A Genetic Platform to Model Sarcomagenesis from Primary Adult Mesenchymal Stem Cells

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ABSTRACT

The regulatory factors governing adult mesenchymal stem cell (MSC) physiology and their tumorigenic potential are still largely unknown, which substantially delays the identification of effective therapeutic approaches for the treatment of aggressive and lethal forms of MSC-derived mesenchymal tumors, such as undifferentiated sarcomas. Here, we have developed a novel platform to screen and quickly identify genes and pathways responsible for adult MSC transformation, modeled undifferentiated sarcoma in vivo, and, ultimately, tested the efficacy of targeting the identified oncopathways. Importantly, by taking advantage of this new platform, we demonstrate the key role of an aberrant LRF–DLK1–SOX9 pathway in the pathogenesis of undifferentiated sarcoma, with important therapeutic implications.

SIGNIFICANCE: The paucity of therapeutic options for the treatment of sarcoma calls for a rapid and effective preclinical assessment of new therapeutic modalities. We have here developed a new platform to deconstruct the molecular genetics underlying the pathogenesis of sarcoma and to evaluate in vivo the efficacy of novel targeted therapies. Cancer Discov; 5(4). 396–409. © 2015 AACR.

INTRODUCTION

Tumorigenesis and stem cell differentiation are frequently tied together, in that genetic and functional loss of genes responsible for cell commitment and differentiation are selected during the tumorigenic process, because undifferentiated cells are often endowed with longer life spans, higher survival, and proliferative potential, and are often resistant to treatment (1).

Adult mesenchymal stem cells (MSC) are known for their ability to self-renew as well as to differentiate into cells of varying mesenchymal lineages, such as chondrocytes, osteoblasts, and adipocytes (2, 3). Importantly, mounting evidence now implicates adult MSCs as the cell of origin of human undifferentiated sarcomas, one of the most aggressive and lethal soft-tissue tumors (4–8). Undifferentiated sarcomas are generally treated by surgical resection (whenever possible), radiotherapy, and chemotherapy; all of these options, however, minimally change the very poor overall survival in patients. Limited genetic and molecular analyses, along with the absence of faithful in vivo models enabling preclinical testing of the targeting of specific tumorigenic pathways, are together the main factors impeding the development of new and more effective therapeutic options. Although specific forms of sarcoma (e.g., osteosarcoma) have been successfully modeled in genetically engineered mice (9–12), current protocols to model undifferentiated sarcomas are generally based on human tumor cell lines transplanted in immune-compromised mice (13), in vitro expanded and spontaneously transformed heterogeneous mouse primary mesenchymal cells (4, 14, 15), or in vitro genetically modified human bone marrow MSCs (16, 17), whose purity and stemness features have recently been debated (3, 18). Although these studies have proved helpful in uncovering aspects of sarcomagenesis, such protocols fail to mirror the real onset and progression of undifferentiated sarcomas because they cannot control key factors such as the type and number of the genetic alterations driving the tumorigenic process or cell of origin (19). In turn, these approaches will hardly allow the discovery of key genetic drivers involved in the onset and progression of undifferentiated sarcomas, and, most importantly, of potential druggable targets with clinical relevance. To fill this void, we have developed a novel ex vivo/in vivo genetic platform that will allow the discovery of genetic drivers responsible for adult MSC transformation and the generation, in vivo, of undifferentiated sarcomas.

RESULTS

Optimized Culture Conditions to Prevent MSCs’ Spontaneous Transformation Lead to the Development of a New Genetic Platform to Model Sarcomagenesis

To model undifferentiated sarcomas, we selectively isolated from the bone marrow (BM) of mice a cell population highly enriched for adult MSCs (BM-MSCs: CD45−PDGFRα+; refs 20, 21), grew them in vitro, in conditions that maintain their stemness properties, and then studied the genetic drivers leading to their transformation. We have
recently described that mimicking in vitro the hypoxic conditions characterizing the natural environment of MSCs within the bone favors the expansion of adult BM-MSCs, while maintaining their stem features (21). This analysis led us to discover that, unexpectedly and in contrast with what has been previously reported, focus-forming cell assays performed in regular oxygen concentrations (20% oxygen; refs. 4, 14, 15, 22), primary adult BM-MSCs cultured in hypoxic conditions (1% oxygen) did not undergo spontaneous in vitro transformation; on the contrary, they showed progressive reduction in the proliferation rate during the culture (Fig. 1B). Moreover, once seeded into scaffolds and implanted subcutaneously in mice, MSCs remained vital even after months, showing the ability to recruit blood vessels within the scaffold, but not to form tumors or to show marks of neoplastic transformation (Fig. 1C).

Loss of p53 has been firmly implicated in the pathogenesis of undifferentiated sarcomas in humans (23). We therefore assessed the impact of p53 inactivation in our model system. Different from wild-type MSCs, primary Trp53KO (p53Δ57) adult MSCs maintained in vitro in hypoxic conditions were characterized by a high proliferation rate even after numerous passages, as evidence of a status of immortalization (Fig. 1D). Surprisingly, however, p53Δ57 MSCs did not show signs of neoplastic transformation in hypoxic growth conditions in vitro, such as the ability to form foci of transformation in the dedicated assay or sizable colonies in soft agar (Fig. 1D). To test their tumorigenic potential in vivo, we next seeded p53Δ57 MSCs into scaffolds (24) and transplanted them subcutaneously in syngeneic C57BL/6 nude mice (first recipients). Two months after the implantation, the scaffolds were collected, cells within them were expanded in hypoxic conditions, and were then used for a second round of implantation (second recipients; Fig. 1E). Similar to wild-type MSCs, p53Δ57 MSCs remained vital within scaffolds. They recruited blood vessels, and they did not show any signs of neoplastic transformation in either first and second recipients, which resulted in the inability to generate tumors in serially transplanted animals (Fig. 1F).

