MYC regulates CSF1 expression via microRNA 17/20a to modulate tumor-associated macrophages in osteosarcoma

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Conflict of interest

The authors declare no conflicts of interest.

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Abstract

Osteosarcoma (OS) is the most common primary bone tumor of childhood. Approximately 20-30% of OS carry amplification of chromosome 8q24, which harbors the oncogene c-MYC and correlates with a poor prognosis. To understand the mechanisms that underlie the ability of MYC to alter both the tumor and its surrounding tumor microenvironment (TME), we generated and molecularly characterized an osteoblast-specific Cre-Lox-Stop-Lox;(LSL)-c-MycT58A;p53fl/+ knockin genetically engineered mouse model (GEMM). Phenotypically, the Myc knockin GEMM had rapid tumor development with a high incidence of metastasis. MYC-dependent gene signatures in our murine model demonstrated significant homology to the human hyperactivated MYC OS. We established that hyperactivation of MYC leads to an immune-depleted TME in OS demonstrated by the reduced number of leukocytes, particularly macrophages. MYC-hyperactivation leads to the downregulation of macrophage-colony-stimulating factor 1 (CSF1), through increased miR-17/20a expression, causing a reduction of macrophage population in the TME of OS. Furthermore, we developed cell lines from the GEMM tumors, including a dTAG-MYC model system, which validated our MYC-dependent findings both in vitro and in vivo. Our studies utilized innovative and clinically relevant models to identify a novel molecular mechanism through which MYC regulates the profile and function of the osteosarcoma immune landscape.
Introduction

Osteosarcoma (OS) is the most common and highly metastatic primary bone tumor in children and adolescents (1). Despite extensive genomic aberrations, OS has no pathognomonic DNA translocation or targetable mutations (2). Thus, no effective molecularly targeted therapies for OS are currently available. However, many OS patients present with genetically defined somatic DNA copy number alterations (SCNAs), such as chromosome 8q24 gains, which is noted in about 20% of OS patients (3, 4). The 8q24 locus harbors the known oncogene c-MYC (MYC), which directly regulates several protein-coding and non-coding genes important for distinct cellular functions, including cell cycle regulation, protein biogenesis, metabolism, signal transduction, transcription, and translation (5, 6). MYC has been found to be deregulated in more than half of human cancers (7). Amplification of the 8q24 region and overexpression of MYC is seen in both high-grade premalignancy and invasive tumors and is associated with poor outcome in different human tumor types, including OS (8-12). Besides its effects on intrinsic tumor cell biology, hyperactivation of MYC leads to alterations in the tumor immune microenvironment (TME) in multiple cancers (13-15).

Macrophages are abundantly present cells in the TME of solid tumors, including OS, and play multifunctional roles in host defense, tissue repair, apoptosis, and tissue homeostasis by releasing a distinct repertoire of growth factors, cytokines, chemokines, and enzymes (16, 17). In mature adults, macrophages are differentiated from peripheral blood monocytes with the help of a cytokine, macrophage stimulating factor (M-CSF or CSF1). CSF1 not only regulates the differentiation of monocytes to macrophages, but also supports monocytes/macrophage’s survival, proliferation, and macrophage motility.
through interaction with its receptor (CSF1R) (18). The role of intratumoral oncogenic MYC in macrophage regulation has been partially explored. MYC has been found to play a key role in alternative macrophage activation (19), but knowledge of the underlying molecular pathways is scarce. Moreover, the role of MYC in the regulation of the macrophage population in the TME of OS is unknown.

We have generated an osteoblast-specific Myc knockin genetically engineered mouse model (GEMM) of OS and molecularly characterized spontaneous OS tumors that arise in this model to identify MYC-dependent intrinsic and extrinsic therapeutic vulnerabilities. The murine molecular profiles were compared with the human tumor transcriptomic profile from the Therapeutically Applicable Research To Generates Effective Treatments (TARGET dataset, https://ocg.cancer.gov/programs/target) and R2: Genomics Analysis and Visualization Platform dataset (20). We observed that hyperactivation of MYC is associated with a diminished leukocyte population, particularly the macrophage subpopulation in the TME of OS. Additionally, we observed the infiltration of the macrophage cells was diminished in the OS TME for both human and mouse tumors. Our results demonstrated the role of cytokine CSF1 in macrophage recruitment to the TME in OS. Subsequently, we identified that MYC-regulated miRs-17/20a downregulate CSF1 expression resulting in the direct downstream effects on intratumoral macrophage recruitment. Additionally, MYC was found to regulate macrophage functions, including phagocytosis, through CSF-1 regulation. This is the first study that identifies a direct molecular mechanism of macrophage regulation in the OS TME by MYC. In addition, our novel Myc knockin GEMM provides valuable resources to improve our
knowledge about the etiology and identification of therapeutic targets for this high-risk subgroup of OS patients.
Results

Development and proteo-transcriptomic characterization of Myc knockin genetically engineered mouse model of osteosarcoma.

Previously, we generated a conditional GEMM of OS to gain an understanding of the molecular pathogenesis of disease development and progression through osteoblast-specific alteration of the Trp53 gene (21). Trp53 is a tumor suppressor and estimated to be mutated, or dysregulated in 80-90% of OS tumors (22). More recently, efforts have been made to categorize OS patients into genetically defined subpopulations, including patients with amplified chromosome 8q24.2 region, which harbors the oncogene c-Myc. To further understand the role of MYC-dependent molecular and cellular tumor intrinsic and extrinsic profiles in OS tumor development and metastasis, we utilized our prior OS GEMM (referred to as the p53 model in the text). We generated a Myc knockin GEMM by crossing the conditional Col 2.3-Cre; Trp53fl/+ mice with Lox-Stop-Lox (LSL) c-MycT58A mice to generate Cre-Lox-Stop-Lox;(LSL)-c-MycT58A;Trp53lfl/+ mice (referred as Myc knockin model in the text) (Figure 1A).

The Myc knockin GEMM developed rapid onset of OS tumors with a median time to sacrifice of approximately 24 weeks vs. 52 weeks for the conditional p53 model (Figure 1B). In addition, we observed a high incidence of pulmonary metastasis (>60%) in the Myc knockin compared to approximately 20% incidence seen in our p53-GEMM. Histological analysis of the primary and metastatic tumors confirmed the OS histology (Figure 1C). MYC IHC staining showed higher protein expression in the tumor tissue of the Myc knockin specimens as compared to the p53 samples (Figure 1D). Also, a significantly higher level of MYC expression was observed both at the mRNA and protein
levels in the tumor tissue samples of Myc knockin GEMMs as compared to the p53 GEMM (Figure 1, E and F).

