***PPARɣ Agonists Enhance ET-743 Mediated Adipogenic Differentiation in a Transgenic Myxoid Round Cell Liposarcoma Model*.**

SUPPLEMENTAL SECTION - TEXT:

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Clinical Trial and Characteristics of ET-743 Treated Patients.

A dose of 1.5 mg/m^2 ET-743, diluted to approximately a 500 mL volume, was administered to patients via a central venous catheter as a 24-hour infusion on Day 1 of each 21 day cycle. All subjects were pretreated with 20 mg of dexamethasone i.v. on Day 1 of each treatment cycle approximately 30 minutes prior to each infusion of ET-743. Each treatment cycle lasted 21 days provided sufficient recovery of any hepatic or hematological toxicity. If the following criteria were not met on day 21, the next cycle was postponed: Platelets ≥100,000 µL, ANC ≥1500µL, Bilirubin ≤ULN, Alkaline phosphatase ≤1.5x ULN, and transaminases, CPK, and other nonhematologic, drug-related effects <Grade 2. A delay of 4 weeks was allowed, and if a persistent lack of recovery was documented, the treatment was stopped. There was no limit to the number of cycles a patient could receive. Treatment was continued until disease progression, unacceptable toxicity, patient refusal, or removal from study by investigator. All patients were evaluated by full medical history, physical examination, full blood count and serum biochemistry and a staging computed tomography (CT) or magnetic resonance imaging (MRI) scan prior to enrollment. Tumor reassessment was carried out every two cycles. The RECIST criteria were used to assess response. Patients were assessed for toxicity at the beginning of every cycle. As toxicity was a secondary endpoint of this study, all adverse events were collected. The inclusion criteria were as follows: Age ≥18 years, unresectable advanced or metastatic histologically proven soft tissue sarcoma (eligibility including desmoplastic small round cell tumor, Ewing's sarcoma, and osteosarcoma), relapsed or progressive disease following standard of care treatment with chemotherapy or intolerant to prior standard of care chemotherapy, hemoglobin ≥8, ANC ≥1500, platelet count ≥100,000, serum creatinine ≤1.5 x upper limit of normal (ULN), total bilirubin ≤ ULN, alkaline phosphatase ≤ 1.5x ULN (≤2.5x ULN if liver metastases are present), AST and ALT ≤ 2.5 x ULN, full recovery from toxicity of previous therapy, and written informed consent to treatment and data collection for research purposes

The patients had an almost 1:1 ratio of men to women, ranged from 25-81 years of age (median 52), were comprised predominantly of liposarcoma and leiomyosarcoma sub-types and represented a heavily pre-treated population: 35 patients had a resection of the primary tumor, 25 patients had previous radiotherapy, and 2/3 of patients previously received at least 2 treatment regimens (Table 1). All 42 patients were treated with the 24-h continuous intravenous schedule at a starting dose of 1.5 mg/m^2. Cycles were repeated every 21 days, with a maximum delay of 28 days for toxicity. Four patients were removed from study due to treatment related toxicities: three due to elevated alkaline phosphatase and one due to a grade 3 fever (Supplemental Table 1&2). Three patients died while on study, all from complications related to

disease. Patients were assessed for tumor response every 2 cycles. Four patients were removed from study before the first tumor assessment was conducted. 16 patients demonstrated progressive disease at the first assessment, and 22 patients demonstrated stable disease (less than a 30% reduction and less than a 20% increase in the sum of the longest diameters of the target lesions). No objective responses were seen. Overall, tumor control (i.e., complete response (CR) , partial response (PR), and stable disease (SD)) was achieved in 21 0f 42 patients, 50%, of the patients.

In agreement with previous response date for ET-743 in the sarcoma population (15), patients with a diagnosis of liposarcoma accounted for the majority (11 of 21; 52%) of all responders (Table 1, Figure 1). Within our liposarcoma group of 16 patients, 11 (69%) patients had partial remission or stable disease following two cycles of ET-743. We further sub-divided our liposarcoma group into either dedifferentiated liposarcoma or myxoid round cell liposarcoma (MRCLS), and noted an obvious improvement in overall survival for those patients with both liposarcoma subtypes treated with ET-743 (black and red lines, Figure 1A) as opposed to patients with other sarcoma diagnoses. A statistical analysis (Chi-square distribution, 4 degrees of freedom, p= 0.0572) suggests that this difference in overall survival is only a trend bordering on statistical significance, but completely in concordance with the preferential liposarcoma focused response data.

Clinical History and Characteristics of MRCLS Patient 1.

Patient 30 presented to us with a large abdominal mass displacing the liver completely into the left abdomen and spanning from the diaphragm to the lower pelvis abutting the urinary bladder. Approximately 1.5 years prior to presenting to us he presented with an almost identical (in both size and location) mass to another institution and underwent an aggressive surgical debulking procedure. Based on the lack of efficacy of adjuvant chemotherapy for soft-tissue sarcomas (16), he was followed with imaging and physical examinations but eventually lost to follow up. He subsequently recurred and received two cycles of doxorubicin/ifosfamide with progression of disease, following which he was referred to us for clinical trial participation.

Clinical History and Characteristics of MRCLS Patient 2.

