Intratumoral heterogeneity is thus generated by a combination of genetic and functional diversities and is consequently highly complex.

Normal tissues are constructed from heterogeneous cell types that are derived from tissue stem cells and they develop in a hierarchical manner. Such heterogeneity is determined by differential gene expression, which is itself under precise and programmed epigenetic control. Recent studies have suggested that tumors also show cellular hierarchy, with a subpopulation of cancer cells having a tumorigenic potential much greater than that of other cancer cells. This highly tumorigenic subpopulation of cells at the top of the hierarchy comprises CSCs and gives rise to progenitors and cells at various levels of differentiation along various lineages in a manner similar to that of normal tissue stem cells (Fig. 1a). Before the CSC theory became widely accepted, tumor heterogeneity was thought to result predominantly from the stochastic accumulation of genetic mutations. Both genetic and epigenetic mechanisms are now thought to contribute to tumor heterogeneity in a parallel manner.

Human CSCs were first identified in AML as the CD34+/CD38− cell subpopulation by transplantation into immunodeficient mice. Since then, several approaches have been adopted to distinguish CSCs from other cancer cells, including surface marker characterization, sphere formation assays, analysis of persistent tumorigenic potential after serial transplantation, and side-population detection. Flow cytometric analysis of surface markers has been applied to the detection of breast CSCs that are enriched within a cell subpopulation that is CD44+CD24−/CD024+. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Cancer Stem Cells and Intratumoral Heterogeneity

Heterogeneity of tumor tissue is highly associated with failure of conventional anticancer therapies. Tumors have been found to be composed of genetically distinct clones of cancer cells that arise in the face of selection pressure imposed by the tumor microenvironment. Even genetically homogeneous tumor cells show different patterns of gene expression. Intratumoral heterogeneity is thus generated by a combination of genetic and functional diversities and is consequently highly complex.

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Cancer stem cells possess the property of “robustness”, which refers to several biological characteristics including resistance to redox stress, the capacity to carry out rapid repair of damaged DNA, the ability to adapt to a hyperinflammatory or
Cancer stem cells possess both self-renewal ability and multilineage differentiation potential, leading to the composition of intratumoral heterogeneity (a). Cancer stem cells possess the property of “robustness,” which is established by a combination of various phenotypes (b). Cancer stem cells are more resistant to various therapeutic interventions, leading to the generation of minimal residual disease (MRD) that is mainly composed by CSCs, and MRD is a major cause of recurrence and metastasis (c). ABC, ATP-binding cassette.

The Niche, a Favorable Microenvironment for CSCs to Maintain their Stemness

Normal tissue stem cells are located within or adjacent to a microenvironment, known as the “niche,” that is favorable for the maintenance of their stemness. Niches are composed of various cell types as well as ECM, cytokines, and growth factors released by the niche cells. For instance, Paneth cells located in intestinal crypts and melanocyte stem cells located in the bulge area of hair follicles form niches for normal intestinal stem cells and hair follicle stem cells, respectively. Cancer stem cells have also been shown to possess niches whose components include endothelial cells, osteoblasts, and ECM molecules composed of osteopontin and hyaluronic acid. In addition, cancer-associated fibroblasts, tumor-assoc-

iated macrophages, undifferentiated mesenchymal stem cells, and immune cells in the tumor stroma serve as niches for CSCs by providing growth factors such as transforming growth factor-β, epidermal growth factor, and hepatocyte growth factor as well as pro-inflammatory cytokines such as tumor necrosis factor-α and various interleukins including IL-1β and IL-6. The inflammatory microenvironment is beneficial for cancer cells in that it results in activation of the NF-κB signaling pathway. The cytokine network not only promotes tumor development but also maintains CSC characteristics that underlie tumor metastasis and recurrence.