Previously published data reported spontaneous transformation of primary MSCs cultured in regular oxygen conditions after several passages (14, 15). We therefore analyzed the spontaneous transformation of p53Δ57 MSC populations, culturing them for 1 month or 4 months in low (1%) or high (20%) oxygen tension, and then performed a focus formation assay. As shown in Fig. 1G, cells cultured for 1 month at 1% oxygen were not able to generate transformed foci, whereas, on the contrary, cells kept at 20% oxygen formed several foci of transformation, which increased in number and size during the culture. Importantly, we also noticed that MSC cultures kept at 20% oxygen showed a significant increase in the number of cells characterized by several (n > 5) nuclear dots of γH2AX, in comparison with the same cells kept at 1% oxygen (Supplementary Fig. S1A), thus defining a condition of increased DNA damage linked to the 20% oxygen condition, the primary cause of genomic instability in replicating cells (25).

Overall, these data led us to hypothesize that loss of p53 functions in human MSCs (hMSC) may be necessary but not sufficient to trigger sarcomagenesis. In addition, in vitro hypoxic growth conditions, by maintaining genomic stability of primary adult p53-null MSCs and by preventing their spontaneous neoplastic transformation, might represent the cornerstone for the development of a tightly controlled genetic platform aimed at identifying specific genetic alterations that, in combination with p53 loss, could dictate adult MSC transformation and development of undifferentiated sarcomas. To test this hypothesis, we decided to challenge our platform with oncogenic stresses previously implicated in sarcomagenesis, and assess their capacity to transform p53-null MSCs. Specifically, in p53-null MSCs maintained in hypoxic conditions, we overexpressed c-MYC (26), KRASG12V (27), and IDH2R172K (13), whereas we knocked down PTEN (28). The expression of c-MYC and KRASG12V, as well as the loss of PTEN (but not the expression of IDH2R172K), was indeed able to trigger p53-null MSC transformation in vitro and represented proofs of principle for the validity of our approach (Fig. 1H and Supplementary Fig. S1B–S1G).

Identification of Novel Genes Involved in Sarcomagenesis through the MSC-Derived Platform

We next tested whether this new platform would be useful in identifying new genes implicated in sarcomagenesis. Because undifferentiated sarcomas have been suggested to originate through the combined deregulation of adult MSCs of genes involved in cellular proliferation/apoptosis (such as p53), and genes implicated in the regulation of stem cell differentiation (29, 30), we decided to couple two known regulators of stem cell maintenance and differentiation with the loss of p53. The
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A. Wild-type BM-MSCs

B. Scaffolds with WT MSCs

C. Scaffolds with WT MSCs

D. p53\(^{ko}\)

E. BM-MSCs

F. p53\(^{ko}\) MSCs

G. 1% O\(_2\) vs. 20% O\(_2\)

H. Number of transformed foci
regulators enrolled in the platform were PML (31) and LRF/Pokemon [encoded by the gene Zbtb7a (Zinc finger and BTB domain-containing protein 7A)], which is emerging as one of the master regulators of differentiation for both hematopoietic and nonhematopoietic stem/progenitor cells, and has been attributed oncogenic or tumor-suppressive functions, depending on the specific cellular context (32). Although PML knockdown in p53-null MSCs did not trigger neoplastic transformation, adult p53-null MSCs knocked down for LRF showed features of neoplastic transformation in vitro (Fig. 1H and Supplementary Fig. S1F–S1G), suggesting a possible role for LRF as a suppressor of sarcomagenesis. To fully determine this new role of LRF/Pokemon in sarcomagenesis, we generated a cohort of p53^αα/αα Zbtb7a^αα/αα mice and derived adult MSCs as previously described (Fig. 1A). Zbtb7a was knocked out in vitro by transducing p53^αα/αα Zbtb7a^αα/αα MSCs with lentiviral vectors containing CRE cDNA, or an empty vector as control (hereafter referred to as p53^αα/αα Zbtb7a^αα/αα Cre or p53^αα/αα Zbtb7a^αα/αα CTR for brevity; Supplementary Fig. S2A). Although p53^αα/αα Zbtb7a^αα/αα CRE cells and p53^αα/αα Zbtb7a^αα/αα CTR cells showed similar anchorage-dependent growth rates (Supplementary Fig. S2B), the anchorage-independent colony-forming capacity of p53^αα/αα Zbtb7a^αα/αα CRE cells, as well as the capacity to form transformation foci in vitro, was significantly higher than that of p53^αα/αα Zbtb7a^αα/αα CTR cells (Fig. 2A and data not shown). Off-target effects of the CRE infection were ruled out upon transduction of p53^αα/αα Zbtb7a^αα/αα CRE cells with the same lentiviral vectors containing CRE cDNA, or an empty vector as control used in p53^αα/αα Zbtb7a^αα/αα cells, and then performing a focus formation assay (Supplementary Fig. S2C).

Finally, to test their tumorigenic potential in vitro, we seeded p53^αα/αα Zbtb7a^αα/αα CRE or p53^αα/αα Zbtb7a^αα/αα CTR MSCs into the scaffolds and implanted them into mice following the same protocol described in Fig. 1E. Two months after implantation, we observed that p53^αα/αα Zbtb7a^αα/αα CRE cells were not able to generate tumors in mice (as expected, only nontransformed mesenchymal cells were recovered from the scaffolds), whereas the p53^αα/αα Zbtb7a^αα/αα CRE cells underwent neoplastic transformation and generated tumors in all the transplanted mice (Fig. 2B). Cells were then recovered from the scaffolds characterized for LRF expression (Supplementary Fig. S2D), and further tested for their neoplastic potential in vitro, and then in vivo in a second round of transplantation. Once again, p53^αα/αα Zbtb7a^αα/αα CRE and p53^αα/αα Zbtb7a^αα/αα CTR cells recovered from the scaffold showed similar rates of anchorage-dependent proliferation (Supplementary Fig. S2E), but only the p53^αα/αα Zbtb7a^αα/αα CRE cells displayed the capacity to form transformed foci (Fig. 2C). Importantly, once transplanted in secondary recipients (second recipients), p53^αα/αα Zbtb7a^αα/αα CRE cells originated tumors larger than 5 mm in less than 2 weeks (7 of 7 mice; 2 nude mice and 5 C57BL/6), whereas none of the 4 mice (2 nude mice and 2 C57BL/6) retransplanted with p53^αα/αα Zbtb7a^αα/αα CTR cells originated tumors (Fig. 2D–G). Histopathologic analysis of scaffolds recovered from mice revealed that p53^αα/αα Zbtb7a^αα/αα CRE cells originated undifferentiated sarcomas, which were able to egress the scaffolds and to invade tissues nearby. On the contrary, only nontransformed mesenchymal cells were present in the scaffolds implanted with p53^αα/αα Zbtb7a^αα/αα CTR cells (Fig. 2F and G).

