Uterine Leiomyosarcoma Tumorigenesis in *Lmp2*-deficient Mice: Involvement of Impaired Anti-oncogenic Factor IRF1

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Abstract. *Background/Aim: Uterine leiomyosarcoma (Ut-LMS) is a highly metastatic smooth muscle neoplasm. We have previously reported that low molecular mass protein2 Lmp2-deficient mice spontaneously developed Ut-LMS, which implicated this protein as an anti-oncogenic candidate. We also suggested that LMP2 may negatively regulate Ut-LMS independently of its role in the proteasome. Initially described as a transcription factor able to activate the expression of interferon-gamma (IFN-γ)-responsive genes, interferon regulatory factor-1 (IRF1) has been shown to play roles in the immune response, and tumor suppression. The aim of this study was to elucidate the molecular mechanism of sarcomagenesis of Ut-LMS using human and mouse uterine tissues. Materials and Methods: The expression of the IFN-γ signal molecules, IRF1 and -2, STAT1, and LMP2, -3, -7 and -10 were examined by western blot analysis, electrophoretic mobility shift assay and immunohistochemistry in human and mouse uterine tissues. Physiological significance of IRF1 in sarcomagenesis of Ut-LMS was demonstrated by xenograft studies. Results: In the present study, several lines of evidence indicated that although treatment with IFN-γ strongly induced the activation of STAT1 as a transcriptional activator, its target molecule, IRF1, was not clearly produced in Lmp2-deficient uterine smooth muscle cells (Ut-SMCs). Conclusion: Defective expression of IRF1 in the IFN-γ-induced signaling molecules may result in the malignant transformation of Ut-SMCs. The modulation of LMP2 may lead to new therapeutic approaches in human Ut-LMS.*

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The uterus is composed of three layers, the uterine endometrium, which serves as a bed for the embryo; the myometrium of the wall, which protects the embryo; and a serous membrane enveloping the uterus. The term uterine tumor generally refers to an epithelial malignant tumor of the uterus, which is roughly classified as a tumor of the uterine cervix or uterine body. Due to the prevalence of medical check-ups, the rate of mortality from malignant tumors of the uterine cervix is now decreasing, and such tumors are commonly detected at a very early stage. In contrast, the mortality rate from malignant uterine tumors is increasing, and these are rarely detected at the initial stages. While most uterine tumors are adenocarcinomas, tumors of the uterine cervix are classified into squamous cancer and adenocarcinomas. Tumors of uterine smooth muscle cells (Ut-SMCs), which develop in the myometrium, have traditionally been divided into benign leiomyoma (LMA) and malignant human uterine leiomyosarcoma (Ut-LMS) based on cytological atypia, mitotic activity, and other criteria. Ut-LMS is relatively rare, having an estimated annual incidence of 0.64 per 100,000 women (1). Ut-LMS accounts for between 2% and 5% of tumors in the uterine body and develops more frequently in the muscle layer of the uterine body than in the uterine cervix. The prognosis of human Ut-LMS is poor, with 5-year survival rate being approximately 35%, although this has been shown to depend on the disease stage (2, 3). When adjusted for stage and mitotic count, human Ut-LMS has a significantly worse prognosis than carcinosarcoma (4). Since human Ut-LMS is resistant to chemotherapy and radiotherapy, surgical intervention is virtually the only means of treatment; therefore, the

development of efficient adjuvant therapies is expected to improve the prognosis of this disease (5-7). The identification of risk factors associated with the development of human Ut-LMS may significantly contribute to the development of preventive and therapeutic treatments.

Cytoplasmic proteins are mostly degraded by a protease complex, which has many substrates consisting of 28 20- to 200-kDa subunits, referred to as the 20S proteasome (8, 9). Proteasomal degradation is essential for many cellular processes, including the cell cycle, regulation of gene expression, and immunological function (10). Interferon-γ (IFN-γ) induces the expression of large numbers of responsive genes, including interferon regulatory factor 1 (*IRF1*) and immunoproteasome subunits, *i.e.*, low-molecular mass polypeptide (LMP) 2, LMP7, and LMP10 (11). A molecular approach to investigating the relationship between IFN-γ and tumor cell growth has been attracting attention. Homozygous mice deficient in *Lmp2* exhibit tissue- and substrate-dependent abnormalities in the biological functions of the immunoproteasome (12). Mouse Ut-LMS was reportedly detected in female Lmp2-deficient mice at 6 months of age or older, and its incidence at 14 months of age was approximately 40% (13). Histological studies of Lmp2-deficient uterine tumors identified the characteristic abnormalities of Ut-LMS (13, 14). These tumors consisted of uniform elongated Ut-SMCs arranged into bundles. The nuclei of the tumor cells varied in size and shape, and mitosis was frequently observed. Marked reductions in body weight have been reported in *Lmp2*-deficient mice that developed Ut-LMS, and these mice died by 14 months of age. *Lmp2*-deficient mice also developed skeletal muscle metastasis from Ut-LMS. Therefore, *Lmp2*-deficient mice with Ut-LMS may die as a result of the tumor mass and metastasis.

In the present study, we investigated the molecular mechanisms underlying sarcomagenesis in murine Ut-LMS involving the defective expression of *Lmp2*.

