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Review

Molecular basis of differentiation therapy for soft tissue sarcomas

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ABSTRACT

Stem cells are undifferentiated precursor cells with the capacity for proliferation or terminal differentiation. Progression down the differentiation cascade results in a loss of proliferative potential in exchange for the differentiated phenotype. This balance is tightly regulated in the physiologic state. Recent studies, however, have demonstrated that during tumorigenesis, disruptions preventing terminal differentiation allow cancer cells to maintain a proliferative, precursor cell phenotype. Current therapies (i.e., chemotherapy and radiation therapy) target the actively proliferating cells in tumor masses, which in many cases inevitably induce therapy-resistant cancer cells. It is conceivable that promising therapy regimens can be developed by treating human cancers by inducing terminal differentiation, thereby restoring the interrupted pathway and shifting the balance from proliferation to differentiation. For example, osteosarcoma (OS) is a primary bone cancer caused by differentiation defects in mesenchymal stem cells (MSCs) for which several differentiation therapies have shown great promise. In this review, we discuss the various differentiation therapies in the treatment of human sarcomas with a focus on OS. Such therapies hold great promise as they not only inhibit tumorigenesis, but also avoid the adverse effects associated with conventional chemotherapy regimens. Furthermore, it is conceivable that a combination of conventional therapies with differentiation therapy should significantly improve anticancer efficacy and reduce drug-resistance in the clinical management of human cancers, including sarcomas.

KEYWORDS: soft tissue tumors, Sarcomas, Osteosarcoma, Mesenchymal stem cells, lineage-specific differentiation, differentiation therapy, tumorigenesis, sarcomagenesis

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INTRODUCTION

Sarcomas are rare neoplasms that arise from connective tissue structures in the body. Each year, there are approximately 15,000 newly diagnosed cases, 80% of which arise in soft tissue (ST) compartments [1-3]. ST sarcomas typically present as a painless enlargement without characteristic clinical symptoms, though pain and loss of function may occur as a result of adjacent structure compression [1]. They can arise anywhere in the body, but tend to occur most commonly in the deep compartments of the extremities, trunk, and abdomen [3]. These deep sarcomas are more aggressive than their superficial counterparts, often metastasizing via hematogenous routes [3-4]. ST sarcomas are traditionally classified by histopathological resemblance to adult mesenchymal tissue, with over 50 subtypes defined by the WHO [3]. The etiology of ST sarcomagenesis is unclear, as most cases arise sporadically without an identifiable cause [3, 5]. However, known risk factors include viral infections, radiation exposure and chronic lymphedema [3, 6-8]. Numerous genetic and epigenetic changes have also been implicated in ST sarcomagenesis, but no specific model of molecular pathogenesis has been established [9].

The remaining 20% of human sarcomas originate in bone, including Ewing's sarcoma, chondro-sarcoma and osteosarcoma [1-2]. Ewing's sarcoma, the second most common malignant bone tumor, primarily affects young children and adolescents [10-11]. Since most patients with clinically apparent disease may also have occult metastases, current therapy regimens involve both surgery and multidrug chemotherapy [10]. Chondrosarcomas are categorized by their location, central or peripheral, as well as their origin, as they are able to arise from nonmalignant lesions such as enchondromas and osteochondromas [12-14]. Low grade chondrosarcomas are locally aggressive but rarely metastasize, and can therefore be treated with surgery alone [15]. On the other hand, high grade chondrosarcomas frequently metastasize, leading to a 10 year survival of only 29% [12]. Chemo- and radiotherapy are largely ineffective as the poor vascularity surrounding the tumor impedes drug delivery. Osteosarcoma (OS) is the most common nonhematalogic malignancy of bone in children and adults, with a peak incidence in the second decade of life [16-18]. OS tumors show a propensity for regions of high bone turnover, and typically arise in the metaphysis of long bones [19-20]. It is associated with a relatively poor prognosis due to its high grade at presentation, chemoresistance, and frequent pulmonary metastases [21-23]. Furthermore, while 80% of OS patients are thought to have micro- or macro- metastatic disease at presentation, current radiographic modalities are only able to identify 10-15% of these patients [20, 24-26]. Given the high suspicion for metastases at the time of diagnosis, current treatment modalities include both surgery and multi-agent chemotherapy [20, 24, 27-29]. However, even with aggressive management, only about 10% of OS patients are able to achieve long-term disease- free survival [25]. Taken together, current treatment strategies are minimally effective in treating these skeletal sarcomas, and there is a critical need to develop novel therapies.

The ineffective therapeutic interventions are, in part, due to the diverse and poorly understood etiology and mechanisms underlying human sarcomas. As will be discussed below, there are numerous genetic and epigenetic changes that contribute to tumorigensis and metastasis in human sarcomas. Studies have recently shown that these diverse molecular changes may frequently lead to a similar cellular outcome: inhibition of mesenchymal stem cell differentiation. Thus, there has been a push to develop novel therapies that target and restore these differentiation defects and inhibit tumorigenesis. In this review, we discuss some of the molecular aspects of MSC differentiation and sarcomagenesis; we then review the molecular mechanisms of current differentiation therapies and their use in the treatment of human sarcomas.

