Targeting of cancer stem cells by differentiation therapy

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Abstract
Chemoresistance is a hallmark of cancer stem cells (CSCs). To develop novel therapeutic strategies that target CSCs, we established osteosarcoma-initiating (OSi) cells by introducing the c-Myc gene into bone marrow stromal cells derived from Ink4a/Arf KO mice. These OSi cells include bipotent committed cells (similar to osteochondral progenitor cells) with a high tumorigenic activity as well as tripotent cells (similar to mesenchymal stem cells) of low tumorigenicity. We recently showed that the tripotent OSi cells are highly resistant to chemotherapeutic agents, and that depolymerization of the actin cytoskeleton in these cells induces their terminal adipocyte differentiation and suppresses their tumorigenicity. We here provide an overview of modulation of actin cytoskeleton dynamics associated with terminal adipocyte differentiation in osteosarcoma as well as discuss the prospects for new therapeutic strategies that target chemoresistant CSCs by inducing their differentiation.

Keywords
actin, adipocyte, cancer stem cell, differentiation, osteosarcoma

1 | INTRODUCTION

Tumors are heterogeneous tissues comprised of various tumor cell types, including cancer stem cells (CSCs), which manifest self-renewal capacity and pluripotency, as well as progenitor cells and more differentiated cells, with a hierarchy similar to that of normal tissues. Given that tumors are maintained by CSCs and that these cells are able to survive conventional anticancer treatments and to evade immune responses, thereby giving rise to therapeutic resistance, disease recurrence, and metastasis, CSCs are thought to be an attractive target for novel therapies aimed at eliminating cancer cells and eradicating cancer. In addition to agents that directly target CSCs for elimination, drugs that induce senescence or terminal differentiation in these cells or that trigger their conversion to other cell types that are sensitive to conventional anticancer treatments have the potential to achieve cancer eradication. Recent evidence has indeed suggested that the plasticity of CSCs can be exploited by differentiation therapy to achieve depletion of the CSC pool. Differentiation therapy has long been considered a potential approach to suppression of tumorigenesis through conversion of undifferentiated cancer cells of high malignancy into differentiated cells of low tumorigenicity. In this review, we address the concept of induction of differentiation in CSCs, including its mechanisms and relevance to potential novel treatment strategies.

2 | CANCER STEM CELLS AND TUMOR HETEROGENEITY

Intratumoral heterogeneity among tumor cells within a given tumor is apparent at both phenotypic and functional levels as a result of genetic alterations, environmental influences, and reversible changes in cellular properties. Heterogeneity of tumor tissue has been accounted for in recent years by a hierarchy-based model in which...
CSCs have the ability both to self-renew and to give rise to differentiated tumor cells and are responsible for the overall organization of a tumor.\textsuperscript{2,5,6}

Cancer stem cells are a subpopulation of tumor cells that possess high tumorigenic activity and represent the top of a hierarchical organization similar to that of normal tissues. Cancer stem cells are thought to arise either from normal tissue stem cells through the acquisition of malignant properties due to mutations or changes in gene expression or from the spontaneous dedifferentiation of tumor cells.\textsuperscript{7,8} The tumor microenvironment has also been implicated in both the generation and maintenance of CSCs.\textsuperscript{6,9} Ever since the first experimental identification of CSCs, the CSC-based hierarchical model has been a major topic of debate in the field of cancer biology as a result of uncertainties concerning the properties of these cells, such as their defining cell surface markers as well as their frequency and plasticity.\textsuperscript{10-14} We believe that the introduction of the concept of CSCs has led to significant advances in cancer research, having provided a better understanding of tumor heterogeneity, as well as served as the basis for the development of novel therapeutic strategies that target subpopulations of tumor cells with a high tumorigenic potential.

Chemoresistance is a key characteristic of CSCs. Much evidence thus now supports the existence of CSCs that are highly resistant to classical anticancer drugs in comparison with other cancer cells. Cancer stem cells are thought to play a key role in disease relapse after termination of anticancer treatment.\textsuperscript{11,15} and targeting of CSCs at the top of the hierarchical organization of tumors is a key potential strategy for eradication of cancer tissue.