Materials and Methods

Chemicals. The following reagents were purchased from Gibco/BRL Life Technologies (Carlsbad, CA, USA): Hank's balanced salt solution (HBSS) with and without Mg^{2+} or Ca^{2+}, Dulbecco's modified Eagle's medium: F12 nutrient mixture (DMEM:F12), antibiotics and FBS. NG-Mono-methyl-L-arginine (L-NMMA), Nw-nitro-L-arginine methyl ester (L-NAME), trypsin (type II), DNase (type IV), and mouse recombinant IFN-γ were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). PromoCell Smooth Muscle Cell Growth Medium 2 and supplement solution (0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 μg/ml insulin) as the smooth muscle cell medium were purchased from PromoCell, Heidelberg, Germany. Mouse recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). Collagenase (type IV) was purchased from Life Technologies (Carlsbad, CA, USA). Monoclonal antibodies against STAT1, IRF1, IRF2, IκBα E2F1, DP1, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against LMP2, LMP7, LMP3, and LMP10 were purchased from Sigma-Aldrich Co. Antibodies to CDK2, CDK4, CDK6, RB, and cyclin D were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Animals and cells. C57BL/6 mice and BALB/c Nude (*nu/nu*) mice were purchased from CLEA Japan, Inc. (Meguro, Tokyo, Japan). *Trp53*-deficient mice (B6.129S2-*Trp53*^{tm1Tyj}/J) were purchased from Charles River Laboratories International, Inc. (Wilmington MA, USA). Lmp2-deficient mice were a generous donation from Dr. Susumu Tonegawa (Massachusetts Institute of Technology, Cambridge, MA, USA). All wild-type, homozygous mice deficient in *Lmp2* were littermates of F10 heterozygous mice generated by backcrossing with C57BL/6J mice.

The same 2 original male *Trp53*$^{-/-}$ mice were bred with 2 female *Lmp2*$^{-/-}$ mice, to generate F1 *Lmp2*$^{+/-}$*Trp53*$^{+/-}$ mice. F1 mice were bred with each other, and the resulting *Lmp2*$^{-/-}$*Trp53*$^{-/-}$ males were bred with *Lmp2*$^{-/-}$*Trp53*$^{+/-}$ or *Lmp2*$^{-/-}$*Trp53*$^{-/-}$ females to generate the *Lmp2*$^{-/-}$*Trp53*$^{-/-}$ mice used in this study. Occasionally, *Lmp2*$^{+/-}$ mice were included in breeding, to maintain the colony. Every mouse was screened by genomic PCR, to determine its genotype.

Genomic PCR. A 1-cm sample from the tip of each tail was digested in 0.5 ml of autoclaved digestion buffer [50 mM Tris, 10 mM EDTA, 100 mM NaCl, 1% Triton X-100 (pH 8.0)] with 0.2 mg/ml proteinase K (Life Technologies, Grand Island, NY, USA) at 55˚C for 16 h. The DNA sample was then boiled for 5 min and diluted 1:5 in sterile water. PCR reaction mixture was combined with 2.5 μl of Ex Taq PCR buffer (Takara Bio Inc., Otsu, Shiga, Japan), 1.0 μl of 50 mM $MgCl_2$, 1.0 μl of each 100 mM dNTP, 0.5 μl of Ex Taq DNA polymerase (all from Takara Bio Inc., Otsu, Shiga, Japan), 1.0 μl of each 25 mM primer and water to achieve a final volume of 25 μl. PCR samples were incubated in a Mastercycler (Eppendorf AG, Barkhausenweg, Hamburg, Germany) at 94˚C for 1 min, followed by 25 of the following cycles: 30 sec at 94˚C, 30 sec at 60˚C and 1.5 min at 72˚C. After these cycles, samples were incubated at 72˚C for 5 min. PCR products were electrophoresed at 90 V in 1.5% agarose gels with 0.5% Tris-Acetate-EDTA buffer solution (TAE). Primers were as follows: wild-type *Trp53* 5'-GTGTTTCATTAGTTCCCCACCTTGAC-3', 5'-ATGGGAGGCTG CCAGTCCTAACCC-3'; mutant *Trp53* 5'-GTGGGAGGGACAA AAGTTCG AGGCC-3', 5'-TT TACGGAGCCCTGGCGCTCGAT GT-3', wild-type *Lmp2* 5'-GGGTGTCAGCGGGGTGA GTAG-3', 5'-CAT GAATGCCATGGATTCAG-3'.

All animal-related procedures were performed in compliance with Guide for the animal care, which is prescribed by The Japanese association of Laboratory Animal Facilities of National University Corporation the National Institute of Health Guide for the Care. These mice were kept in a specific pathogen-free environment at Shinshu University's animal facilities, in accordance with institutional guidelines (approval no. 03–28–008).

Fresh mouse embryonic fibroblasts (MEFs) were obtained from C57BL/6 or *Lmp2*-deficient embryos at 13.5 days by standard methods. *Stat1*-deficient MEFs were a generous donation from Dr. David E Levy (New York University, New York, NY, USA). *Ifn-γR*-deficient MEFs were a generous donation from Dr. Tagawa (The University of Tokyo, Minato-ku, Tokyo, Japan). Cytosolic and nuclear extracts were prepared from 1×10^7 cells and treated with or without

250 U/ml of mouse IFN-γ (Pepro Tech, Inc., Rocky Hill, NJ, USA) for 1 to 2 h for the periods indicated in each figure, essentially as described previously. (14). The cells were collected by centrifuging for 10 min at 1200 r.p.m., 130 × *g* washed in 5 ml of ice-cold PBS, and centrifuged again for 5 min at 12000 r.p.m. 13,000 × *g* at 4˚C. The cells were pelleted and washed once in 400 μl of buffer A [10mM HEPES, pH 7.8; 10 mM KCl; 2 mM $MgCl_2$; 1 mM DTT; 0.1 mM EDTA; complete protease inhibitor cocktail (Hoffmann-La Roche Ltd., Basel, Switzerland)] and were vigorously mixed for 1 h at 4˚C and centrifuged for 5 min at 12,000 r.p.m. 13,000 × *g*. The supernatant was collected as cytosolic extract and stored at −80˚C. Pelleted nuclei were resuspended in 40 μl of buffer C (50 mM HEPES, pH 7.8; 50 mM KCl; 300 mM NaCl; 0.1 mM EDTA; 1 mM DTT; 10% (v/v) glycerol), mixed for 2 h at 4˚C, and centrifuged for 5 min at 12000 r.p.m. 13000 × *g* at 4˚C. The supernatant containing the nuclear proteins was harvested and stored at −80˚C.