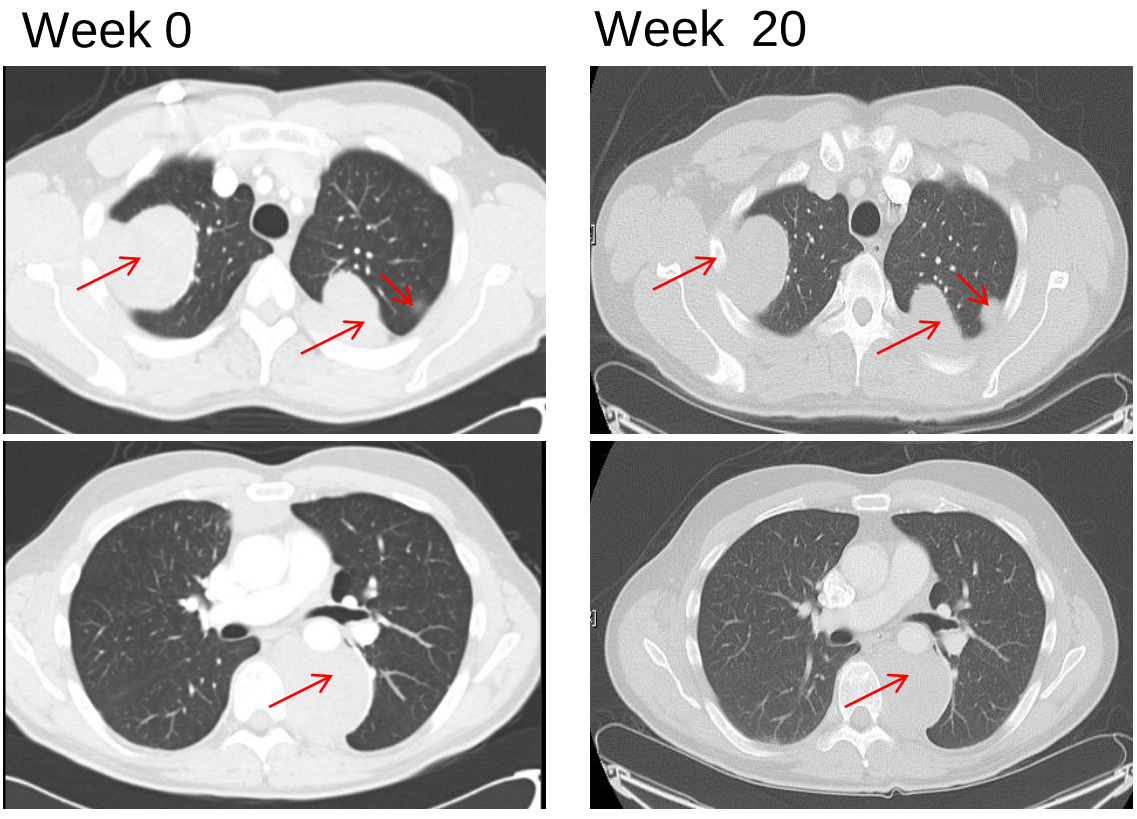
To contrast and compare the atypical differentiation based response observed for MRCLS patient 1, we show the more typical prolonged stable disease type of response observed in 4 of 7 of our other MRCLS patients (Table 1) treated with ET-743. Patient "2" underwent a resection of a 6cm thigh lesion in 2003. Pathologic examination and split probe

testing confirmed TLS:CHOP MRCLS. Five months before presentation to us he developed chest pain. Eventual work-up identified multiple lung lesions. Biopsy and split probe testing confirmed TLS:CHOP MRCLS. Pathology of this patient is now shown as Fig 1Diii. The patient received doxorubicin and ifosfamide for six cycles with progression of disease before being subsequently enrolled on ET-743. The patient remains stable as depicted in Supplemental Figure 1 after 20 weeks of treatment. Although some of the images suggest that this patient's disease burden may have decreased, the change is not sufficient for a partial response (change in 20% via RECIST criteria). Of note, there are no obvious radiological changes suggesting differentiation despite stable disease.

Generation of stable "infectants" of p53null MSCs expressing TLS:CHOP:

