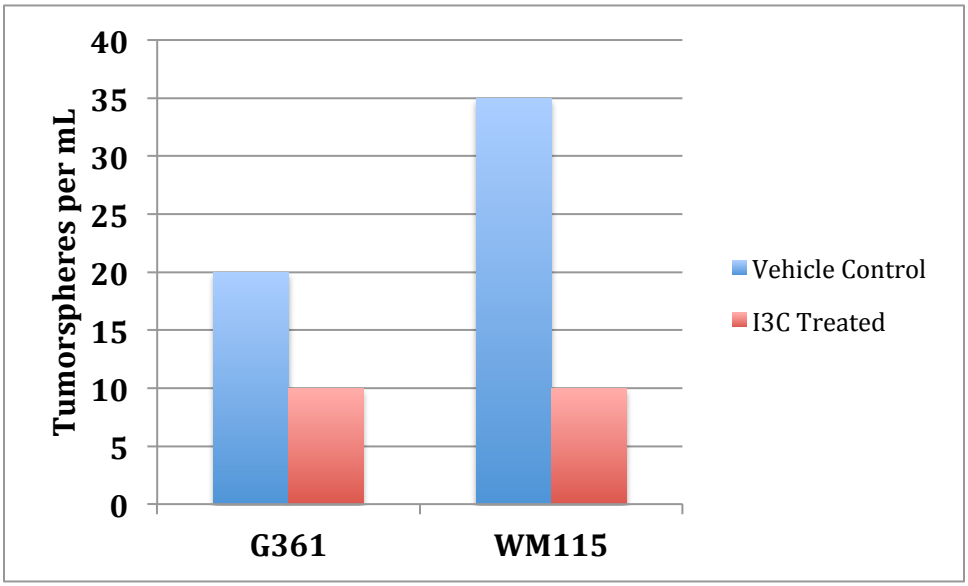
Earlier studies have demonstrated a correlation between tumor initiation and adherent free suspensions. The ability of cells to initiate melanosphere growth in suspension media has been suggested to define the cancer stem cell. Therefore, the presence of tumorsphere growth can be utilized to identify a side population of cells in a tumor to elucidate tumor initiation capabilities. Cancer cells that lack stem cell properties have limited sphere-forming potential due to telomere loss, cellular senescence, and inactivation of self-renewal signaling pathways as a result of differentiation.

G361 and WM115 cells were plated at a density of 10,000 cells per well in tumorsphere culture conditions and incubated with or without I3C. Only one dose of I3C was administered in tumorsphere culture media at day 0 and after 6 days, tumorsphere formation was assessed visually by phase microscopy and quantified. Only G361 and WM115 cells were capable of melanosphere formation, suggesting the melanoma stem cell like tumorigenic potential of the two cell lines. I3C ablated spheroid formation in G361 cells twofold; while WM115 cells were affected approximately more than three fold by the treatment of I3C.

Figure 25

I3C inhibits formation of melanoma spheroids in G361 and WM115 cells.

10,000 G361 cells or WM115 per well were plated in serum free nonadherent tumorsphere culture conditions and treated with the indicated indole-3-carbinol or the vehicle control. Treatment was administered at day 0 and incubated for six days. Tumorsphere formation was quantified in three independent experiments by determining the total number of tumorspheres in each cell culture well.


40
35
30
25
20
15
10
5
0
Tumorspheres per mL
G361
WM115
Vehicle Control
I3C Treated

Molecular Profiling of WM115 and G361 cells reveal presence of cancer stem cell markers

We profiled the responses of mutationally distinct melanoma cell lines and investigated which cell lines are capable of forming spheroids, whether commonly used cancer stem cell markers were enriched in these spheroids, and whether they were responsive to I3C. Cancer stem cell markers are upregulated in cells cultured as spheres. To investigate the enrichment of cancer stem cell markers in spheroids versus adherent suspension cells, we sorted cells on the basis of cancer stem cell markers, CD133, CD20, and ALDH1. We analyzed the expression of cell surface markers, CD133 and CD20, through fluorescence activated cell sorting (FACS) and compared levels of functional ALDH1 activity through the ALDEFLUOR assay, amongst five distinct cell lines under adherent and nonadherent conditions.