Cell culture. The myometria of eight 10-week-old *Lmp2*-deficient mice were removed, cleaned of fat and blood vessels, and rinsed thoroughly in HBSS containing 1% antibiotics (penicillin and streptomycin) and 20 mM HEPES. The mouse Ut-LMS tissues of eight 8-month-old *Lmp2*-deficient mice were removed, cleaned of fat and blood vessels, and rinsed thoroughly in HBSS containing 1% antibiotics (penicillin and streptomycin) and 20 mM HEPES. We scraped off the endometrial tissue, and the remaining myometrial tissues or mUt-LMS tissues were subjected to the enzymatic dispersion of cells. Briefly, we washed the tissues three times with HBSS and incubated them in enzyme solution in HBSS containing collagenase (0.1%), DNase I (0.02%), protease (0.01%), and trypsin (0.01%) for 10 min at 37˚C with shaking. The supernatant was discarded, replaced with fresh enzyme solution, and incubated for another 10 min. During this incubation period, the tissue pieces were gently broken up by drawing the mixture slowly through a large-bore siliconized pipette. This procedure was continued for 30 s every 3 min. The mixture was then centrifuged at 20 × *g* for 5 min and supernatant was removed and saved. Fresh enzyme solution was added to the remaining pieces, and the above process was repeated three times. After enzyme digestion, the combined supernatant solution containing cells was filtered through a 100-mm nylon mesh and centrifuged at 430 × *g* for 10 min. The cell pellet was washed in 1:1 DMEM:F12 nutrient medium (GIBCO, Carlsbad, CA, USA) containing 10% FCS, 1% antibiotics, and sodium bicarbonate. These cells were resuspended in culture medium, PromoCell Smooth Muscle Cell Growth Medium 2 (PromoCell) with FCS (final concentration, 5%) plus SmGM-2 SingleQuots (0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 μg/ml insulin; CAMBREX, MD, USA), and plated on 10-cm dishes. We removed the medium 24 h later, and the myometrial cells were washed and incubated in fresh medium containing 0.1% BSA and 1% antibiotic solution. We identified 95% of the cells in culture as smooth muscle cells by the detection of α-smooth muscle actin (α-SMA) as a smooth muscle cell marker using immunohistochemical methods.

Isolation of mouse embryonic fibroblasts (MEFs). The following two steps were performed under aseptic conditions. Pregnant mice were anesthetized by the intraperitoneal administration of a mixture of xylazine (10 mg/kg) and ketamine (70 mg/kg), and were sacrificed at 13 or 14 day post-coitum. The uterine horns were quickly dissected, rinsed in 70% (v/v) ethanol, and placed into a falcon tube containing PBS without $Ca^{2+}Mg^{2+}$ (Gibco). The following steps were carried out in a tissue culture hood under aseptic conditions using sterile instruments. Each embryo was separated from its placenta and embryonic sac, and the head and red organ were dissected. The tissue was finely minced using a sterile razor blade until it could be pipetted. One milliliter of solution S [0.05% trypsin/EDTA (Gibco, Invitrogen), including 100 units Kunitz units of DNase I], was added per embryo, which were then incubated for 15 min at 37˚C. To inactivate trypsin, one volume of MEF culture medium (components to make 500 ml of media, all components mixed and filtered): 450 ml of DMEM, 50 ml of FBS (10% v/v), 5 ml of 200 mM L-glutamine (1/100 v/v), and 5 ml of penicillin-streptomycin (1/100 v/v) were added to the cell suspending solution. The cells were centrifuged at a low speed (300 × *g*) for 5 min, and the supernatant was then carefully removed. MEFs were placed in flasks. Ideally, cells were 80-90% confluent after 48 h and, at this stage, the majority of P0 cells were frozen for future usage.

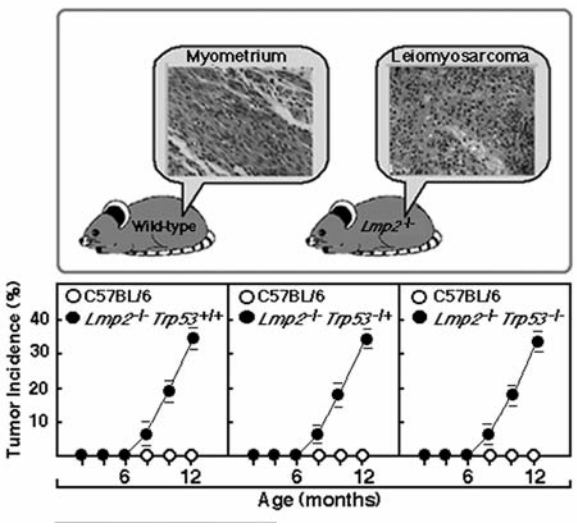


Figure 1. *The p53 protein is crucial in multi-cellular organisms, in which it regulates the cell cycle and, thus, functions as a tumor suppressor, preventing cancer. More than 50% of human tumors contain a mutation or deletion in the TP53 gene. To increase the tumor incidence and better-assess the role of the systemic expression of Trp53 in response to initiation of mouse Ut-LMS tumorigenesis, Lmp2-deficient mice were bred with Trp53-deficient mice to create $Lmp2^{-/-}Trp53^{-/-}$ mice and closely matched control $Lmp2^{-/-}Trp53^{+/+}$ mice. However, no significant differences were observed in the tumor incidence between these three genetically-modified mouse groups.*

