Cancer Stem-like Cells Derived from Chemoresistant Tumors Have a Unique Capacity to Prime Tumorigenic Myeloid Cells

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Abstract

Resistance to anticancer therapeutics greatly affects 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. Furthermore, we identified the IFN-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 IRF5/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 affects the properties of CSCs in their niche microenvironments. Cancer Res; 74(10): 2698–709. ©2014 AACR.

Introduction

Resistance to anticancer modalities poses serious obstacles that must be addressed to improve the clinical prognosis for patients with cancer. 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 immunologic remodeling within tumor tissues (1–4). Coordinated intrinsic and extrinsic pressures force tumor cells to accommodate to stressful microenvironments and coopt 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 (CSC) are indispensable as a main source of tumorigenicity and anticancer drug resistance (5). Although it remains largely unclear whether chemoresistance further modifies the phenotypic and functional manifestations of CSC, previous studies have revealed that the low sensitivity to cytotoxic therapies of CSC 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. IFN 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 biologic character of CSCs in distinct niche microenvironments.

Materials and Methods

Mice and tumor cell lines

NOD–SCID (nonobese diabetic/severe combined immunodeficient) animals were purchased from Charles River Laboratories. All experiments were conducted under a protocol approved by the animal care committees of Hokkaido University (Sapporo, Japan).

Tumor cell lines (MDA-MB-231 and HCT116) were obtained from the American Tissue Culture Collection. All cell lines described above were obtained 1 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 cancers (NSCLC) 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 (1 μg/mL) for 96 hours, 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 antibody (BD Biosciences) 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-CD14 antibody (BD Biosciences) and ALDEFOUR reagents (VERITAS), 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.

**Sphere-forming assay**

For sphere-forming assays, CSC-R, CSC-N, or bulk MDA-MB-231 cells primed with myeloid cells (1,000 cells/well) were cultured in ultra-low attachment culture dishes (Corning) in serum-free medium. Dulbecco’s Modified Eagle Medium/F-12 serum-free medium was supplemented with 20 ng/mL epithelial growth factor and 10 ng/mL basic fibroblast growth factor (bFGF)-2 (PeproTech). Digestion 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 × 10^6/well at start point) were cultured for 48 hours, 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 interleukin (IL)-1β, 4 pg/mL; IL-6, 2 pg/mL; IL-8, 2 pg/mL; IL-10, 7.8 pg/mL; IL-12 p40, 15 pg/mL; TNF-α, 15 pg/mL; TGF-β, 19 pg/mL; GM-CSF (granulocyte macrophage colony-stimulating factor), 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 and CCL20, 7.8 pg/mL.

**Quantification of cytokine mRNA by real-time PCR**

The mRNA was isolated from CSC-R, CSC-N or these cells (colony-stimulating factor-1 receptor), C/EBPα, and PU.1] or polarization (AGB-1, Relm-α, IL-4R) or effector functions (IL-10, IL-12p70, CXCL9, IFN-γ) were quantified by real-time PCR (RT-PCR) using SYBR Green Gene Expression Assays (Applied Biosystems).

**Luciferase reporter assay for NF-κB and IFN-stimulated response element**

CSC-R or CSC-N from MDA-MB-231 or HCT116 was treated with taxane or CDDP, respectively, and transfected with control or IRF5 siRNA (5′GrArCrUrCrUrGUUrCrArArATT3′) for 24 hours. The cells were then transiently transfected with firefly luciferase reporter plasmid encoding NF-κB or IFN-stimulated response element (ISRE) and control Renilla luciferase plasmid (1 ng) for 20 hours. 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-MB-231 or HCT116 was subjected to Western blotting to quantify the protein levels of IRF5 by using antibodies for human IRF5 antibody (Cell Signaling Technology). β-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 hours, and then cocultured with CD14^+ monocytes for 96 hours. The myeloid cells primed with CSC-R were isolated from cocultured cells and further cultured for 24 hours to obtain cancer 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 (200 μg/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 × 10^6/mice) in conjunction with intravenous administration of CD68+ macrophage (1 × 10^6/mice) isolated from the peripheral blood of the healthy donor. The tumor growth was measured on the indicated days, and numbers of human macrophages were evaluated in tumors from each mouse.

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

EpCAM^+ CD133^+ CSCs obtained from primary patients with NSCLC were transfected with control or IRF5 siRNA for 48 hours, and then cocultured with CD14^+ monocytes isolated from...
autologous peripheral blood leukocytes (PBL) for 96 hours, CD11b+ myeloid cells were isolated from the cocultured cells for in vitro tumor growth assays. For the in vivo primary tumor experiments, bulk NSCLC tumor cells (1 × 107/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. A P value of <0.05 was considered as statistically significant. *, P < 0.05; **, P < 0.01; ns, not significant.

