Cancer stem-like cells derived from chemoresistant tumors have a unique capacity to prime tumorigenic myeloid cells

Tsunaki Yamashina 1, Muhammad Baghdadi 1, Akihiro Yoneda 1, Ichiro Kinoshita 2, Shinya Suzu 3, Hirotoshi Dosaka-Akita 2 & Masahisa Jinushi 1

1 Research Center for Infection-associated Cancer, Institute for Genetic Medicine, Hokkaido University, Sapporo, 060-0815, Japan
2 Department of Medical Oncology, Hokkaido University Graduate School of Medicine, Sapporo, 060-0815, Japan
3 Center for AIDS research, Kumamoto University, Kumamoto, 860-0811, Japan
Grant support

This study was partially supported by a Grant-in-Aid for Scientific Research and Scientific Research for Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and the Ministry of Health, Labor and Welfare (M. J.).

A conflict of interest disclosure

All authors have no conflicts of interest to declare.

Correspondence author

Masahisa Jinushi, MD, PhD
Institute for Genetic Medicine, Hokkaido University
Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido, 060-0815, Japan
Tel: 81-11-706-6073
Fax: 81-11-706-6071
E-mail: Jinushi@igm.hokudai.ac.jp

Running Title: Characteristics of chemoresistant CSCs

Key Words: Cancer stem cells, Chemoresistance, Myeloid cells, M-CSF, Interferon regulatory factor-5
Abstract

Resistance to anticancer therapeutics greatly impacts the phenotypic and functional properties of tumor cells, but how chemoresistance contributes to the tumorigenic activities of cancer stem-like cells remains unclear. In this study, we found that a characteristic of cancer stem-like cells from chemoresistant tumors (CSC-R) is the ability to produce a variety of proinflammatory cytokines and to generate M2-like immunoregulatory myeloid cells from CD14+ monocytes. Further, we identified the interferon-regulated transcription factor IRF5 as a CSC-R-specific factor critical for promoting M-CSF production and generating tumorigenic myeloid cells. Importantly, myeloid cells primed with IRF5+ CSC-R facilitate the tumorigenic and stem cell activities of bulk tumors. Importantly, the activation of IRF-5/M-CSF pathways in tumor cells were correlated with the number of tumor-associated CSF1 receptor+ M2 macrophages in patients with non-small lung cancer. Collectively, our findings show how chemoresistance impacts the properties of cancer stem-like cells in their niche microenvironments.
Introduction

Resistance to anticancer modalities poses serious obstacles that must be addressed in order to improve the clinical prognosis for cancer patients. Recent studies have revealed that multiple mechanisms enable the development of resistance to anticancer therapies through genetic alterations and environmental modifications such as vascular and immunological remodeling within tumor tissues (1-4). Coordinated intrinsic and extrinsic pressures force tumor cells to accommodate to stressful microenvironments and co-opt multiple strategies for survival, invasion and distant metastasis, further enhancing tumorigenicity and worsening clinical prognosis. Accumulating evidence has established that rare populations termed cancer stem cells (CSCs) are indispensable as a main source of tumorigenicity and anticancer drug resistance (5). While it remains largely unclear whether chemoresistance further modifies the phenotypic and functional manifestations of CSCs, previous studies have revealed that the low sensitivity to cytotoxic therapies of CSCs derives mainly from cellular quiescence and multidrug transporter activity (6).

In this study, we elucidate an unexpected property of CSCs derived from chemoresistant tumors (CSC-R). Although such CSCs are identical to untreated CSCs in self-renewal and phenotypic properties, they have a unique ability to produce various proinflammatory mediators that act to generate tumorigenic myeloid cells. Interferon-regulatory factor-5 (IRF5) plays a critical role as
a CSC-R-specific transcription factor that facilitates M-CSF production and promotes myeloid cell-mediated tumorigenic activities. Our findings provide novel mechanisms whereby resistance to anticancer therapies changes the biological character of CSCs in distinct niche microenvironments.
Materials and Methods

Mice and tumor cell lines

NOD-SCID animals were purchased from Charles River. All experiments were conducted under a protocol approved by the animal care committees of Hokkaido University.

Tumor cell lines (MDA-MB-231 and HCT116) were obtained from the American Tissue Culture Collection (ATCC). All cell lines described above were obtained one year before being used in experiments and authenticated by the Central Institute for Experimental Animals (Kawasaki, Japan) for interspecies and mycoplasma contamination by PCR within 3 months before the experiments.

Patient samples

The clinical protocols for this study were approved by the committees in the Institutional Review Board of Hokkaido University Hospital (Approval number: 10-0114). Pleural effusion cells were obtained from patients with stage IV non-small cell lung carcinomas after written informed consents had been obtained. The cells were isolated by Ficoll-Hypaque density centrifugation, and further purified as EpCAM+ epithelial cells and CD14+ monocytes from tumor tissues or pleural effusion.

Generation of chemoresistant tumor cells

To generate chemoresistant tumor cells, MDA-MB-231 cells or HCT116 cells were treated
with taxane or CDDP at low concentrations (1μg/mL) for 96 h, respectively. The cells were extensively washed three times and then treated with the identical drug at same concentrations for additional five passage times. The generation of chemoresistant cell variants of MDA-MB-231 or HCT116 cells was confirmed by the near-complete resistance to cell death (90% or more) by the treatment with taxane or CDDP, respectively.