Treatment with proteasome inhibitors. Influence of proteasome inhibitors on the expression of IRF1 and IRF2 were examined with MEFs treated with proteasome inhibitors. MG 115 (N-Cbz-Leu-Leu-Nva-CHO) and MG 132 (N-Cbz-Leu-Leu-Nle-CHO) were purchased from Calbiochem (San Diego, CA, USA). MG 115 or MG 132 were added in the culture media at the concentration of 50 mM and incubated with MEFs for 6 h, and then incubated further with 5 mM MG 115 or MG 132 for another 18 h at 37˚C. After the 24 h treatment with MG115 or MG132, MEFs were stimulated with

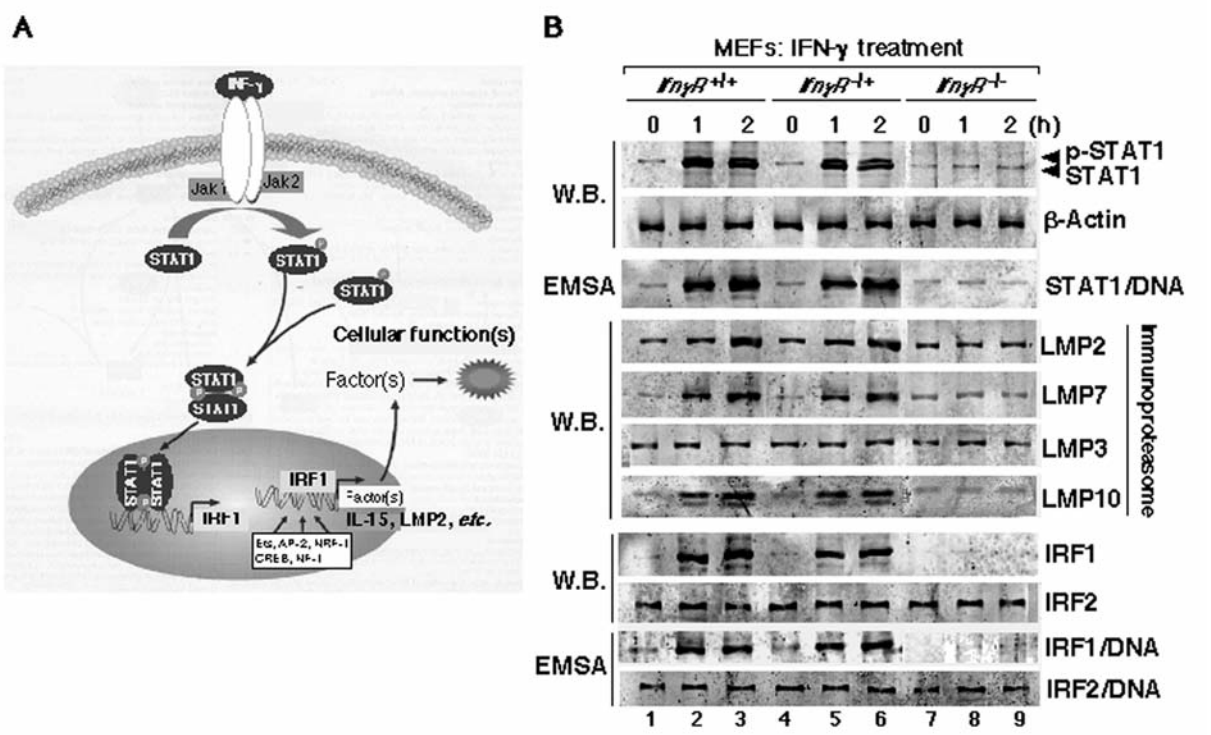


Figure 2. *Defective IRF1 expression in the IFN-γ signal cascade in mouse embryonic fibroblasts (MEFs) derived from IFN-γ receptor-deficient mice. A: Key role of the IFN-γ signaling pathway on LMP2 expression in human myometrium. After the binding of IFN-γ to the type II IFN receptor, Janus activated kinase 1 (JAK1) and JAK2 are activated and phosphorylate signal transducer and activator of transcription 1 (STAT1) on the tyrosine residue at position 701 (Tyr701). The tyrosine-phosphorylated form of STAT1 forms homodimers that translocate to the nucleus and bind to IFN-γ-activated site (GAS) elements that are present in the promoters of IFN-γ-regulated genes. The phosphorylation of Ser727 is not essential for the translocation of STAT1 to the nucleus or for the binding of STAT1 to DNA, but is required for full transcriptional activation. IfnγR1, Ifn-γ receptor subunit 1; IfnγR2, Ifn-γ receptor subunit 2. B: Defect in IFN-γ-induced IRF1 expression in MEFs, which were isolated from homozygous or heterozygous mice deficient in IfnγR, and wild-type mice. Cytosolic extracts were prepared from MEFs treated with IFN-γ (250 U/ml) for the individual times indicated, and 50 μg of cytosolic extracts was resolved using a 10% sodium dodecyl sulfate-polyacrylamide gel. The expression levels of p-STAT1, STAT1, LMP2, LMP7, LMP3, LMP10, IRF1, IRF2, and β-actin were examined by western blotting analysis with appropriate antibodies. The DNA-binding activities of IRF1 and IRF2 were examined using an electrophoretic mobility shift assay (EMSA).*

TNFα (20 ng/ml) or IFN-γ (250 U/ml) for varying period indicated in Figure 5, and then the expression of appropriated proteins were analyzed by western blotting experiments.