Further, we performed molecular characterization of the GEMM models by analyzing the tumor tissue samples using whole tumor RNA-sequencing and total proteome analysis. We performed a cross-species transcriptomic comparison of the GEMM molecular signatures with human OS tumors using the OS TARGET data set and R2 Genomic Analysis dataset. We noted that 2743 genes were differentially expressed at the transcriptional level (p<0.05) between Myc knockin and p53 tumors, in which 1055 were downregulated and 1688 upregulated (Supplemental Figure 1A). Subsequently, a comparison of gene set enrichment analysis (GSEA) using differentially expressed genes (DEGs) from Myc knockin and the p53 GEMM model tumors to the OS TARGET dataset was performed. This demonstrated our Myc knockin GEMM closely resembles the high-MYC expressing human OS tumor subtype with 3147 positively and negatively enriched overlapping gene sets between mouse and human samples (Supplemental Figure 1B). Specifically, we identified concurrent alterations in the innate and adaptive immune response, myeloid and leukocyte mediated immunity, macrophage-migration, chemotaxis, differentiation, and CSF signaling, for our GEMM and human OS TARGET data set (Figure 1G and Supplemental Table 1). Besides immune-related signatures, which had significant negative normalized enrichment scores (NES) for both our murine and human MYC-hyperactivated datasets, we also observed common enrichment scores in numerous other gene sets/pathways. Besides enrichment of MYC target genes (HALLMARK MYC TARGETS V1 and V2), these include positive enrichment in DNA replication, RNA processing and splicing, and amino acid metabolism, while there is
common negative enrichment in cell-cell adhesion, oxidative phosphorylation, fatty acid metabolism, and antigen presentation (Supplemental Table 1). In summary, based on histopathology and proteo-transcriptomic analysis we provide evidence of a strong correlation between the phenotypic and molecular profiles of the murine Myc knockin model and high MYC-expressing patient OS tumors.

*Molecular characterization of OS syngeneic mouse models and cell lines.*

To develop resources for additional molecular and therapeutic studies, we generated and characterized GEMM-derived OS cell lines and syngeneic tumor models. Supplemental Figure 2A represents the schematic diagram of how the OS cell lines and the syngeneic mouse models were generated. Similar to the GEMM, the expression of MYC was significantly increased at the protein and mRNA levels in cell lines ($p<0.001$ and $p<0.01$) and syngeneic tumor models ($p<0.05$ and $p<0.05$) derived from the Myc knockin as compared to the p53 tumors (Figure 2, A-D). The Myc knockin-derived cells were more proliferative than the p53-driven cells in vitro (Figure 2E). Further, we analyzed the MYC phosphorylation status at Ser62 position in Myc knockin-derived cell lines as it contributes to the stabilization of MYC protein. We observed that all Myc knockin cell lines have phosphorylated Ser62 MYC (Supplemental Figure 2B). Other than Ser62 phosphorylation, threonine at amino acid position 58 also plays an important role in MYC protein stability as a phosphorylation site for subsequent ubiquitination recognition (23). This Myc knockin model has the point mutation T58A, thus, it lacks any phosphorylation at that position, which has been previously demonstrated in another Myc knockin model (24). We observed mice injected intratibially with the Myc knockin syngeneic cell lines
rapidly developed tumors with a high incidence of metastatic disease like the spontaneous Myc knockin GEMMs. Tumors were palpable in 1-2 weeks after injection and sacrificed at roughly 3-4 weeks, with 60-80% of syngeneic mice developing metastatic tumors primarily to the lung. In the case of p53-driven cell lines, those injected mice took approximately 2-3 weeks to develop a palpable tumor and the time of sacrifice ranged from 6-12 weeks. We also noticed that the p53 cells injected in syngeneic mice were less metastatic in nature over the experimental time course, with only 10-20% of the mice developing metastasis. These results demonstrate the aggressive nature of the Myc knockin model, and the utility of the syngeneic cell lines derived from the GEMM to recapitulate tumor development and progression, which can be used as valuable resources for downstream molecular and pharmacological studies.

**MYC suppresses immune cell infiltration to the tumor microenvironment in osteosarcoma.**

GSEA analysis of Myc knockin and patient transcriptomic data identified that innate and adaptive anti-tumor immune response-related pathways were significantly downregulated in the high MYC murine and human tumors as compared to low MYC tumors (Supplemental Table 1). Specifically, we observed that the expression of *Ptprc* (CD45, a pan hematopoietic cell marker) was significantly lower both at the transcript (p<0.05) and protein level (p<0.05) in the Myc knockin tumors (Figure 3, A-B). Immunohistochemistry (IHC) for CD45 on tumor tissue samples also showed a significant reduction in their expression on the Myc knockin tumors as compared to the corresponding p53 tumors (Figure 3C).
To further validate our findings, we found a negative correlation between MYC and PTPRC mRNA expression in the human TARGET (r=-0.36, p<0.0006) and Kujjer datasets (r=-0.25, p=4.3x10^-3) (Figure 3D and Supplemental Figure 3A). In addition, high PTPRC mRNA expression was associated with a better prognosis compared to low PTPRC expression in the TARGET data set (p<0.05) and trended toward significance in the Kujjer datasets (p=0.059) (Figure 3E and Supplemental Figure 3B).

To further analyze the immune sub-population, we performed a CIBERSORT analysis of our RNA-seq data that gave us the fraction of 22 immunocyte types in OS tumor samples (Supplemental Figure 4). We observed a significant reduction in the macrophage population in the Myc knockin tumor samples (p<0.01) (Figure 3F). The expression of Cd68 mRNA transcript, a macrophage marker, was also significantly decreased in the Myc knockin GEMM tumor tissue samples as compared to the p53-driven tumors (p<0.05) (Figure 3G). We confirmed this reduction of macrophage population in the TME of OS with IHC staining on paraffin-embedded GEMM tumor tissue samples using F4/80 antibody, which is a specific mouse macrophage marker (Figure 3H), which showed reduced macrophage population in the TME of Myc knockin tumor. In addition, we observed that Cd68 mRNA expression was negatively correlated with the Myc mRNA expression in the TARGET (r=-0.38, p<0.0005) and Kujjer datasets (r=-0.17, p=0.05) (Figure 3I and Supplemental Figure 3C), and has prognostic significance, with higher CD68 expression being associated with improved patient survival (Figure 3J and Supplemental figure 3D). These findings not only further define a prominent role for
macrophages in OS tumor biology but also validate our novel murine *Myc* knockin OS model for its ability to recapitulate human OS.

After identifying the direct association between elevated *Myc* expression and decreased immune infiltration, we applied FACS analysis on an orthotopic model using syngeneic murine high and low MYC expressing cell lines to analyze the immune landscape of OS tumor (gating strategies is shown in Supplemental Figure 5). The total CD45+ cells (hematopoietic cells) (p<0.001), as well as the macrophage population (p<0.001), were significantly lower in the *Myc* knockin samples as compared to the p53-driven syngeneic tumor tissue samples (Figure 3, K and L). In summary, we identified that MYC regulates the OS TME by modulating immune cell populations, specifically macrophages. Additionally, macrophage-associated genes, *PTPRC* and *CD68*, are related to poor patient’s outcome.

*Myc hyperactivation downregulates the cytokine CSF1 in the tumor immune microenvironment of osteosarcoma.*

To explore the mechanism underlying the alteration of the macrophage population in *Myc* knockin OS TME, we investigated molecular alterations in macrophage maturation and recruitment. Our proteo-transcriptomic analysis identified significant downregulation in the expression of *Csf1* in the *Myc* knockin OS tumors as compared to the p53-driven tumor both at the protein (p<0.05) and transcript level (p<0.001) (Figure 4, A and 4B). Moreover, IHC staining for CSF1 confirmed lower protein expression in *Myc* knockin GEMM tumors as compared to the p53-driven tumors (Figure 4C). To validate this finding
in human samples, we investigated the correlation between CSF1 and MYC mRNA expression in human OS tumors using the TARGET data set and available institutional patient-derived xenograft (PDX) samples. Interestingly, we observed a negative correlation between the CSF1 and MYC expression for both the TARGET data set (r=-0.37, p<0.05) and the OS PDX samples (Figure 4, D and E). We also observed that a higher transcript expression level of CSF1 was correlated with a good prognosis for OS patients (Figure 4F). Besides CSF1, IL-34 is an important ligand of CSF1R, so we further investigated the mRNA expression of Il-34 in the Myc knockin and p53-driven tumor tissue samples. We noticed there was no significant difference in the Il-34 expression between the Myc knockin and p53-driven tumor samples (Supplemental Figure 6), so subsequently focused on the regulation and role of Csf1 in hyperactivated MYC OS tumorigenesis.