Molecular biology of human sarcomas

Soft tissue sarcomas

The genetic changes associated with ST sarcomas can be dichotomized into two entities; one containing balanced translocations with specific genetic alterations and simple karyotypes, and a second characterized by extensive chromosomal

rearrangements with non-specific genetic alterations and complex karyotypes [3-4, 30]. Rhabdomyosarcoma is the most common ST sarcoma in children and adolescents, and is characterized by embryonal, alveolar, and pleomorphic subtypes [3-4]. The embryonal subtype is the most common but displays no consensus epigenetic modifications, while the alveolar and pleomorphic subtypes contain genetic translocations between FOXO1A/FOX4 and PAX3/PAX7 [31-35]. Leimyosarcomas are relatively rare, accounting for only 5-10% of ST sarcomas [3]. They display extensive tumor heterogeneity with no specific genetic aberrations [36-37]. Liposarcomas are classified into well differentiated, dedifferentiated, myxoid/round cell and pleomorphic subtypes [3-5]. The myxoid variant is characterized by a specific t(12;16) (q13;p11) translocation involving the DDIT3 and FUS genes and has a more aggressive round cell variant that is genetically identical [3, 38]. Fibroblastic tumors display two distinct clinical presentations depending on the time of presentation [4, 39-40]. Infantile fibrosarcomas are generally congenital, less aggressive, and result from fusion between the ETV6 and NTRK3 genes [39-40]. Adult fibrosarcomas have greater metastatic potential and include entities such as inflammatory myofibroblastic tumor, fibromyxoid sarcoma, and dermatofibrosarcoma protuberans, all of which display characteristic chromosomal abnormalities [41-55]. Synovial sarcomas occur in young adults around large joints, and have a consistent chromosomal translocation t(x;18) (p11.2; q11.2) [56-57]. Other soft tissue sarcomas with characteristic genetic aberrations include, but are not limited to, epitheliod sarcoma, clear cell sarcoma, desmoplastic small round cell tumor, malignant fibrous histiocytoma, alveolar soft part sarcoma, and gastrointestinal stromal tumor (GIST).

Non-Soft tissue sarcomas

Non-soft tissue sarcomas include Ewing's sarcoma, chondrosarcoma and osteosarcoma. On a molecular level, Ewing's sarcoma is defined by a translocation between the EWS gene on chromosome 22 and one of three ETS-like genes, most commonly FLI-1, on chromosome 11. The resultant EWS/FLI-1 fusion protein blocks cellular differentiation while promoting proliferation, thus leading to tumorigenesis [11, 58-60]. Molecular profiling experiments have also demonstrated that Ewing's tumors frequently resemble MSC progenitor cells, and expression of the EWS/FLI protein in MSCs prevents lineage specification, though the mechanisms of differentiation arrest are still unclear [61]. Chondrosarcomas are characterized by a variety of genetic aberrations that facilitate tumor growth and metastasis [62]. Cytogenetic studies show that 96% of central chondrosarcomas have alterations in the p53 or Rb pathways [63]. Cell cycle modulators such as CDK4, INK4A and MDM2 are frequently modified alongside signaling pathways, such as PI3K-AKT and SRC [64-68]. These pathways are currently being investigated as possible targets for novel therapeutic regimens against chondro-sarcoma. The molecular pathogenesis of OS is still poorly understood, though certain genetic and acquired conditions predispose patients to developing OS [69-70]. These include mutations in tumor suppressor genes (p53, Rb), oncogenes (MDM2, c-Myc), and signaling pathways (TGFβ) [71-90]. Furthermore, studies have suggested that these genetic and epigenetic modifications block osteoblastic differentiation, causing OS cells to mimic their precursor mesenchymal stem cell progenitors [19, 21, 24, 91-92].

Mesenchymal stem cell differentiation

Mesenchymal stem cells (MSCs) are pluripotent bone marrow stromal cells that can differentiate into myogenic, chondrogenic, osteogenic and adipogenic lineages [92-95]. Differentiation along these pathways is a complex, tightly controlled process that is regulated by a variety of endogenous and environmental factors. Furthermore, as MSCs progress through each successive stage of differentiation, they lose their proliferative capacity in exchange for a differentiated phenotype.