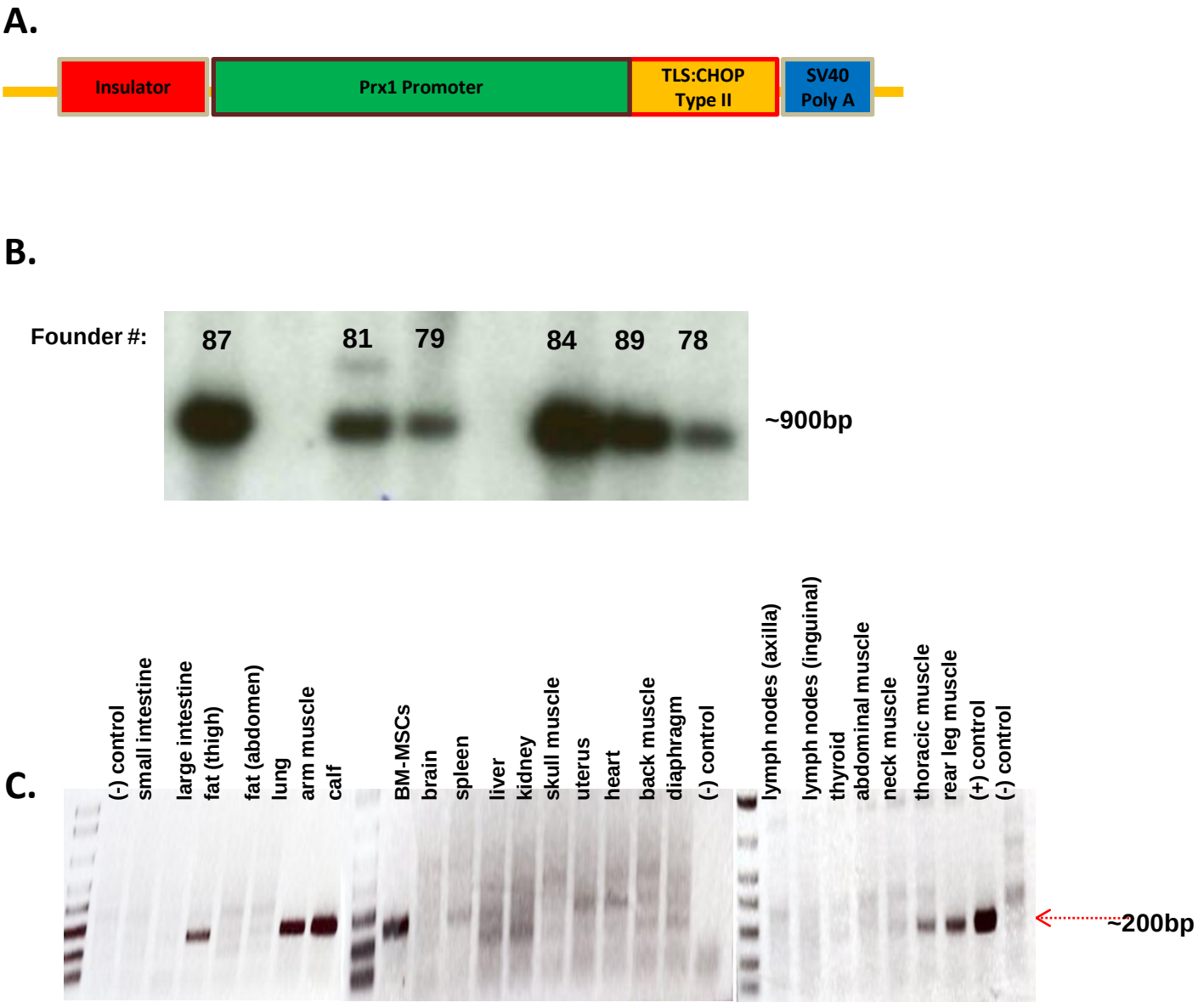
TLS-CHOP type II construct in pCR4-TOPO vector (Invitrogen) was provided by PharmaMar industry (Spain) and was subsequently cloned into the CMV-promoter based pLENTI 6.2 vector (Invitrogen). MS). Transfection into 293FT cells and subsequent infection of MSCs were performed with the ViraPower system (Invitrogen). Clones were selected for blasticidin resistance and verified for TLS:CHOP expression. Four positive expressing clones are shown in Supplemental Figure 4A. A204 (rhabdomyosarcoma cells) induced with tunicamycin 24 ug/ml 2 hours (a known stress activator resulting in CHOP/GADD133 upregulation) is used as a control for the antibody.

p53nullMSCs expressing lentivector based TLS:CHOP were then expanded and inoculated into NSG mice to generate xenografts as described in the main text. Mice were either followed for three weeks from initial tumor nodule formation (Supplemental Figure 4Bi), treated two times with ET-743 (days 1 and 5; Supplemental Figure 4Biii) and immediately sacked, or treated four times (days 1, 5, 9, 13; Supplemental Figure 4Bv)) and then immediately sacked. Tumors were paraffin embedded and assayed via IHC for TLS:CHOP. As can be seen in Supplemental Figure 4B, treatment with ET-743 results in a rapid downregulation of TLS:CHOP at the protein level which appears to be directly proportional to the number of ET-743 doses. Concurrent with downregulation of TLS:CHOP adipogenic differentiation is observed via Oil-Red-O staining. The effects however are not as dramatic as observed in the transgenic mice as the latter are treated for a much longer period of time.



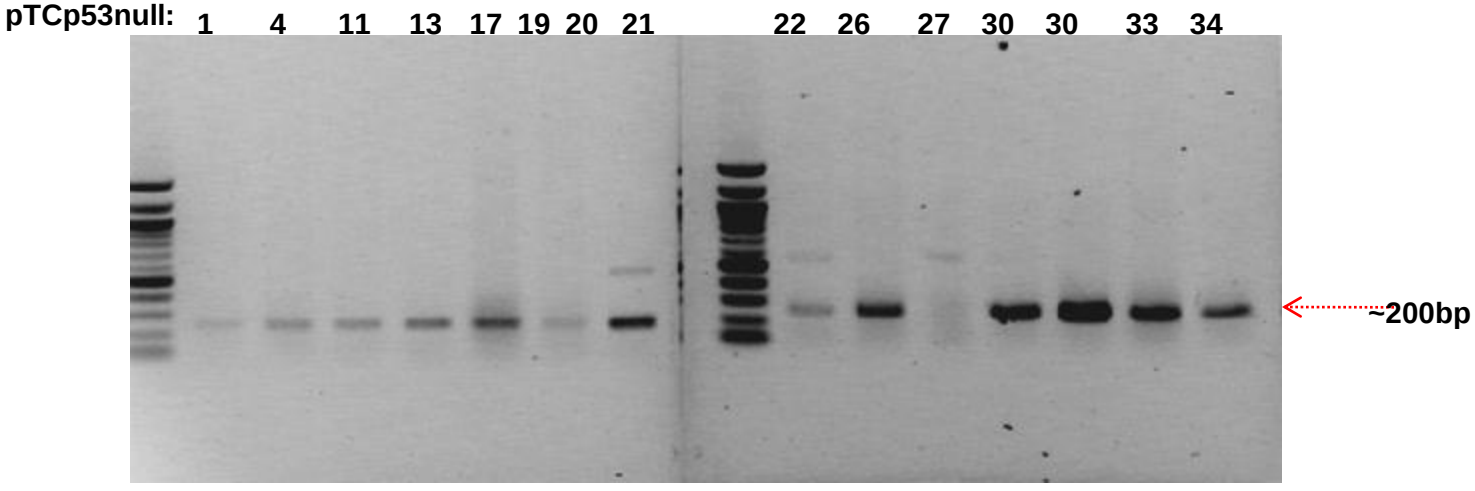
Supplemental Figure 1: CT imaging of MRCLS patient 2 (Figure 1Diii) treated with ET-743 showing stable disease without evidence of differentiation. Red arrows indicated tumor.