To prove that MYC modulates CSF1 expression in OS, we used transient knockdown of the Myc transcript levels in Myc knockin cell lines and the MYC-dTAG protein degradation system using the murine F331 cell lines generated in our laboratory. The dTAG-degradation system allows real-time selective degradation of a target protein as a useful alternative to genetic methods for target validation. For the dTAG system, we stably expressed the FKBP12F36V-MYC{T58A} construct in a low-Myc-expressing murine OS cell line (F331) described here as F331-dTAG-Myc cell line. Application of dTAG induces rapid, reversible, and selective degradation of FKBP12F36V-MYC{T58A} fusion protein both in vitro and in vivo (Supplemental Figure 7).
After knockdown of the *Myc* transcript via siMyc, we observed a significant upregulation in the *Csf1* expression when compared to the corresponding scrambled control (p<0.05) (Figure 4G). Furthermore, our complementary MYC-dTAG protein degradation model showed upregulation of *Csf1* upon exposure of the F331-dTAG-Myc cells to dTAGv1 or dTAG-13 compounds. The *Csf1* expression level was significantly upregulated after the MYC degradation both at the transcript and protein levels (Figure 4, H and I). Therefore, MYC negatively regulates the expression of CSF1. Subsequently, we were interested in investigating MYC-mediated mechanisms of CSF1 suppression. Additionally, to understand the role of MYC overexpression in OS regulating *Csf1r* expression in TAMs, we analyzed their expression at transcription and protein levels. CSF1R expression was significantly lower in the *Myc* knockin tumor compared to the p53-driven tumor samples (Supplemental Figure 8A and 8B). Subsequently, we performed co-culture in vitro studies using RAW 264.7 cells, which are an established mouse macrophage cell line, and OS cells to assess the effect of MYC expression on expression of *Csf1r* in the population. We observed a significant reduction in the expression of *Csf1r* when RAW 264.7 cells were cultured with hyperactivated *Myc* knockin cell lines compared to the p53-driven cell lines (Supplemental Figure 8C).

*Myc represses Csf1 expression by empowering the miR-17/20a axis.*

To investigate the *Myc*-dependent mechanism of regulation of *Csf1* expression, we noted TargetScan predicted a binding region (1059-1065) on *Csf1* mRNA for miR17-92 family members (Supplemental Figure 9 A, B) (25), which are MYC-mediated microRNAs. MYC regulates the expression of several microRNAs, including the
polycistronic miR-17-92 cluster by binding to their promoter region in both humans and rodents (26-28). For a better understanding of miR-17-92 cluster activities, we focused on the function of mir-17 and mir-20a, two members of the miR-17-92 cluster. The expression of miR17-5p and Mir20a-5p was significantly higher in the GEMM tumor tissue samples isolated from the Myc knockin compared to the p53-driven (p<0.01) (Figure 5A). Further, we validated the MYC-dependent regulation of miR-17-5p/20a-5p expression using transient knockdown of MYC in mouse Myc knockin-derived cell lines and the MYC-dTAG protein degradation system using the F331-dTAG-Myc cells. The expression of miRNA-17-5p/miR20a-5p after transient MYC knockdown and via the dTAG-MYC-degradation resulted in the downregulation of the miR17-5p/20a-5p expression (p<0.01) (Figure 5, B and C), thus confirming a role for MYC in the regulation of miR17-5p and miR20a-5p expression in OS.

Further, to examine the role of miR17-5p/20a-5p on the Csf1 regulation we performed both gain and loss-of-function studies using microRNA-17/20a inhibitors and mimics. Myc knockin cell lines, which have significantly elevated levels of miR17-5p/20a-5p expression were treated with the inhibitors, whereas the p53-driven cell lines in which miR17-5p/20a-5p expression was lower were used with mimic treatment. As shown in Figure 5D, after the treatment with the miR-17-5p/20a-5p inhibitors, the expression of Csf1 was significantly upregulated (p<0.05), whereas in the case of miR17/20a mimics reversed these effects and led to a downregulation of Csf1 expression (p<0.05) (Figure 5E). We established that miR-17-5p/20a-5p is causally responsible for at least part of the mechanism by which MYC regulates the Csf1 expression in OS.
Myc mitigates macrophage cell infiltration to the TME of OS and their phenotypic function.

After identifying that the macrophage population was significantly diminished in the Myc knockin OS tumors, we were interested in investigating the role of MYC in dictating this cellular microenvironmental feature. First, we examined the effects of elevated intrinsic MYC OS levels on the migration and proliferation of RAW 264.7 cells, using a transwell co-culture assay in an in vitro set-up (Figure 6A). The sarcoma cells, either Myc knockin or p53-driven, were cultured in the bottom chamber, and RAW 264.7 cells were seeded in the top chamber, and the migratory potential of RAW 264.7 cells were monitored. In the wells with the Myc knockin OS cells, we observed significantly lower amounts of macrophage migration compared to p53-driven OS cells (Figure 6B). To confirm the role of MYC in this migration, we used two independent loss-of-function models, including siMyc and direct protein degradation via the dTAG system. A significant increase in the macrophage migration was observed after siMyc treatment compared to the scramble control-treated OS cells (Figure 6C). Migration was also increased after direct MYC protein degradation using the dTAG system (Figure 6D). These results demonstrate that MYC negatively regulates the macrophage cell infiltration to TME of OS.

After confirming the role of MYC in macrophage migration, we examined their involvement in dictating the macrophage function, particularly their polarization and phagocytosis. To show the effect of MYC on the polarization of macrophage cells, RAW 264.7 cells were cultured in the conditioned media collected from the cell culture supernatants of the Myc knockin and p53-driven cell lines (Schematic diagram shown in
Figure 6E). In a control experiment, RAW 264.7 cells were stimulated in vitro either with cytokines, LPS and IFN-γ, or IL-4 and IL-13 to transform into the M1 and M2-like macrophage subpopulations, respectively. The morphology of the transformed cells was analyzed, as well as the M1 and M2-related genes (Cd86 and Arg1, respectively) were quantified at the transcriptional level (Supplemental Figure 10 A-C). We observed an upregulation in the expression of Arg1, in the RAW 264.7 cells cultured in the Myc knockin cell lines conditioned media compared to the p53-driven cell lines (Figure 6F). Moreover, we observed similar gene expression changes when we treated the RAW 264.7 cells with IL-4 and IL-13 cytokines (Supplemental Figure 10B). Cd86 expression was not significantly different when it was compared between the experimental groups (Supplemental Figure 10D). To confirm the MYC-dependent macrophage transformation, we cultured RAW 264.7 cells in the conditioned media collected from the siMyc knockdown OS cell culture supernatant and compared it with the siScr control. A significant reduction in the Arg1 expression (Figure 6G) and enhancement in the Cd86 was observed in the RAW 264.7 cells cultured in the MYC knockdown OS supernatant culture media (Supplemental Figure 10E).

Lastly, we analyzed if enhanced MYC expression in OS affects the phagocytic nature of macrophage cells. To establish the role of Myc in macrophage phagocytosis, we used transient gene and protein knockdown via siMyc and the dTAG protein degradation system. RAW 264.7 cells were cultured in the conditioned media collected from Myc knockin-cell lines treated with siMyc or siScr control. To complement this, RAW 264.7 cells were cultured in the conditioned media collected from either dTAG-v1 or
DMSO treated 331-dTAG-Myc cells. A significant enhancement in the phagocytosis was observed for the RAW 264.7 cells cultured in the conditioned media from siMyc (p<0.05) and dTAG-v1 (p<0.05) treated cells compared to the corresponding controls (Figure 6, H and I).