3 | TRANSDIFFERENTIATION THERAPY FOR NONCANCER DISEASES

Transdifferentiation is defined as the conversion of one mature somatic cell type to another, and many cell types have been found to be capable of this process.\textsuperscript{16,17} Forced expression of lineage-specific transcription factors has been shown to induce the transdifferentiation of fibroblasts into neurons,\textsuperscript{18} cardiomyocytes,\textsuperscript{19} or endothelial cells.\textsuperscript{20,21} In addition to transcription factors, microRNAs have been found to trigger transdifferentiation through posttranscriptional regulation.\textsuperscript{22}

Disorders of differentiation underlie some pathological conditions and noncancer diseases, and transdifferentiation-based therapies that correct differentiation abnormalities are thus under development. Strategies for the induction of therapeutic transdifferentiation that do not rely on viral vectors for overexpression of transcription factors are under investigation for ischemic heart disease.\textsuperscript{23} An approach based on the activation of innate immune signaling by stimulation of Toll-like receptors (TLRs) has thus been found to be effective for the conversion of fibroblasts into cardiomyocytes. Activation of such signaling through TLRs and the transcription factor nuclear factor-kB triggers epigenetic changes such as DNA demethylation and chromatin modification that affect cellular plasticity. For orthopedic disorders such as osteoporosis and bone dysplasia, transdifferentiation of myoblasts or adipocytes into osteoblasts also has the potential to provide a novel therapy.\textsuperscript{17,24} Many studies have now provided a proof-of-concept for therapeutic transdifferentiation.

The production of differentiated cells from embryonic stem cells, induced pluripotent stem cells, or dedifferentiated cells has also been thought to be a potential effective strategy for differentiation therapy. Although transdifferentiation was originally defined as a process by which differentiated cells undergo a change in lineage without going through an intermediate cell stage, it remains to be determined whether transdifferentiation does actually occur without passage through progenitor cell or undifferentiated cell stages.

4 | CANCER STEM CELL DIFFERENTIATION OR CONVERSION

Similar to normal tissue stem cells, some CSCs might also have the potential to differentiate into cell lineages other than the original lineage from which the tumor arose.\textsuperscript{2} Such plasticity of CSCs is considered to be a promising therapeutic target.\textsuperscript{7} The concept of CSC differentiation therapy encompasses induction of terminal differentiation in CSCs or of their conversion into nonstem cells that are sensitive to conventional anticancer treatments. Differentiation therapy has thus been under consideration as a means to suppress tumorigenesis through conversion of undifferentiated cancer cells of high malignancy into differentiated cells with low tumorigenicity. Cancer stem cell differentiation therapy could attenuate the malignant potential of a tumor or suppress its aggressive behavior. It also offers a therapeutic strategy to deplete the CSC pool and eradicate cancer.

All-trans retinoic acid has been established as an effective treatment for patients with acute promyelocytic leukemia. It elicits terminal granulocytic differentiation in the leukemia cells by impairing transcriptional repression of genes necessary for differentiation.\textsuperscript{25} Although much research has been done, limited evidence is available at present regarding the applicability of such a strategy to solid tumors.\textsuperscript{26-28} A better understanding of the mechanisms underlying CSC differentiation or conversion as well as improvements in cellular reprogramming technology that increase the efficiency and quality and reduce the risk of this promising therapeutic approach will benefit many patients in the future.

5 | ESTABLISHMENT OF INDUCED CSCS

To develop novel therapeutic strategies that target chemoresistant CSCs, we set out to isolate subpopulations of tumor cells that acquire chemoresistance for study as CSCs. We established induced CSCs (iCSCs) from mouse somatic stem or progenitor cells of various tissues by forced expression of a set of defined factors.\textsuperscript{5} Retroviral transduction with oncogenic driver genes such as Myc or Ras has thus given rise to several types of iCSCs capable of forming tumors.
after transplantation in recipient mice. We have established iCSC mouse models of osteosarcoma,29 choriocarcinoma,30 glioblastoma,31 ovarian cancer,32 and leukemia-lymphoma.33 These iCSCs possess the abilities to undergo both self-renewal and differentiation, and the iCSC-derived tumors in mice show phenotypes similar to those of the corresponding human cancers, including excessive growth, heterogeneity, and invasive or metastatic abilities.

6 | OSTEOSARCOMA CSC MODEL

Osteosarcoma is the most common primary malignant bone cancer in childhood and adolescence34 and comprises heterogeneous cell types including CSC-like cells, progenitor cells, and differentiated cells.35 Although chemotherapy regimens based on doxorubicin or cisplatin have improved the survival rate of individuals with osteosarcoma, 20% to 30% of patients are refractory to these conventional treatments.36-38 The mechanisms underlying the survival of chemoresistant cells and which types of tumor cells are responsible for such resistance are unclear.

We have established osteosarcoma-initiating (OSi) cells by introducing the gene for c-Myc into bone marrow stromal cells of Ink4a/Arf KO mice. Mice injected with these OSi cells develop lethal osteosarcoma with undifferentiated and osteo- or chondro-like differentiated areas.29 These OSi cells include AX cells, which have a bilineage (osteogenic and chondrogenic) differentiation potential similar to that of osteochondro progenitor cells, as well as AO cells, which have a trilineage (adipogenic, osteogenic, and chondrogenic) differentiation potential similar to that of mesenchymal stem cells (MSCs).

