Involvement of tumor suppressors PTEN and p53 in the formation of multiple subtypes of liposarcoma

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Liposarcoma (LPS) is a type of soft tissue sarcoma that mostly occurs in adults, and in humans is characterized by amplifications of MDM2 and CDK4. The molecular pathogenesis of this malignancy is still poorly understood and, therefore, we developed a mouse model with conditional inactivation of PTEN and p53 to investigate these pathways in the progression of the disease. We show that deletion of these two tumor suppressors cooperate in the formation of multiple subtypes of LPS (from well-differentiated LPS to pleomorphic LPS). In addition, progression of the tumors is further characterized by the expression of D cyclins and CDK4/6, which allow for continued cell division. Microarray analysis also revealed novel genes that are differentially expressed between different subtypes of LPS, which could aid in understanding the disease and to unravel potential new therapeutic targets.

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Abbreviations: LPS, liposarcoma; WDLPS, well-differentiated liposarcoma; DDLPS, dedifferentiated liposarcoma; MLPS, myxoid/round cell liposarcoma; PLPS, pleomorphic liposarcoma; WDLPS, well-differentiated liposarcoma; DDLPS, dedifferentiated liposarcoma; MLPS, myxoid/round cell liposarcoma; PLPS, pleomorphic liposarcoma; MScs, mesenchymal stem cells; IHC, immunohistochemistry; LPL, lipoprotein lipase; HGF, hepatocyte growth factor; MDM2, mouse double minute 2 homolog; p53, tumor protein p53; Pten, phosphatase and tensin homolog; PI3K, phosphatidylinositol-3 kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; CDK, cyclin-dependent kinases; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; CEBP delta, CCAAT/enhancer-binding protein delta; Fabp4, fatty acid binding protein 4; adipocyte; Lpl, lipoprotein lipase; Pparg, peroxisome proliferator-activated receptor gamma; Lxn5, iroquois homeobox 5; Meis2, meis homeobox 2; Edf1, early B-cell factor 1; Sox17, SRY (sex determining region Y)-Box 17; Lep, leptin (murine obesity homolog); Ntrk2, neurotrophic tyrosine kinase receptor, type 2; Nkd2, naked cuticle homolog 2; Fzd4, frizzled class receptor 4; Vegla, vascular endothelial growth factor A; G0s2, G0/G1 switch 2; Gorf, glutamic-oxaloacetic transaminase 1; Aurka, aurora kinase A; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; SUV39H1, suppressor of variegation 3–9 homolog 1; CREB, cAMP response element-binding protein

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and \( Pten \) together results in tumor formation in ~85% of mice with tumors representing all four major subtypes of LPS observed in humans. A gene expression microarray analysis of these tumor types indicates many differences in gene expression and unravels a new possible method of diagnosis of these tumors. This mouse model also demonstrates a role for the MDM2 protein in the formation of some LPS cell types even in the absence of the \( p53 \) functions. Once \( p53 \) and PTEN functions are eliminated, the next step in the transformation pathway of LPSs is the enhanced production of some D cyclins and CDK4/6, leading to continual signaling for cell division. Interestingly this is a pathway observed when \( p53 \) knockout mice produce T-cell lymphomas.17

Results

High penetrance of LPS formation initiated by \( PTEN \) and \( p53 \) deficiency. Accumulating evidence points to important roles for the \( p53 \) and PI3K/PTEN pathway in the development of LPSs. In this study, we examined the consequences of deletion of \( Pten \) and \( p53 \) in adipose tissue of mice. Adenovirus-cre was either injected into the adipose tissue surrounding the ovary (gonadal fat pad) or surrounding the testes resulting in either deletion of exons 2–10 in \( p53^{\text{fox/fox}} \) mice,18 or exon 5 in the \( Pten^{\text{fox/fox}} \) mice.19 Injection of adenovirus-cre into adipose tissue of either \( p53^{\text{fox/fox}} \), \( Pten^{+/+} \) mice or \( p53^{+/+}, Pten^{\text{fox/fox}} \) mice led to no tumor formation, whereas injection of \( p53^{\text{fox/fox}} \), \( Pten^{\text{fox/fox}} \) mice led to >85% of mice with tumor formation (Figure 1). Tumors in the \( p53^{\text{fox/fox}} \), \( Pten^{\text{fox/fox}} \) mice were detected by palpation as early as 81 days post injection and were 50% penetrant at 153 days. Thus, deletion of both alleles of \( p53 \) and \( Pten \), in combination, results in highly penetrant LPS development in this mouse model.