**Flow cytometry**

The expression levels of CD44 and ALDH1 for CSC-R and CSC-N were evaluated with anti-human CD44 Ab (BD Bioscience) and ALDEFOUR reagents (VERITAS), respectively. For myeloid cell analysis, monocytes stimulated with M-CSF, with supernatants of CSC-R or with those of CSC-N were stained with anti-CD206 Ab and anti-CD68 Ab (BD Bioscience). The cell viability of tumor cells treated cytotoxic drugs was examined by staining with annexin-V/propidium iodide staining according to the manufacturer’s instructions (BD Bioscience). The cells were subjected to flow cytometry using a FACS Caliber.

**Sphere forming assay**

For sphere forming assays, CSC-R, CSC-N or bulk MDA-MB-231 cells primed with myeloid cells (1,000 cells per well) were cultured in ultra-low attachment culture dishes (Corning) in serum-free medium. DMEM/F-12 serum-free medium was supplemented with 20ng/mL epithelial growth factor and 10ng/mL basic-fibroblast growth factor-2 (PeproTech).
and cell passage were performed every 3 days, and the sphere forming colonies were counted after three serial passages.

**Measurement of cytokine and chemokines**

CSCs, non-CSC or bulk cells from treatment-naïve or chemoresistant tumors (MDA-MB-231 or HCT116) (1 x 10^6/well at start point) were cultured for 48 h, and the protein levels of cytokines and chemokines were quantified by ELISA using supernatants obtained from cultured CSC-R and CSC-N according to the manufacturer’s instructions (BD Biosciences). Detection limit for cytokines: IL-1β, 4 pg/mL; IL-6, 2 pg/mL; IL-8, 2 pg/mL; IL-10, 7.8 pg/mL; IL-12p40, 15 pg/mL; TNF-α, 15 pg/mL; TGF-β, 19 pg/mL; GM-CSF, 9.4 pg/mL; M-CSF, 16.2 pg/mL; IFN-α, 15 pg/mL; IFN-γ, 10 pg/mL; CCL2, 2.3 pg/mL; CCL19, 7.8 pg/mL; CCL20, 7.8 pg/mL.

**Quantification of cytokine mRNA by RT-PCR**

The mRNA was isolated from CSC-R, CSC-N or these cells after transfection with control or IRF5 siRNA. The mRNAs of genes associated with myeloid cell differentiation (CSF1R, C/EBPα, and PU.1) or polarization (ARG-1, Relm-α, IL-4R) or effector functions (IL-10, IL-12p70, CXCL9, IFN-γ) were quantified by real-time PCR using SYBR Green Gene Expression Assays (Applied Biosystems).

**Luciferase reporter assay for NF-κB and ISRE**

CSC-R or CSC-N from MDA-MB-231 or HCT116 were treated with taxane or CDDP,
respectively, and transfected with control or IRF5 siRNA (5’rGrArCuCrCuUrGuUrCrArArATT3’) for 24 h. The cells were then transiently transfected with firefly luciferase reporter plasmid encoding NF-kappaB or ISRE and control renilla luciferase plasmid (1ng) for 20h. The luciferase activities of lysates from the cells were shown as a ratio of firefly reporter intensities to control renilla intensities.

**Immunoblotting**

CSC-R or CSC-N from MDA-MB231 or HCT116 was subjected to Western blotting to quantify the protein levels of IRF-5 by using antibodies for human IRF5 Ab (Cell Signaling Technologies). Beta-actin was used as a loading control to check the integrity of each sample.

**RNA interference**

The siRNA-mediated knockdown was performed by On-target plus SMART pool reagents designed to target human IRF5 (Thermo-Dharmacon). The human IRF5 or control siRNAs were transfected into tumor cells by Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions.

**Myeloid cell-mediated tumorigenic activities**

CSC-R, CSC-N or bulk tumor populations from MDA-MB-231 cells were transfected with control or IRF5 siRNA for 48 h, and then co-cultured with CD14⁺ monocytes for 96 h. The myeloid cells primed with CSC-R were isolated from co-cultured cells and further cultured for
24 h to obtain culture supernatant. Bulk MDA-MB-231 or HCT116 cells treated with the supernatant of CSC-R-primed myeloid cells were subjected to chemotherapy-induced apoptosis and sphere formation analysis.

**In vivo** tumorigenic activities of CSCs mediated by human myeloid cells

After depletion of endogenous myeloid cells by clodronate liposome (200μg/mouse) intraperitoneally administered twice a week, the CSC-N or CSC-R isolated from HCT-116 cells were injected subcutaneously into NOD-SCID mice at small doses (1 x 10^5/mice) in conjunction with intravenous administration of CD68^+ macrophage (1 x 10^6/mice) isolated from healthy donor’s peripheral blood. The tumor growth was measured on the indicated days, and numbers of human macrophages were evaluated in tumors from each mice.

**In vivo** tumorigenic activities of primary NSCLC tumors

EpCAM^+CD133^+ CSCs obtained from primary NSCLC patients were transfected with control or IR5 siRNA for 48h, and then co-cultured with CD14^+ monocytes isolated from autologous PBL for 96 h. CD11b^+ myeloid cells were isolated from the co-cultured cells for *in vivo* tumor growth assays. For the *in vivo* primary tumor experiments, bulk NSCLC tumor cells (1 x 10^5/mouse: n=5) were inoculated subcutaneously into NOD-SCID mice in combination with intravenous transfer of autologous CD11b^+ myeloid cells. Two days before tumor challenge, NOD-SCID mice were treated with clodronate intravenously to remove endogenous
macrophages. Tumor growth was measured on the indicated days.