Myogenesis results in the formation of vertebrate muscle from myoblastic precursor cells [95]. MSC commitment to the myogenic pathway is a two step process influenced by numerous signaling molecules, genes and growth factors [96-98]. First, primitive mesoderm differentiates into myoblastic cells, which is regulated by the

MyoD family of transcription factors, bone morphogenetic proteins (BMPs), Hedgehog and Wnt signaling pathways [99]. Myoblasts then undergo terminal differentiation into myocytes and coalescence into multinucleate myofibers, a process that is induced by the Mef2 and MyoD transcription factors [95-98]. Commitment to the adipogenic lineage is characterized by MSC differentiation into preadipocytes. These cells, while phenotypically identical to MSCs, have lost their ability to differentiate into other cell types [95]. Preadipocytes then further differentiate into terminal adipocytes, a process that is largely controlled by the nuclear hormone receptor PPARγ and BMPs 2, 4, 7 and 9 [21, 94, 100-109]. Chondrogenic differentiation is influenced both by extracellular mechanical interactions and multiple cytokines and growth factors [95]. For example, the TGFβ and fibroblast growth factor (FGF) family of cytokines stimulate chondrogenesis via Smad and MAPK signaling pathways [110-114]. These, along with other chondrogenic stimulators, lead to downstream activation of the Sox9 transcription factor, inducing expression of chondrogenic markers [64-67]. Finally, osteogenesis is primarily under the control of a master regulatory gene, Runx2 [95, 115-117]. Runx2 interacts with transcriptional activators and repressors, such as Rb, MAPK and histone deacetylases, to induce expression of the osteogenic phenotype [118-119]. In addition to Runx2, other important osteogenic regulators include the Wnt proteins and TGFβ/BMP pathways [94, 120-123]. Wnt signaling proceeds via the LRP5 and LRP6 co-receptors and leads to downstream activation of β-catenin, another osteogenic stimulator [95, 124-126]. BMPs, in particular BMP-2 and BMP-9, initiate downstream signaling via the Smad pathway, resulting in the activation of osteogenic specific genes [21, 24, 94-95, 100, 121, 127-131].

MSC differentiation and cancer

Mesenchymal stem cells are unique precursor cells that maintain pluoripotency and can give rise to different types of tissues. They are defined by their capacity for self-renewal, proliferation and differentiation. A critical aspect of stem cell biology the regulation of the balance between proliferation and terminal differentiation, as a disruption of this balance in favor of proliferation is associated with tumorigenesis [132]. Recently, the theory of "cancer stem cells" has emerged, in which a small subset of stem cells fail to undergo terminal differentiation and maintain their proliferative capacities, serving as the driving force behind tumor proliferation and regeneration [132]. This notion is supported by the fact that both cancer cells and stem cells have tremendous proliferative and regenerative capacity, display similar phenotypic markers, and consist of a heterogenous population of cells at various stages of differentiation [132]. Though MSC differentiation is a highly complex and intricate pathway, as MSCs pass through each successive stage of differentiation, they uniformly lose their proliferative capacity. Defects in this differentiation pathway can arrest MSCs in a highly proliferative state, with the ability to regenerate future tumorigenic progeny. By preventing terminal differentiation, sarcoma cells can mimic their precursor MSC phenotype and retain the capacity for uncontrolled proliferation. Thus, it is conceivable that cancer therapies inducing terminal differentiation may serve as an attractive alternative to treatment regimens.

Molecular mechanisms of differentiation therapies

Though many sarcomas may be the result of differentiation defects in MSCs, current cancer therapies only target the proliferative component of tumorigenesis and fail to restore the differentiation defects that are present. These therapies are often associated with significant morbidity due to the nonspecific targeting and destruction of non-cancerous cells. For example, patients with osteosarcoma are typically treated with chemotherapy regimens using a combination of cisplatin, doxorubicin, isofosfamide or methotrexate [20, 24, 27-29]. These therapies expose patients to long term toxicities including hearing loss, cardiomyopathy, sterility, and hypomagnesemia [20, 133-138]. Soft tissue sarcomas use similar chemotherapy regimens in addition to radiation therapy, which can be associated with nausea/vomiting, diarrhea, epidermal desquamation and carcinogenicity. Furthermore, though effective initially, these therapies often give way to eventual drug resistance.

An alternative strategy is to overcome tumorigenesis by promoting terminal differentiation in tumor cells, thereby decreasing and/or eliminating the tumor cells' proliferative potential. Such differentiation agents would also avoid the severe side effects often associated with typical chemotherapy regimens. Below, we discuss the molecular mechanisms for some of the differentiation therapies that have shown promise in treating human sarcomas.

PPARγ

The perixosome proliferator-activated receptors (PPARs) are ligand activated transcription factors that play a role in a variety of processes such as diabetes, obesity, cancer, inflammation and atherosclerosis [139]. Three PPAR subtypes have been identified, PPARα, PPARβ, and PPARγ, and each contains 4 unique domains, AB, C, D, and E [21, 140-141]. The primary activity is carried out by the E domain, as it is responsible for ligand binding, transactivation and dimerization [21, 141-143]. Upon ligand binding, PPARs form a heterodimer with the 9 cis-retinoid X receptor (RXR) and modulate transcription via PPAR response elements (PPREs) [142, 144]. This transcriptional regulation is modulated by co-activators, co-repressors and signaling pathways such as PRIP, C/EBP, PRIP, Hsp-72, NFκB, STAT, and AP-1 [142, 145-151].