Because our platform identified Lrf as a tumor-suppressor gene in triggering the transformation of MSCs, we investigated the expression of LRF through immunohistochemistry analyses on a comprehensive human tissue microarray (TMA) containing 45 undifferentiated mesenchymal tumors (fibrosarcomas (F) and 20 malignant fibrous histiocytomas (M.F.H)), compared with 4 normal fibrous tissues (N.F.T., Fig. 2H). All normal fibrous tissues (4 of 4) as well as human adult MSCs (Supplementary Fig. S2F) were characterized by LRF expression, whereas, in sharp contrast, malignant undifferentiated sarcomas were strongly negative (41 of 45 cases of fibrosarcomas and 19 of 20 cases of malignant fibrous histiocytomas).

Overall, these results demonstrate that our platform may be an important tool to identify new oncogenes and tumor suppressors involved in the development of human sarcomas.

**LRF Is Essential for MSC Commitment and Differentiation**

We next investigated the biologic processes through which LRF loss could trigger sarcomagenesis. LRF/Pokemon has been described in the stemness maintenance/differentiation in different cell lineages (32); however, nothing is known about its role in adult MSCs. For this reason, we first investigated the possibility that LRF could trigger sarcomagenesis by blocking the differentiation capacity of MSCs. Accordingly, we derived MSCs from Zbtb7a-floxed mice (Zbtb7a^lox/lox) and investigated their commitment/differentiation ability toward osteoblasts, chondrocytes, and adipocytes upon LRF knockout in vitro through transduction with CRE-lentiviral vector (hereafter referred to as CRE cells or CTR cells). Despite the profound reduction of Lrf expression (Supplementary Fig. S3A), CRE cells remained similar to CTR cells in terms of morphology, size, and number of the CFU-F colonies generated (Supplementary Fig. S3B), as well as in numbers of cells undergoing senescence (Supplementary Fig. S3C) or apoptosis (Supplementary Fig. S3D and S3E). However, as shown in Fig. 3A, CRE cells displayed differentiation defects when compared with CTR cells. CRE cells generated only 30% of the Oil-Red-O–positive CFU-F colonies generated by CTR cells in response to adipogenic induction (Fig. 3A), and, accordingly, the expression of Pparg and Fabp4 during differentiation was significantly lower in CRE cells than in CTR cells (Fig. 3B). Similarly, CRE cells treated with osteogenic-stimulating factors originated fewer ALP osteoblasts than CTR cells (Fig. 3C), and CRE cells expressed lower levels of Alp and osteocalcin (Oc) mRNAs compared with controls (Fig. 3D). Finally, regarding chondrogenesis, CRE cells originated chondrocyte micromasses surrounded by less extracellular matrix (as shown by Toluidine blue staining) than CTR cells. Interestingly, CRE cells expressed significantly higher amounts of Col2α2 in comparison with CTR cells, although the expression of markers of terminal differentiation, such as Col9α and Col10α, was significantly reduced, suggesting that chondrocyte progenitors are favored in their initial commitment, yet they fail to reach a mature terminal differentiation (Fig. 3E).

Finally, to corroborate our findings in hMSCs, LRF was knocked down through specific shRNAs, taking advantage of commercially available hMSCs cultured under the same conditions used for mouse MSCs. The ability of shLRF and shCTR hMSCs to originate mature adipocytes was evaluated as previously described for mouse MSCs. Both shLRF- and shCTR-transduced hMSCs behaved comparably in culture, without displaying features of apoptosis or senescence.
Role of LRF in Adult MSCs

**Figure 2.** LRF loss in MSCs leads to formation of mesenchymal tumors. **A,** soft-agar assay for detecting anchorage-independent cell growth of p53KO/Zbtb7aΔF-CTR and p53KO/Zbtb7aΔF-CRE MSCs. Representative pictures of the colonies are shown on the left, and the quantification on the right is shown as average of three biologic independent replicates ± SEM. **B,** schematic overview of the experimental design is shown on the left, and the percentage of mice with a tumor bigger than 0.5 cm³ is shown on the right. p53KO/Zbtb7aΔF-CTR cells were not transformed, and only scaffolds with mesenchymal cells were recovered after transplantation (n = 2, CTR, n = 2 CRE). **C,** detection of the transformation status of p53KO/Zbtb7aΔF-CTR or p53KO/Zbtb7aΔF-CRE cells. Pictures of the foci are shown on the left, and the quantification of the transformed foci is shown on the right. The quantification on the right is shown as pooled from three independent experiments, mean ± SEM. **D,** tumors generated by p53KO/Zbtb7aΔF-CTR or p53KO/Zbtb7aΔF-CRE cells transplanted within scaffolds into second recipient mice. Representative pictures of mice are shown on the left, and the percentage of mice with a tumor bigger than 0.5 cm³ (scaffold size used as control) is shown on the right (n = 4 CRE, n = 7 CRE). **E,** sizes of tumors generated by p53KO/Zbtb7aΔF-CTR or p53KO/Zbtb7aΔF-CRE cells transplanted within scaffolds into second recipient mice. Pictures of the collected tumors are shown on the left, and the relative size of tumors is shown on the right (scaffold size used as control). **F** and **G,** tumors generated by p53KO/Zbtb7aΔF-CRE cells were compared with p53KO/Zbtb7aΔF-CTR cells collected from first recipients, seeded into scaffolds, and then transplanted into second recipients. Hematoxylin and eosin staining showing the morphology of collected tumors or mesenchymal cells on top of the scaffold. p53KO/Zbtb7aΔF-CTR cells were not able to generate tumors in vivo, and only scaffolds with mesenchymal cells were recovered after transplantation, whereas p53KO/Zbtb7aΔF-CRE cells originated undifferentiated sarcomas. (†, scaffold; scale bars, 20 μm). **H,** LRF expression in human undifferentiated sarcomas. (scale bars, 30 μm). ***P < 0.01; ****P < 0.001; t test.
Figure 3. LRF is a key factor for MSC commitment and differentiation. A, MSC differentiation into mature adipocytes. Adipocytic colonies stained with Oil-Red-O are shown on the left, and the relative quantification of adipocytic colonies (positive for Oil-Red-O) among the total number of colonies (CFU-F) is shown on the right. The quantification on the right is shown as average of five independent experiments ± SEM. B, Lrf, Pparg, and Fabp4 relative mRNA expression in CTR cells and CRE cells during adipogenesis. Results are shown as one representative experiment out of two independent biological replicates. C, MSC differentiation toward mature osteoblasts. Representative pictures of ALP+ cells are shown on the left, and the relative number of ALP+ cells is shown on the right. The quantification on the right is the average of three independent experiments ± SEM. D, Lrf, Alp, and Oc relative mRNA expression in CTR cells and CRE cells during osteogenesis. Results are shown as one representative experiment out of two independent biological replicates. E, MSC differentiation toward chondrocytes. Representative images of chondrocytes originated from CTR cells and CRE cells stained with Toluidine blue are shown on the left (n = 2 independent biological replicates). Relative mRNA expression levels of Lrf, Col2, Col9, and Col10 are shown on the right. Results are shown as average of two independent biological replicates ± SEM. F, morphology of hMSCs transduced with shCTR or shLRF and induced to undergo adipogenesis, at day 7 of the differentiation process. Magnification shows in detail fully differentiated adipocytes. H, hMSC differentiation into adipocytes, at day 15 of the differentiation process. Adipocytes stained with Oil-Red-O are shown on top, and the relative mRNA expression of FABP4 is shown in the graph. *, P < 0.05; **, P < 0.01; ***, P < 0.001; t test.