**Statistical analysis**

Statistical analysis was performed using the paired student t test, and the degree of statistical correlation was evaluated from the Pearson correlation coefficients analysis. $p<0.05$ was considered as statistically significant. * $p<0.05$, ** $p<0.01$, ns: not significant.
Results

Characterization of cancer stem cells derived from chemoresistant tumors

Cytotoxic chemotherapy manipulates multiple signaling cascades including DNA damage pathways, cell death machineries, oncogenic signaling and chromatin remodeling-associated events (1, 7). CSCs serve as the apex of cellular hierarchy responsible for tumor initiation and progression, but whether biological alterations linked with chemoresistance further modulate the tumorigenic activities of CSCs remains largely unknown. To define this, we generated chemoresistant variants of MDA-MB231 breast cancer cells and HCT116 colon cancer cells through repetitive exposure to low doses of taxane and cisplatin, respectively (8). CSCs were isolated from these chemoresistant and sensitive variants based on their identical CSC marker expression (Figure 1A). CSCs derived from chemoresistant variants (CSC-R) were refractory to cytotoxic therapies compared to CSCs from untreated tumor cells (CSC-N). However, the frequencies of CSCs were nearly identical with similar expression levels of CSC markers (ALDH1 and CD44) on naïve and chemoresistant tumor cells (Figure 1B), and there were little differences between CSC-N and CSC-R on the self-renewal activities as shown by similar levels of sphere forming activities (Figure 1C). Importantly, the chemoresistant cell variants generated from the treatment-naïve CD44− non-CSC subsets did not exhibit the phenotypic and functional properties of CSCs (Supplementary Figure 1). Thus, chemoresistance
does not render non-CSC with plasticity to undergo trans-differentiate into CSCs.

Together, our findings potentially suggest that CSC-N undergoes intrinsic genetic and/or epigenetic alternation to transform into the CSC-R upon chronic exposure with cytotoxic drugs, but further studies should be required for defining this possibility by comprehensive genetic analysis.

**Proinflammatory profile of cancer stem cells derived from chemoresistant tumor cells**

Inflammatory signals play a critical role in the regulation of the stem cell characteristics and tumorigenic activities of CSCs through autocrine and paracrine-mediated mechanisms (9-11). We therefore next evaluated the possibility that chemoresistance modifies inflammatory profiles in CSCs from MDA-MB-231. To our surprise, we found that mRNA levels of various cytokines and chemokines, such as IL-1β, IL-6, IL-8, IL-12p40, TNF-α, M-CSF and CCL2 were produced in CSC-R subsets at higher amounts compared to the bulk tumor cell counterparts, although bulk chemoresistant cells could produce proinflammatory mediators at higher levels than CSC-N or treatment-naïve bulk tumor cells (Figure 2A and Supplementary Figure 2A). We also confirmed the higher protein levels of several cytokines (M-CSF, IL-1β, IL-6 and TNFα) in CSC-R compared to bulk or non-CSC populations (Figure 2B and data not shown).

Consistent with its proinflammatory activities, the transcriptional activity of NF-κB was higher...
in CSC-R compared to CSC-N (Figure 2C). We observed similar trends in CSC-R from HCT-116 (Supplementary Figure 2A). Furthermore, the chemotherapy-naïve CSCs from MDA-MB-231 cells did not produce pro-inflammatory cytokines such as M-CSF when they were treated with cytotoxic drug taxane for a brief period, suggesting that acute stress responses changes evoked by exposure of cytotoxic drugs had little effects in modifying inflammatory profiles of CSCs (Supplementary Figure 3A). Taken together, our findings suggest that chemoresistance rendered CSCs with the ability to triggers autocrine activation of inflammatory signals, which subsequently resulted in the production of proinflammatory mediators.

**CSC-R prime monocytes to differentiate into M2 macrophages**

Given the characteristic proinflammatory cytokine profiles of CSC-R, we focused our study on the effects of CSC-R-mediated inflammatory signals on phenotypic and functional properties of macrophages, since myeloid cells have a tremendous impact on the regulation of tumorigenic activities and anticancer drug resistance (12, 13). Macrophages express several immunoregulatory genes that are closely linked with impaired tumor immunosurveillance (14). We found that CSC-R culture supernatants induced several factors characteristic of M2 macrophages, such as arginase-1, CSF1 receptor, IL-4R, Relm-α and IL-10, C/EBPα and PU.1 (Figure 3A and Supplementary Figure 2B). In contrast, there was little difference between CSC-
R and CSC-N in the expression of IL-12p70, IFN-γ and CXCL9, which are associated with M1 phenotypes (Figure 3B). Furthermore, the treatment with CSC-R-derived soluble factors upregulated the M2 macrophage marker CD206 mannose receptor at comparable levels to the treatment with M-CSF. However, bulk chemoresistant cells could generate M2 macrophages at higher levels than CSC-N or treatment-naïve tumor counterparts (Figure 3C). It is notable that bulk tumor cells are capable for differentiating the M2 macrophages from CD14+ monocytes, but non-CSCs had little ability in differentiating into M2 macrophages compared to non-treatment control (Figure 3C). Thus, it is highly likely that the M2-macrophage differentiation by the bulk tumors relies mainly on the CSCs contained within the whole populations, although it remains probable that chemoresistance itself differentiate M2 macrophage by CSC-independent fashion. Furthermore, supernatant of the chemotherapy-naïve MDA-MB231 CSC-N treated with taxane overnight had little effect in generating CD206+ M2-macrophages, suggesting that acute stress responses evoked by exposure of cytotoxic drugs do not render CSCs with the ability to induce macrophages with immunosuppressive phenotypes (Supplementary Figure 3B).