To contrast and compare the atypical differentiation based response observed for MRCLS patient 1, we show the more typical prolonged stable disease type of response observed in 4 of 7 of our other MRCLS patients (Table 1) treated with ET-743. Patient "2" underwent a resection of a 6cm thigh lesion in 2003. Pathologic examination and split probe testing confirmed TLS:CHOP MRCLS. Five months before presentation to us he developed chest pain. Eventual work-up identified multiple lung lesions. Biopsy and split probe testing confirmed TLS:CHOP MRCLS. Pathology of this patient is now shown as Fig 1Diii. The patient received doxorubicin and ifosfamide for six cycles with progression of disease before being subsequently enrolled on ET-743. The patient remains stable as depicted in Supplemental Figure 1 after 20 weeks of treatment. Although some of the images suggest that this patient's disease burden may have decreased, the change is not sufficient for a partial response (change in 20% via RECIST criteria). Of note, there are no obvious radiological changes suggesting differentiation despite stable disease.



Supplemental Figure 2: (A) Schematic of transgenic expression vector showing TLS:CHOP (Type II) under the mesodermal specific promoter Prx1 enhancer element and flanked 5' by an additional insulator element and 3' with the SV40 polyadenylation signal. (B) Positive founders as identified via Southern analysis using a PCR generated probe using a PCR synthesized template using primers Prx1 Forward: TAGATCGTAGAGAGCC; TLS:CHOP Reverse: ACTGAGTTCCATAGCCTG (~900bp). (C) RT-PCR showing expression pattern in tissues of 10 week old positive Prx1-TLS:CHOP transgenic mice using a forward TLS and a reverse CHOP primer set: F': CAGCCAGCAGCCTAGCTATG R: TGTCCCGAAGGAGAAAGGCAATG, as previously described in (10).

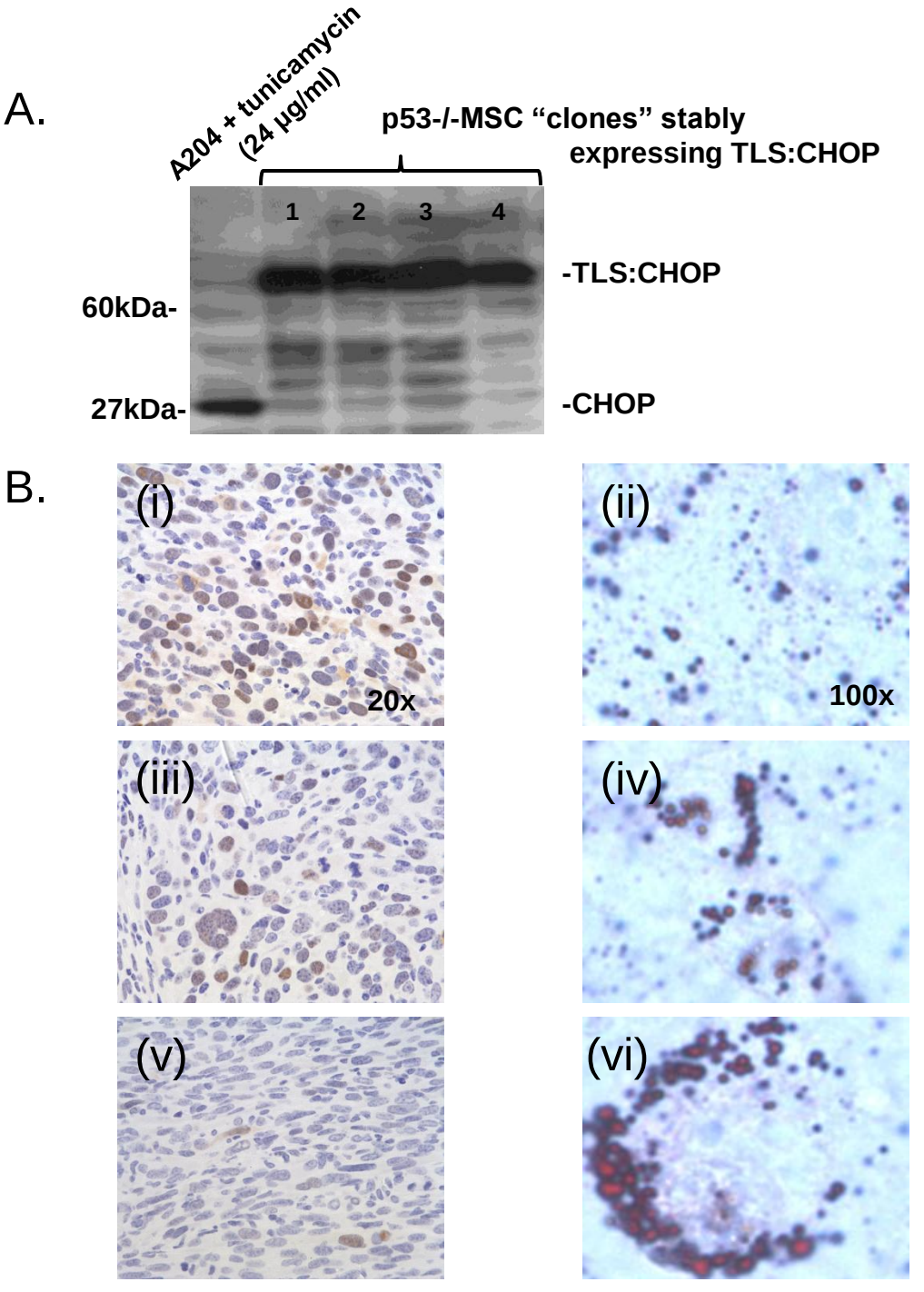
A.