Of particular interest is PPARγ, a crucial regulator of both adipogenesis and osteogenesis in MSCs [152-153]. PPARγ is able to bind fatty acid derivatives and induce differentiation of preadipocytes into terminal adipocytes [21, 142, 154]. Overexpression of PPARγ in fibroblasts activates the adipogenic cascade, while PPARγ knockout mice are unable to form any adipose tissue [155-157]. Furthermore, activating mutations of PPARγ in humans results in increased adipogenesis and weight gain, while mutations decreasing PPARγ activity result in lower BMIs [158-159]. PPARγ shunts MSC differentiation toward adipogenesis and away from osteogenesis [160-166]. This ability of PPARγ to induce terminal differentiation has led to its use in various malignancies such as breast cancer, leukemia, gastric cancer, prostate cancer and liposarcoma [167-175]. For example, treatment of end stage prostate cancer with the PPARγ agonist troglitazone leads to PSA stabilization [176]. In other studies, treatment of osteosarcoma (OS) cells with PPARγ agonists troglitazone and ciglitazone leads to decreased OS cell proliferation, increased susceptibility to apoptosis, and increased expression of differentiation markers such as alkaline phosphatase (ALP) [18, 177]. This ability of PPARγ agonists to induce terminal differentiation of tumor cells has led to its expanded use in the treatment of human sarcomas, as will be discussed later.

Retinoids

Retinoic acid (RA) plays a critical role in embryonic development, differentiation and maintenance of vital organ function in the adult [178-179]. It has been linked to diverse biological functions including vision, immunologic development, heart patterning, forelimb induction, and critical steps of embryonic patterning [178-181]. RA ligands function by binding to one of six nuclear receptors (RARα, RARβ, RARγ, RXRγ, RXRβ, and RXRγ), which are members of the steroid/thyroid hormone superfamily [182]. The most abundant form of RA, all-trans-retinoic acid (ATRA), signals via the RAR subfamily of receptors while the RXR receptor family is activated by 9-cis-retinoic acid (9CRA) [181, 183]. Upon ligand binding, these receptors form homodimers or heterodimers that bind to DNA sequences called retinoic acid response elements (RAREs) [182]. RA binding to the hetero-/homo-dimeric RAR/RXR receptors leads to recruitment of various co-activators and initiation of transcription [181-182, 184].

Studies have demonstrated that retinoids are able to induce terminal differentiation in various cell lines. Treatment of hepatocyte progenitor cells (HPCs) with either ATRA or 9CRA results in decreased expression of early progenitor markers and increased expression of late hepatocyte markers [185]. Furthermore, ATRA and 9CRA are able to induce glycogen synthesis and storage in HP14.5 progenitor cells, suggesting that RA may be capable of inducing terminal differentiation in HPCs [185]. Treatment of myoblast progenitor C2C12 cells with ATRA and 9CRA results in increased expression of late

myogenic markers, myoblastic fusion, and abundant presence of muscle fibers, strongly suggesting a crucial role of RA signaling in myogenic differentiation [186]. These differentiation modulating effects make retinoids prime candidates in cancer therapies [187]. These effects have been demonstrated in oral leukoplakia, head and neck cancer, breast cancer, and skin cancer [188-192]. However, the most significant anti-tumor activity of RAs has been shown in acute promyelocytic leukemia (APML) and hepatocellular carcinoma (HCC). In HCC, phosphorylation of RXR impairs its function and causes uncontrolled cell growth [187], and acyclic retinoid inhibits this phosphorylation, thus inducing apoptosis [187]. In APML, a chromosomal translocation between RAR and promyelocyte leukemia protein (PML) creates a dominant negative protein that interferes with the normal function of RAR and/or PML, thereby arresting cell maturation [193-194]. Oral administration of ATRA rescues this defect by inducing differentiation of leukemic cells into mature neutrophils and leads to remission rates upwards of 90% [187, 195-198]. The anti-cancer ability of the retinoid compounds can largely be attributed to their ability to induce terminal differentiation, a property that has recently been investigated for the treatment of human sarcomas.

Histone deacetylase inhibitors

Histone acetylation/ deacetylation is invariably linked to the modulation of gene transcription [199-200]. Furthermore, the addition or removal of acetyl groups to proteins other than histones can have a dramatic impact on protein stability and activity [201-203]. There are currently four classes of histone deacetylases (HDACs), each with a high degree of homology. Histone deacetylase inhibitors (HDACIs) are a diverse group of compounds that inhibit the action of HDACs, thereby modulating both gene transcription as well as protein function. HDACIs can be classified as hydroxamic acid derivatives (vorinostat, trichostatin), small-chain fatty acids (sodium butyrate, valproic acid), benzamides or cyclic tetrapeptides (depsipeptide)[204]. Several mechanisms of action have been proposed for HDACIs effects on tumor cells [204]. In leukemias, HDACIs induce expression of the apoptotic TRAIL and FAS pathways, thereby triggering selective tumor death [205-206]. There is also an increased accumulation of reactive oxygen species (ROS) in these leukemic cells when treated with HDACIs [207]. Other studies suggest that HDACIs play a key regulatory role in cell cycle progression [204]. In mouse embryonic fibroblasts, inactivation of Hdac3 leads to DNA damage and apoptosis, and treatment of leukemic cells with HDACIs leads to activation of the DNA damage response and apoptosis [208]. Finally, recent studies show that HDACIs may function by modulating the retinoic acid signaling pathway [209]. Treatment of cancer cells with HDACIs activates RA signaling, especially when combined with exogenous RA. Furthermore, cells deficient for RAR isoforms (α, β, γ) are more sensitive to the anti-tumor effects of HDACIs, which can be reversed by exogenous administration of RARα [209].