The generation of therapy-resistant cell lines was largely dependent on the numbers of treatment with low-dose chemotherapy (more than 4 times). Therefore, we next setup the experiments by utilizing CSC-N of HCT116 cells treated with low-dose CDDP for 72 h on
various treatment / passage cycles. We found that the CSC-N generate M-CSF and prime M2 macrophages in concomitant with acquisition of chemoresistant phenotype (Supplemental Figure 3C and 3D). To further address whether CSC-R differs from CSC-N in their unique activities of priming M2-macrophages in vivo, CSC-N or CSC-R isolated from HCT-116 cells were injected subcutaneously into NOD-SCID mice at small doses (1 x 10²/mice) in conjunction with intravenous administration of CD14⁺ monocytes (1 x 10⁶/mice) isolated from healthy donor’s peripheral blood, and the tumor formations were evaluated in vivo. In this experiment, NOD-SCID mice were pre-treated with clodronate liposome to remove endogenous macrophages. Although the CSC-R-driven tumors were grown at greater levels than CSC-N tumors, their tumorigenicity was positively correlated with the presence of myeloid cells, since its tumor growth was accelerated by adoptive transfer of human monocytes. In marked contrast, the transfer of human monocytes had little effects on the CSC-N-derived tumor growth (Figure 3D). More importantly, human CD68⁺ macrophages expressing the putative M2 marker CD163 and CD206 were infiltrated into the tumor tissues of CSC-R at greater levels than those of CSC-N (Figure 3E). In contrast, the frequencies of CD163lowHLA-DRhigh M1 macrophages were comparable in tumors from CSC-R and CSC-N (Figure 3E). Together, these results further support our hypothesis that CSC-R specifically regulates infiltration and differentiation of immunoregulatory M2-macrophages.
Interestingly, the supernatants of CSC-R contributed to the induction of CD33<sup>+</sup>HLA-DR<sup>low</sup> myeloid-derived suppressor cells (MDSCs) at higher levels than those of CSC-N or non-CSC counterparts. In addition, the induced MDSCs were homogenously CD14<sup>+</sup>CD15<sup>−</sup> monocytic subsets when treated with either CSC or non-CSCs from chemoresistant or treatment-naïve cells (Supplementary Figure 4). Again, CSCs-N acutely treated with chemotherapy did not induce M2-like macrophages, suggesting that CSCs could not acquire an ability to prime tolerogenic myeloid cells by chemotherapy-mediated acute stress responses (Supplementary Figure 3B).

Together, these results suggested that soluble factors released from CSC-R may regulate the differentiation of immunosuppressive macrophages from monocyte precursors, which have a great impact on tumorigenicity.

**Upregulation of IRF5 promotes M-CSF production from CSC-R**

In addition to NF-κB-mediated inflammatory signals, transcriptional activities mediated by interferon-stimulated response elements (ISRE) were detected in CSC-R at much higher levels than those in CSC-N (Figure 4A). In contrast, type-I interferon and transcriptional activities of interferon-γ-activation sequences (GAS) were detected in CSC-R at levels similar to those in CSC-N upon chemotherapy (Figure 4A).

Given that IFNs signal through JAK/STAT pathways to induce interferon-stimulation genes
(ISG), which are under control of ISRE and GAS elements, we hypothesized that CSC-R have a unique ability to stimulate ISRE-mediated transcriptional activities using autocrine IFN and/or JAK/STAT-independent mechanisms (15). Interferon-regulatory factors (IRFs) serve upstream of ISREs, which have distinct and overlapping roles for IFN-γ/GAS-related signals. To evaluate the role of IRFs in the regulation of ISRE transcriptional activities, we employed specific siRNA gene knockdown (KD) of each of the IRF genes (IRF1-9) in HCT-116 CSC-R and then treated the cells with cisplatin for 24 h. In this analysis, we identified IRF5 as a critical factor which is specifically expressed in CSC-R and regulate ISRE activities. The knockdown of IRF5, but not that of other IRF families, diminished the transcriptional activities of ISRE in CSC-R (Figure 4B). In contrast, ISRE activities remained unchanged in CSC-N regardless to the inhibition of IRF members including IRF5 (Figure 4B). We also confirmed that the mRNA levels of IRF5 expression were detected in CSC-R at much higher levels than parental tumors or CSC-N, and the protein levels of IRF5 were also higher in CSC-R from MDA-MB231 and HCT116 compared to the CSC-N counterparts (Figure 4C and D).

To define the functional relevance of IRF5 to the immune-modulatory effects of CSC-R, we next examined whether IRF5 either regulates selective sets of cytokines or, instead, generally affects inflammatory signals. The siRNA knockdown of IRF5 suppressed M-CSF production from CSC-R but not non-CSC-R or CSC-N, whereas IRF5 had little impact on the regulation of
other soluble mediators in CSC-R (Figure 4E and data not shown). Importantly, IRF5 inhibition substantially reduced the ability of CSC-R to induce several factors critical for M2 macrophage differentiation in CD14+ monocytes, but addition of M-CSF recovered the expression levels of M2 factors in macrophages primed with IRF5-KD CSC-R at comparable levels to those primed with control CSC-R, further indicating that IRF5-mediated M-CSF of CSC-R is critical for inducting M2 macrophage-associated factors (Figure 4F).