In addition, we determined if MYC-regulated CSF1 modulates macrophage functions including their migration, phagocytosis, and proliferation in the OS TME. To examine the role of MYC-mediated CSF1 in macrophage cell migration, we used a transwell co-culture model (Figure 6A). As shown in Supplemental Figure 11A, after transient knockdown of Csf1 expression in OS cells, we observed a significant reduction in the RAW 264.7 cell migration towards the OS cells compared to the scramble control. p53-derived cell lines were used for the Csf1 knockdown experiment as their expression was relatively higher. After confirming the role of CSF1 in the RAW 264.7 migration, we performed a rescue experiment using the F331-dTAG-Myc cell line to determine the role of MYC-dependent CSF1 in macrophage cell migration. Specifically, we used the dTAG-v1 to degrade the MYC protein followed by transient knockdown of the Csf1 gene to examine their role in macrophage cell migration. Supplemental Figure 11B shows after dTAG-V1 treatment, migration of macrophages increased, and later decreased followed by the Csf1 knockdown. We also observed that macrophage proliferation was significantly enhanced in the presence of CSF1 (Supplemental Figure 11C). We conclude that MYC was sufficient to regulate the CSF1 expression in the OS tumor, which orchestrates the migration of macrophages in the TME of OS.
In summary, we demonstrated that intratumoral MYC dictates environmental macrophage cell migration and their functions, particularly their polarization and phagocytic properties in the TME of OS.

Selective in vivo pharmacological degradation of Myc improves immune cell infiltration to the TME of OS.

To validate the MYC-dependent regulation of the immune infiltration and macrophage functions, we used the dTAG protein degradation approach in vivo. F331-dTAG-Myc cells were injected intratibially into the C57BL/6 mice and upon detection of palpable tumor, mice were randomized to receive treatment either with dTAG-v1 or vehicle control for two weeks intravenously via retro-orbital injection. Figure 7A shows the schematic diagram for the dTAG-v1 treatment. Tumor volume was significantly reduced after two weeks of dTAG-v1 treatment when compared to the placebo control group (Figure 7B). As shown in Figure 7C, after two weeks of treatment, overall CD45+ cell populations were significantly enhanced in the dTAG-v1 treated group as compared to the placebo control. The macrophage population was also significantly enhanced after the treatment compared to the placebo control group (Figure 7D). An effective MYC-protein degradation was noticed after dTAG-v1 treatment (Figure 7E). The analysis of Mir17/20a transcript expression showed a significant reduction after the dTAG-v1 treatment (Figure 7F). Together, these data support the involvement of MYC in the immune-suppressive tumor microenvironment in OS by regulating the macrophage population (Figure 7G).
Discussion:

Despite surgical advancements and multi-drug systemic chemotherapy, the overall survival for OS patients has had minimal improvements over the last three decades (29). The rarity of this disease, tumor cell heterogeneity, and the lack of targetable robust oncogenic mutations make it very challenging to study and cure a significant number of patients with OS. While there is a lack of identifiable targetable mutations, OS has recurrent chromosomal copy number alterations encompassing gains and losses of key oncogenes or tumor suppressor genes. Amplification of c-MYC is seen in a significant portion of OS tumors and conveys an overall poor prognosis. We have established and characterized a novel conditional osteoblast-specific Myc knockin GEMM to understand the pathophysiology of OS tumors and the role of MYC in the TME modulation. Moreover, we demonstrated the critical role of MYC in regulating macrophages in the TME and its impact on the generation of more aggressive tumors.

MYC oncogene is considered a master regulator of many processes, including cell cycle entry, ribosome biogenesis, and metabolism and it’s expression is dysregulated in >50% of human cancers (10, 30). We previously generated a conditional osteoblast-specific OS GEMM by altering the Trp53 status (21, 31). However, the Trp53fl/+; Myc knockin GEMM we have generated and comprehensively characterized has high Myc expression both at the mRNA and protein level with accelerated osteosarcomagenesis and metastatic potential compared to the previously reported Trp53fl/+ OS GEMM model (21). We subsequently generated murine OS cell lines and applied them towards
orthotopic syngeneic tumor models for additional molecular and therapeutic studies. Our novel *Myc* knockin OS GEMM closely resembles the high-MYC expressing human OS subtype and has significant homology to genomic and biological phenotypes seen in these tumors. Through the integration of innovative murine models of OS and bioinformatics analysis of human OS datasets, we have identified a novel immune-regulatory function of MYC in OS tumor biology.

We established that hyperactivation of MYC suppresses immune cell infiltration, including macrophages in the OS TME. Recent studies elucidate the role of MYC in TME modulation as well as in the host immune response in multiple tumor types (14, 32, 33). The macrophage has been reported to be the most abundant immune cell infiltrate to the TME of solid tumors, including OS (34), and is involved in regulating other immune cell functions and matrix remodeling that leads to tumor-suppressing or promoting microenvironment (35, 36). Recently, Lee et al. reported similar findings in the triple-negative breast cancer tumor, where they found elevated *MYC* expression was associated with lower overall immune cell infiltration, including the macrophages both in the mouse models and patient data (37). MYC has been found to be associated with immune-suppressive TME in lung and pancreatic cancer models (32).

The role of a macrophage in tumor progression for OS remains to be fully elucidated, in part due to the contrasting roles they play depending on their polarization. The excessive macrophage infiltration in the TME and its association with the patient's clinical outcome depends on the cancer diagnosis. The abundance of macrophages in
the TME of colorectal and gastric cancer is associated with a good prognosis whereas is the opposite in breast, head and neck, glioma, melanoma, and prostate cancers confer the worst prognosis (38-40). Macrophages are highly plastic cells and can polarize into different subpopulations, such as M1 or M2-like macrophages, depending on the microenvironmental signals in the TME. On the one hand, M2-like macrophage promotes the immune-suppressive TME by recruiting regulatory T cells (Tregs), inhibiting the T cell function by controlling the expression of programmed cell death ligand 1 (PD-L1), cytokines IL-10 and TGFβ (41, 42), and inhibits macrophage phagocytosis by upregulating the expression of SIRT-α (43). On the other hand, M1-like macrophages take part in the adaptive anticancer immune response through enhancing antigen presentation and activation of adaptive immunity and enhanced phagocytosis (35). We noted an inverse correlation between the hyperactivation of Myc and the abundance of macrophage populations in the TME of OS of our murine model. The human TARGET and R2 datasets also show a negative enrichment of the macrophage population in the abundance of MYC expression additionally, the higher macrophage population was associated with the OS patient's good prognosis. Similar to our finding, the abundance of macrophages was associated with reduced metastasis and improved survival in high-grade osteosarcoma (44). However, it should be noted that the overall macrophage population and patient outcome depend on the tumor type. The levels of CD68, a macrophage marker, correlated with an adverse prognosis in glioblastoma, kidney renal clear cell carcinoma, hepatocellular carcinoma, lung squamous cell carcinoma, thyroid carcinoma, and a favorable prognosis in colorectal cancer, OS and kidney chromophobe
We think the heterogeneity of phenotype behind the inconsistency of these functions in various cancer types.