Accumulating evidence thus supports the importance of a cellular niche for maintenance of the stem cell pool. Lineage tracing has suggested that Paneth cells are required for the support not only of Lgr5-expressing normal stem cells in the intestine but also of APC-mutant adenoma-initiating cells. Furthermore, melanocytic stem cells localized to the secretory portion of sweat glands in the volar skin are responsible for the development of acral melanoma associated with amplification of the driver oncogene CCND1, which encodes cyclin D1. These findings suggest that some CSCs are derived from normal tissue stem cells and share their niche.

Glioma stem cells reside in contact with endothelial cells, in what is referred to as a perivascular niche. On the other hand, a hypoxic or perinecrotic microenvironment has been found to be advantageous for the survival and proliferation of other types of CSCs. Both HIF1 and HIF2α are activated under hypoxic conditions and promote the stem-like properties of cancer cells. Furthermore, HIF2α was recently shown to contribute in a cooperative manner with the intracellular domain of CD44 generated by γ-secretase to the acquisition of radiosensitivity by glioma stem cells in a perivascular niche rich in osteopontin.

The concept of the niche is important in terms of the “seed and soil” theory of tumor dissemination proposed by Paget, which stipulates that the distant metastasis of cancer cells is dependent on the site of the primary tumor. Such organ-selective tumor metastasis is thought to be established by homing of CSCs present among CTCs to a premetastatic niche.
Dissociation
the identification of CSCs clinically
microenvironment. This plasticity of CSCs is thought to hinder
makes them possible to show hyperadaptation to the tumor
more, CXCL12, an osteoblast-secreted cytokine, interacts with
its receptor CXCR4, located on the prostate cancer cell sur-
mary tumor site.(46) Circulating tumor cells can colonize their
dissemination, seeding not only distant sites but also their pri-
tation for expansion of the primary tumor as well as establish-
universality and factors for CTCs. Such cancer
original tumors of origin because the primary lesion provides a
antibodies to CXCR4 were found to be effective for prevention
promote CSC survival and proliferation. Indeed, neutralizing
metastasis of tumor cells occurs by way of several distinct steps: dissociation of cancer cells from the primary tumor after they have undergone epitelial-mesenchymal transition (1) is followed by their intravasation (2), circulation in the blood as CTCs (3), extravasation (4), and homing to the premetastatic niche and colonization of the metastatic site (5). Organ-selective tumor metastasis may depend on whether the premetastatic niche is a favorable microenvironment for circulating cancer stem cells.

Molecular Mechanisms Underlying Plasticity of CSCs

Originally, CSCs were defined as a static entity and thought to stably locate at the top of the hierarchy among tumor cells. However, it is becoming more acceptable that CSCs undergo dynamic and reversible changes depending on the surrounding microenvironment. This is referred to as the dynamic stemness model. Epigenetic changes induced by various factors including chronic inflammation, excessive redox stress, and hypoxic stimuli enhance the plasticity of the transition between CSCs and non-CSCs. Indeed, the dual nature of CSCs makes them possible to show hyperadaptation to the tumor microenvironment. This plasticity of CSCs is thought to hinder the identification of CSCs clinically in vivo.

Importantly, a treatment-induced transient decrease in the extent of cancer heterogeneity has been found to reflect enrichment of CSCs. Such CSC enrichment is caused by not only selection of therapeutic resistant CSCs but also induction of CSC properties in non-CSCs. For example, long-term treatment with vemurafenib, a drug targeted to the V600E active mutant form of the protein kinase BRAF, upregulates expression in melanoma of the histone demethylase Jumonji AT-rich interactive domain 1B protein, an enzyme that is highly expressed in slow-cycling melanoma cells with stem-like properties. Cancer stem cells are generally slow-cycling or dormant under unfavorable conditions, which is why melanoma CSCs exposed to vemurafenib lose their addiction to onco-

Cross-talk between signaling pathways and reversible epige-
netic changes are thus thought to give rise to adaptive resis-
tance to exogenous stress or anticancer treatment. It is there-
fore expected that combination therapies with two or more molecularly targeted drugs will be required for eradica-
tion of cancer.