Results
Characterization of CSCs 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 biologic alterations linked with chemoresistance further modulate the tumorigenic activities of CSCs remains largely unknown. To define this, we generated chemoresistant variants of MDA-MB-231 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 (Fig. 1A). CSCs derived from chemoresistant variants (CSC-R) were refractory to cytototoxic therapies compared with 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 (Fig. 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 (Fig. 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 Fig. S1). Thus, chemoresistance does not render non-CSC with plasticity to undergo transdifferentiate 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 CSCs 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 with 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 (Fig. 2A and Supplementary Fig. S2A). We also confirmed the higher protein levels of several cytokines (M-CSF, IL-1β, IL-6, and TNFα) in CSC-R compared with bulk or non-CSC populations (Fig. 2B and data not shown). Consistent with its proinflammatory activities, the transcriptional activity of NF-xB was higher in CSC-R compared with CSC-N (Fig. 2C). We observed similar trends in CSC-R from HCT-116 (Supplementary Fig. S2A). Furthermore, the chemotherapy-naïve CSCs from MDA-MB-231 cells did not produce proinflammatory 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 Fig. S3A). 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, because myeloid cells have a tremendous impact on the regulation of tumorigenic activities and anticancer drug resistance (12, 13). Macrophages express several immunomodulatory 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-I, CSF1R, IL-4R, Relm-α and IL-10, C/EBPα, and PU.1 (Fig. 3A and Supplementary Fig. S2B). 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 (Fig. 3B). Furthermore, the treatment with CSC-R–derived soluble factors upregulated the M2 macrophage marker CD206 mannose receptor at comparable levels with the treatment with M-CSF. However, bulk chemoresistant cells could generate M2 macrophages at higher levels than CSC-N or treatment-naïve tumor counterparts (Fig. 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 with nontreatment control (Fig. 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-MB-231 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 Fig. S3B).

The generation of therapy-resistant cell lines was largely dependent on the numbers of treatment with low-dose chemotherapy (more than four times). Therefore, we next setup the experiments by using CSC-N of HCT116 cells treated with low-dose CDDP for 72 hours on various
treatment/passage cycles. We found that the CSC-N gene-rate M-CSF and prime M2 macrophages in concomitant with acquisition of chemoresistant phenotype (Supplementary Fig. S3C and S3D). To further address whether CSC-R differs from CSC-N in their unique activities of priming M2-macro-

phages 
in vivo 
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CSC-N or CSC-R isolated from HCT-116 cells were injected subcutaneously into NOD–SCID mice at small doses (1×10²/mice) in conjunction with intravenous admin-

istration of CD14⁺ monocytes (1×10⁶/mice) isolated from 

the peripheral blood of the healthy donor, and the tumor formations were evaluated 
in vivo 
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In this experiment, NOD–SCID mice were pretreated with clodronate liposome to remove endogenous macrophages. Although the CSC-R--driv-

en tumors were grown at greater levels than CSC-N tumors, their tumorigenicity was positively correlated with the pres-

ence of myeloid cells, because its tumor growth was accel-

erated by adoptive transfer of human monocytes. In marked contrast, the transfer of human monocytes had little effects 
on the CSC-N--derived tumor growth (Fig. 3D). More impor-

tantly, 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 (Fig. 3E). In contrast, the frequencies of CD163→HLA-DRhigh M1 macrophages were comparable in tumors from CSC-R and CSC-N (Fig. 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⁺HLA-DRlow myeloid-derived suppressor cells (MDSC) at higher levels than those of CSC-N or non-CSC counterparts. In addition, the induced MDSCs were homogeneously CD14⁺CD15⁻ monocyctic subsets when treated with either CSC or non-CSCs from chemoresistant or treatment-

naive 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 cytotxic 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. * , P < 0.05.

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

In addition to NF-κB--mediated inflammatory signals, tran-

scriptional activities mediated by ISREs were detected in CSC-

R at much higher levels than those in CSC-N (Fig. 4A). In con-

trast, type I IFN and transcriptional activities of IFN-γ activation sequences (GAS) were detected in CSC-R at levels 
similar to those in CSC-N upon chemotheraphy (Fig. 4A).