When cancer stem cell markers, such as CD133 and CD20 were analyzed, in both adherent cells and spheroids, tumorspheres were enriched for ALDH-1 expression. However, CD133 expression which was normally higher in adherent cells for G361 cells and WM115 over I3C irresponsive cell lines, such as Sk-Mel-28 and Sk-Mel-2 cells, or the I3C responsive Sk-Mel-30 cell line.

We analyzed the molecular and functional phenotype of adherent cells and melanoma spheroids by sorting for melanoma-associated markers. Gene expression profiling and spheroid modeling allow for a greater grasp of melanosphere behavior and allowed for enrichment of cancer stem cells with biomarker expression and enhanced self-renewal capabilities. Out of the wild type PTEN and wild type p53 phenotypic cell lines, only G361 cells were able to form spheroids in nonadherent media. Even though Sk-Mel-30 which show a reasonably strong response to I3C expressed small levels of CD133, CD20, and functional ALDH1 at 1%, 2%, and 8% respectively, it was not able to form melanosphere enriched with stem cell factors. However, the WM115 cell line,, which were able to form melanospheres in nonadherent suspension and expressed stem cell markers CD133, CD20, and ALDH1 in both adherent and spheroid conditions, interestingly did not change between two conditions. As a result, it could be hypothesized that the WM115 cell lines, as a well-recognized metastatic and malignant melanoma cell line, has reached a “capacity” in expression of melanosphere markers. However, when quantifying the number of tumorspheres per mL in media, WM115 cells were able to form almost double the number of spheroids of that of the G361 cells after 4 days.

Figure 26

Quantification of cancer stem cell markers, CD133, CD20, and ALDH-1.

Cell surface expression of CD133 and CD20 in G361, Sk-Mel-28, Sk-Mel-30, Sk-Mel-2, and WM115 cells were quantified by flow cytometry of 500,000 cells in triplicate independent cell cultures. ALDH1 activity was measured through the ALDEFLUOR kit by STEMCELL BIOLOGIES. G361, Sk-Mel-28, Sk-Mel-30, Sk-Mel-2, and WM115 cells were quantified by flow cytometry of 500,000 cells in triplicate independent cell cultures.

		Adherent			Spheroid		
Cell Line	Phenotype	CD133	CD20	ALDH1	CD133	CD20	ALDH1
G361	Wild Type PTEN Wild Type p53 B-Raf V600E	7.6%	1%	9.2%	8%	7.5%	78.7%
Sk-Mel-28	A499G PTEN Mutant p53 B-Raf V600E	2%	3%	1%	--	--	--
Sk-Mel-30	Wild Type PTEN Wild Type p53 B-Raf V600E	1%	2%	8%	--	--	--
Sk-Mel-2	Wild Type PTEN Mutant p53 Wild Type B-Raf	0%	0%	0%	--	--	--
WM115	PTEN Deletion Wild Type p53 B-Raf V600D	5.2%	3.1%	22.2%	5.9%	3.5%	82.1%

Western Blot analysis identifies selective modulation of the G361 and WM115 cells

To verify the molecular mechanism of indole-3-carbinol regulation, G361 and WM115 cells were treated with the vehicle control, DMSO, I3C, and the potent indole derivative, 1-benzyl-I3C. WM115 and G361 cells were selectively responsive to I3C. Levels of MITF-M decreased with I3C and 1-benzyl-I3C treatment, but in WM115 cells, only after 1-benzyl-I3C treatment was MITF-M signal lost. MITF-M is a "lineage survival" oncogene that is required for both tissue-specific tumorigenesis and progression and elevated MITF-M levels correlate with decreased overall patient survival. In the null PTEN line, WM115, no PTEN changes were detected but, in G361 cells, protein levels of PTEN, a tumor suppressor, increased upon I3C and 1-benzyl-I3C treatment. This effect was similar for the Axin protein, which remain unchanged in WM115 cells, but increased in G361 cells after I3C and the more potent 1-benzyl-I3C derivative. Wnt levels decreased in G361 cells, but not in WM115 cells, identifying a different molecular mechanism to ablate tumor initiating cells for WM115 cells. The control protein, actin, remain unchanged.

Figure 27

I3C and 1-benzyl-I3C selectively modulate G361 and WM115 cells.

G361 and WM115 cells were treated with 15 μM 1-benzyl-I3C, 200 μM I3C, or DMSO vehicle control for 48 hours. Total cell lysates were subjected to SDS-PAGE and the levels of MITF-M, B-catenin, Wnt, PTEN, Axin and Actin (loading control) was monitored by western blot analysis.