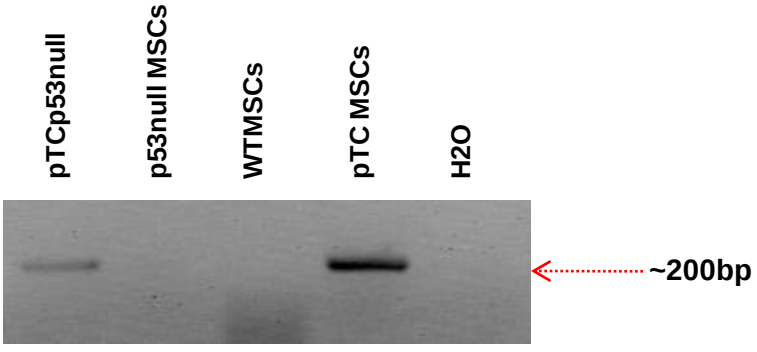
B.

	Sarc:Prx1-D	Sarc:Non-Prx1-D	Lymp:Prx1-D	Lymp:Non-Prx1-D
p53+/-	12	14	6	18
p53-/-	1	4	12	34
PRX-TLS:CHOPxp53+/-	28	9	2	7
PRX-TLS:CHOPxp53-/-	15	6	5	22

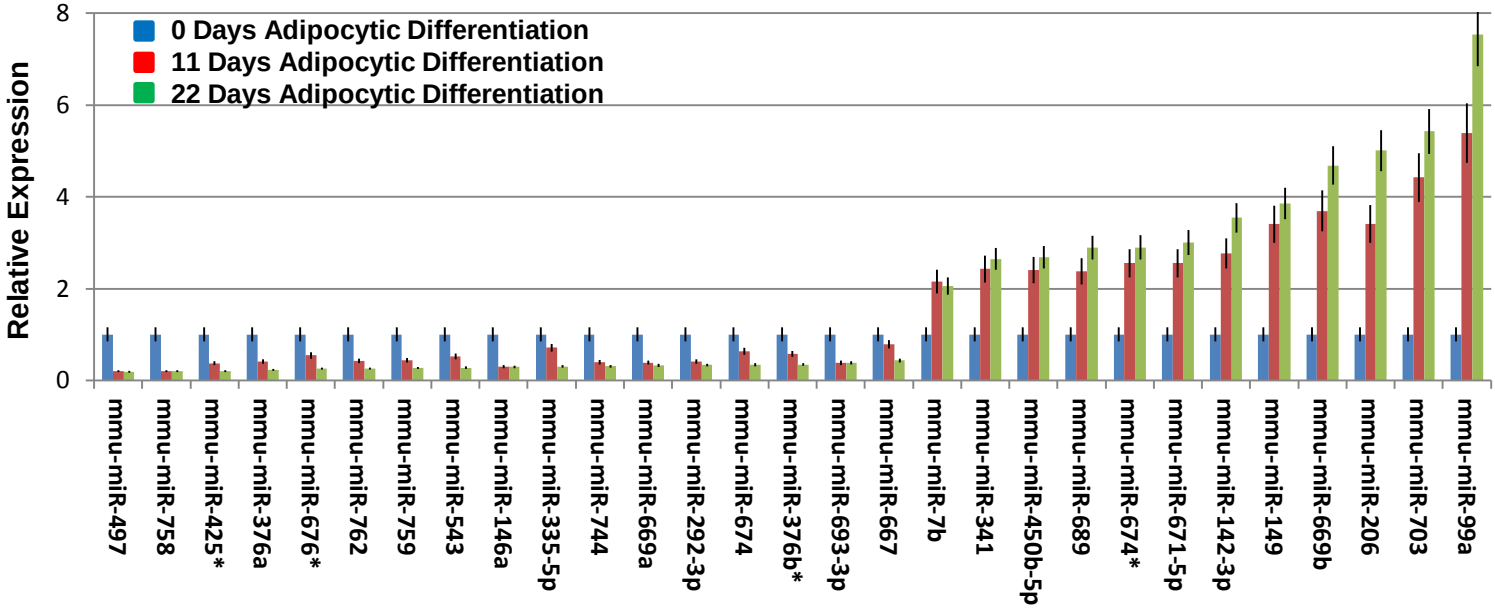
Supplemental Figure 3: (A) Detection of TLS:CHOP (type II) fusion product in the sarcomas of pTCp53null mice. RT-PCR showing expression pattern in FNA-diagnosed sarcomas in pTCp53null mice. Each lane represents one mouse. Each number at the top of each lane refers to the pre-assigned designation to the sacrificed mouse. Lanes in which the 200bp fusion PCR product is missing indicates a sarcoma forming in the absence TLS:CHOP. PCR was performed from extracted RNA using a forward TLS and a reverse CHOP primer set: F': CAGCCAGCAGCCTAGCTATG R: TGTCCCGAAGGAGAAAGGCAATG, as previously described in (10). (B) Table form of data presented in Figure 2A.