Development of multiple subtypes of LPS in \( p53^{\text{fox/fox}}, Pten^{\text{fox/fox}} \) mice. Histological analysis of the LPSs, which formed in the \( p53^{\text{fox/fox}}, Pten^{\text{fox/fox}} \) mice, showed that all four main subtypes, WDPLS, DDPLS, MLPS and PLPS, were represented (Figure 2a). Interestingly, within the same tumor one or two different subtypes could be found. Evidence points to mesenchymal stem cells (MSCs) being the target cell for the development of sarcomas and LPSs from which multiple types of these tumors are possible.20 Approximately, 80% of the tumors examined had a dedifferentiated subtype component, whereas the pleomorphic subtype represented the least number of cases (Figure 2b). Marker analysis to verify the histologically identified subtypes showed that WDPLS and MLPS, which have been classified as being more mature adipocytic sarcomas, were characterized by high expression of adipocytic markers (LPL) when compared with DDPLS and PLPS (Figure 2c, upper panels). In the contrary, DDPLS and PLPS subtypes, which are classified as being more immature LPSs, display stronger and more diffuse expression of MSC markers (HGF) than WDPLS and MLPS (Figure 2c, lower panels, and data not shown).21

Expression of cell cycle regulation genes in multiple subtypes of \( p53^{\text{fox/fox}}, Pten^{\text{fox/fox}} \) LPS tumors. Cell cycle regulation in human cancer is often deregulated resulting in unscheduled proliferation, genomic instability and chromosomal instability leading to aneuploidy. Cyclin-dependent kinases (CDK) and cyclins are the main driving forces of cell cycle regulation and they are in turn regulated by \( p53 \) and PTEN. Therefore, we wanted to investigate some of the molecular consequences of \( p53 \) and PTEN loss on these proteins in LPS. Here we found that there is an aberration in the G1–S checkpoint with the upregulation of CDK4, CDK6, Cyclin D1 and Cyclin D3 in all four of the subtypes of LPS (WDLPS, DLLPS, MLPS and PLPS) (Figure 3 and data not shown). The available evidence suggests that Cyclin D1 and Cyclin D3 can be regulated by PTEN,22,23 however, this is the first time it has been shown that they are overexpressed in the absence of both \( p53 \) and PTEN in a mouse model of LPS. These results suggest that alterations of this pathway are essential for the oncogenic process of the different subtypes of LPS.
MDM2 and C/EBP delta showed similar patterns of expression around lipoblasts (Supplementary Figure 1), which allows for a potential explanation for the restricted MDM2 expression pattern and dedifferentiation from WDLPS to DDLPS.

Bioinformatics analysis of differentially expressed genes in two of the biological types of LPS. To understand the possible progression of WDLPS to DDLPS, we analyzed gene expression profiles by microarray to identify pathways and genes that might be differentially expressed. This would aid in understanding the molecular consequences for the loss of p53 and PTEN pathways in different subtypes of LPS. The differentially expressed genes between WDLPS and DDLPS are shown in a Heat Map (Figure 5, expression data for all samples in Supplementary file 2). While this list of genes is not statistically significant due to limited number of samples and multiple hypotheses testing, it suggests candidate genes in differentiation between the two subtypes as well as potential new markers of tumorigenesis and therapeutic targets. Table 1 shows the upregulated genes in each subtype compared with the other subtype that have a greater than threefold change in expression and $P$ value $< 0.01$ (genes that have a twofold change but of particular importance are marked in the table). Many of the genes that are of interest, in distinction of the two subtypes, relate to the control of lipid, fatty acid, amino acid and carbohydrate metabolism (Fabp4, Lpl, Pgm5, Ldr, Got1, Ech1), insulin-like growth factor pathway (Igf2, Igf1, Foxo1), transcription factors (Foxc2, Sox6, Pparg, Lnx5, Meis2, Ebf1, Sox17), obesity-related genes (Lep, Ntrk2), Wnt signaling (Nkd2, Fzd4) or genes related to cell proliferation and apoptosis (Vegfa, G0s2). Two genes that are p53 regulated or regulate p53, Got1 and Aurora, respectively, might help to explain targets that are of interest in the formation of LPSs in the absence of p53. These data suggest potentially novel genes that can be used to distinguish between WDPLS and DDLPS subtypes and can also provide insight into LPS pathogenesis and progression.
Discussion