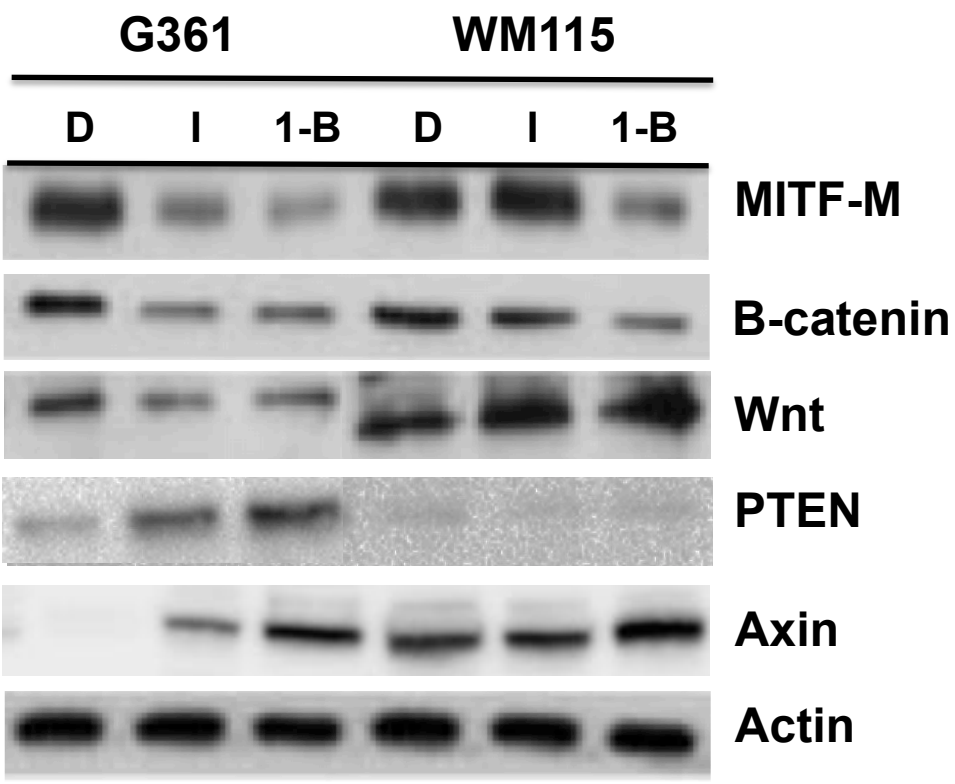
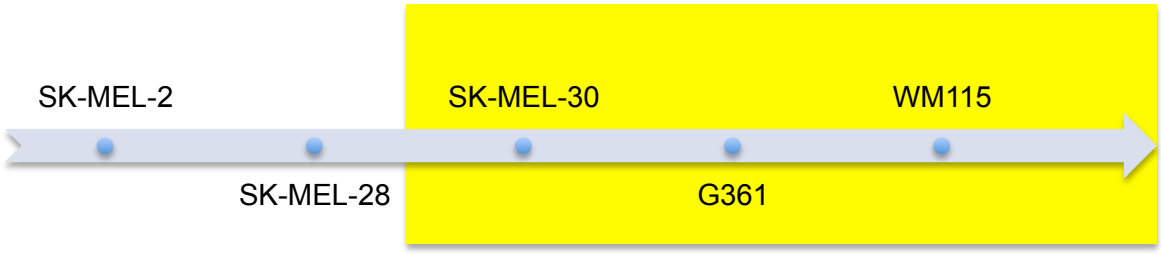

G361
WM115
D
I
1-B
D
I
1-B
MITF-M
B-catenin
Wnt
PTEN
Axin
Actin

Figure 28

Summary of varying tumorigenic potential of human melanoma cell lines.



Increasing Stem Cell Character

Discussion

The aim of this study is to investigate whether established melanoma cell lines contain subpopulations of cells that aggregate as cancer stem cells and to understand their responsiveness to the phytochemical, indole-3-carbinol. Previous research in our laboratory detected a G1 cell cycle arrest of G361 melanoma cells, which retain a wild type p53 and wild type PTEN, molecular phenotype and no response from Sk-Mel-28 cells, which possess mutant PTEN, p53, and mutant B-RAF. Melanomas show phenotypic heterogeneity both *in vivo* and *in vitro*, suggesting an origin from a cell with multilineage differentiation abilities [23, 24]. Melanoma cells retain their morphologic and biological plasticity despite repeated cloning and contribute to increased resistance to therapy selection.