Supplemental Figure 4: (A) Western blot analysis showing detection of TLS:CHOP in p53-/-MSC "clones" stably expressing TLS:CHOP. CHOP upregulation as a result of tunicamycin treatment is used as a control for the antibody. (B) IHC of TLS:CHOP in xenografts from NSG mice inoculated subcutaneously with p53-/-MSC "clones" stably expressing TLS:CHOP after either no treatment and three weeks of observation (i), two doses of ET-743 treatment (iii), or four doses of ET-743 treatment (v). (ii, iv, vi) represent Oil-Red-O stained sections of corresponding treatments.



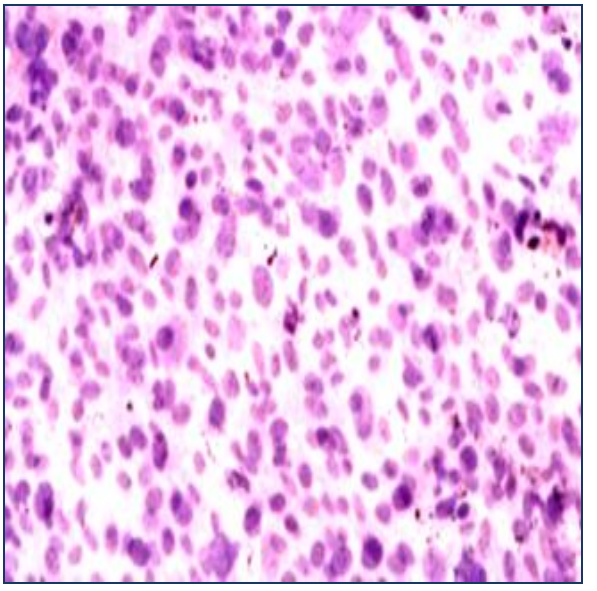
Supplemental Figure 5: Validation and detection of TLS:CHOP fusion product in the MSCs of pTCp53null mice. PCR was performed from extracted RNA using using a forward TLS and a reverse CHOP primer set: F': CAGCCAGCAGCCTAGCTATG R: TGTCCCGAAGGAGAAAGGCAATG, as previously described in (10). MSCs extracted from pTC mice were used as a positive control. MSCs extracted from p53null and wild-type (WT) mice MSCs were used as negative controls for TLS:CHOP expression. MSCs were extracted as described in the Materials and Methods.



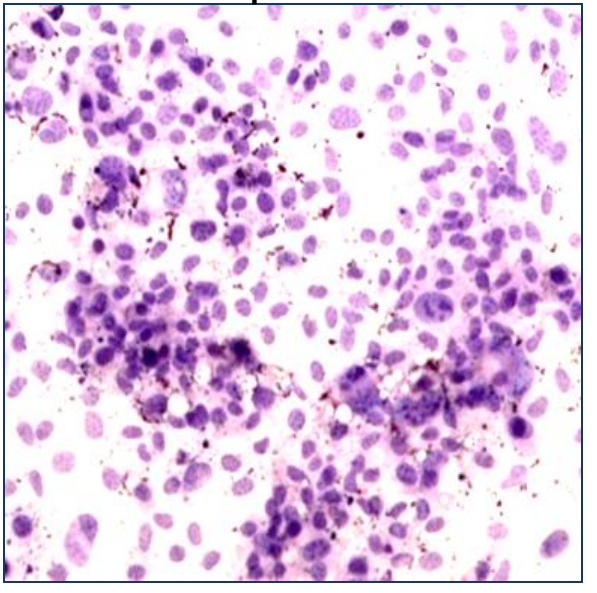
Supplemental Figure 6: Validation of miRNA expression of an adipocytic differentiation gene panel during adipocytic differentiation (identified as described in the main text).