High-level amplification of the MDM2 and CDK4 genes have become markers for studying human LPSs, implicating deregulation of both the p53 pathway and cell cycle regulation as necessary aspects for tumor progression. However, researchers have also seen that other points in the p53 pathway can be deregulated such as p53 itself with or without MDM2 amplification. In addition, another widely mutated gene in cancer, PTEN, a regulator of the PI3K-AKT pathway, has been found in multiple lipomas (benign adipocytic neoplasms) and AKT activation has been found in human LPSs. This finding is of significance owing to the many drugs that have been found to target the PI3K-AKT-mTOR pathway and the lack of drugs currently effective for the treatment of LPSs or that are still in the clinical trial period. In this study, we developed a mouse model of LPS that demonstrates the combined effect of p53 and PTEN deletion in the generation of these tumors and its potential utility in the development of novel therapeutics.

The interaction of the p53 and PI3K/AKT/PTEN pathways is well documented. Likewise, combinatorial deletion or inactivation of members from each pathway is also very common. In this mouse model, we observe that when either alleles of p53 or Ptten were deleted individually it did not result in any tumor formation but in combination several different subtypes of LPS were formed. It could be that inactivation of both pathways in a stem cell or progenitor type cell could lead to the formation of the different subtypes of LPS (WDLPS, DDLPS, MLPS and PLPS). Two previous studies, have suggested this possibility as well as a way for the progression of tumorigenesis.

In addition, one of the mechanisms that could be the driving force for tumor progression, in the absence of p53 and PTEN, is cell cycle regulation. In this model of LPS, the loss of p53 and PTEN promotes the abnormal expression of D cyclins and CDK4/6. CDKs are bound by regulatory subunits called cyclins that are in turn made at certain times during the cell cycle and regulate enzymatic activity. Mutations that deregulate these CDK-cyclin complexes lead to continued proliferation or inappropriate re-entry into the cell cycle. In this model, altered expression of these cell cycle regulation proteins could be one of the driving forces of uncontrolled growth and LPS tumorigenesis. Specifically, the increased expression of the G₁–S phase regulators CDK4 and cyclin E would serve to enhance the G₁–S transition.

Figure 3  Immunohistochemical analysis of cell cycle markers in WDLPS and DDLPS subtypes. There is a deregulation in the G₁–S checkpoint with the upregulation of CDK4, Cyclin D1 and Cyclin D3. Scale bar corresponds to 50 μm

Figure 4  Immunohistochemical analysis of MDM2 in WDLPS, DDLPS and MPLS subtypes. MDM2 protein is expressed at very high levels in WDLPS and DDLPS sections in areas with lipoblasts (a and b) and absent in DDLPS sections lacking lipoblasts (c) and MLPS (d)
It is of some interest that p53 knockout mice develop thymic lymphomas. These tumors select for a common series of mutations in a specific order. After the gatekeeper mutation of p53, then Pten deletions are commonly selected for and this is followed by Cyclin D1, D2, D3 and Cdk-6 amplifications and overexpression. The LPSs described here have demonstrated an identical series of events, suggesting a common pathway to diverse cancers following a p53 mutation.

Other mechanisms involved in this tumor model could be regulating the dedifferentiation of tumor subtypes. MDM2 has been shown to exert a p53-independent role on the initiation of adipocyte differentiation through controlling CREB-dependent transactivation, as well as the determination of cell fate of MSCs, another p53-independent function. Adipocytes, osteoblasts, myocytes and chondrocytes all differentiate from MSCs. In the current study, MDM2 expression was found in areas of lipocytes in both WDLPS and DDLPS p53\textsuperscript{flox/flox}, Pten\textsuperscript{flox/flox} tumors. Therefore, in this model MDM2 might be directing these cells toward a more dedifferentiated cell type, a hypothesis that still needs to be further tested.