Treatment of tumor cells with HDACIs results in histone hyperacetylation and modulation of various proteins associated with the tumorigenic phenotype [210-211]. In both mouse models and cell culture, HDACIs induce growth arrest, differentiation, and apoptosis of tumor cells [204]. For example, combination treatment using both HDACI with RA demonstrated enhanced neuroblastoma differentiation and inhibition of tumorigenesis [212]. Currently, HDACIs are in phase I and phase II clinical trials for a range of malignancies [210-211, 213]. These trials have shown promise in treatment of Hodgkin's lymphoma, Non-Hodgkin's lymphoma, acute myeloid leukemia (AML) and T-Cell lymphoma [214-216]. A phase IIb study evaluated the treatment of advanced, refractory cutaneous T-cell lymphoma (CTCL) with the HDACI vorinostat and showed that 30% of patients demonstrated clinical response. These results led to the FDA approval of vorinostat as a second-line therapy for progressive, recurrent CTCL [217-218]. Given the high success rate of HDACIs in cancer treatment and their activation of the RA pathway, it is no stretch to also imagine their potential efficacy in the treatment of human sarcomas.

Trabectedin

Trabectedin, also known as ET-743, is a new marine derived alkaloid that has recently been

developed as an anti-cancer agent. From 2007-2009, it obtained marketing authorization from the European Commission for treatment of advanced soft tissue sarcomas and relapsed platinum-sensitive ovarian cancer [219]. Unlike traditional alkylating agents that bind the DNA major groove, trabectedin functions by inserting into the DNA minor groove [219]. It displays multiple mechanisms of anti-tumor therapy, which can be classified into four categories; DNA repair, transcription regulation, modulation of tumor microenvironment, and differentiation induction. Studies have demonstrated that cells with deficient nucleotide exchange repair (NER) machinery are up to 10 times less sensitive to trabectedin therapy [219-222]. Trabectedin is able to bind to both the major and minor groove regulating multiple transcription factors, including E2F, SRF, and TBP [223]. Trabectedin is also able to modulate the tumor microenvironment by inhibiting the production of proinflammatory mediators such as CCL2, IL-6, CXCL8 and VEGF, thus creating a hostile environment to tumor progression [224-225]. Finally, trabectedin is able to induce terminal differentiation and thereby inhibit proliferation of cancer cells [226-227]. As will be discussed later, this ability to induce terminal differentiation has been successfully employed in the treatment of certain human sarcomas.

Differentiation therapies for human sarcomas

Since human sarcomas may be the result of defects in terminal differentiation, the aforementioned therapies are attractive anti-cancer agents since they target the critical differentiation defect that underlies sarcomagenesis. Furthermore, such differentiation therapies avoid the morbidity that is associated with current chemotherapy regimens that cause nonselective death of both healthy and cancerous tissues. Many studies have investigated differentiation therapies for human sarcomas, as they hold significant potential as alternative anti-cancer agents to our current chemotherapy regimens (Table 1). Below, we discuss some of the differentiation therapies that have been developed for various human sarcomas.

Liposarcoma

Liposarcoma is the most common soft tissue malignancy in adults, accounting for nearly 20% of all sarcomas in this age bracket [228]. They are classified by histologic subtypes including well differentiated, dedifferentiated, myxoid and pleomorphic. Typically, the histologic subtype also correlates with prognosis, and the average 5 year survival can be as low as 25% depending on the type of tumor [168]. Since conventional chemotherapy only has a success rate of 10% in metastatic liposarcoma, and these same liposarcomas show evidence of dedifferentiation, differentiation therapies are an attractive alternative [229]. Adelmant *et al.* found that human translocation liposarcoma (TLS) expresses a CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) fusion oncoprotein that results from a t(12;16) translocation[230]. This fusion protein blocks adipocyte differentiation by inhibiting adipocyte genes and can be rescued by addition of PPARγ2 [230]. This and other similar studies have led to the development of potential liposarcoma therapies focused on inducing adipocyte differentiation.