As CSF1/CSF1R has been identified as the principal pathway that controls macrophage survival and differentiation from progenitor or circulating monocytes to macrophages (50, 51) we examined the role of CSF1 in MYC-associated macrophage regulation in the TME of OS and identified a novel MYC-miR-17-92-CSF1 axis that directly contributes to this alteration in TME. MYC regulates the MiR-17/20a expression by directly binding to their UTR region (52, 53). The miR17-92 family is overexpressed in various human cancers, including lung, breast, colon, B-cell lymphomas, gastric, and retinoblastomas, where they regulate several genes important for cell cycle progression and metastasis (54-57).

Furthermore, we demonstrated MYC-dependent macrophage polarization, phagocytosis, and migration to the TME of OS. Currently, two different macrophage-centered approaches are in clinical trials. One includes the elimination of tumor-associated macrophages and the other re-polarizing tumor-promoting macrophages into pro-inflammatory M1-like macrophages. The blocking of the CSF1/CSF1R axis either by targeting CSF1 or CSF1R is in clinical trials to eliminate the tumor-promoting macrophage population (58). But the results are contradictory, in a recent breast cancer clinical trial, the use of neutralizing anti-CSF1R and anti-CSF1 antibodies, along with the small-molecule inhibitors of CSF1R showed an enhancement in the metastasis without altering primary tumor growth (59). Another study observed a strong correlation between the
clinical chemotherapy response and a higher expression of CD4/CD68/CSF1R gene signatures in OS (60). These results indicate that the role of the CSF1/CSF1R system is far more complex than it seems and requires further investigation as a therapeutic target.

Since CSF1 regulates macrophage proliferation, function, and infiltration to the TME. The association of the macrophage population abundance with the disease prognosis dramatically depends on the tumor subtypes, so targeting the CSF1/CSF1R pathway may vary greatly depending on the tumor subtypes. So, before targeting the macrophage or CSF1/CSF1R pathway, one must consider the tumor sub-type and its genetic drivers. We noticed in the case of Myc knockin OS that CSF1 expression was low and had a more aggressive and metastatic tumor. So, targeting CSF-1 might not be a rational approach in this subpopulation of patients. The other approach which might be clinically beneficial for OS patients and effective therapy development is the use of drugs that can polarize the M2-like macrophage population to M1-macrophage. Based on the available preclinical and clinical data, for example, mifamurtide showed some benefits in OS treatment and has approval from the European Medicines Agency (EMA), but further investigations are required to define its role in the treatment of OS patients (61). Other potential molecules that could be used to transform the M2-like macrophage phenotype to M1-like phenotypes include linear 3-O-methylated galactan, CD40 agonist, zoledronic acid, statins, trabectedin, and TLR ligands (e.g., imiquimod and CpG) (62-65). But all these approaches require additional investigation before being used in the clinical setup.
We and others think that innate immune cells, especially macrophages, play an essential role in inhibiting the initiation and development of cancer (66), and reorienting and polarization of tumor-associated macrophages towards the M1-like macrophage is the holy grail of macrophage-mediated cancer therapy (67, 68). Our preclinical mouse model will help in screening the small molecules including the immune modulators, macrophage polarizers, and a combination of drugs to target this aggressive tumor subtype and provide more insight into the OS tumor biology. Future studies also include further dissecting the role of macrophages in MYC-driven osteosarcoma through evaluating the cell-cell communication between the macrophage and OS cells as well as modulation of the macrophage population. In addition, additional mechanistic studies assessing the effects of stably altering miR-17/92 expression in osteosarcoma cells to determine the in vivo effects of these miRs. Presently, systemic administration of anti-miR therapy is very exploratory and results can often be difficult to interpret or make definitive conclusions.

Overall, our study is the first to successfully demonstrate a MYC-dependent regulation of the OS tumor microenvironment. We have identified a novel molecular mechanism in which MYC-hyperactivation leads to the downregulation of Csf1 through increased miR-17-92 expression, resulting in diminished macrophage presence in the TME of OS. Thus, perturbations of direct MYC activity, or downstream effectors, such as miR-17/92 family members, can have potent effects on enhancing the tumor immune microenvironment and therapeutic benefit for this high-risk group of OS patients.
Conclusion

Our studies using a newly developed c-Myc knockin OS GEMM and cell line models identified that hyperactivation of c-MYC is sufficient to inhibit the CSF1 expression associated with the macrophage proliferation and localization to the TME of OS. We established the involvement of MYC-dependent miR17/20a in the regulation of Csf1 resulted in those phenotypes. Thus, we identified a novel molecular mechanism through which MYC regulates the CSF1 expression. This leads to alterations in the macrophage population and an immunosuppressive tumor microenvironment.

Material methods

Generation of Myc knockin GEMM Myc knockin genetically engineered mouse model (GEMM) were generated by crossing Col2.3-Cre/Trp53\(^{fl/+}\) and Lox-Stop-Lox (LSL)-Myc\(^{T58A}\) mice, which were developed and obtained from the Sears lab (Oregon Health and Science University) (24). Col2.3-Cre/Trp53\(^{fl/+}\) mouse model was generated by crossing mice expressing Cre recombinase under the transcriptional regulation of the osteoblast-specific promoter Col2.3 with Trp53-floxed mice represented as p53-driven-mouse (21). Genotyping primer details are given in Supplemental table 2A. The Institutional Animal Care and Use Committee approved the experimental protocol (AN-5225). All experiments were performed per relevant guidelines and regulations.

Generation of an OS syngeneic mouse model and cell lines Primary murine tumor OS cell lines were generated by dissociating the GEMM OS tumor using Miltenyi Tumor
Dissociation Kit (Catalog no- 130-096-730). OS cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% pen/strep, and maintained at 37 °C with 5% CO₂. All cell lines were routinely checked for Mycoplasma contamination. Alkaline phosphatase staining (NBT/BCIP, Catalog no-11697471001, Roche, USA) was performed according to the manufacturer’s instruction to confirm the osteosarcoma cell type. A syngeneic mouse model was generated by injecting 1x10⁶ OS tumor cells intratibially into C57BL/6 mice.

**Cell proliferation assays** Growth assays were performed by plating 1000 cells per well in a 96-well dish. Cell growth was assessed daily by the addition of the Cell Counting Kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer’s instructions (CCK-8 assay kit; Dojindo Laboratories). Each cell line was plated in triplicate, and the value presented represents the average of the samples.

**Transfection of siRNA in OS cell lines** In vitro transfections were performed in six-well plates (2 × 10⁵ cells) for OS cells derived from the Myc knockin and p53-driven -GEMM, using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. For the transfection experiments, cells were plated 24 hours before the experiment. For each candidate gene, two predesigned gene-specific siRNA (Sigma Aldrich) were tested in parallel with scrambled control (Sigma Aldrich) and a blank with the transfection agent only. The most effective siRNA for Myc and Csf1 were selected (Catalog no- SASI_Mm01_00157478 and SASI_Mm02_00308073, Sigma Aldrich, USA) for further experiments. Cells for mRNA evaluation were harvested 48 hours post-treatment, while those for protein evaluation were harvested at 72 hours.
**Quantitative real-time PCR** Total mRNA and microRNA were extracted by using the RNeasy mini kit (Qiagen) and miRNeasy mini kit respectively, as recommended by the manufacturer. In brief, after removing the medium, cells were washed twice with cold PBS and lysed in RLT lysis buffer (Qiagen) for 5 minutes at RT. For tumor tissue samples, around 30 mg of tumor sample was homogenized in either triazole for microRNA isolation or RLT lysis buffer for total RNA isolation using the tissue homogenizer. Lysates were stored at -20ºC before the RNA isolation. RNAs were quantified using Thermo Scientific™ NanoDrop 2000. 1µg RNA was used for cDNA synthesis using qScript cDNA SuperMix (Quantabio, Beverly, MA, USA). Real-time PCR with iQ SYBR Green SuperMix (Bio-Rad) was performed using gene-specific primer pairs (Qiagen/sigma Aldrich) utilizing the StepOnePlus real-time PCR machine (Applied Biosystems). The hsa-miR-17-5p and 20a-5p miRCurry LNA miRNA PCR Assay kits were used to quantify the miRNA expression. The relative mRNA expression was calculated with the ΔΔCT method. A list of primers is given in Supplemental table 2B.