LRF Promotes MSC Commitment through the Transcriptional Repression of DLK1

On the basis of these data showing that LRF/Pokemon governs the commitment capacity of MSCs, and having excluded a possible role of two well-characterized pathways regulated by LRF/Pokemon, ARF (33) and NOTCH1 (34), in this process (data not shown), we aimed to understand which pathways are regulated by LRF in MSCs, to identify possible new players involved in the genesis of undifferentiated sarcoma.
and, in turn, potential targets for therapy. Considering that oncogenic and tumor-suppressive pathways are often wired to regulate cell-lineage commitment and differentiation in physiological conditions (35), we focused our attention on the DLK1 (Delta-like-1)-SOX9 (SRY sex determining region Y-box 9) pathway, which is known to be critical in the determination of mesenchymal lineages (36–40). In particular, we focused on SOX9, as it has been shown to be involved in the differentiation process of MSCs (41), in addition to being recently described as functionally antagonized by LRF in prostate tumorigenesis (42, 43). CTR cells and CRE cells were first analyzed by RT-qPCR for the expression of SOX9 transcriptional target genes (44, 45); both genes were overexpressed in CRE cells as compared with CTR cells (Fig. 4A). Similarly, the knockdown of LRF in hMSCs resulted in the upregulation of SOX9 activity (Fig. 4B). Next, to address the critical question of whether LRF mediates MSC commitment through SOX9, CRE cells were knocked down for SOX9 (Supplementary Fig. S4A) and induced to differentiate. CTR-shSCR cells, CRE-shSCR cells, and CRE-shSOX9 cells originated a comparable number of colonies as detected by crystal violet, whereas downregulation of SOX9 levels and activity did not rescue the capacity of CRE cells to differentiate into mature adipocytes (Fig. 4C and Supplementary Fig. S4B and S4C). Similarly, downregulation of SOX9 in CRE cells negligibly rescued the ability of MSCs to differentiate into mature osteoblasts (Fig. 4D).

Another critical regulator of MSC commitment is DLK1 (46–50). We therefore decided to investigate the possibility that LRF could regulate DLK1 activity, and through it the commitment of MSCs. Intriguingly, CRE cells showed a significant increase in Dlk1 expression compared with CTR cells (Fig. 4E). Similarly, knockdown of LRF resulted in the upregulation of DLK1 in hMSCs (Fig. 4F). To determine whether LRF could directly regulate DLK1 expression, we cloned the Dlk1 promoter sequence into a luciferase construct and performed in vitro transactivation assays. This analysis revealed that LRF efficiently repressed the basal promoter activity in a dose-dependent manner (Fig. 4G), and, among the six putative LRF consensus regions within the promoter of Dlk1, we discovered that only the two consensus sequences (indicated as 4 and 5 in Supplementary Fig. S4D) closest to the transcriptional starting site were necessary for LRF repression of Dlk1 transcription. In addition, the ability of LRF to bind the Dlk1 promoter was confirmed by performing electrophoretic mobility shift assays (EMSA; Fig. 4H) and chromatin immunoprecipitation (ChIP; Fig. 4I) in the 3T3L1 mouse mesenchymal cell line. To functionally validate the LRF–DLK1 axis in MSC commitment, we knocked down DLK1 in CRE cells and induced the generated cells to differentiate toward adipocytes and osteoblasts. As expected, CRE-shSCR cells failed to differentiate compared with CTR-shSCR cells; but, critically, the concomitant inactivation of DLK1 (CRE-shDLK1 cells) rescued their defects of adipogenesis (Fig. 4J and K and Supplementary Fig. S4E and S4F) and osteogenesis (Fig. 4L and M).