GROWTH MEDIA - STAINED W/ ORO

MSCs from pTCp53null mice

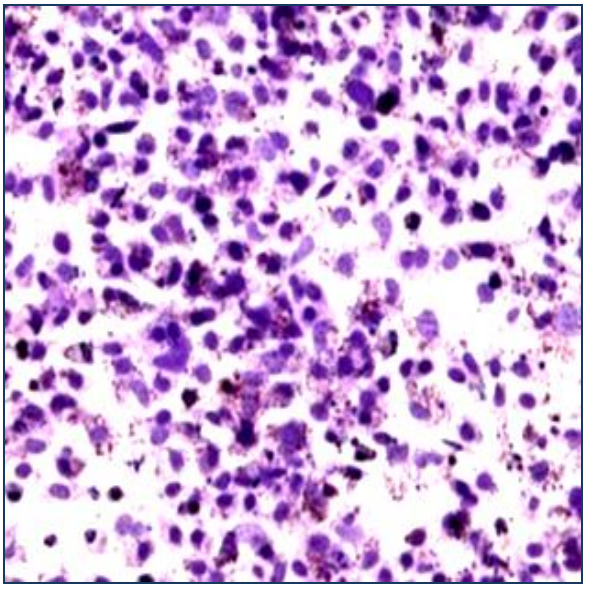


MSCs from p53null mice

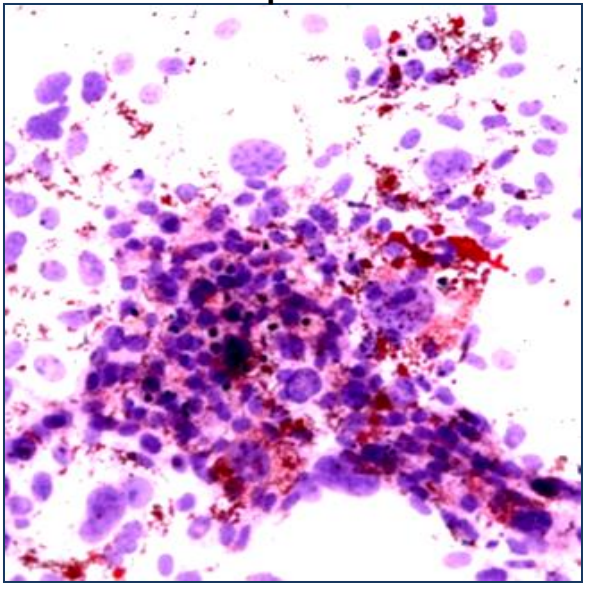


ADIPOGENIC MEDIA - STAINED W/ ORO

MSCs from pTCp53null mice



MSCs from p53null mice



Supplemental Figure 7: Oil Red O staining of MSCs from pTCp53null mice and from p53 null mice after 22 days of adipocytic differentiation medium.

	Type of Response		
Sarcoma Histopathology	**SD**	**POD**	**NE**
Myxoid Round Cell Liposarcoma	5	2	0
Dedifferentiated Liposarcoma	6	3	0
Leiomyosarcoma	7	6	2
High Grade Undifferentiated Sarcoma	1	2	2
Synovial Sarcoma	1	3	0
Osteosarcoma	1	0	0
Alvelolar Soft-Parts Sarcoma (ASPS)	0	0	1

Supplemental Table 1: Type of Response per Sarcoma Sub-Type.
SD=stable disease; POD=progression of disease; NE=not evaluable..

Frequently Observed Toxicities				
AE Term	Percentage of Patients			
	Grade 1	Grade 2	Grade 3	Grade 4
Elevated Alkaline Phosphatase	40.5	14.3	2.4	0
Elevated ALT	35.7	16.7	19	2.4
Elevated AST	40.5	11.9	4.8	2.4
Hypoalbuminemia	19	2.4	0	0
Fatigue	28.6	11.9	4.8	0
Nausea	23.8	14.3	11.9	0
Vomiting	4.8	14.3	4.8	0
Anemia	28.6	35.7	16.7	2.4
Hyponatremia	11.9	2.4	2.4	0
Hypocalcemia	19	14.3	2.4	0
Hypokalemia	11.9	2.4	4.8	0
Neutropenia	16.7	31	23.8	16.7
Leukopenia	35.7	59.5	33.3	11.9
Thrombocytopenia	19	9.5	9.5	7.1
Hyperglycemia	21.4	7.1	2.4	0
Anorexia	11.9	0	0	0

Supplemental Table 2: Frequently observed toxicities in patients treated with ET-743.

MRLCS Case:	p53 Mutation
1 (D,E), SD	Ex7; Codon 225; GTT(Val)>GCT(Ala)
2 (F), SD	Not Detected
3 (G), SD	Not Detected
4 (H), SD	Not Detected
5 (I), SD	Not Detected
6 (J), POD	Not Detected
7 (K), POD	Not Detected

Supplemental Table 3: p53 genotype (exons5-9) of MRCLS patients shown in Figure 1D. Note: letters in parenthesis following each case number indicate corresponding histology shown in Figure 1D-K. SD=stable disease. POD=progression of disease.

As stated in the main text, genomic DNA was extracted from frozen tumor tissues from primary surgeries and mutational analysis was performed for p53 exons 5-9. Genomic DNA was extracted using standard Genomic DNA was extracted using the standard proteinase K digestion and phenol/chloroform extraction methods. Single-strand conformation polymorphism and direct sequencing p53 (exons 5–9) were carried out using primer sequences and PCR conditions as previously described (31). In agreement with the immunohistochemistry, a p53 mutation (Ex7; Codon 225; GTT(Val)>GCT(Ala)) was detected only in tumor genomic DNA from MRCLS patient. Although we cannot rule out the possibility that mutations in p53 were not present in the non-analyzed portions of p53, the mutational analysis coupled to the IHC analysis strongly suggests that p53 was aberrant only in this patient. Of note this specific p53 mutation in MRCLS was previously reported (13).