Given that IFNs signal through Janus-activated kinase (JAK)/

STAT pathways to induce IFN stimulation genes, which are under control of ISRE and GAS elements, we hypothesized that
CSC-R have a unique ability to stimulate ISRE-mediated transcrip-
tional activities using autocrine IFN and/or JAK/STAT–
independent mechanisms (15). 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 used 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 hours. In
this analysis, we identified IRF5 as a critical factor, which is
specifically expressed in CSC-R and regulates ISRE activities.
The knockdown of IRF5, but not that of other IRF families,
diminished the transcriptional activities of ISRE in CSC-R (Fig.
4B). In contrast, ISRE activities remained unchanged in CSC-N
regardless to the inhibition of IRF members, including IRF5
(Fig. 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-MB-231 and HCT116 com-
pared with the CSC-N counterparts (Fig. 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 (Fig. 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 with those primed with control CSC-R, further indicating that IRF5-mediated M-CSF of CSC-R is critical for inducing M2 macrophage-associated factors (Fig. 4F).

The IRF5-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 antibody (Fig. 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 (Fig. 4G). Collectively, these findings demonstrated that the IRF5–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 CD105.
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 IRF5, M-CSF production, and M2 macrophage differentiation, compared with CD44+CD133+ CSC-N, further validating the importance of the IRF5–M-CSF pathway in defining CSC-R to initiate immunosuppressive myeloid cells (Supplementary Fig. S5). Interestingly, M-CSF was detected in the CSC-N from HCT116 cells, unlike those from MDA-MB-231 cells. However, M-CSF was produced from HCT-116–derived CSC-N by an IRF5-independent manner, because the knockdown of IRF5 had a little impact on basal levels of M-CSF in HCT116-CSC-N (Supplementary Fig. S5). Therefore, the baseline levels of M-CSF were valuable among different cell lines, but chemoresistance conferred CSCs capable of selectively using 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 prestimulated 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 IRF5-KD CSC-R sensitized bulk HCT116 tumor cells to apoptotic cell death upon CD95 treatment at levels comparable with untreated cells (Fig. 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-MB-231 cells, but knockdown of IRF5 in CSC-R decreased the macrophage-mediated sphere-forming activities of MDA-MB-231 cells (Fig. 5B). In addition, treatment with anti-CSF1R–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 (Fig. 5B). Although the supernatants of CSC-N had a little ability in promoting sphere formation of bulk MDA-MB-231 cells, they support the sphere formation in the presence of recombinant M-CSF at similar extent with the CSC-R supernatants (Supplementary Fig. S6).

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 used clinical samples of patient refractory to anticancer therapies after extensive intervention by multiple rounds of chemotherapy and EGFR–TKI (tyrosine kinase inhibitor; gefitinib). The CD14+ monocytes isolated from peripheral blood of these patients were cocultured with EpCAM+CD133+ autologous CSCs transfected with control or IRF5 siRNA for 48 hours. 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 (Fig. 5C). These results suggest that CSC–activated myeloid cells specifically expand the resident populations of CSCs, but they did not undergo the transdifferentiation from non-CSC to CSCs or increased tumorigenicity by CSC-independent manner.

We next evaluated whether the IRF5–M-CSF axis in CSCs promote *in vivo* tumorigenicity by using these primary NSCLC tumors. To do so, the bulk tumor populations were injected subcutaneously into NOD–SCID mice in low numbers (1 × 10^6/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. Cotransfer of CSC–primed monocytes resulted in larger tumor formation compared with 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 (Fig. 5D). Overall, these findings provide clear evidence that IRF5–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 NSCLC to check the mRNA levels of IRF5 and M-CSF in EpCAM+ epithelial cells. We also analyzed total numbers of 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 (Fig. 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 was not correlated with total numbers of CD68+ macrophages (Fig. 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 (Fig. 6). Together,
these findings support the notion that the IRF5/M-CSF pathway positively regulates tumorigenic myeloid cells and CSC activities in patients with cancer.

**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 biologic 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 with those from untreated counterparts. CSC-R promoted M-CSF production through an

![Figure 5](image-url)
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 affect prognosis and therapeutic responses in patients with cancer.

IRF5 has been considered a proinflammatory mediator but under specific conditions in which 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 biologic 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 reexamine IRF5's role in tumor biology.

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-CSF-mediated mechanisms. Thus, transcription factors that have been known as proinflammatory mediators might be changed to an immune suppressor under specific conditions in which therapeutic interventions could reconstruct the complex genetic and epigenetic networks in tumor cells.
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 immunologic 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 immunomediators affect 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 nontransformed 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Jinushi

Development of methodology: M. Jinushi

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References