**LRF Acts as an Oncosuppressor of Mesenchymal Tumorigenesis by Controlling the Activity of DLK1 and SOX9**

Both DLK1 and SOX9 have been described as oncogenes in several tumor types (36–39). Because we have demonstrated that LRF negatively controls both the expression of DLK1 and the activity of SOX9 in primary nontransformed MSCs, and that, in p53-null MSCs, LRF acts as a tumor-suppressor gene, we next used our platform to investigate the possibility that DLK1 and SOX9 deregulation might be responsible for transformation of MSCs and sarcomagenesis after LRF loss. p53F/F Zbtb7aF/F-CRE cells or p53F/F Zbtb7aF/F-CTR cells collected from scaffolds (first recipient) were grown in hypoxia for 7 days and then transduced with shRNA to silence Dlk1 or Sox9. Four different cellular types were then obtained: p53F/F Zbtb7aF/F-CRE-shSCR, p53F/F Zbtb7aF/F-CRE-shSCR, p53F/F Zbtb7aF/F-CRE-shDLK1, and p53F/F Zbtb7aF/F-CRE-shSOX9 (in the figures indicated only as CTR-shSCR, CTR-shSCR, CRE-shDLK1, and CRE-shSOX9 for brevity; Fig. 5A and Supplementary Fig. S5A and S5B). Cells were then used in both in vitro and in vivo experiments to test their tumorigenic potential. In an anchorage-dependent proliferation assay, all the cell types showed similar division rates (Fig. 5A), but their capacity to form transformed foci in culture was significantly different. Although p53F/F Zbtb7aF/F-CRE-shSCR cells were extremely prone to form foci of transformation, downregulation of Dlk1 (p53F/F Zbtb7aF/F-CRE-shDLK1) or Sox9 (p53F/F Zbtb7aF/F-CRE-shSOX9) in p53F/F Zbtb7aF/F-CRE cells significantly limited their oncogenic potential in vitro (Fig. 5B and Supplementary Fig. S5C). To analyze in vivo the possible implications of DLK1 and SOX9 hyperactivity in the tumorigenic process induced by the absence of LRF, we transplanted scaffolds containing CTR-shSCR, CRE-shSCR, CRE-shDLK1, or CRE-shSOX9 cells subcutaneously in the flank of recipient mice, and then evaluated their tumorigenic potential. One month after implantation, 4 of 4 mice implanted with scaffolds containing p53F/F Zbtb7aF/F-CRE-shSCR cells, 4 of 5 mice implanted with scaffolds containing p53F/F Zbtb7aF/F-CRE-shDLK1 cells, and 3 of 5 mice implanted with scaffolds containing p53F/F Zbtb7aF/F-CRE-shSOX9 cells developed visible tumors, whereas no tumors were observed in mice implanted with scaffolds containing p53F/F Zbtb7aF/F-CRE-shSCR cells (0/4; Fig. 5C). Although 80% of mice implanted with p53F/F Zbtb7aF/F-CRE-shDLK1 and 60% of mice implanted with p53F/F Zbtb7aF/F-CRE-shSOX9 MSCs developed tumors, these lesions were significantly smaller than the p53F/F Zbtb7aF/F-CRE-shSCR tumors (Fig. 5D), and only a few transformed multinucleated cells were present within the scaffolds containing p53F/F Zbtb7aF/F-CRE-shDLK1 cells or p53F/F Zbtb7aF/F-CRE-shSOX9 cells (Fig. 5E), revealing that both of these two oncogenes participate in undifferentiated sarcoma formation from MSCs.

**DISCUSSION**

Adult MSCs have been proposed as the cell of origin of human undifferentiated sarcomas, one of the most aggressive and lethal soft-tissue tumors (4–8). However, a comprehensive analysis of the molecular mechanisms dictating the onset and progression of undifferentiated sarcomas is still missing, consequently limiting the generation of preclinical models faithfully recapitulating the human disease and, more importantly, the development of new and effective therapeutic options for this lethal tumor. A major current hurdle in studying and modeling the pathogenesis of sarcoma is represented by the need to deliver the appropriate genetic perturbation(s) to a specific
LRF regulates the differentiation process of MSCs through the repression of DLK1. A, relative mRNA expression levels of Lrf, Mia, and Col2α2 in CTR cells and CRE cells. Results are shown as one representative experiment out of two independent biologic replicates. B, relative mRNA expression levels of Lrf, H19, and Mia in hMSCs transduced with shCTR or shLRF. C, adipogenesis potential of CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Sox9 cells. Adipocytic colonies stained with Oil-Red-O (top) or crystal violet (bottom) are shown on the left, and the relative quantification of adipocytic colonies is shown on the right. The quantification on the right is presented as average of three independent experiments ± SEM. D, osteogenesis potential of CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Sox9 cells. Adipocytic colonies stained with Oil-Red-O (top) or crystal violet (bottom) are shown on the left, and the relative quantification of adipocytic colonies is shown on the right. The quantification on the right is presented as average of three independent experiments ± SEM. E, Lrf and Dlk1 relative mRNA expression levels of CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Sox9 cells. Relative mRNA expression of LRF, Dlk1, and H19, and Mia are shown as average of three biologic independent replicates ± SEM. F, relative mRNA expression levels of LRF and Dlk1 in hMSCs transduced with shCTR or shLRF. G, luciferase assay for detecting the activity of LRF on the Dlk1 promoter region. Results are shown as average of five biologic independent replicates ± SEM. H, relative mRNA expression levels of Pparg and Fabp4 in adipocytes derived from CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Dlk1 cells. Results are shown as average of three biologic independent replicates ± SEM. I, ChIP of the LRF at the Dlk1 promoter region. Results are shown as average of three biologic independent replicates ± SEM. J, adipogenesis potential of CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Dlk1 cells. Adipocytic colonies stained with Oil-Red-O (top) or crystal violet (bottom) are shown on the left, and the relative quantification of adipocytic colonies is shown on the right. The quantification on the right is presented as average of three biologic independent replicates ± SEM. K, relative mRNA expression levels of Pparg and Fabp4 in adipocytes derived from CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Dlk1 cells. Results are shown as average of three biologic independent replicates ± SEM. L, osteogenesis potential of CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Dlk1 cells. Adipocytic colonies stained with Oil-Red-O (top) or crystal violet (bottom) are shown on the left, and the relative quantification of adipocytic colonies is shown on the right. The quantification on the right is presented as the average of three biologic independent replicates ± SEM. M, Alp and Dc relative mRNA expression in osteoblasts derived from CTR-sh SCR cells, CRE-sh SCR cells, and CRE-sh Dlk1 cells. Results are shown as average of three biologic independent replicates ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, t test.
Role of LRF in Adult MSCs