PPARγ and retinoid X receptor α (RXRα) form a heterodimeric complex that functions as a crucial regulator of adipocyte differentiation [168]. Furthermore, PPARγ is expressed at high levels in most liposarcoma subtypes, and addition of synthetic PPARγ agonists induces terminal differentiation in liposarcoma cells [168, 231]. In a study by Tontonoz *et al.*, addition of the PPARγ ligand pioglitazone induced differentiation of cultured human liposarcoma cells [168]. When cells were simultaneously treated with RXR-specific ligands, there was an additive effect on liposarcoma cell differentiation characterized by accumulation of lipid, withdrawal from the cell-cycle, and expression of adipocyte genes [168]. Demetri *et al.* showed that administration of troglitazone to patients with high-grade liposarcoma demonstrated histobiochemical evidence of adipocyte differentiation *in vivo* [231]. Biopsies showed extensive lipid accumulation and NMR-detectable triglycerides, while treatment was associated with increased expression of mRNA transcripts characteristic of

Table 1. Potential differentiation therapies for soft tissue sarcomas.

Sarcomas	Ligand	Anti-tumor Mechanism	References
Liposarcoma	Pioglitazone (PPARγ)	Lipid accumulation, cell-cycle withdrawl, adipocytic gene expression	Tontonoz *et al*. [168]
	RXR Ligands	Additive effect when Pioglitazone combined with RXR Ligands	Tontonoz *et al*. [168]
	Troglitazone (PPARγ)	Histochemical evidence of lipid accumulation, NMR-detectable triglycerides, increased adipocytic mRNA expression	Demetri *et al.* [231]
	Trabectedin	Myxoid Liposarcoma: Detachment/inhibition of FUS-CHOP from target promoters, activation of CAAT/enhancer pathway	Forni *et al.* [232]
Chondrosarcoma	Depsipeptide (HDACI)	Increase in late markers COL2A1, Aggregan, COL10A1	Sakimura *et al.* [233]
	15d-PGJ (PPARγ)	Dose dependent inhibition of cell proliferation, increased apoptosis	Nishida *et al.* [238]
	Pioglitazone (PPARγ)	Apoptosis in OUMS-27 cells, morphologic cellular changes	
	Rosiglitazone	Inhibits MMP 1 and MMP 13 expression, blunts collagen destruction	Nishida *et al.* [237]
	LG268 (RXR Ligand)	Additive effect when LG268 combined with Rosiglitazone	
Rhabdomyosarcoma	ATRA	Change in cellular morphology, increased Troponin T expression	Barlow *et al.* [240] Gabbert *et al*. [242]
	9 CRA	Myofilament formation, contact inhibition, formation of myotube-giant cells	

Table 1 continued..

	GR-891, QF-3602 (5-FU)	Myogenic protein expression, morphologic changes at 6 days	Marchal *et al.* [245] Melguizo *et al.* [246]
	Ara-C	*In Vitro* cell differentiation, parallel growth inhibition	Crouch *et al.* [247]
	Actinomycin D	Increase in desmin and alpha-actinin, ultrastructural myogenic changes	Prados *et al.* [241] Melguizo *et al.* [246] Marchal *et al.* [248]
Ewing's Sarcoma	FK228 (HDACI)	Upregulation of p21, inhibition of HDAC	Sakimura *et al.* [250]
	si-EWS-FLI1 (siRNA)	Decrease in invasiveness, proliferation, and migration	Ouchida *et al.* [251] Chansky *et al.* [252]
Osteosarcoma	Troglitazone Ciglitazone Pioglitazone	Increased susceptibility to apoptosis, decreased proliferative capacity, increased ALP activity	Haydon *et al.* [18, 177]
	9 cis-retinoic acid All-trans retinoic acid	Morphologic differentiation, inhibited anchorage dependent growth, additive with PPAR ligands	Haydon *et al.* [18, 177]
	Tamoxifen Raloxifene 17-β Estradiol	Increased apoptosis, increased osteoblastic differentiation markers	Kallio *et al.* [255]
	1,25-dihydroxyvitamin D3	Decreased cell proliferation, increased ALP, OCN	Nozaki *et al.* [256] Zenmyo *et al.* [257]
	Parathyroid hormone related peptide (PTHrP)	MAPK activation, elevated ALP, Type 1 Collagen	Carpio *et al.* [258]

adipocyte differentiation [231]. In addition, there was a marked reduction in cellular proliferation and increased apoptosis [231]. Finally, a study by Forni *et al.* found that trabectedin successfully induced cellular differentiation in myxoid liposarcoma by inhibiting the FUS-CHOP oncogene [232]. Trabectedin caused detachment of the FUS-CHOP protein from target promoters (Forni) and also activated the CAAT/enhancer binding protein-pathway leading to morphologic changes characteristic of terminal differentiation [232].

Chondrosarcoma

Chondrosarcomas are the second most common primary skeletal malignancy. Since they are highly resistant to conventional chemotherapy, surgery remains the mainstay of treatment [233]. To date, no adjuvant therapy exists for inoperable cases, and there is a critical need to develop novel therapeutic strategies [12, 234]. Histochemical analyses have shown that chondrosarcomas display a range of phenotypes ranging from poorly to highly differentiated [235]. Those exhibiting a mature and terminally differentiated phenotype display minimal proliferation, while dedifferentiated chondrosarcomas are more aggressive with increased proliferation [236]. The stage of differentiation also correlates to prognosis, supporting the possibility that differentiation therapies for chondrosarcoma may be a novel therapeutic strategy [235].