Western blotting. Equal amounts of proteins (20 mg) were size-fractionated using 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride membrane (PVDF). The blots were allowed to air dry and then placed in blocking buffer (1% BSA in 10 mM Tris buffer with 100 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature. The blots were then incubated with specific primary antibodies for 1 h at room temperature. All primary antibodies were mouse monoclonal or rabbit polyclonal antibodies, obtained from several suppliers, and used at different final dilutions (1:500-1:1000) in the blocking buffer. These antibodies were raised using the following proteins as immunogens: IRF1 (37.3 kDa), IRF2 (39.5 kDa), STAT1 (87.2 kDa), LMP2 (23.4 kDa), LMP7 (30.3 kDa), LMP3 (29.1 kDa), LMP10 (29.1 kDa), β-actin (41.7 kDa), CDK2 (39.0 kDa), CDK4 (33.8 kDa), CDK6 (37.0 kDa), cyclin D (33.4 kDa), RB (105.4 kDa), E2F1 (46.3 kDa), and $p21^{CIP1}$ (17.8 kDa). The blots were washed three times for 30 min each with wash buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween-20, pH 7.5) and then incubated with an alkaline phosphatase-conjugated goat-anti-mouse IgG antibody or anti-rabbit IgG antibody (Promega, Madison, WI, USA) diluted in 5% non-fat milk in wash buffer. PVDF membranes were washed with wash buffer three times for 30 min, Western Blue Stabilized Substrate (Promega) was then added, and membranes were then incubated at room temperature until proteins were appeared as blue color bands.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from cells treated and untreated with IFN-γ, as described above. EMSA was performed as previously described (15), using DNA probes containing the STAT1-binding sequence or IRFE. The DNA sequences of these synthetic oligonucleotides for the STAT1-binding site or IRFE were as follows. STAT1: 5'-AAGCATTCCTGTA AGGACT-3', IRF-E: 5'-GGAAGCGAAAA TGAAATTGACT-3'.