**Western blot analysis** After removing the medium, cells were washed twice with cold PBS and lysed in ice-cold RIPA lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate) supplemented with protease inhibitors and phosphatase inhibitors (Roche) for 15 minutes at 4ºC. Lysates were harvested and centrifuged at 13000 g for 10 minutes to remove the cell debris, the supernatant was collected, and the protein concentration was determined using the bicinchoninic acid assay (Pierce) and stored at -20ºC before the analysis. For tumor tissue samples, around 50 mg of tumor sample was homogenized in the RIPA lysis buffer using the tissue homogenizer and the supernatant was collected similarly as described above for the cell lines. 30µg of protein
was electrophoresed in a 4-15% precast gel (Mini-PROTEAN® TGX™) SDS-PAGE under reducing conditions. iBlot 2 dry blotting system was used for transferring the protein to the PVDF membrane. After blocking the blot with 5% BSA in PBS, the blots were probed overnight with antibodies (cMYC 1:1000, ab32072, Abcam, CSF1 1:1000, ab233387, Abcam, Phospho-cMYC Ser 62 1:1000, #13748, CST, GAPDH 1:1000 EMD Millipore, Billerica, MA, USA, AB2302). Blots were incubated with the appropriate secondary HRP-conjugated antibodies for 1 h, and the signal was detected utilizing Millipore Immobilon Western chemiluminescent HRP substrate (Millipore). Quantification and statistics Western blots were visualized and quantified using Image J software version 1.53e. Statistical significance was determined by Student’s t-test.

**Immunohistochemistry** Primary as well as metastatic tumor tissue samples from the *Myc* knockin GEMM were fixed in 10% formalin at the time of tumor harvest, paraffin-processed, and sectioned. For immune-histochemical analysis, sections were de-paraffinized, rehydrated, and boiled in a microwave for 20 minutes in 10 mM citrate buffer (pH 6.0) for antigen retrieval. VECTASTAIN Elite ABC kit following the manufacturer’s instructions was used for visualization. For control, IgG isotype was used instead of primary antibody, wherever indicated. Tumor sections were stained with hematoxylin and eosin and analyzed with the help of Baylor core pathology for OS tumor characterization. IHC slides were visualized using the phase-contrast microscope.

**SymphonyFACS analysis** Tumors were excised from the syngeneic mice and dissociated using the gentleMACS™ Dissociator (Miltenyi Biotec) according to the manufacturer’s protocol. Cells were passed through the 70µm cell strainer to remove cell
clumps. RBCs were lysed using 1X RBC lysis buffer (eBiosciences, Thermofisher, Catalog no- 00-4300-54). After centrifugation at 1200 rpm for 5 minutes, the supernatant was discarded, and cells were re-suspended into 1mL of FACS buffer (PBS with 5% FBS) followed by the staining with 0.1% fixable viability stain (BD Biosciences, catalog no- 565388) along with the 10 µl of brilliant stain buffer plus (Catalog no-566385) for 10 minutes at 4ºC. Cells were surface-stained with a pre-mixed fluorescence conjugated mAb cocktail for 30 minutes at 4ºC in the dark. The cocktails were prepared with CD45- BUV805 (Catalog no-748370), F4/80-BV 421 (Catalog no-565411), CD11B PE-CF594 (Catalog no-562287), CD3 BV711 (Catalog no- 740665), CD19 BUV 395 (Catalog no- 565965), and Siglec-F PE (Catalog no- 552126) . After washing, cells were fixed in 2% PFA for 30 minutes at 4ºC and finally re-suspended in the 0.5 ml of FACS buffer and stored at 4ºC before analysis. Data were acquired on five laser FACSymphony (BD FACSymphony™ A5 Cell Analyzer). Analysis was performed using BD FACSDiva software v. 6.0 and FlowJo 10.8.0 (Tree Star, Inc, Ashland, OR). Gating strategies for immune cells were used as follows: Total leukocytes CD45+, Macrophages CD45+ CD11B+ F4/80+.

dTAG protein degradation system A Nobel degradation tag (dTAG) system was used to selectively target the MYC-protein degradation. The dTAG system provides a linker that links FKBP12-F36V fused with MYC-protein to ubiquitin ligase and then degrades it through the ubiquitin-proteasome system as described previously by Nabet et al. (69). We have generated a syngeneic murine OS cell line (F331-dTAG-Myc), which was originally derived from a Col2.3-Cre/Trp53fl/+ GEMM tumor, with a low level of endogenous c-MYC, but stable overexpression of the FKBP12F36V-MYCT58A construct.
Commercially available dTAG-v1 (Tocris, Bio-Techne Corporation, catalog no- 6914) and dTAG-13 (Tocris, Bio-Techne Corporation, Cat. No. 6605) at a concentration range from 10nM to 1μM were used to selectively degrade MYC-protein.

**In vivo MYC-protein degradation** \(1 \times 10^6\) viable F331-dTAG-MYC cells expressing FKBP12F36V-MYCT58A were injected into 4 weeks-old C57BL/6 mice through intra-tibial injection. Before injection, cells were tested for mycoplasma contamination. Mice were routinely monitored for the tumor and after the confirmation treated for two weeks either with the vehicle control (n = 3 biologically independent mice) or dTAG-v1 (2 mg/kg, n = 4 biologically independent mice) through the retro-orbital injection. The dTAG-v1 was formulated in a 20% solutol (Sigma), 5% DMSO in 0.9% sterile saline. After two weeks of treatment, tumors were harvested and dissociated for the FACSsymphony analysis using the gentleMACS™ dissociator (Miltenyi Biotec). Protein and RNA samples were prepared from the tumor samples and stored at -80°C before analysis. All experiments were adherent to institutional standards.

**Tumor-conditioned media collection** Both Myc knockin and p53-driven OS cell lines were cultured separately in DMEM supplemented with 10% FBS and 1% Pen/Strep. Once grown to 90% of confluence, media were discarded and rinsed with PBS. Cells were then incubated with fresh DMEM without any supplement for 24 hours; the conditioned media (CM) was collected and centrifuged at 1200 RPM for 5 minutes to remove cell debris. The supernatant was filtered with a 0.20 μm syringe filter and stored at ~20°C before use.
also collected conditioned media from the Myc and Csf1 siRNA knockdown cell lines as well as the corresponding Scr control cells.

**Co-culture experiment**

The GFP-labeled murine macrophage cell, RAW 264.7 (Gifted by Prof. Ananth V Annapragada, Texas Children’s Hospital, Baylor College of Medicine, Houston, TX) were co-cultured with the murine OS cells in DMEM supplemented with 10% FBS and 1% of pen/strep and maintained in a humidified atmosphere with 5% CO₂ at 37°C. Before co-culture, RAW-264.7 cells were transiently transfected with GFP plasmid (Addgene, Plasmid #176015), and GFP-positive cells were FACS sorted using the BD FACSAria™ II Cell Sorter. Myc knockin and p53-driven OS cells were co-cultured separately with GFP-labeled RAW 264.7 cells at a ratio of 1:4 (RAW 264.7: Tumor cells) for 72 hours. After co-culture, GFP-labeled-RAW 264.7 cells were FACS sorted by BD FACSAria™ II Cell Sorter and Csf1R mRNA expression was analyzed in the RAW-264.7 cells using semi-quantitative qPCR.