### Table 1 Upregulated genes, with a greater than threefold change in expression, in WDLPS and DDLPS as compared with each other

| Gene Title          | Fold change |
|---------------------|-------------|
| Lep (Leptin)        | 151.98      |
| Aspa (Aspartoacylase)| 58.72       |
| Adh1 (Alcohol dehydrogenase 1A) | 35.11 |
| Cd36 (CD36 molecule) | 25.57       |
| Sunc1 (Succinate receptor) | 21.78 |
| Cyp4b1 (Cytochrome P450, family 4, subfamily B, polypeptide 1) | 20.87 |
| Fabp4 (Fatty-acid binding protein 4, adipocyte) | 15.48 |
| Zfp423 (Zinc finger protein 423) | 13.77 |
| Fata2 (Fibroblast growth factor receptor 2) | 11.79 |
| G0s2 (G0/G1 switch 2) | 10.03 |
| Ablim3 (Actin-binding LIM protein family, member 3) | 9.31 |
| Pparg (Peroxisome proliferator-activated receptor gamma) | 9.16 |
| Ebi3 (Early B-cell factor 3) | 6.92 |
| Fox2 (Forkhead box C2 (MFH-1, mesenchyme forkhead 1)) | 6.03 |
| Fgt13 (Fibroblast growth factor 13) | 5.77 |
| Pgm5 (Phosphoglucomutase 5) | 5.47 |
| Ntrk2 (Neurotrophic tyrosine kinase, receptor, type 2) | 5.12 |
| Lpl\* (Lipoprotein lipase) | 4.37 |
| Igf2\* (Insulin-like growth factor 2) | 4.18 |
| Srx10 (Caspase recruitment domain family, member 10) | 4.09 |
| Sox6 (SRY (sex determining region Y)-box 6) | 4.05 |
| Ebf1 (Early B-cell factor 1) | 3.29 |
| Igf1 (Insulin-like growth factor 1) | 3.27 |
| Sox5 (SRY (sex determining region Y)-box 17) | 3.13 |
| Ech1 (Erythroblast specific protein) | 3.04 |
| Fzd4\* (Frizzled class receptor 4) | 2.47 |
| Foxo1\* (Forkhead box O1) | 2.01 |

Genes of interest include those that regulate lipid, fatty acid and carbohydrate metabolism, cell proliferation, apoptosis, transcription, insulin-like growth factor pathway, Wnt signaling and obesity

* P-value ≤ 0.02. # Fold change between 2 and 3

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Other mechanisms involved in this tumor model could be regulating the dedifferentiation of tumor subtypes. MDM2 has been shown to exert a p53-independent role on the initiation of adipocyte differentiation through controlling CREB-dependent transactivation, as well as the determination of cell fate of MSCs, another p53-independent function. Adipocytes, osteoblasts, myocytes and chondrocytes all differentiate from MSCs. In the current study, MDM2 expression was found in areas of lipocytes in both WDLPS and DDLPS p53\textsuperscript{flox/flox}, Pten\textsuperscript{flox/flox} tumors. Therefore, in this model MDM2 might be directing these cells toward a more dedifferentiated cell type, a hypothesis that still needs to be further tested. Another
The potential way that MDM2 might direct cells toward a dedifferentiated phenotype is through epigenetic reprogramming. MDM2-dependent degradation of RB increased levels of p53, leading to repression of p53 target genes. Similarly, MDM2 interacts with the histone methyltransferase SUV39H1 and can in certain instances lead to repression of p53 target genes and potentially epigenetic reprogramming.

To begin to understand how WDLPs and DDLPS differ molecularly in this mouse model in the presence of p53 and PTEN deletions we ran gene expression microarray analysis to compare the two different LPS tumor types. Our investigation would hopefully lead to the discovery of novel deregulated pathways, potential new therapeutic targets and pathways involved in the dedifferentiation process of the subtypes. Some deregulated genes and pathways were similar to a previous human study of WDLPs and DDLPS and involved in development and adipogenesis could be another key pathway that should be investigated in LPS.