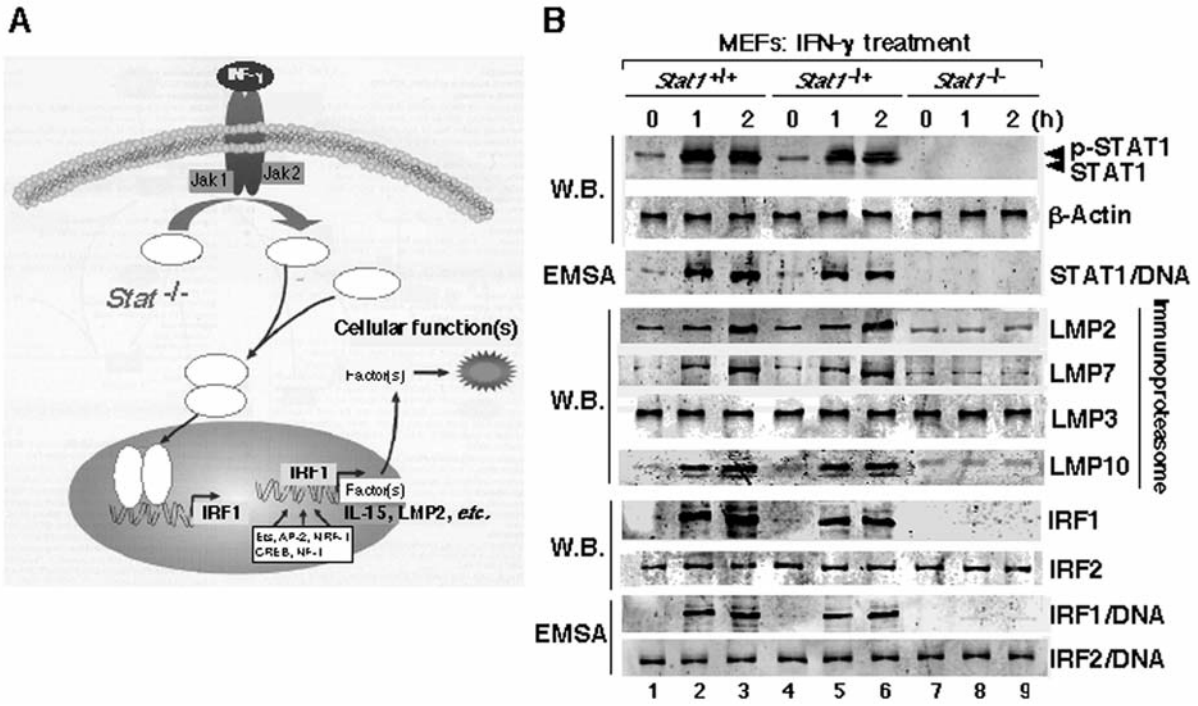


Figure 3. *Signal transducer and activator of transcription 1 (Stat1) was essential for IFN1-induced IRF1 expression. A: The key role of the IFN-γ signaling pathway on LMP2 expression in the human myometrium. After the binding of IFN-γ to the type-II IFN receptor, Janus activated kinase 1 (JAK1) and JAK2 are activated and phosphorylate STAT1 on the tyrosine residue at position 701 (Tyr701). The tyrosine-phosphorylated form of STAT1 forms homodimers that translocate to the nucleus and bind to IFN-γ-activated site (GAS) elements that are present in the promoters of IFN-γ-regulated genes. The phosphorylation of Ser727 is not essential for the translocation of STAT1 to the nucleus or the binding of STAT1 to DNA, but is required for full transcriptional activation. IFNγR1, IFN-γ receptor subunit 1; IFNγR2, IFN-γ receptor subunit 2. B: Defect in IFN-γ-induced IRF1 expression in mouse embryonic fibroblasts (MEFs), which were isolated from homozygous or heterozygous mice deficient in Stat1, and parental mice (wild-type). Cytosolic extracts were prepared from MEFs treated with IFN-γ (250 U/ml) for the individual times indicated, and 50 μg of cytosolic extracts was resolved using 10% SDS-PAGE. The expression levels of p-STAT1, STAT1, LMP2, LMP7, LMP3, LMP10, IRF1, IRF2, and β-actin were examined by western blotting analysis with appropriate antibodies. The DNA-binding activities of IRF1 or IRF2 were examined using an electrophoretic mobility shift assay (EMSA).*

DNA transfection and isolation of Irf1 stable transformants. Primary murine Ut-LMS cells were co-transfected with the pcDNA1 control (2 μg) or *Irf1* expression vector (pcDNA1-IRF1) (2 μg), which were a generous donation from Dr. Tadatsugu Taniguchi (The University of Tokyo School of Medicine, Bunkyo-ku, Tokyo, Japan), by FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's recommendation in serum-free PromoCell Smooth Muscle Cell Growth Medium 2 at 2.0 μg DNA per 1×10^6 cells, which were plated on 6-well tissue culture dishes the previous day. Transfected cells were grown in PromoCell Smooth Muscle Cell Growth Medium 2 with FCS plus (final concentration 5%) for 48 h before the addition of the selection antibiotic, G418 sulfate (Gibco). Selected murine Ut-LMS were maintained in DMEM with 10% FCS with G418 sulfate at 0.4 mg/ml for the pcDNA1 plasmid. The transfected G418-resistant colonies were isolated as previously described (16). IFN-γ was added when the plates were 60% confluent, for 48 h prior to harvesting. After the IFN-γ treatment, cells were scraped from the plates. Viable cells were counted by trypan blue exclusion (17).

Xenograft studies. Nude mice (BALB/cSlc-*nu/nu*, female, 7-8 weeks old; Japan SLC, Meguro, Tokyo, Japan) were injected intracutaneously with 1×10^7 cells of the mUt-LMS-pcDNA1 clone or mUt-LMS-pIRF-1 clone with BD Matrigel Matrix (BD Biosciences, MA, USA) in 5 mg/ml of culture medium (PromoCell Smooth Muscle Cell Growth Medium 2) containing 5% FCS and PromoCell supplement solution plus SmGM-2 SingleQuots (CAMBREX) at a final volume of 100 μl. Tumor formation was assessed every day. Seven weeks after the injection, the tumors were dissected for reverse transcription-polymerase chain reaction analysis (RT-PCR) and western blotting experiments. Tumor volumes were calculated as (L×W×W)/2, where W represents the width and L the length. These mice were kept in a specific pathogen-free environment at Shinshu University's animal facilities, in accordance with institutional guidelines (approval no. 03–28–008). Statistical analysis was performed on mean tumor volumes at the end of the study using Dunnett's test.

RT-PCR. The expression of *Irf1* and the β-actin transcripts was examined using RT-PCR. All tested cell types were both untreated