Furthermore, it has recently been reported that relatively dif-
fferentiated progenitor cells outside of the intestinal crypt niche are responsible for intestinal tumorigenesis through the activation of Wnt or NF-xB signal transduction. Given that Paneth cells correspond to the “cellular niche” for Lgr5-positive intestinal stem cells which have been shown to form adenoma by cell-lineage tracing analysis. Remarkably, aberrant expression of the bone morphogenetic antagonist leads to altering cell fate determination by promoting the dedifferentiation of Lgr5-negative progenitor cells. It is also notable that the constitutive activation of Wnt or the NF-xB signaling pathway leads to emergence of CSCs derived from non-stem cells in normal tissue. These reports strongly suggest that the origin of CSCs is not necessarily a normal tissue stem cell.

Novel CSC-Targeted Therapies: Targeting of CSC Quiescence

The dormant status of CSCs has long been thought to reduce their susceptibility to chemotherapy. Mitotic inhibitors such as paclitaxel and vincristine preferentially kill proliferating cancer cells during M phase of the cell cycle. Antimitabolite drugs...
such as 5-fluorouracil, 6-mercaptopurine, and methotrexate damage cancer cells during S phase. Topoisomerase inhibitors such as irinotecan (CPT-11) and etoposide (VP-16) interfere with the separation of DNA strands during DNA replication and transcription. These agents, however, exhibit antitumor effects only when tumor cells are under proliferative conditions. By striking contrast, CSCs in the quiescent state (G₀ phase of the cell cycle) are thus refractory to such conventional antitumor drugs whose action is dependent on operation of the cell cycle.

The cell cycle status of CSCs is determined by many factors. Endogenous cyclin-dependent kinase inhibitors and Fbw7 can contribute to the cell cycle delay or arrest manifest in CSCs. Fbw7 is a component of E3 ubiquitin ligase and promotes the ubiquitin-proteasome-dependent degradation of several proto-oncogene products such as c-Myc, cyclin E, Notch, and JUN. Thus, Fbw7 inactivation triggers “awakening” of quiescent CSCs in the niche (Fig. 3). A recent study uncovered an important role for the cyclin-dependent kinase inhibitor p57, which is expressed at a high level in HSCs, in the maintenance of quiescence in these cells. Ablation of p57 in HSCs was thus found to induce the aberrant proliferation of these cells in bone marrow and consequent HSC exhaustion.

Fbw7 has also attracted much attention as a potential novel target for CSC elimination. This protein is a subunit of a ubiquitin ligase responsible for the degradation of the proto-oncoprotein c-Myc. In addition to the stabilization of c-Myc, Fbw7 shows antitumor function through regulation of the NOTCH1–CCL2 axis in tumor stroma.

Many patients with CML treated with imatinib, a drug targeted to the oncogenic fusion protein produced by the Philadelphia chromosome, eventually develop acquired resistance to this tyrosine kinase inhibitor. Chronic myeloid leukemia stem cells that are resistant to this agent as a result of their quiescence, and which are responsible for MRD, express Fbw7 at a high level, which results in the degradation of c-Myc by the ubiquitin–proteasome system and cell cycle arrest. Ablation of Fbw7 in imatinib-resistant CML cells was found to markedly enhance the antitumor effect of this drug in mice, with the loss of Fbw7 expression resulting in molecular stabilization of c-Myc and consequent induction of cell proliferation (Fig. 3).

Intriguingly, heterogeneity of Fbw7 expression has been detected at the invasive front of solid tumors such as gastric and breast cancer, which show a collective cell migration pattern. This heterogeneity might reflect coincidence of dormant and proliferative cancer cells at the invasive front.

The “locked-out” therapeutic strategy combining Fbw7 inhibition with conventional anticancer agents to lock CSCs out of G₀ dormant phase is thus potentially effective for overcoming the low susceptibility of CSCs to anticancer drugs, but its possible side-effects will need to be fully investigated before its clinical implementation. The inhibition of Fbw7 and consequent upregulation of c-Myc might promote tumor cell proliferation before the combined modality therapy is able to eliminate CSCs. By contrast, “locked-in” therapy might be expected to prevent further tumor growth as well as relapse due to MRD if the proliferative potential of CSCs remains inactivated for the lifetime of the patient.