The IRF-5-dependent effects in priming M2-macrophage differentiation were largely dependent on M-CSF from CSC-R because the CSC-R-mediated induction of CD206+ macrophages was substantially diminished by the treatment with anti-M-CSF neutralizing Ab (Figure 4G). Moreover, the treatment with recombinant M-CSF proteins increased CD206 expression on monocytes treated with CSC-N at similar levels to those with CSC-R, and abrogated the suppressive effect of IRF5 siRNA in priming M2-like macrophages by CSC-R (Figure 4G). Collectively, these findings demonstrated that IRF-5-M-CSF pathway specifically regulated by CSC-R plays a critical role in priming immunoregulatory myeloid cells.

Colon CSCs have been defined as the cells expressing multiple cell-surface markers other than CD44, such as CD133 and CD166 (16). We found that the frequencies of CD133+CD44+ populations were similar on naïve tumor cells and their chemoresistant variants. Furthermore, CD44+CD133+HCT116-CSC-R displayed higher expression of IRF-5, M-CSF production and
M2-macrophage differentiation, compared to CD44+CD133+ CSC-N, further validating the importance of IRF5-M-CSF pathway in defining CSC-R to activate immunosuppressive myeloid cells (Supplementary Figure 5). Interestingly, M-CSF was detected in the CSC-N from HCT116 cells, unlike those from MDA-MB231 cells. However, M-CSF was produced from HCT-116-derived CSC-N by an IRF5-independent manner, since the knockdown of IRF5 had a little impact on basal levels of M-CSF in HCT116-CSC-N (Supplemental Figure 5). Therefore, the baseline levels of M-CSF were valuable among different cell lines, but chemoresistance conferred CSCs capable of selectively utilizing IRF5 for M-CSF production.

Taken together, these results identified IRF5 as a critical transcriptional factor in CSC-R that suppresses the induction of tumorigenic and immunosuppressive macrophages by promoting M-CSF production.

**CSC-R-derived IRF5 renders myeloid cells competent to promote tumorigenicity and chemoresistance**

Several studies have revealed that IRF5 plays an antitumor role through the induction of cell death programs by cell-intrinsic and immune mediated mechanisms (17-19). Our findings that IRF5 is selectively activated in CSC-R suggest that this transcription factor may potentially have unique functions distinct from those it has in chemosensitive tumors and immune cells. To
define the functional significance of IRF5-regulated CSC-R/macrophage cross-talk, bulk HCT116 colon cancer cells were treated with supernatants from human macrophages pre-stimulated with CSC-R or IRF5-KD CSC-R, and then subjected to *in vitro* chemotherapy to induce apoptosis. The supernatant from macrophages primed with CSC-R suppressed apoptosis. In contrast, supernatant from macrophages stimulated by IRF-5 KD CSC-R sensitized bulk HCT116 tumor cells to apoptotic cell death upon CDDP treatment at levels comparable to untreated cells (Figure 5A). The importance of IRF5-regulated pathways in CSC/macrophage interaction was further confirmed using MDA-MB-231 breast cancer cells (data not shown).

We next evaluated the role of IRF5 in long-term tumor sphere forming activity, which is a common characteristic of CSCs. The supernatant of CSC-R-primed macrophages increased sphere numbers and diameters in bulk MDA-MB231 cells, but knockdown of IRF5 in CSC-R decreased the macrophage-mediated sphere forming activities of MDA-MB231 cells (Figure 5B). In addition, treatment with anti-CSF1 receptor neutralizing antibodies completely suppressed sphere formation in HCT-116 cells primed by either control CSC-R or IRF5-KD CSC-R, suggesting that M-CSF produced by CSC-R-stimulated macrophages plays a critical role in inducing the stem cell activities of bulk tumor cells (Figure 5B). Although the supernatants of CSC-N had a little ability in promoting sphere formation of bulk MDA-MB231 cells, they support the sphere formation in the presence of recombinant M-CSF at similar extent.
with the CSC-R supernatants (Supplemental Figure 6). These results further support our hypothesis that IRF5-mediated M-CSF of CSC-R plays an indispensable role in activating tumorigenic activities of bulk tumor cells by triggering CSC properties.

To further define the contribution of CSC-R-specific IRF5 to the regulation of in vivo tumorigenic activities in clinically relevant settings, we utilized clinical samples of patient refractory to anticancer therapies after extensive intervention by multiple rounds of chemotherapy and EGFR-TKI (gefitinib). The CD14^+ monocytes isolated from peripheral blood (PBM) of these patients were co-cultured with EpCAM^+CD133^+ autologous CSCs transfected with control or IRF5 siRNA for 48 h. Interestingly, the CSC marker ALDH1 expression were substantially increased in bulk primary NSCLC cells stimulated by the CSC-primed monocytes, whereas CSC-primed monocytes had little effects on ALDH1 expression in the CSC-depleted bulk tumor cells (Figure 5C). These results suggest that CSC-R-activated myeloid cells specifically expand the resident populations of CSCs, but they did not undergo the trans-differentiation from non-CSC to CSCs or increased tumorigenicity by CSC-independent manner.

We next evaluated whether IRF-5-M-CSF axis in CSCs promote in vivo tumorigenicity by utilizing these primary NSCLC tumors. To do so, the bulk tumor populations were injected subcutaneously into NOD-SCID mice in low numbers (1 x 10^2 / mice) in conjunction with the CSC-primed monocytes, and the in vivo tumor formations were evaluated at the indicated times.
To exclude the involvement of endogenous macrophages, NOD-SCID mice were pretreated with clodronate liposomes to remove endogenous macrophages before all procedures. Co-transfer of CSC-primed monocytes resulted in larger tumor formation compared to those of non-CSC-primed monocytes or untreated tumor cells. Importantly, tumor growth was markedly suppressed by adoptive transfer of CSC-IRF5 KD-primed monocytes (Figure 5D). Overall, these findings provide clear evidence that IRF-5-mediated regulation of CSC-myeloid cell interactions serves as a critical pathway supporting tumorigenicity and chemoresistance.