A RESEARCH ARTICLE

Figure 5. LRF plays a role as an oncosuppressor gene in mesenchymal tumors through DLK1 and SOX9. **A**, schematic overview of the experimental design is depicted on the left, and the growth curve of p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CTR-shSCR, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shSCR, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shDLK1, and p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shSox9 cells is shown on the right. Data show one representative experiment out of three independent biologic replicates. **B**, detection of transformation status of p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CTR-shSCR, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shSCR, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shDLK1, and p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shSox9 cells. Pictures of the transformed foci are shown on the left, and their quantification on the right is presented as average of three biologic independent replicates ± SEM. **C**, experimental design for in vivo sarcomagenesis is shown on the left, and the percentage of mice with tumor is shown on the right. Representative pictures of tumors generated by p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CTR-shSCR, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shSCR, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shDLK1, and p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shSox9 cells implanted into recipient mice (n = 4 CTR-shSCR, n = 4 CRE-shSCR, n = 5 CRE-shDLK1, n = 5 CRE-shSox9) are shown on the left, and the relative size of tumors is shown on the right (scaffold size used as control). **D**, representative pictures showing the morphology of collected tumors or mesenchymal cells on top of the scaffold, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CTR cells were not able to generate tumors in vivo and only scaffolds with mesenchymal cells were recovered after transplantation, whereas p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE cells, p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shDLK1, and p53<sup>−/−</sup>Zbtb7<sup>−/−</sup>-CRE-shSox9 cells originated undifferentiated sarcomas (*, the scaffold; scale bars, 20 μm). **F**, schematic overview of the genetic platform for discovering new oncopathways involved within the sarcomagenesis process and new targeted therapies. **G**, schematic overview of LRF involvement as a tumor-suppressor gene in undifferentiated sarcomas. The LRF–DLK1 and LRF–SOX9 pathways are examples of results obtained with the application of the genetic platform. *, P < 0.05; ***, P < 0.001; t test.
putative cell of origin (19). Here, we used primary adult mouse bone marrow MSCs sorted according to the expression of specific markers and tested for stemness potential. By modifying their culturing condition, we have developed a new experimental ex vivo/in vitro platform to deconstruct the molecular genetics underlying the pathogenesis of undifferentiated sarcomas. Comparable findings were obtained using commercially available unsorted hMSCs. It must be noted, however, that recent reports question the use of unsorted hMSCs collected from bone marrow and selected solely on their ability to adhere to culture dishes for their inherent heterogeneity (3). Our approach nevertheless rests on the discovery that culturing mouse MSCs and hMSCs in hypoxic conditions prevents their spontaneous propensity to transform. As we recently reported, MSCs reside in bone marrow areas characterized by low oxygen concentration. Compared with other stromal cells within the endosteal bone marrow, MSCs express higher levels of hypoxia-inducible factor-1α (HIF1α) and HIF2α transcripts, showing a distinctive hypoxic profile (21). Therefore, the enforced switch from an anaerobic to an aerobic environment due to culturing in a 20% oxygen condition could determine excessive levels of reactive oxygen species (ROS) and, in turn, an increasing amount of unrepaired DNA damage, a prelude to genetic instability and neoplastic transformation. By preventing the spontaneous transformation of wild-type as well as p53-null MSCs, this new approach will turn out to be very useful to (i) quickly assess the relevance of specific genetic alterations within the tumorigenesis process of MSCs; (ii) characterize oncogenic or oncosuppressive functions and molecular pathways controlled by the newly identified genetic alterations; and (iii) evaluate, preclinically, both in vivo and in vitro (in mice implanted with scaffolds), the efficacy of novel targeted therapies toward the eradication of this lethal disease (Fig. 5F). The relevance of this new approach is based on its ability to control the cell of origin of the disease, its simplicity, and velocity. Furthermore, it can easily be coupled with the use of shRNA/overexpressing vectors/libraries in vitro, or the new developing system CRISPR/Cas9 for genome editing of both coding and noncoding genes to be tested individually or in combination. Importantly, we demonstrate that, by using this new drug discovery platform, specifically, we have defined a role for LRF as tumor-suppressor gene in adult MSCs and identified LRF as a key factor in the control of the early steps of adult MSC commitment. In addition, we have characterized two evolutionarily conserved oncosuppressive mechanisms regulated by LRF in adult MSCs: its ability to transcriptionally repress DLK1 expression and to inhibit SOX9 activity (Fig. 5G). DLK1 is a transmembrane protein that, once cleaved by the enzyme TACE/ADAM17, releases the soluble factor fetal antigen-1 (FAI; ref. 51). Thus, the use of neutralizing antibodies against DLK1 (52), alone or in combination with inhibitors of SOX9 downstream factors, may offer a window of opportunity for the development of novel therapeutic strategies for this lethal form of cancer.

METHODS

Mice

Transgenic Zbb7α+/− mice were generated as described previously (34); p53−/− mice were purchased from The Jackson Laboratory, and generated as described previously (53). All the experimental animals were kept in a C57BL/6J pure background. In some specific experiments, immunodeficient mice were used (The Jackson Laboratory; B6.Cg-Foxn1nu/j). Animal experiments were performed in accordance with the guidelines of the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Cell Lines and hMSCs

The 3T3-L1 cells were purchased from the ATCC (ATCC-CL-173) and cultured following the vendor’s directions. Cell lines were tested for Mycoplasma (MycoAlert; Lonza), but not further authenticated. hMSCs were purchased from Lonza (PT-2501). According to the vendor, cells were positive for CD105, CD166, CD29, and CD44, and tested negative for CD14, CD34, and CD45. Cells were cultured and induced to differentiate following the vendor’s directions, but were not further authenticated. Cells were maintained in culture with MSC basal medium (Lonza; PT-3238) supplemented with growth factors (Lonza; PT-3001). During adipogenesis, cells were cultured in adipogenic induction medium (Lonza; PT-3102B), followed by adipogenic maintenance medium (Lonza; PT-3102A), according to the vendor’s instructions.