New CSC-Targeted Therapies: Targeting of CSC Resistance to Oxidative Stress

The adhesion molecule CD44, which binds to osteopontin and hyaluronic acid, has recently been identified as a CSC marker. Alternative splicing of the CD44 gene results in the generation of various CD44 isoforms, which are classified as either CD44 standard or CD44v isoforms according to the absence or presence of sequences encoded by variant exons. The isoforms CD44v8–10 and CD44v6 have been shown to enhance the metastatic potential of colon cancer and melanoma cells, respectively. CD44v6 interacts with c-Met, a receptor tyrosine kinase that binds hepatocyte growth factor, and thereby increases the potential of melanoma cells to migrate to the brain. Epithelial splicing regulatory protein 1 (ESRP1), an RNA binding protein, as well as heterochromatin protein 1γ, an epigenetic modulator, contribute to the alternative splicing of CD44 pre-mRNA. Three-dimensional culture experiments have revealed that both normal and cancer cells change the splicing pattern of CD44 to upregulate CD44v

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expression during the formation and maintenance of organoids or spheroids in ECM,\(^{(75,76)}\) suggesting that expression of variant forms is associated with epithelial organization.

We have shown that CD44v including sequences encoded by variant exons 8, 9, and 10 (CD44v8–10) interacts with and stabilizes the protein xCT at the cell membrane. This latter protein, together with CD98 heavy chain, forms an antiporter known as system Xc(–) that exchanges intracellular glutamate for extracellular cysteine.\(^{(77)}\) Cysteine as well as glycine and glutamate are essential substrates for synthesis of GSH. CD44v8–10 thus promotes GSH synthesis by increasing the import of cystine and thereby increasing the intracellular concentration of cysteine.\(^{(14)}\) The elimination of ROS by GSH inhibits the activation of p38 MAPK signaling\(^{(78)}\) and thereby prevents ROS-induced senescence, apoptosis, or differentiation of cancer cells. The CD44v8–10–xCT–GSH axis thus protects CSCs from redox stress (Fig. 4).

Regulation of oxidative stress is thought to be important not only for therapeutic resistance but also for the metastatic potential of cancer. Highly metastatic 4T1 mouse breast cancer cells include a subpopulation positive for CD44v8–10, and ESRP1-depleted 4T1 cells form significantly fewer nodules and smaller metastatic foci in lungs after their injection into mammary fat pads compared with control 4T1 cells. Furthermore, microarray analysis revealed that ESRP1-positive cancer cells were more undifferentiated than ESRP1-negative cells, consistent with the notion that CD44v8–10-positive cancer cells have characteristics of CSCs, serving as the cell of origin for metastatic lesions in this model.\(^{(70)}\) Differential expression of ESRP1 may thus determine CSC robustness by affecting resistance to oxidative stress.

The expression of ESRP1 is regulated by epigenetic factors. Trimethylation of lysine 4 residue on histone H3 (H3K4me3) is recognized in the promoter region of ESRP1 gene of CD44v8–10-positive 4T1 cells. In contrast, H3K27 residues in the promoter region of this gene are trimethylated in CD44v8–10-negative 4T1 cells.\(^{(79)}\) Those 4T1 cells positive for ESRP1-induced CD44v8–10 expression also have greater tumorigenic potential compared with those negative for such expression. Indeed, clinical data suggest that ESRP1 is a marker for poor prognosis in breast cancer.\(^{(79)}\) Epigenetic regulation of ESRP1 expression is therefore a potential therapeutic target. However, given that ESRP1 regulates the alternative splicing of p120 and fibroblast growth factor receptor genes in addition to that of the CD44 gene,\(^{(70,80)}\) the altered regulation of alternative splicing by ESRP1 might also give rise to unwanted side-effects.