**Clinical significance of IRF5-M-CSF pathways**

Finally, we sought to determine whether our observations could be verified in the clinically relevant settings. To do so, we obtained pleural effusion cells from patients with stage IV non-small cell lung cancer (NSCLC) to check the mRNA levels of IRF5 and M-CSF in EpCAM⁺ epithelial cells. We also analyzed total numbers of CSF1 receptor (CSF1R)-positive macrophages in NSCLC tumors, which have been known as tumorigenic and immunosuppressive subsets in human cancers (20, 21). We found that mRNA levels of IRF5 were highly correlated with those of M-CSF in tumor tissues in patients with NSCLC (Figure 6). Moreover, there were positive relationships between the number of CSF1R⁺CD68⁺ macrophages and levels of IRF5 or M-CSF in NSCLC tumors, whereas the activation of IRF5-M-CSF
pathways were not correlated with total numbers of CD68+ macrophages (Figure 6). Finally, the expression levels of CSC maker ALDH1 mRNA was also correlated with the those of IRF5, M-CSF, as well as CSF1R+ cell numbers (Figure 6). Together, these findings support the notion that IRF5/M-CSF pathway positively regulates tumorigenic myeloid cells and CSC activities in cancer patients.
Discussion

Although intrinsic genetic and epigenetic alterations serve as a mainstay leading to generalized chemoresistance in transformed cells, it remains largely unclear whether these chemoresistant phenotypes further modulate the biological properties of specialized subsets of tumorigenic populations. In particular, CSCs have emerged as the main tumor-initiating and propagating cells and are responsible for acquiring chemoresistance through multidrug transporter activities and cellular quiescence. Therefore, it is critical to clarify whether altered drug sensitivities modulate other functional properties of CSCs. Here we provide the first evidence that CSCs isolated from therapy-resistant tumors have unique tumorigenic properties compared to those from untreated counterparts. CSC-R promoted M-CSF production through an IRF5-dependent mechanism, and IRF5-mediated M-CSF further facilitated the tumorigenicity of bulk tumor cells by activating tumorigenic myeloid cells. These findings offer a major advance in understanding the molecular machineries whereby responses to anticancer therapeutics serve as a critical factor in regulating the functional plasticity of CSCs and may greatly impact prognosis and therapeutic responses in cancer patients.

IRF5 has been considered a tumor suppressive factor that activates apoptosis-related signaling pathways in transformed cells and creates antitumor inflammatory microenvironments through immune cells (17-19). Moreover, deletions of chromosome 7q32 are associated with
down-regulation of IRF5 function, disease progression and poor prognosis in patients with marginal-zone lymphoma. Genetic polymorphisms of IRF5 may serve as biomarkers to predict clinical responses to immunotherapy and chemotherapy in patients with melanoma and hematological malignancies, respectively (22-24).

Previous study revealed that IRF5 serves as a lineage marker for M1-type macrophages, and macrophages promote the generation of immunogenic Th1 or Th17 subsets by an IRF5-dependent manner (25). In marked contrast, we demonstrate that IRF5 expressed on chemoresistant CSCs contributes to the differentiation of M2-like macrophages by M-CAS-mediated mechanisms. Thus, transcription factors that have been known as a proinflammatory mediator might be changed to an immune suppressor under specific conditions where therapeutic interventions could reconstruct the complex genetic and epigenetic networks in tumor cells. Moreover, these “transformation” of cross-talks between tumor cells and immune cells may dramatically change phenotypic properties and biological actions of identical genes and their pathways. Furthermore, chemoresistance may modify the tumor microenvironments and create a specialized chemoresistant niche that further amplifies the tumorigenic and therapy-resistant behaviors of tumor cells (1-4). Thus, it is essential to re-examine the molecular and functional dynamics of various regulators identified as “tumor suppressors” according to therapeutic interventions.
We demonstrated that IRF5-dependent induction of M-CSF is a key step in the generation of

tumorigenic myeloid cells by CSC-R. Although the molecular mechanisms by which CSC-R
regulate M-CSF production remain to be defined, several transcription factors, such as PU.1,
C/EBP-α and NF-κB, may coordinate with IRF5 to regulate specific sets of cytokines and
growth factors. Moreover, PU.1 associates with various types of IRFs to amplify its
transcriptional activities, which greatly modifies the immunological functions of myeloid cells
in multiple ways (26-29). Thus, it is tempting to speculate that IRF5 may cooperate with various
myeloid cell differentiation factors in CSC-R and that such cooperation may be critically
involved in the genetic and epigenetic control of M-CSF expression.

We also found that CSC-R have unique propensities to activate inflammatory signals thus
inducing multiple sets of cytokines and chemokines. Among them, TNF-α, IL-6 and IL-8 have
emerged as tumorigenic mediators that link various inflammatory signals with oncogenic
cascades to create tumorigenic microenvironments (30-32). In addition, CCL2 supports the
recruitment of CCR2+ inflammatory monocytes into tumor tissues, which serves as a key step in
generating tumorigenic macrophages (33, 34). Thus, it is important to address how the distinct
and coordinated actions of these inflammatory mediators impact the tumorigenicity and
anticancer drug responses of CSCs within the privileged tumor microenvironments.