We also have some clues as to transcription factors that might be working to induce dedifferentiation through epigenetic mechanisms. SOX6 is an important developmental gene as well as involved in differentiation. It is also known to suppress cyclin D1 activities by interacting with beta-catenin and HDAC1, thus, leading to a decrease in acetylated H3 and H4 at the cyclin D1 promoter. SOX6 is misexpressed in the tumor subtypes although it is unknown if it works in conjunction with MDM2 to promote these processes.

Genes that are p53 regulated or regulate p53 might aid in the investigation of mechanisms responsible for LPS formation when p53 and PTEN are deleted in adipose tissue. Got1 and Aurora were found to be upregulated in DDLPS. Through Got1 and Aurora might be a novel regulator of glucose production and inhibit the use of glucose in pathways that promote tumorigenesis. Likewise, Aurora has been shown to be important not only in the p53 pathway but also in the Pten/Akt pathway. Specifically, Aurora regulates ESC pluripotency through phosphorylation-mediated inhibition of p53-directed gene expression. Inhibition of Aurora in the absence of p53 made cells more susceptible to mitotic arrest and slippage. Similarly, Aurora inhibition can downregulate Akt and promotes significant cell death.

In summary, we have shown that combined deletion of p53 and PTEN leads to the formation of several subtypes of LPS in a mouse model of the disease. Molecular mechanisms involved in the progression include deregulation of cell cycle genes leading to uncontrolled cell proliferation as well as upregulation of MDM2, which might promote dedifferentiation and epigenetic reprogramming. Finally, gene expression microarray analysis has given us not previously known genes that can be further investigated in exploring these hypotheses.

Materials and Methods

Animal model. All animal study protocols were approved by the University of Medicine and Dentistry of New Jersey Institutional Animal Care and Use Committee review board. Conditional alleles for Pten (Pten\(^{\text{fl}^{\text{flox}}/\text{fl}^{\text{flox}}}\)) (Lesche et al. 1999) and p53 (Tg53\(^{\text{Tat}}\)) (Jonkers et al. 1998) mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA. In all, 8–10-week-old mice were used for injection of an adenovirus expressing cre recombinase (AdSCMV-Cre, University of Iowa’s Vector Core Facility (http://www.uiowa.edu/~gene) into adipose tissue. Concentrated virus (25 µl; 4 X10^9 PFU/ml) was mixed with 20 µl of Dulbecco’s modified eagle medium (D-MEM). Mice were monitored for tumor development by palpitation at weekly intervals. Kaplan–Meier analysis was generated using GraphPad software.

Histological analysis. Adipose tissue and tumor samples were harvested and fixed in 10% formalin overnight. The samples were embedded in paraffin and sectioned. The sections were subjected to hematoxylin and eosin staining or immunohistochemical staining following the standard avidin-biotin protocol. The following antibodies were used: MDM2 (clone S1814 Abcam, Cambridge, MA, USA), LPL (Abcam ab21336), HGF (Novus Biologicals NBP1-19182, Littleton, CO, USA), CDK4 (Abcam ab7955), CDK6 (Novus Biologicals NB100-91722), Cyclin D1 (Cell Signaling 297B, Danvers, MA, USA), Cyclin D3 (Cell Signaling 2938), CEBP delta (antibodies-online, Atlanta, GA, USA; ABIN233849).

Gene expression microarray. Total RNA was isolated from frozen tissue using Trizol (Life Technologies) followed by clean-up with the Qiagen RNeasy kit and subjected to the 2100 Bioanalyzer from Agilent Technologies. cDNA samples were generated and labeled using Affymetrix 1-cycle expression kit (Affymetrix, USA), LPL (Abcam ab21336), HGF (Novus Biologicals NBP1-19182, Littleton, CO, USA), CDK4 (Abcam ab7955), CDK6 (Novus Biologicals NB100-91722), Cyclin D1 (Cell Signaling 297B, Danvers, MA, USA), Cyclin D3 (Cell Signaling 2938), CEBP delta (antibodies-online, Atlanta, GA, USA; ABIN233849).

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