Sulfasalazine, a drug given for the treatment of rheumatoid arthritis or inflammatory bowel disease,\(^{(81)}\) inhibits the transport activity of xCT. Given that targeting of xCT with sulfasalazine increased the sensitivity of CD44v8–10-positive cancer cells to ROS,\(^{(14)}\) we are currently carrying out clinical trials using this drug for patients with advanced gastric adenocarcinomas and non-small-cell lung cancer without driver gene mutations. Furthermore, the combination of sulfasalazine and auranofin, both disease-modifying antirheumatic drugs, were reported to inhibit cysteine uptake by xCT transporter and compensatory-activated Nrf2-dependent anti-ROS machinery.\(^{(82)}\)

Of note, CSCs of head and neck squamous cell carcinoma that had survived treatment with an epidermal growth factor receptor-targeted drug, cetuximab, were found to be sensitive to the induction of death by sulfasalazine. The cetuximab-resistant cancer cells tended to be undifferentiated and CD44v8–10-positive, whereas sulfasalazine-resistant cancer cells were relatively differentiated and CD44v8–10-negative.\(^{(83)}\) Combination therapy with both drugs might therefore prove effective for the elimination of heterogeneous tumor tissue.

In addition to being more susceptible to redox stress, CD44v-negative cancer cells tend to show a higher level of ROS-induced signaling by the Wnt/β-catenin pathway compared with CD44v-positive cancer cells. Furthermore, there is an inverse relation between the expression of CD44v8–10, which is a CSC marker, and that of c-Myc, which is a target of canonical Wnt signaling.\(^{(66)}\) This negative relation between CD44v8–10 expression and ROS-induced canonical Wnt signaling might support the survival and proliferation of CSCs under the evolutionary selection pressure of oxidative stress in

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**Fig. 4.** Function of CD44 variant isoform (CD44v) in promoting resistance to oxidative stress. Alternative splicing of the CD44 gene results in the generation of multiple protein isoforms. CD44v8–10 is overexpressed in epithelial cancer stem cells, and their colocalization with the xCT subunit of system Xc(–), a glutamate/cystine antiporter, promotes the uptake of cystine and the consequent synthesis of the antioxidant glutathione, which reduces reactive oxygen species (ROS).
the tumor microenvironment. Collectively, these various observations suggest that CD44v is not only a promising diagnostic or prognostic marker but also a therapeutic target for various types of cancer. Given that CD44v expression reflects the cellular heterogeneity of tumor tissue, novel therapeutic strategies targeted to CD44v would be expected to reduce both the cellular heterogeneity of tumor tissue, novel therapeutic strategies, and thereby to limit relapse and distant metastasis.

Conclusions

The development of molecularly targeted drugs to destroy CSCs has been pursued as a “silver bullet” for eradication of cancer composed of heterogeneous cell populations. However, such strategies would not be expected to be successful if they do not take into account the roles of stromal cells such as cancer-associated fibroblasts and tumor-associated macrophages as well as reversible transitions between CSCs and non-CSCs. Furthermore, inhibition of a single signal transduction pathway to which CSCs are addicted eventually becomes ineffective as a result of the activation of alternative survival pathways, and consequently induces the paradoxical enrichment of CSCs in MRD after chemotherapy. This phenomenon of adaptive resistance highlights the importance of simultaneous blockade of multiple signaling pathways. The therapeutic approaches to overcome the robustness of CSCs and their incorporation into regimens that simultaneously target both CSCs and non-CSCs are urgently required.

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Abbreviations

CD44v CD44 variant
CML chronic myeloid leukemia
CSC cancer stem cell
CTC circulating tumor cell
CXCR4 C-X-C chemokine receptor type 4
ECM extracellular matrix
ESRP1 epithelial splicing regulatory protein 1
Fbxw7 F-box and WD40 repeat domain-containing protein 7
GSH glutathione
HIF hypoxia-inducible factor
HSC hematopoietic stem cell
IGF insulin-like growth factor
Lgr5 leucine-rich repeat-containing G-protein coupled receptor 5
MRD minimal residual disease
NF-κB nuclear factor-κB
ROS reactive oxygen species

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