MSC Maintenance

MSCs were derived from C57BL/6 wild-type, Zbb7α+/−, and p53+/p53−/− mice. Long bones were collected, crushed, and digested with collagenase II (1 mg/mL) for 1-hour shaking at 37°C. Recovered cells were washed and FACScelled as CD45−, CD105−, CD166−, CD29−, and CD44−, and cultured at the density of 1,000 cells in a T25 flask. MSCs were cultured using complete MesenCult medium (STEMCELL Technologies) and maintained in a humidified chamber with 5% CO2 and 1% O2. Half medium was changed every 3 days. After 7 days in culture at 1% O2, cells formed visible CFU-F colonies; at this point, cells were split once they reached 80% confluence.

MSC Differentiation

MSCs were cultured using complete MesenCult medium (STEMCELL Technologies) and differentiated in a humidified chamber with 5% CO2 and 1% O2. For inducing the differentiation, the MesenCult medium was changed with specific medium for each differentiation (see below). During the differentiation process, cells were maintained in regular oxygen concentration, 5% CO2 and 37°C. For adipocyte differentiation, MSC colonies were treated with StemXVivo Osteogenic/Adipogenic medium (R&D Systems; CCM007) plus adipogenic supplements (R&D Systems; CCM011). Medium was changed every other day for 7 days. Mature adipocytes were identified with Oil-Red-O (Sigma) following the manufacturer’s procedure. Briefly, Oil-Red-O stock solution was prepared by solubilizing Oil-Red-O powder in isopropanol (0.35 g/100 mL isopropanol), stirring it overnight. Cells were washed once in PBS and then fixed with 10% formalin (Sigma) for 30 minutes at room temperature. The working Oil-Red-O solution was prepared by mixing three parts stock solution with two parts dH2O. Fixed cells were washed once to remove the formalin with water and then treated for 5 minutes with 60% isopropanol; then they were treated for 5 minutes with the working solution. After the treatment, cells were washed with water to eliminate Oil-Red-O precipitates. For osteocyte differentiation, medium was changed with StemXVivo Osteogenic/Adipogenic medium (R&D Systems; CCM007) together with osteogenic supplement (R&D Systems; CCM009). Medium was changed every 3 days for 20 days. Mature osteoblasts were stained with a Leukocyte Alkaline Phosphatase kit (Sigma; cat. no. 85L3R) according to the manufacturer’s procedures. For chondrocyte differentiation, 150,000 MSCs were pelleted in StemXVivo Chondrogenic medium (R&D Systems; CCM005) with Chondrogenic Supplement (R&D Systems; CCM006). Tubes were then incubated at 37°C and 5% CO2 with loosened cap. Medium was changed every 3 to 4 days for 20 days. Micromasses were then collected, embedded into paraffin, sectioned, and stained with Toluidine blue.
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Flow Cytometry
Cells were analyzed using LRSII (BD, Pharmingen) and sorted using FACSVARIA II (BD, Pharmingen). The following antibodies were used: anti-CD45 FITC, anti-CD31 FITC, anti-Ter119 FITC, anti-Sca1 Pacific Blue, anti-PDGFRRo PE (all purchased from BioLegend), and Annexin V–PE (BD, Pharmingen).

Immunofluorescence
Cells were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS, and permeabilized with PBS, Triton X-100 0.2% for 10 minutes. Blocking before antibodies was performed in PBS, Triton X-100 0.2% and 10% PBS for 30 minutes. Primary antibody p-histone H2AX (Cell Signaling Technology) was incubated overnight in blocking buffer, and an anti-Rabbit-488 was used as secondary antibody.

Immunohistochemistry and Human Tumor Samples
A TMA of human fibrous tissue, fibromas, fibrosarcomas, and malignant fibrous histiocytomas was purchased from US Biomax, Inc. (SO2084). IHC was performed on 5-mm paraffin sections with the avidin–biotin–peroxidase method. The following primary antibody was used: LRF (Bethyl Laboratory; cat. no. A300-549) 1:400 for the avidin–biotin–peroxidase method. The following primary antibodies were used: LRF (Bethyl Laboratory; cat. no. A300-549) 1:400 for the avidin–biotin–peroxidase method. The following primary antibodies were used: LRF (Bethyl Laboratory; cat. no. A300-549) 1:400 for the avidin–biotin–peroxidase method. The following primary antibodies were used: LRF (Bethyl Laboratory; cat. no. A300-549) 1:400 for the avidin–biotin–peroxidase method.

Luciferase Assay
One day before transfection, cells were plated into a 24-well plate at a density of 70% to 80%. The cells were transfected with the plasmid DNA (pGL3-Luc-DLK1 promoter and pDNA3-LRF) and Lipofectamine 2000 (Invitrogen) for 24 hours according to the manufacturer’s recommendation. Forty-eight hours after transfection, cells were lysed and analyzed for luciferase activity using the Dual-Luciferase Assay System (Promega). pRL-SV40-Renilla was used a control for transfection efficiency.

EMSA
For EMSA, ST3L1 cells were resuspended in lysis buffer (10 mmol/L Tris·HCl, pH 7.5, 1 mmol/L EDTA, 0.5% Nonidet P-40, 150 mmol/L NaCl, 1 mmol/L dithiothreitol, 10% glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors). After 20 minutes on ice, extracts were centrifuged at 16,000 × g for 20 minutes at 4°C to remove cell debris. Protein concentration in supernatants was determined using a Bio-Rad protein assay. A 26-mer DNA oligonucleotide containing the Dlk1 promoter sequence with putative LRF binding site (5′-GGCTCCTGGAGGCTTGCTTGTTTTC-3′) was end-labeled with [32P] (10 μmol/L oligonucleotide, 1 μL kinase buffer 10 μL of the kinase (10 U/μL) was end-labeled with [32P] (40 mmol/L, 5 μmol/L MgCl2, 50 mmol/L dithiothreitol), 2 μL ATP (ATP (Y-32P), 5,000 Ci/mmol), and 0.5 μL of the kinase (10 U/μL)] and annealed to the complementary strand. For binding reactions, 30 μg of whole cell extract was added to gel shift buffer (20 mmol/L HEPES pH 8, 25 mmol/L KCl, 0.1 mmol/L EDTA, 2 mmol/L MgCl2, 0.5 mmol/L dithiothreitol, 0.025% Nonidet P-40, 2 mmol/L spermidine, 10% glycerol, 0.1 mg/mL acetylated bovine serum albumin, 120 ng of double-stranded poly(dI-dC)] containing the labeled oligonucleotide in a final volume of 30 μL. Reactions were incubated for 30 minutes at room temperature, and electrophoresed on a nondenaturing 4% Tris–HCl–glycine-7.5% PAGE gel. Autoradiograms were scanned, and the amount of LRF binding was quantified using ImageJ.