In conclusion, we provide a novel pathway whereby responsiveness to anticancer drugs
determines the plasticity and biologic properties of CSCs in part through the paracrine regulation of tumor-associated non-transformed cells. Comprehensive analysis of CSC-niche interactions upon exposure to various types of anticancer agents should clarify the molecular and cellular pathways that govern CSC functions and reveal new therapeutic options in clinically relevant settings.
References


Figure Legends

Figure 1 Phenotypic and functional analysis of CSC-R

(A) Isolation of CSC populations from chemoresistant (CSC-R) or untreated MDA-MB-231 breast cancer cells or HCT-116 colon cancer cells (CSC-N). The cell viability of CSC-R and CSC-N was assessed upon cytotoxic chemotherapy treatment (taxane for MDA-MB-231 and cisplatin for HCT-116). (B) CSC-R or CSC-N from MDA-MB-231 or HCT-116 cells were treated with cytotoxic drugs (CTX) (taxane for MDA-MB-231 and cisplatin for HCT-116) for 24 h, and the ALDH1⁺CD44⁺ CSC frequencies in bulk tumor cells, as well as expression of ALDH1 or CD44 was assessed by flow cytometry. (C) The CSC-R, CSC-N and their non-CSC counterparts from MDA-MB-231 and HCT116 cells were cultured in serum-free medium supplemented with EGF and bFGF in ultra-low attachment culture dishes for three passages, and the numbers of formed spheres generated per 1,000 cells were determined. We repeated three experiments with all similar results.

Figure 2 Proinflammatory profiles of CSC-R

(A) The mRNA levels of cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p40, TNF-α, TGF-β, GM-CSF, M-CSF, IFN-α, IFN-γ) or chemokines (CCL2, CXCL19, CXCL20) in CSC-R or CSC-N from MDA-MB-231 were quantified by RT-PCR. (B) The protein levels of M-CSF were
measured in culture supernatants from bulk cells, CSC or non-CSC subsets of chemoresistant and treatment-naïve MDA-MB-231 cells. (C) CSC-R and CSC-N (MDA-MB-231 or HCT-116) were transfected with a p-NF-κB reporter plasmid in the presence or absence of cytotoxic agents (taxane for MDA-MB-231, CDDP for HCT-116), and luciferase assays were performed to measure NF-κB activities in cell lysates. We repeated five experiments with all similar results.

**Figure 3 CSC-R contribute to tumorigenic myeloid cell differentiation**

(A) CD14+ monocytes were isolated from peripheral blood leukocytes (PBL) and untreated (-) or treated for 48 h with 20 % culture supernatant of CSC-R or CSC-N from MDA-MB-231 cells. The mRNA levels of genes associated with polarization of immunoregulatory macrophages (Arginase-I (ARG-I), colony-stimulating factor-1 receptor (CSF1R), IL-4 receptor (IL-4R), Relm-α, and IL-10) or with myeloid lineage differentiation (C/EBPα and PU.1) were measured by quantitative RT-PCR. Similar results were obtained from three experiments, and the means ± SEM are shown. (B) The mRNA levels of genes associated with polarization of immunostimulatory macrophages (IL-12p70, CXCL9, IFN-γ) were measured by quantitative RT-PCR. (C) CD14+ monocytes were isolated from peripheral blood leukocytes (PBL) and treated for 48 h with recombinant M-CSF (100 ng/mL) or 20 % culture supernatant of Bulk cells, CSCs or non-CSC subsets of chemoresistant or treatment-naïve MDA-MB231 cells. The
expression levels of CD206 and CD68 were assessed by flow cytometry. Representative data (left panel) and statistical analysis are shown. We repeated four experiments with all similar results. (D) CSC-N or CSC-R (1 x10^7/mice) were inoculated into clodronate-pretreated NOD-SCID mice (n=3 per group) with or without CD14^+ monocytes obtained from healthy donor’s peripheral blood (1 x10^6/mice). The growth curves of each tumor are shown. (E) Total number (x10^3), as well as the frequencies (%) of CD68^+ total macrophages, CD163^+CD206^+ M2 macrophages or CD163^lowHLA-DR^high M1 macrophages infiltrating into tumor tissues was evaluated by flow cytometry.

**Figure 4 Selective activation of IRF5 in CSC-R**

(A) CSC-R and CSC-N (MDA-MB-231 or HCT-116) were transfected with p-ISRE or p-GAS reporter plasmids in the presence or absence of cytotoxic agents (taxane for MDA-MB-231, CDDP for HCT-116), and luciferase assays were used to measure the transcriptional activities using cell lysates. (B) CSC-R and CSC-N (HCT-116) were transfected with control siRNA or siRNA for IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8 and IRF9 in the presence of a p-ISRE reporter plasmid for 48 h, and then treated with CDDP for 24 h. Luciferase assays were performed to measure transcriptional activities using the cell lysates. (C, D) The mRNA (C) or protein levels (D) of IRF5 in CSC, non-CSC or their bulk populations of chemoresistant or
treatment-naïve cells were evaluated by RT-PCR or western blot, respectively. (E) CSC-R, CSC-N or their non-CSC counterparts were transfected with control or IRF5 siRNA for 48 h. The protein levels of M-CSF in the culture media were measured. (F) CSC-R or CSC-N was transfected with control or IRF5 siRNA for 48 h. In addition, M-CSF (10μg/mL) was added to the IRF5 siRNA-transfected cells. The mRNA levels of CSF1R, Arginase-I (ArgI) or Relm-α in macrophages primed with CSCs were measured by RT-PCR. (G) CSC-R, CSC-N or bulk tumor cells (HCT-116) were transfected with control or IRF5 siRNA for 48 h and then treated with anti-M-CSF neutralizing Ab (clone 26730), control Ig or recombinant M-CSF for 24 h. The 20 % supernatant of each tumor subsets were used for treating with CD14+ monocytes isolated from peripheral blood leukocytes (PBL) for 48 h. The CD206 expression on CD68+ macrophages were determined by flow cytometry. We repeated three experiments with all similar results.