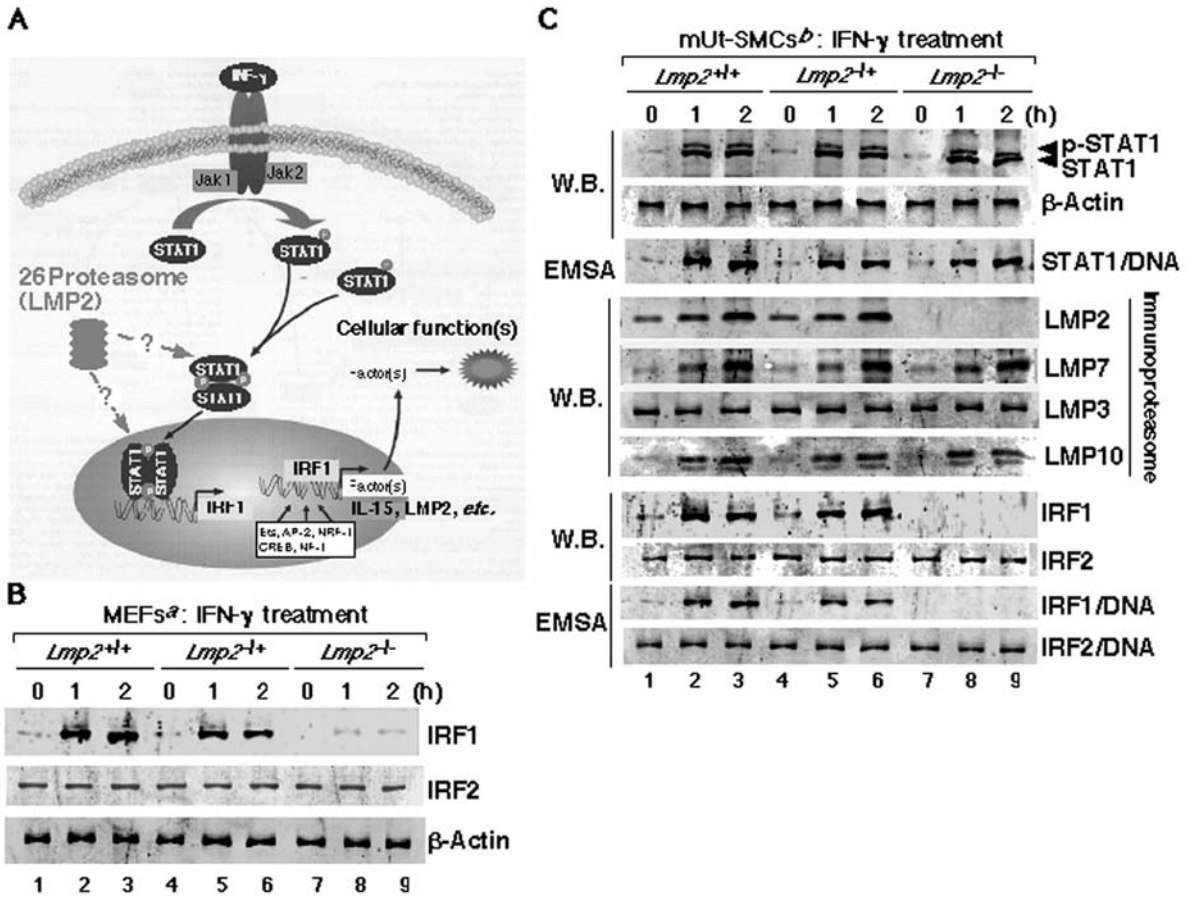


Figure 4. *Defective IRF1 expression in the IFN-γ signal cascade in mouse uterine smooth muscle cells derived from Lmp2-deficient mice. A: The key role of the IFN-γ signaling pathway on LMP2 expression in the human myometrium. After the binding of Ifn-γ to the type-II Ifn receptor, Janus activated kinase 1 (JAK1) and JAK2 are activated and phosphorylate signal transducer and activator of transcription 1 (Stat1) on the tyrosine residue at position 701 (Tyr701). The tyrosine-phosphorylated form of Stat1 forms homodimers that translocate to the nucleus and bind to Ifn-γ-activated site (GAS) elements that are present in the promoters of Ifn-γ-regulated genes. The phosphorylation of Ser727 is not essential for the translocation of Stat1 to the nucleus or for the binding of Stat1 to DNA, but is required for full transcriptional activation. IfngR1, Ifn-γ receptor subunit 1; IfngR2, Ifn-γ receptor subunit 2. (B) Defect in Ifn-γ-induced Irf-1 expression in mouse embryonic fibroblasts (MEFs), which were isolated from homozygous or heterozygous mice deficient in Lmp2, and parental mice (wild type). Cytosolic extracts were prepared from MEFs treated with Ifn-γ (250 U/ml) for the individual times indicated in the Figure, and 50 mg of cytosolic extracts was resolved using 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The expression levels of Irf-1, Irf-2, and β-actin were examined by western blotting analysis with appropriate antibodies. (C) Defect in Ifn-γ-induced Irf-1 expression in mouse uterine smooth muscle cells (mUt-SMCs), which were isolated from the uterii of homozygous or heterozygous mice deficient in Lmp2, and parental mice (wild type). Cytosolic extracts were prepared from mUt-SMCs treated with Ifn-γ (250 U/ml) for the individual times indicated in the figure, and 50 μg of cytosolic extracts was resolved using 10% SDS-PAGE. The expression levels of p-Stat1, Stat1, Lmp2, Lmp7, Lmp3, Lmp10, Irf-1, Irf2, and β-actin were examined by western blotting analysis with appropriate antibodies. The DNA binding activities of Irf-1 or Irf-2 were examined using an electrophoretic mobility shift assay (EMSA). The details of MEFs and mUt-SMCs clones are indicated in Table I.*

and treated with 250 units/ml human or mouse IFN-γ (Pepro Tech, Inc., NJ, USA) for 48 h before the RNA harvest. Total RNA was prepared from 5×10^6 cells using TRIzol reagent (Invitrogen Co.) according to the manufacturer's protocol. The RNA was reverse transcribed with the Superscript II enzyme (Invitrogen Co.), single-strand cDNA was used for amplification of *Irf1* or *β-actin* DNA fragments by PCR analysis, following a program of 35 cycles of 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 1.5 min with an additional 5 min for the extraction of the transcripts. The PCR-amplified products were run on 2.0% agarose gels as described previously (18). *Irf1*: forward primer 5'-CAGAGGAAAGA GAGAAAGTCC-3', reverse primer 5'-CACACGGTGACAGT GCTGG-3'; β-actin: forward primer 5'-TCCGGAGA CGGG GTCA-3', reverse primer 5'-CCTGCTTGCTGATCCA-3'. These experiments were conducted at Shinshu University in accordance with local guidelines (approval no. 4737, no. 150, and no. M192).

Tissue collection. A total of 51 patients aged between 32 and 83 years who were diagnosed with smooth muscle tumors in the uterus were selected from pathological files. Serial sections were cut from