Mesenchymal stem cells are pluripotent cells that have the ability to give rise to osteoblasts, chondrocytes, skeletal muscle cells, and adipocytes.29 The differentiation fate of a particular lineage is determined by growth factors, ECM proteins, cytokines, and cell-to-cell contact,40,41 with differentiation being executed through the action of various transcription factors (Figure 1). The differentiation of MSCs into preadipocytes during adipogenesis is induced by the action of insulin, dexamethasone, cyclic AMP, and bone morphogenetic protein 4.42 Transcription factors that contribute to adipocyte differentiation include CCAAT/enhancer binding protein-α (C/EBPα), C/EBPβ, and peroxisome proliferator-activated receptor-γ (PPARγ), the latter of which controls the expression of various adipocyte-specific genes that leads to terminal adipocyte differentiation. Skeletal muscle cell differentiation is regulated by MyoD and myogenin, osteoblast differentiation by Runx2 and Osterix, and chondrocyte differentiation by Sox9.

Consistent with this notion, we found that the proportion of AO-like cancer cells expressing PPARγ is increased in human recurrent osteosarcoma tissue compared with primary tumor tissue. The induction of terminal adipocyte differentiation might thus provide a novel approach to suppression of chemoresistant AO-like CSCs in osteosarcoma.

7 | ACTIN CYTOSKELETON DYNAMICS AND ADIPOCYTE DIFFERENTIATION

Mechanical or physical stimuli, such as matrix stiffness and cytoskeletal tension, regulate cell fate determination.43 Mesenchymal stem cells thus differentiate into osteoblasts when seeded on rigid matrices, into myoblasts when grown on matrices with intermediate stiffness, and into adipocytes when cultured on soft matrices.44 Mesenchymal stem cells also differentiate into osteoblasts when allowed to flatten and spread by plating on large islands, whereas they differentiate into adipocytes when they are confined to a round shape by plating on small islands.45 Moreover, when MSCs were patterned into 3 different geometric shapes and cultured with a mixture of adipogenic and osteogenic media, cells with a flower shape characterized by large convex curves along each edge preferentially adopted an adipogenic lineage, those with a pentagonal shape characterized by straight lines for each edge differentiated into both adipocytes and osteoblasts, and those with a star shape characterized by concave edges and sharp points at the vertices favored an osteogenic fate.46 Such mechanical cues...
have been found to influence the activity of RhoA–ROCK (Rho-kinase) intracellular signaling, which in turn regulates dynamics of the actin cytoskeleton, and ROCK-induced cytoskeletal tension and downstream effects have been found to regulate the lineage commitment of MSCs.

Signaling by the small GTPase RhoA and the kinase ROCK negatively regulates adipocyte differentiation of MSCs or preadipocytes. However, some studies have suggested that this regulation of adipogenesis is attributable to a cytoskeleton-independent action of RhoA-ROCK signaling. We attempted to clarify the role of RhoA-ROCK signaling in adipocyte differentiation. We found that, before induction of adipogenesis, dedifferentiated fat (DFAT) cells, a preadipocyte cell line derived from isolated mature adipocytes, manifest a fibroblastic morphology with well-developed actin stress fibers associated with a high level of RhoA activity. Exposure of the cells to an adipogenic cocktail resulted in the rapid depolymerization of actin stress fibers as a result of downregulation of RhoA activity, with this effect then allowing expression of PPARγ and consequent adipogenic differentiation. We also recently found that Rac1, which is a downstream effector of the insulin-PI3K signaling pathway, promotes the formation of adipocyte-associated cortical actin structures, which is essential for completion of adipocyte differentiation, suggesting that actin cytoskeleton dynamics play a key role in regulation of the overall adipocyte differentiation program.

8 | REGULATION OF ADIPOCYTE DIFFERENTIATION BY ACTIN CYTOSKELETON DYNAMICS

Molecular mechanisms by which dynamics of the actin cytoskeleton directly affect cell differentiation through the control of gene expression have been identified. The transcriptional coregulators Yes-associated protein (YAP) and transcriptional coactivator with PDZ binding motif (TAZ) have been identified as mechanotransducers that mediate the effects of actin cytoskeleton dynamics or ECM stiffness, with RhoA-ROCK signaling being essential for the nuclear translocation and function of these factors. Yes-associated protein and TAZ were found to be localized to the nucleus and active in MSCs subjected to strong mechanical forces by culture on a large adhesive area or on a rigid matrix, resulting in the induction of osteogenic differentiation. In contrast, YAP and TAZ are inactive and localized to the cytoplasm in MSCs subjected to weak mechanical forces by culture on a small adhesive area or a soft matrix, resulting in the induction of adipogenic differentiation.