To assess whether the decrease in cell proliferation due to I3C was either cytostatic or cytotoxic, we determined the effect of I3C on cell cycle progression. Cell cycle analysis by quantification of DNA content indicates the strongest G1 cell cycle arrest in G361 cells, followed by a cell cycle arrest in Sk-Mel-30 cells. Genotypically, both cells exhibit wild type PTEN and wild type p53, highlighting sensitivity to indole-3-carbinol. This is contrasted by Sk-Mel-28 cells and Sk-Mel-2 cells which exhibit a distinct mutant PTEN and a p53 mutation, respectively. They showed no response to indole-3-carbinol in cell cycle studies. In the dose response study, Sk-Mel-28 cells minutely began to respond to I3C treatment, at 300 μM treatment at 48 hours, indicating a minute cellular signaling response. WM115 cell line is resistant to many types of therapeutic molecules including Vemurafenib [25] As a result, I3C can be a promising malignant melanoma-initiating cell therapy due to its ability to ablate not only typical B-raf mutation melanomas, but also Vemurafenib-resistant, but notoriously malignant melanomas [26, 27]. Our findings indicate I3C as a potential therapeutic for melanomas that express low levels of wild type PTEN protein in a background of either wild type BRAF or BRAF (V600E) by disrupting the NEDD4-1 mediated ubiquitination of PTEN and enhancing the total levels of this tumor suppressor protein.

Previous research in our laboratory has shown I3C and the synthetic derivative (1-benzyl-I3C) strongly upregulates PTEN protein levels without altering the level of PTEN transcripts in wild type PTEN expressing cell lines. In WM115 cells, which display a null PTEN genotype and a mutant B-raf V600D mutation, I3C and 1-benzyl-I3C still conferred a strong apoptotic response. This suggests the I3C response in melanoma stem cells is different than those cell lines that display a wild type PTEN, wild type p53, and mutant B-raf V600E mutation [4, 5, 8].

We profiled the responses of mutationally distinct melanoma cell lines and investigated which cell lines are capable of forming spheroids, whether commonly used cancer stem cell markers were enriched in these spheroids, and whether they were responsive to I3C. Cancer stem cell markers are upregulated in cells cultured as spheres. To investigate the enrichment of cancer stem cell markers in spheroids versus adherent suspension cells, we sorted cells on the basis of cancer stem cell markers, CD133, CD20, and ALDH1. Upregulation of CSC markers such as ALDH1 and CD133 is consistent with the hypothesis

that growth of melanospheres in nonsuspension media enriches for CSCs markers. Only G361 cells and WM115 cells were capable for spheroid formation consistent with their higher expression of CD133, CD20, and ALDH-1 over I3C non-responsive cell lines such as Sk-Mel-28 and Sk-Mel-2 cells, as well as the I3C responsive cell line, Sk-Mel-30. This is indicative of a less active melanoma stem cell phenotype in the Sk-Mel-28, Sk-Mel-2, and Sk-Mel-30 cell lines. Melanoma spheres were significantly enriched for expression of ALDH-1, but not CD133 and CD20.

Melanomas are a significant cause of mortality due to their resistance to current therapies. This can be due to the presence of heterogeneous population of cancer cells that are poorly characterized, with a side subpopulation of transformed stem cells. These cells, also known as melanoma stem cells or melanoma initiating cells, possess the ability to self-renew, differentiate into diverse progenies, and drive continuous growth. Given this, it is critical to molecularly understand the responsiveness of melanoma to various nonconventional therapies as well [28, 29].

These tumor-initiating cells are distinct from malignant cancer cells or non-malignant stem cells due to their self-renewing capability, propensity to differentiate into actively proliferating tumor cells, elevated expression of stem cell biomarkers, resistance to conventional therapies, and low proliferative rates (similar to a stem cell capacity). Targeting and personalizing therapies to these cells can not only greatly ablate tumor size but also attack tumor metastasis and tumor initiation of a melanoma tumor, reducing poor prognosis and high rates of remission [1, 29-31]. This is especially pertinent due to the multiclonal origins of a heterogeneous population of bulk tumor cells and rare cancer stem cells. Agents such as indole-3-carbinol that enhance sensitivity of these therapy-resistant tumor stem cells to current or emerging anti-proliferative drugs could potentiate higher inhibition abilities as well.

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