**Monocyte differentiation to M1/M2-like macrophages** An established mouse macrophage cell line, RAW 264.7 was used for the polarization/differentiation experiment. For M₀ to M1-like-macrophage differentiation, RAW 264.7 cells were treated with IFN-γ (20 ng/mL) and LPS (100 ng/mL) whereas for the M2-like macrophage transformation it was treated with IL-4 (20 ng/mL) and IL-13 (20ng/mL) for 48 hours. Before the cytokines treatment 5x10⁵ RAW 264.7 cells were seeded in a 6-well plate containing DMEM, 10% FBS media, and 1% Pen/strep overnight.
**Migration assay** Migration assays were analyzed in a 24-well Boyden chamber. 1x10^6 tumor cells were seeded on the bottom chamber containing the complete media with 10% FBS whereas the 5x10^4 RAW264.7 cells on the top chamber were suspended in 100 µl serum-free media. After incubation at 37°C for 48 hours, cells were fixed and stained with 0.1% crystal violet. Random fields were quantified using ImageJ.

**RNA-sequencing library preparation and sequencing** Tumor tissue samples collected from the GEMMs, both Myc knockin and p53-driven, were used for RNA sequencing. RNA samples underwent quality control assessment using the RNA tape on Tapestation 4200 (Agilent) and were quantified with Qubit Fluorometer (Thermo Fisher). The RNA libraries were prepared and sequenced at the University of Houston Seq-N-Edit core per standard protocols. RNA libraries were prepared with QIAseq stranded total RNA library kit (Qiagen) using 500 ng input RNA. mRNA was enriched with Oligo-dT probes attached to pure mRNA beads (Qiagen). RNA was fragmented, reverse transcribed into cDNA, and ligated with Illumina sequencing adaptors. The size selection for libraries was analyzed using the DNA 1000 tape Tapestation 4200 (Agilent). The prepared libraries were pooled and sequenced using NextSeq 500 (Illumina); generating ~10 million 2×76 bp paired-end reads.

**RNAseq analysis** Paired-end reads were trimmed using trimGalore software (https://github.com/FelixKrueger/TrimGalore), mapped using STAR (70) against the UCSC mm10 genome build, and quantified with featureCounts (71). Differential expression (DE) analysis was performed using DESeq2 R package (1.28.1) (72). The p-values were adjusted with Benjamini and Hochberg's approach for controlling the false
discovery rate. Significantly differentiated genes between the comparisons were identified by applying the criteria of adjusted p-value< 0.05 and fold change exceeding 1.5x. Pathway enrichment analysis was carried out using the GSEA (http://software.broadinstitute.org/gsea/index.jsp) software package; significance was achieved for adjusted q-value<0.25.

RNA-Seq original data for this paper were deposited to GEO under accession number GSE231821.

**Global proteomic analysis** Global proteomic analyses were performed through the BCM Proteomics Core for frozen tumor samples isolated from the Myc knockin and p53-driven GEMM. Tumor tissues were crushed on a liquid nitrogen-cooled steel block with mechanical action. The homogenized tissues were then transferred to Eppendorf tubes and resuspended in 50 µl of ammonium bicarbonate + 1 mM CaCl₂, snap-frozen in liquid nitrogen, and thawed at 42 °C. This freeze/thaw step was repeated 3 times, and then the samples were boiled at 95 °C for 2 min with vortexing at 20 s intervals and kept for proteolytic digestion. After isolation, protein concentrations were measured with the Bradford assay. 50 µg of total protein was processed via 2-step trypsin digestion. First, proteins were digested with a 1:20 solution of 1 µg/µl trypsin/protein in ABC solution (50 mM ammonium bicarbonate, 1 mM CaCl₂) overnight at 37 °C with shaking. Next, additional digestion was carried out with a 1:100 solution of 1 µg/µl trypsin/protein for 4 h in the same conditions. After the addition of 10% formic acid at 1:10 volume to neutralize the reaction, an equal volume of 80% acetonitrile + 0.1% formic acid was added to extract the peptides. Peptides were centrifuged at 10,000 × g, and the peptide concentration of
the supernatant was measured using the Pierce Quantitative Colorimetric Peptide Assay (Cat# 23275, Thermo Fisher Scientific). 50 μg of the peptide was vacuum dried and stored at 4 °C before resuspension for fractionation (if applicable) and sequencing.

TARGET and R2 dataset analysis The TARGET Osteosarcoma patient RNAseq dataset (phs000468) was downloaded from dbGAP. Paired-end sequencing reads were trimmed using trimGalore (https://github.com/FelixKrueger/TrimGalore), mapped using STAR alignment software against the human genome build UCSC hg38, and quantified with featureCounts. Differential expression analysis and GSEA were performed as described above under RNAseq analysis. Survival analysis was performed using patient clinical data plotted in Graphpad Prism (version 9.3.1). The secondary osteosarcoma dataset (GSE33382) was analyzed using the R2:Genomics Analysis and Visualization Platform (http://r2.amc.nl) (20).

Patient-Derived Xenografts: The osteosarcoma patient-derived xenografts analyzed in the study were acquired through our institutional protocol H-32668. The PDXs were previously reported (73).

Statistical analysis The student’s two-sample t-test was used to determine if there was a statistical difference between the means of two groups (e.g., control and experimental groups). P-values were two-sided, and a p-value < 0.05 was considered statistically significant. Quantified data shown represent at least three independent experiments. Data were represented as mean ± SEM. Log-rank (Mantel-Cox) tests were performed for the Kaplan-Meier analyses.
Study approval

The Baylor College of Medicine Animal Care and Use Committee approved the experimental protocol (AN-5225).

Author contributions

BKN contributed to coordinating and designing the study; developing, acquiring, analyzing, and interpreting the data; drafted the manuscript.

TDP contributed to the acquisition, analysis, and interpretation of the data and reviewed and edited the manuscript.

LK and RS contributed to the acquisition and analysis of the data.

AD, NR, and CC participated in reviewing and editing the manuscript.

JTY participated in the coordination and design of the study, interpretation of data, revision, and review of the manuscript.

All authors read and approved the final manuscript.