Megakaryoblastic leukemia 1 (MKL1, also known as MAL or MRTF-A) is a transcriptional coregulator of serum response factor, and the binding of MKL1 to monomeric (globular, or G) actin produced as a result of the depolymerization of filamentous (F) actin prevents its translocation to the nucleus and thereby inhibits its transcriptional function. We examined whether the control of MKL1 translocation by actin cytoskeleton dynamics contributes to the regulation of adipocyte differentiation. The induction of adipocyte differentiation in multipotent DFAT cells was associated with the rapid depolymerization of F-actin, resulting in an increase in the G-actin concentration. Although MKL1 was exclusively localized to the nucleus before adipogenic induction, it was predominantly cytoplasmic after such induction. We also examined the effects of the actin-depolymerizing agents latrunculin A (LatA), which increases the concentration of G-actin, and swinholide A (SwinA), which increases the concentration of a dimeric form of actin that does not interact with MKL1. Both LatA and SwinA induced depolymerization of F-actin stress fibers and increased the cellular abundance of G-actin monomers or actin dimers, respectively. Whereas LatA induced the cytoplasmic sequestration of MKL1 and the expression of PPARγ, SwinA did not alter the nuclear localization of MKL1 and did not induce PPARγ expression, suggesting that MKL1 contributes to suppression of the expression of PPARγ in the nucleus and that the increase in the cytoplasmic abundance of G-actin triggers adipocyte differentiation by preventing the nuclear translocation of MKL1 (Figure 2).

Mechanical forces have also been shown to regulate gene expression through global chromatin remodeling. Growth of cells on rigid substrates promotes actin polymerization, which in turn induces nuclear localization of MKL1 as well as cytosolic localization of histone deacetylase 3 (HDAC3). In contrast, growth of cells on soft substrates promotes actin depolymerization, which induces cytosolic localization of MKL1 as well as nuclear localization of HDAC3. The mechanism by which dynamics of the actin cytoskeleton drive adipocyte differentiation might thus involve not only MKL1-mediated transcriptional regulation but also epigenetic modification. In addition, MICAL-2, an atypical actin-regulatory protein, induces depolymerization of nuclear actin through redox modification of methionine, and the resulting oxidized G-actin is unable to bind to MKL1, resulting in nuclear retention of and consequent transcriptional regulation by MKL1. Redox-mediated actin remodeling is thus another potential means for control of adipocyte differentiation through regulation of MKL1 in a RhoA- and ROCK-independent manner.
PROPOSED DIFFERENTIATION THERAPY TARGETED TO OSTEOSARCOMA STEM CELLS

On the basis of our observations, we believe that differentiation therapy has the potential to be effective against chemoresistant cancer, including solid tumors. In our osteosarcoma model, AO cells show a high level of resistance to conventional chemotherapeutic agents such as doxorubicin, whereas AX cells are eliminated by doxorubicin treatment.\textsuperscript{42} We also found that the clinically administered ROCK inhibitor fasudil elicited terminal adipocyte differentiation in AX cells through negative regulation of MKL1 associated with actin cytoskeleton dynamics.\textsuperscript{42} Fasudil treatment attenuated tumorigenesis by AX cells,\textsuperscript{42} indicating that the conversion of these bona fide CSCs into terminally differentiated adipocytes results in tumor suppression. Given that human osteosarcoma tumors are
composed of heterogeneous cell types, including chemoresistant stemlike tumor cells, we propose a novel therapeutic option for such tumors (Figure 3): treatment with doxorubicin to eliminate AX-type chemosensitive tumor cells, followed by treatment with fasudil to convert the remaining AO-type chemoresistant cells into terminally differentiated adipocytes. The combination of conventional chemotherapy and differentiation therapy might also provide a potential treatment approach for other types of heterogeneous solid tumors that include chemoresistant CSCs.

10 | CONCLUSION

Our findings suggest a new therapeutic strategy for heterogeneous osteosarcoma based on the combination of conventional chemotherapy and the induction of cell differentiation-conversion through modulation of actin cytoskeleton dynamics in chemoresistant osteosarcoma stem cells. The clinical application of such a therapeutic strategy will be facilitated by the development of quantitative biomarkers for assessment of the effects of differentiation inducers or the establishment of imaging systems to monitor the differentiation state of tumor cells in the human body.

CONFLICT OF INTEREST

Hideyuki Saya serves as a consultant to RBI and received research grants from Daiichi-Saynkyo, Esai, Nihon Noyaku, and JSR.

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How to cite this article: Arima Y, Nobusue H, Saya H. Targeting of cancer stem cells by differentiation therapy. Cancer Sci. 2020;111:2689-2695. https://doi.org/10.1111/cas.14504