In one study, addition of the HDACI Depsipeptide caused cell cycle arrest, growth inhibition and apoptosis in chondrosarcoma cell lines [233]. Depsipeptide increased the expression of the a1 chain of type II collagen (COL2A1) and upregulated the expression of late chondrogenic markers aggregan and COL10A1 [233]. Multiple other studies confirmed that Depsipeptide significantly inhibited tumor growth through the induction of terminal differentiation [233]. Chondrosarcomas also demonstrate a striking response when exposed to differentiation agents such as PPARγ and RXR ligands [237-239]. Treatment of the chondrosarcoma line OUMS-27 by 15d-PGJ, the most potent endogenous ligand for PPARγ, causes a dose dependent inhibition of cell proliferation and an increase in apoptotic activity [238]. Incubation of OUMS-27 cells with pioglitazone also induced apoptosis, as measured by TUNEL and flow cytometry [237]. In another study, the PPARγ ligand rosiglitazone inhibited the expression of matrix metalloproteinases (MMP) 1 and 13 and blunted collagen destruction by SW-1353 chondrosarcoma cells [239]. There was an additive inhibitory effect when rosiglitazone was combined with the RXR ligand LG268 [239]. These results suggest that differentiation agents not only inhibit cell proliferation and tumor growth, but also play a role in preventing local migration and eventual metastasis of chondrosarcoma cells.

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a sporadic soft tissue sarcoma that occurs primarily in childhood and adolescence. Despite the use of surgery, radiation and chemotherapy, the typical 5-year survival rate is still only 70% [240]. The classical cytotoxic treatment of RMS is often associated with significant morbidity [241]. Furthermore, there is a high incidence of multidrug resistance [241]. Thus, novel therapeutic approaches are necessary to improve on current treatment outcomes, such as differentiation therapies.

Barlow *et al.* examined the ability of ATRA and 9CRA to promote differentiation of five RMS cell lines by examining the expression of myogenic proteins. Treatment with both 9CRA and ATRA resulted in suppression of cell proliferation and altered cell cycle progression [240]. Differentiating effects were also observed based on the change in cellular morphology and increased expression of Troponin T [240]. In a second study, BA-HAN-1C rhabdomyosarcoma cells exposed to retinoic acid showed time- and dose-dependent changes in cell differentiation and cell growth [242]. Exposure resulted in formation of thick and thin myofilaments that exhibited contact inhibition and a significant increase in the number of differentiated myotube-like giant cells [242]. TE-671-1-A is a clonal human rhabdomyosarcoma cell line that demonstrates evidence of myogenic differentiation when exposed to similar therapies [243]. Treatment of TE-671-1 A with either retinoic acid or sodium butyrate resulted in a statistically significant induction of differentiation with a parallel

inhibition of proliferation [243]. Other novel differentiation agents have also been developed for RMS that act independently of the RA or PPAR pathways. The pyrimidine derivative GR-891 is a 5-fluorouracil (5-FU) derivative that shows low toxicity [244]. Treatment of RD rhabdomyosarcoma cells with GR-891 resulted in myogenic differentiation at 6 days characterized by myogenic protein expression and morphologic changes representative of muscular maturation [245]. Treatment of RMS cells with another 5-FU derivative, QF-3602, showed similar results suggesting that 5-FU derivatives hold promise as potential differentiating therapies in RMS [245-246]. Ara-C, an antitumor agent that induces differentiation in acute myelogenous leukemia, has also shown a differentiation effects on different RMS cell lines with an inhibition of *in vitro* and *in vivo* proliferation [247]. Finally, multiple studies have shown that actinomycin D, a drug of choice in the treatment of RMS, induces differentiation of RMS cells [241, 246, 248]. Treatment with actinomycin D causes increased expression of differentiation markers desmin and α-actinin, ultrastructural changes indicating myogenic differentiation and corresponding inhibition of cell proliferation [241, 246, 248].

Ewing's Sarcoma

Ewing's sarcomas are highly aggressive round cell tumors of bone and soft tissue that primarily affect children and young adults [60-61]. The majority of these tumors harbor a t(11;22) translocation that results in the expression of an EWS-FLI-1 fusion protein [60-61]. Molecular profiling studies indicate that Ewing's tumors originate from mesenchymal progenitor cells, and expression of the fusion protein leads to a block in terminal MSC differentiation [59-61]. EWS-FLI-1 can bind Runx2, a master regulator of osteogenesis, to block the expression of osteoblastic specific genes and osteogenic differentiation [61]. Gene expression analyses show that Ewing's tumors resemble their MSC progenitors, and that silencing of EWS-FLI-1 results in the ability of Ewing's cell lines to terminally differentiate when exposed to appropriate differentiation cocktails [60]. Furthermore, when EWS-FLI-1is expressed in murine C2C12 cells, a myoblastic precursor cell line, there is a profound block in myogenic differentiation [249]. These studies suggest that expression of the EWS-FLI-1 fusion protein causes blocks in terminal MSC differentiation, and these differentiation defects likely underlie the pathogenesis of Ewing's sarcoma. There has been reasonable success in inhibiting Ewing's pathogenesis by targeting this fusion protein and restoring the capacity for cellular differentiation.