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References


Figure 1. Development and proteo-transcriptomic characterization of Myc knockin genetically engineered mouse model of osteosarcoma. (A) Schema of generation of Myc knockin GEMMs (B) Kaplan-Meier curve showing accelerated OS development in the Myc knockin (n=15; red) vs. heterozygous Trp53fl/+ (n=85; blue), Log-rank (Mantel-Cox) test were performed for the Kaplan Meier analyses (C) H&E of the primary tumor (left panel) and associated lung lesions (right panel). (D) IHC staining with MYC in the paraffin-embedded tumor tissue samples showed higher expression in the Myc knockin specimen compared to p53-driven GEMMs tumor; quantified expression is shown in the right panel. (E) Western blot demonstrating increased MYC protein expression in Myc knockin tumors compared to Trp53fl/+ driven tumor; quantified expression is shown in the right panel. (F) Relative mRNA expression analyzed by the RNA sequencing demonstrated increased Myc mRNA expression in Myc knockin (n=5) tumors compared to p53-driven (n=4) tumor sample (G) Gene Set Enrichment Analysis (GSEA) comparison between GEMM tumor tissue samples and the high MYC vs. low MYC expressing human OS model using the OS-TARGET data set. (**p<0.001)
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Figure 2. Proteo-transcriptomic characterization of OS syngeneic mouse models and cell lines. (A) Western blot demonstrating increased MYC protein expression in Myc knockin cell lines compared to p53-driven cell lines, quantified expression is shown in the right panel (B) Western blot demonstrating increased MYC protein expression in Myc knockin syngeneic mouse tumor tissue compared to p53-driven samples, quantified expression is shown in the right panel (C) qPCR demonstrating increased Myc mRNA expression in Myc knockin (n=4) derived cell lines as compared to Trp53fl/+ (n=4) driven tumor cell lines (D) qPCR demonstrating increased Myc mRNA expression in Myc knockin syngeneic mouse (n=4) compared to Trp53fl/+ (n=4) driven mouse. (E) Myc knockin and p53-driven cell proliferation (the lighter line is for low Myc cell lines and darker lines for high Myc cell lines). (*p<0.05,**p<0.01).
Figure 3. Myc suppresses immune cell infiltration into the osteosarcoma tumor microenvironment. (A) Relative mRNA expression was analyzed by the RNA sequencing, demonstrating reduced Ptprc (Cd45) mRNA expression in Myc knockin (n=5) tumors compared to p53-driven (n=4) tumor samples. (B) Protein expression analyzed by the total proteome analysis using mass spectroscopy demonstrating reduced PTPRC (CD45) protein in Myc knockin tumors compared to p53-driven tumor sample. (C) IHC staining with CD45 in the paraffin-embedded tumor tissue samples showing lower expression in the Myc knockin tumors (right panel) compared to p53-driven tumor (left panel) GEMMs. (D) Negative correlation between the MYC and PTPRC mRNA expression in human OS TARGET dataset patients. (E) Kaplan-Meier curve of human OS TARGET dataset for PTPRC expression with top quartile or bottom quartile samples. (F) Distribution of macrophage population in the tumor tissue sample of the Myc knockin (n=4) and p53-driven (n=4) GEMMs analyzed by CIBERSORT. (G) Relative mRNA expression analyzed by the RNA sequencing demonstrating reduced Cd68 mRNA expression in Myc knockin (n=5) tumors compared to p53- (n=4) driven tumor samples. (H) IHC staining with F4/80 (macrophage marker) in the paraffin-embedded GEMM tumor tissue samples showing lower expression in the Myc knockin tumors (right panel) compared to the p53-driven tumor (left panel) GEMMs. (I) Negative correlation between the MYC and CD68 mRNA expression in human OS TARGET dataset patients. (J) Kaplan-Meier curve of human OS TARGET dataset for CD68 expression with top quartile or bottom quartile samples. (K) Relative immune cell populations (Hematopoietic CD45+) in the syngeneic mouse tumor tissue samples analyzed by FACSsymphony. (L) Relative macrophage populations (% of total CD45+ cells) in the syngeneic mouse tumor tissue samples analyzed by FACSsymphony. (*p<0.05, **p<0.01, ***p<0.0001), Log-rank (Mantel-Cox) test were performed for the Kaplan Meier analyses.
Figure 4. MYC association with CSF1 expression. (A) Western blot of CSF1 protein expression in Myc knockin tumors compared to the p53-driven tumor. The quantified expression is shown in the right panel. (B) The RNA sequencing analyzed relative mRNA expression, demonstrating reduced Csf1 mRNA expression in Myc knockin (n=5) tumors compared to the p53-driven (n=4) tumor samples. (C) IHC staining for CSF1 in paraffin-embedded GEMM tumor tissue samples in the Myc knockin tumors (lower panel) compared to p53-driven tumor (upper panel). (D) MYC and CSF1 mRNA expression in the PDX samples of the OS. (E) Negative correlation between the MYC and CSF1 mRNA expression in human OS TARGET dataset patients. (F) Kaplan-Meier curve of human OS TARGET dataset for CSF1 expression with top quartile (n=22) or bottom quartile (n=22) samples, Log-rank (Mantel-Cox) test were performed for the Kaplan Meier analyses. (G) Csf1 expression upon transient knockdown of Myc in Myc-knockin murine OS cell lines. (H) Csf1 mRNA expression after dTAG-13 and v1 treatment. (I) Western blot of MYC and CSF1 protein expression after dTAG-13 and v1 treatment; blot quantification is shown in the right panel. (*p<0.05, **p<0.01, p<0.0001).
Figure 5. MYC represses Csf1 expression through regulation of miR-17/20a. (A) qPCR for miR17-5p and -20a-5p expression in Myc knockin tumors and p53-driven GEMM tumor sample (n=3). (B) qPCR for miR17-5p/20a-5p expression after Myc siRNA treatment compared to the Scr control (n=3). (C) miR17-5p/20a-5p expression after dTAG-V1 treatment (n=3). (D) Csf1 mRNA expression after miR17-5p/20a inhibition treatment as compared to the negative inhibitor control (n=3). (E) Csf1 mRNA expression after miR17-5p/20a mimic treatment compared to the negative mimic control (n=3). (*p<0.05, **p<0.01, ***p<0.001).
Figure 6. MYC mitigates intratumoral macrophage cell infiltration and phenotypic function. (A) Schematic diagram showing the coculture set-up. 100,000 OS tumor cells were seeded on the bottom chamber, and 50,000 RAW 264.7 cells were seeded on the upper chamber and allowed to migrate for 48h. (B) Transwell migration of RAW 264.7 cells towards the Myc knockin cell lines (upper panel) compared to the p53-driven cell line (lower panel), quantified RAW-264.7 cell migration is shown in the graph. (C) Migration of RAW 264.7 cells in the Myc knockin cell line after siMyc (lower panel) as compared to the Scr-control-treated (upper panel) osteosarcoma cells, quantified RAW-264.7 cell migration is shown in the graph. (D) Migration of RAW 264.7 cells after dTAG-V1 treatment (right panel) compared to control (left panel) in the F331-dTAG MYC cell line, quantified RAW RAW264.7 cells migration is shown in graph. (E) Schematic diagram showing the effect of cell culture supernatant on the RAW 264.7 cell polarization. (F) Expression of Arg1 (M2-macrophage marker) in the RAW 264.7 cells cultured in the supernatant collected from the Myc knockin cell lines (n=3) as compared to p53-driven (n=3) samples. (G) Expression of Arg1 in RAW 264.7 cells cultured in the conditioned media after siMyc knockdown (n=3). (H&I) Macrophage phagocytosis after siMyc and after MYC-protein degradation (dTAG-v1). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
Figure 7. Selective in vivo pharmacological degradation of MYC protein levels and its effects on the OS immune landscape. (A) Schematic diagram showing the tumor cell injection and dTAG-v1 drug treatment. (B) Tumor volume after two weeks of control (n=3) and dTAG-v1 treatment (n=4). (C) Analysis of intratumoral FACS analysis for CD45 population after control (n=3) and dTAG-v1 treatment (n=4). (D) Macrophage population analysis after two weeks of dTAG-v1 treatment. (E) Western blot analysis for MYC expression after two weeks of the dTAG-v1 treatment. Blot quantification data are shown in below left panel. (F) Expression of miR-17/20a expression after control and dTAG-v1 treatment (n=3/cohort). (G) Schematic diagram showing the MYC-dependent regulation in the TME of OS. (*p<0.05, **p<0.01).