**Figure 5 CSC-R-derived IRF5 supports myeloid cell-mediated tumorigenic activities**

(A) CSC-R (MDA-MB-231) transfected with control or IRF5 siRNA were co-cultured with monocytes obtained from PBL at 1:5 ratios for 96 h. The CD68+ myeloid cells were isolated from the co-cultured cells and cultured for 24 h to obtain supernatants. Bulk MDA-MB-231 cells were untreated (-) or treated with 20 % supernatants of myeloid cells primed with control
or IRF5-KD CSC-R in the presence of CDDP (20 μg/mL) for 24 h. The cell viability was analyzed by quantifying annexin-V/PI-positive cells. (B) The supernatants of myeloid cells primed with CSC-R (MDA-MB-231) were obtained as described above. Bulk MDA-MB-231 cells were untreated (-) or treated with 20 % supernatants of the CSC-R-primed myeloid cells with control Ig or anti-CSF1 receptor neutralizing Ab (10 μg/mL) in ultra-low attachment plates, and the numbers of formed spheres generated per 1,000 cells were determined. (C) The CSC or non-CSC obtained from chemoresistant NSCLC cells were transfected with control or IRF5 siRNA (IRF5i) for 48 h. The bulk cells (Bulk) or those depleted of CSCs (Bulk(CSC-)) were untreated (-) or stimulated with 20 % supernatants of the monocytes primed by the supernatant of myeloid cells primed with CSC (Monocytes (CSC)) or non-CSC (Monocytes (non-CSC)) for 24h. The expression of CSC markers ALDH1 was evaluated by ALDEFLOUR assays. (D) EpCAM^+CD133^+ CSCs or non-CSCs obtained from NSCLC patients were transfected with control or IRF5 siRNA for 48 h, and then co-cultured with autologous CD14^+ monocytes. The CSC or non-CSC-primed monocytes were isolated from the co-cultured cells and used for intravenous transfer into clodronate-pretreated NOD-SCID mice (n=4 per group) with subcutaneous injections of autologous bulk NSCLC tumors. The tumor growth was measured on the indicated days. The experimental procedures (upper panel) and tumor growth curve (lower panel) are shown. Similar results were obtained in three independent experiments.
Figure 6. Clinical significance of IRF5/M-CSF regulation of tumorigenic myeloid cells

The CD45⁺ immune or EpCAM⁺ epithelial cell suspensions were isolated from bulk populations of pleural effusions obtained from stage IV NSCLC patients (n=12). The mRNA levels of IRF5, M-CSF and ALDH1 were quantified by RT-PCR and total frequencies of CD68⁺ or CSF1R (CD115)⁺CD68⁺ macrophages were evaluated by flow cytometry, and the correlation coefficients of each parameter were analyzed.
Figure 1

A. Isolation of CSCs
- MDA-MB231: CD44^{high}CD24^{low}
- HCT116: CD44^+

CSC-N

CSC-R

Generation of chemoresistant variant
- MDA-MB231 → Taxane
- HCT116 → Cisplatin

B.

<table>
<thead>
<tr>
<th>ALDH1+CD44+</th>
<th>ALDH1</th>
<th>CD44</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB231</td>
<td>CSC-N</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>CSC-R</td>
<td>ns</td>
</tr>
<tr>
<td>HCT116</td>
<td>CSC-N</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>CSC-R</td>
<td>ns</td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>CSC-N</th>
<th>CSC-R</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Non-CSC-N</th>
<th>Non-CSC-R</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>MDA-MB231</th>
<th>HCT116</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSC-R</td>
<td>ns</td>
</tr>
<tr>
<td>non-CSC-R</td>
<td>ns</td>
</tr>
<tr>
<td>non-CSC-N</td>
<td>ns</td>
</tr>
</tbody>
</table>
Figure 5

A

Monocytes (CSC-R)

(-)

Control siRNA

IRF-5i

CDDP

PI

Annexin-V

Annexin-V cells (%)

B

Monocytes (CSC-N)

Monocytes (CSC-R)

Control Ig

αCSF1R

Control siRNA

IRF-5i

Sphere number (x 10^3)

Tumor volume (mm^3)

Control

IRF-5i

Days

C

Bulk

Bulk

(CSC-)

ALDH1+ cells (%)

Bulk

(CSC-)

ALDH1

Bulk

(CSC-)

ns

(-)

control

IRF5i

control

IRF5i
Cancer stem-like cells derived from chemoresistant tumors have a unique capacity to prime tumorigenic myeloid cells

Tsunaki Yamashina, Muhammad Baghdadi, Akihiro Yoneda, et al.

_Cancer Res_ Published OnlineFirst March 17, 2014.

**Updated version**  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-2169

**Supplementary Material**  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/03/17/0008-5472.CAN-13-2169.DC1

**Author Manuscript**  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

---

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.