Gene Symbol	RefSeq ID	Primer1	Primer2	cDNA amplicon size
A2M	NM_175628	ATGGCCTTTCTTGTGTCCC	TCTAAATGACGAGGCTGTGC	125
AOC3	NM_009675	AAAAGCCATATCCTCTGCCC	TGGTGAGAAGTTTGGGAACC	130
APOD	NM_007470	CTCGCTGGGATCTTCTCAAT	CCACAGCCAAAGGACAAAAT	118
APOE	NM_009696	GAGCTGATCTGTCACCTCCG	GGACTTGTTTCGGAAGGAGC	129
ASPN	NM_025711	CACAGCCAAAAGCAGTAGCA	TGCAGTTAAGTAGTACAGGGTGGA	122
CCL2	NM_011333	GGGATCATCTTGCTGGTGAA	AGGTCCCTGTCATGCTTCTG	127
CDH13	NM_019707	ATGTGTAACACTTTCCGCTGG	CTTCTAGTCGGGCAAGATGC	129
CEBPA	NM_007678	GTCACTGGTCAACTCCAGCA	TGGACAAGAACAGCAACGAG	128
CES1	NM_021456	TTCCTGGGGTCTATCGTCTG	ATGTTCGGTGTCCCATCTGT	117
CHRDL1	NM_031258	TATTCGCATGACTTGCTGGT	GCCGAGTCAGATGTCCAAGT	124
COL11A1	NM_007729	TCTCCCGTGATCAGGAACTG	TCACAAAACCCCTCGATAGAA	127
COL13A1	NM_007731	GGAGTCCAGGTCTTCCAGTG	CCAACTGCTGGACGAGAAAT	119
COMP	NM_016685	GGACAGTTGTCACAGGCATC	CCTGCGACGACGACATAGAT	124
CORIN	NM_016869	TCGCTCCAGTCATCACAGTC	TCTCACTGCAGCAGGAACAT	130
DPT	NM_019759	AAAATTGCCACTCACGATCC	CTGGTGGGAGGAGATCAACA	122
FABP4	NM_024406	TCCCCATTTACGCTGATGAT	TGGAAGCTTGTCTCCAGTGA	119
FBLN1	NM_010180	TCATCTCCAGCTTCAGGACA	GGGAACCTTCGAGACTCCTT	121
FBXO9	NM_023605	TCATCTCCAGCTTCAGGACA	GGGAACCTTCGAGACTCCTT	125
FRZB	NM_011356	ACAGGCTTACATTTGCAACG	GTGTGCATCTCTCCTGAGGC	113
GDF15	NM_011819	GTAGGCTTCGGGGAGACC	GAACTCAGAACCAAGTCCCG	127
GPR21	NM_177383	CAGTGTCTTCAGGCCAAGTG	TGGCTTTTGTTTGGATTTCA	128
HP	NM_017370	CAGTGTCTTCAGGCCAAGTG	TGGCTTTTGTTTGGATTTCA	118
IBSP	NM_008318	CCTCTTCGGAACTATCGCAG	CGGCCACGCTACTTTCTTTA	118
IER3	NM_133662	GACACACCCTCTTCAGCCAT	GAAGGGTGCTCTACCCTCG	130
IGF2	NM_010514	TGAGAAGCACCAACATCGAC	ACTTCAGCAGCTCCCACTTC	129
IL6	NM_031168	TGGTACTCCAGAAGACCAGAGG	AACGATGATGCACTTGCAGA	128
KRT18	NM_008489	GGAGGTCCACTGAAATGGTG	TCGCCATCTCTGACTCTTCC	120
LBP	NM_008489	GGAGGTCCACTGAAATGGTG	TCGCCATCTCTGACTCTTCC	120
LEP	NM_008493	TGAAGCCCAGGAATGAAGTC	CAGGATGACACCAAAACCCT	119
LPL	NM_008509	TCAGCTGTGTCTTCAGGGGT	TTTGGCTCCAGAGTTTGACC	115
MAFF	NM_010755	GCTTGACCTTCAGGGCTTT	GACAAGCACGCACTGAGC	112
MMP13	NM_008607	TAGATGGGAAACATCAGGGC	TGATGAAACCTGGACAAGCA	123
OLFML2A	NM_172854	CCTCTGAGGTCATCCGAACC	AGTTGAGGCTTCTGCTGGTG	111
OLFML2B	NM_177068	GTTCGGATTTCTTCCACGTC	AAGCTGCATTCGGTTACCAC	119
OLR1	NM_138648	GGCAGAGGATGACCAGAGTC	ACAAGATGAAGCCTGCGAAT	123
PCK1	NM_011044	TCTGGATGGTTTTAATGGCA	TGCCTGGATGAAGTTTGATG	122
PDE1A	NM_001009978	ATTCTCAAGCACTGAGCGGT	CTTTGCAGCTGCCATTCAT	112
PLAC8	NM_139198	ACGAACGAATCCAGGTTGAG	AGACTCAACCCCAGACCACA	126
PLIN	NM_175640	GACACCACCTGCATGGCT	ACCATGCAAACCACAGCAT	122
PPARG	NM_011146	TCTTCCATCACGGAGAGGTC	GATGCACTGCCTATGAGCAC	145
PRELP	NM_054077	GCCAAGATGAGGAGAAGTGG	TGGGGAGGAACAGAAGAGTG	117
PRKAR2B	NM_011158	TTCTCTGATCATCGGTTTTGG	ATAAACCGGTTCACAAGGCG	112
PTGDS	NM_008963	ACCACTGACACGGAGTGGAT	CCTCAGGAAAAACCAGTGTGA	114
RABGAP1	NM_146121	CTGCTCCCTCATGGTATGGT	CAGCTCCCTAAAAGATACCGC	126
RARRES2	NM_027852	TTTACCCTTGGGGTCCATTT	AAGCTCCAGCAGACCAACTG	117
RBP4	NM_011255	GGAGTACTGCAGAGCGAAGG	TGAAGATCCTGCCAAGTTCA	118
S100A4	NM_011311	AACTTGTCACCCTCTTTGCC	TCAGCACTTCCTCTCTCTTGG	120
SEPP1	NM_009155	GGGGCTTTGTAACAAGCAGA	AAGCTAGTCCGAAGGGGTTG	129
SERPINF1	NM_011340	GTCAGGGGCAGGAAGAAGAT	GTCCCCATGATGTCAGATCC	122
SLC7A5	NM_011404	GCCTTCACGCTGTAGCAGTT	TCTTCGCCACCTACTTGCTC	124
SORBS1	NM_009166	GGTCCTGGAGTTCTGAGCTG	AAGTTACAGAGCCCGTGAGG	120
TNFAIP6	NM_009398	CCCGAGCTTCTCTGTGGTAT	TCCTCCTTTGCTTATGCGTC	123

Supplemental Table 4: Primers used to determine expression values for mouse adipocytic differentiation gene set.