Chromatin Immunoprecipitation
ChIP was performed as described previously (54) with the Magna CHIP G Chromatin Immunoprecipitation Kit (Millipore) using a hamster monoclonal antibody against PKM2 (Rabbit polyclonal anti-PKM2, Cell Signaling Technology; cat no. 9352), mouse monoclonal anti-β-actin (Sigma-Aldrich), and mouse monoclonal anti-HSP90 (BD Biosciences).

Focus Formation Assay
MSCs were seeded at a concentration of 1 × 10^6 cells per well in a 6-well plate and cultured for 10 days in complete medium (DMEM + 10% FBS) at 37°C. The formed visible foci were fixed with 10% formalin for 30 minutes, they were stained with crystal violet.

In Vivo Sarcomagenesis
As previously described, 3-D scaffolds made with reticulated polycarbonate polyurethane urea matrix (Synthecom) were used (24). Briefly, scaffolds (5 mm × 2 mm) were put into the wells of a 96-well plate previously coated with DMEM containing 1% of low-melting agarose and 10% FCS. The cells were then plated in 6-well plates previously coated with DMEM containing 1% of low-melting agarose and 10% FCS. The number of colonies was scored 3 weeks later, and quantification was done using ImageJ.

Western Blot Analysis
For Western blot analysis, cell lysates were prepared with RIPA buffer. The following antibodies were used: hamster anti-LRF clone 13E9, rabbit polyclonal anti-e-cPARP (Cell Signaling Technology; cat no. 9532), mouse polyclonal anti-β-actin (Sigma-Aldrich), and mouse monoclonal anti-HSP90 (BD Biosciences).

Senescence Detection
MSCs were cultured in hyphaemia, before and for 4 days after the transduction. After the transduction, cells were collected and seeded 10 × 10^5 cells per well in a 12-well plate. Cells were then treated with SA-β-galactosidase overnight. Senescence was detected using a senescence detection kit (Cellbiochem) following the manufacturer’s protocol.

Plasmids
The entire Dlk1 mouse promoter sequence was obtained by PCR from a BAC clone. PFW (5′ ccagccagccagccagcaaggtt, Dlk1 4.5 Rev: ccacagcagcagcaagagatg. The 3T3L1 cells were co-transfected with formaldehyde for 5 minutes and terminated with 0.125 of mmol/L glycine. Cells were sonicated to generate chromatin with an average size of 500 bp. Monoclonal anti-LRF antibody was first incubated with a hamster bridging antibody (Jackson ImmunoResearch Laboratories), followed by the addition of the 3T3L1 chromatin. Immunoprecipitated chromatin was assayed by quantitative PCR using the Dlk1-specific primers.

Viral Vectors
Lentiviral vector expressing CRE, shCTR, and shLRF, and retroviral vectors containing c-MYC and KRAS G12V were obtained from Addgene. Viral vectors of control as well as containing shRNA against LRF, PML, PTEN, Dlk1, and SOX9 were obtained from Open Biosystems. Vectors containing IDH2 R172K were cloned in our laboratory. All the viral particles were produced by transfecting 293T cells with packaging vectors of second generation.

Anchorage-Independent Cell Growth
A soft-agar colony formation assay was carried out by seeding 1 × 10^4 MSCs in DMEM containing 0.4% low-melting agarose and 10% fetal calf serum (FCS). The cells were then plated in 6-well plates previously coated with DMEM containing 1% of low-melting agarose and 10% FCS. The number of colonies was scored 3 weeks later, and quantification was done using ImageJ.
(5′-cgctgagcagcaactgacgcc-3′) primers were used. The fragment was cloned into a PGL3-Luc enhancer (Promega) in Sal I and Not I sites. All the mutants were obtained using the QuickChange Mutagenesis Kit (Stratagene) and confirmed by sequencing. The mutated version of this plasmid was generated by using the DlI and 3′-untranslated region (3′-UTR) as template and modifying the putative LRF binding sites using the QuickChange II XL Site-Directed Mutagenesis Kit.

The mutagenic primers used were as follows:

- site Mutant 1AB forward 5′-gggacagcaagctgacgcc-3′ reverse 5′-ctccttctatcactgtcgt-3′
- site Mutant 2 forward 5′-gggacagcaagctgacgcc-3′ reverse 5′-ccggtgagaggaagtacccagg-3′
- site Mutant 3 forward 5′-gggacagcaagctgacgcc-3′ reverse 5′-ctccttctatcactgtcgt-3′
- site Mutant 4 forward 5′-gggacagcaagctgacgcc-3′ reverse 5′-ctccttctatcactgtcgt-3′

All the mutants were obtained by using the QuickChange Mutagenesis Kit (Stratagene) and confirmed by sequencing. The mutated version of this plasmid was generated by using the DlI and 3′-untranslated region (3′-UTR) as template and modifying the putative LRF binding sites using the QuickChange II XL Site-Directed Mutagenesis Kit.

RNA Extraction and RT-PCR

Cellular RNA was extracted using Quick-RNA MicroPrep (Zymo Research; R1050) and then retrotranscribed to cDNA using an iScript cDNA Synthesis kit (Bio-Rad; 170-8890). All the analyzed mRNA was detected using TaqMan FAM-conjugated probes (Applied Biosystems). Each target was run in triplicate, and expression level was normalized to mouse β2-microglobulin. Details are provided in Supplementary Experimental Procedures.

Statistical Analysis

Data were analyzed using an unpaired t test (GraphPad Prism; GraphPad Software, Inc.). Values of P < 0.05 were considered statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001; t test).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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