In one study, EWS-FLI-1 was shown to inhibit p21 expression and modulate histone acetylation/ deacetylation by upregulating HDAC activity [250]. Addition of a novel HDACI, FK228, resulted in reversal of this phenomenon, upregulation of p21 expression and inhibition of HDAC activity [250]. FK228 levels also correlated with a decrease in EWS-FLI-1 mRNA expression, growth inhibition, and increased apoptosis *in vivo* [250]. In other studies, stable Ewing's sarcoma lines expressing antisense EWS-FLI-1 expression plasmids show loss of anchorage independent growth and tumorigenicity [251]. Expression of EWS-FLI-1 siRNA is correlated with decreased cell proliferation and increased apoptosis [252]. Furthermore, knockdown of EWS-FLI-1 expression abrogates invasiveness in SK-ES Ewing's sarcoma cells by inhibiting the CXCR4 chemokine receptor [252]. These results demonstrate the necessity of the EWS-FLI-1 fusion protein in Ewing's tumorigenicity and emphasize the importance of targeting this protein as potential therapy for Ewing's sarcoma. Although no "magic bullet" has yet been developed, successful therapy will rely on inhibition of EWS-FLI-1 function. Since this fusion protein has been tightly linked to inhibition of terminal cell differentiation, such therapies will likely restore the capacity for tumor cells to undergo cellular differentiation.

Osteosarcoma

We and others have extensively documented that osteosarcoma (OS) cells share many similar features to undifferentiated osteoprogenitors [18, 24, 121]. The late osteogenic markers osteopontin (OPN) and osteocalcin (OCN) are highly expressed in mature osteoblasts, but show minimal levels in primary OS tumors and OS cell lines [94, 121-123]. On the other hand, CTGF is a marker of the earliest stages of osteogenic

differentiation and demonstrates elevated basal expression in OS cells [102]. STAT3, a marker for embryonal stem cells, is expressed at high levels in OS cells and represents an independent prognostic factor for disease-free survival [253]. The more aggressive OS phenotypes typically demonstrate early defects in differentiation, while less aggressive tumors tend to share features with MSCs that have progressed further along the differentiation cascade [21, 92]. Furthermore, Runx2 and Wnt signaling are frequently altered in human osteosarcoma [91]. These results suggest that a lack of terminal differentiation may not only be responsible for OS, but may also contribute to its malignant potential. Therapies that induce terminal differentiation can rescue these critical defects in and prevent OS tumorigenesis.

The nuclear receptor subfamily of proteins has shown promising ability as differentiation therapy for OS. Various PPARγ agonists are able to prevent proliferation and induce differentiation in OS tumor cells [18, 177]. Exposure of OS cells to PPARγ agonists causes decreased proliferation, increased susceptibility to apoptosis, and an increased expression of differentiation markers such as alkaline phosphatase [21]. Treatment with 9CRA or ATRA also induces differentiation and growth inhibition [254]. When these retinoids are combined with troglitazone, a potent PPARγ agonist, there is a strong synergistic effect on cellular apoptosis and differentiation [177]. Other successful nuclear receptor therapies include estrogen receptor antagonists and 1,25-dihydroxyvitamin D3 (1,25(OH)2D3). Treatment of U2OS cell lines with Raloxifene, 17-beta estradiol and SERMS causes decreased cell proliferation with an increase in osteoblast differentiation markers [255]. 1,25(OH)2D3 is able to induce differentiation of MG63 OS cells through a p21 dependent pathway that results in increased expression of the differentiation markers ALP and OCN [256-257]. Another interesting possibility for OS differentiation therapy is parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP). Both of these ligands transducer their signal via a G protein mediated pathway that leads to the eventual phosphorylation of protein kinase A and/or protein kinase C [258]. In a study by Carpio *et al.*, treatment of MG63 OS cell lines with PTHrP resulted in increased expression of ALP and type 1 collagen, suggesting that the tumor cells underwent osteoblastic differentiation [258]. Collectively, these therapies can not only induce terminal differentiation, but also obviate the need for chemotherapy and avoid some of the toxicities and chemoresistance associated with current OS therapeutic regimens.

CONCLUSION

Human sarcomas encompass a diverse set of pathologies caused by various genetic and epigenetic modifications. Though no consensus mechanism can broadly account for sarcomagenesis, studies have shown that the molecular changes lead to a common inhibition of terminal mesenchymal stem cell differentiation. Current treatment regimens for sarcomas focus primarily on targeting the proliferative component of tumor cells, and are associated with significant morbidity due to non-selective death of health tissue. Since many human sarcomas are a result of defects in MSC differentiation, therapies aimed at restoring this critical defect are an attractive alternative. Differentiation therapies for human sarcomas have shown great promise in inhibiting tumor progression and invasion, and should be further explored in the development of successful therapeutic agents for human sarcomas.

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