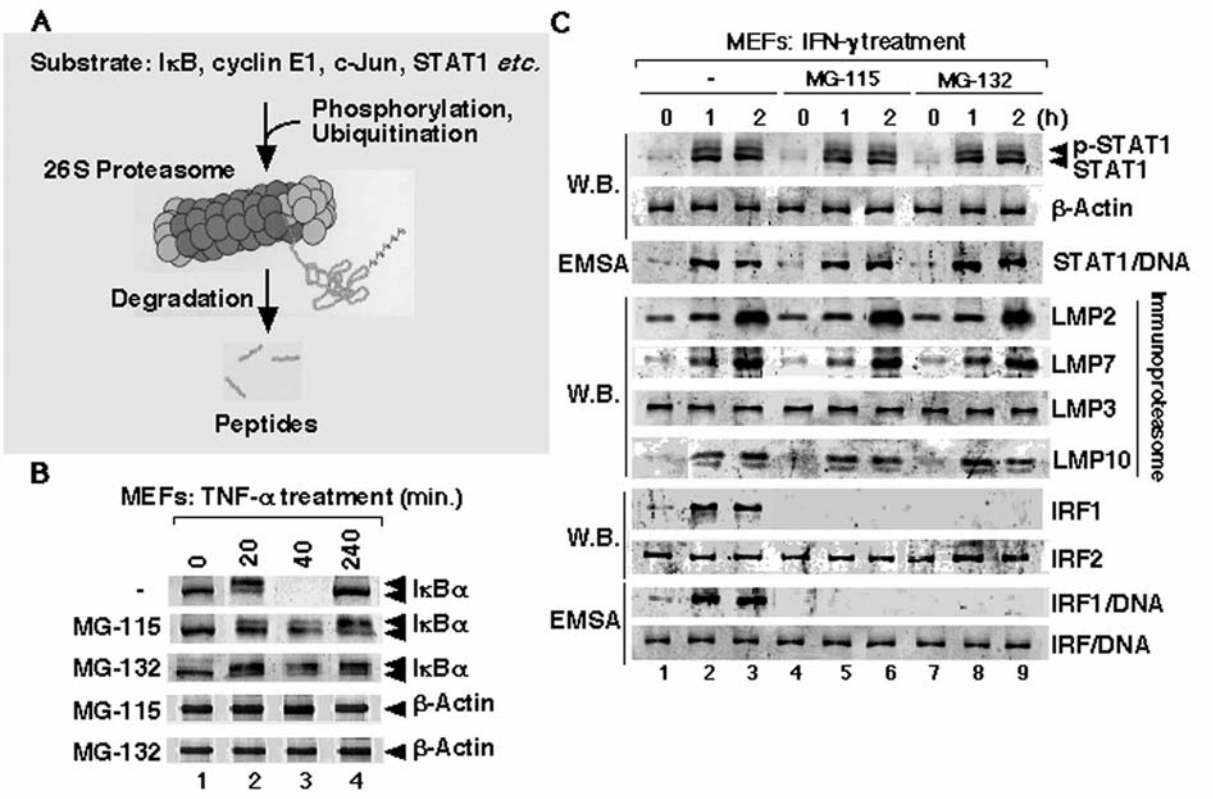


Figure 5. *Defective expression of IRF1 in the IFN-γ-inducible cascade in proteasome-inhibitor-treated mouse embryonic fibroblasts (MEFs). A: Proteasomes are very large protein complexes inside all eukaryotes and archaea as well as some bacteria. In eukaryotes, they are located in the nucleus and cytoplasm. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. Enzymes that carry-out such reactions are called proteases. Proteins are tagged for degradation with a small protein called ubiquitin. The tagging reaction is catalyzed by enzymes called ubiquitin ligases. Once a protein is tagged with a single ubiquitin molecule, this is a signal to other ligases to attach additional ubiquitin molecules. The result is a poly-ubiquitin chain that is bound by the proteasome, allowing it to degrade the tagged protein. B: MEFs derived from wild-type C57BL/6 mice were treated with (+) or without (−) 50 μM mM MG115 or MG132, which are proteasome inhibitors. After the treatment with the proteasome inhibitors, the stimulation of MEFs with TNF-α (20 ng/ml) or IFN-γ (250 U/ml) was also performed for the indicated times at 37˚C. Whole-cell lysates were analyzed by SDS-PAGE, and were then subjected to western blotting analysis with antibodies to IκBα or β-actin. The positions of unphosphorylated (lower arrowheads) and phosphorylated (upper arrowheads) IκBα are indicated. C: Defect in IFN-γ-induced IRF1 expression in MEFs which were isolated from wild-type C57BL/6 mice. Cytosolic and nuclear extracts were prepared from MEFs treated with IFN-γ (250 U/ml) for the individual times indicated, and 50 μg of cytosolic extracts were resolved by 10% SDS-PAGE. The expression levels of p-STAT1, STAT1, LMP2, LMP7, LMP3, LMP10, IRF1, IRF2, and β-actin were examined by western blotting analysis with appropriate antibodies. The DNA binding activities of IRF1 and IRF2 were examined using an electrophoretic mobility shift assay (EMSA). The details of MEFs are indicated in Table I.*

at least two tissue blocks from each patient for hematoxylin and eosin staining and immunostaining. All tissues were used with the approval of the Ethical Committee of Shinshu University (approval number: no. 150) after obtaining written consent from each patient. The pathological diagnosis of uterine smooth muscle tumor was performed using established criteria with some modifications (14). Briefly, usual LMA was defined as a tumor showing typical histological features with a mitotic index (MI) [obtained by counting the total number of mitotic figures in 10 high-power fields (HPFs)] of <5 mitotic figures per 10 HPFs. Cellular LMA was defined as a tumor with significantly increased cellularity (>2000 myoma cells/HPF) and a MI<5, but without cytologic atypia. Bizarre LMA was defined as a tumor either with diffuse nuclear atypia and a MI<2 or with focal nuclear atypia and a MI<5 without coagulative tumor cell necrosis. Tumor of uncertain malignant potential (UMP) was defined as a tumor with no mild atypia and a MI<10, but with coagulative tumor cell necrosis. LMS was diagnosed in the presence of a MI>10 with either diffuse cytologic atypia, coagulative tumor cell necrosis, or both. Of the 51 smooth muscle tumors, 23 were diagnosed as LMA, two were bizarre LMA, and 32 were LMS.

Immunohistochemistry (IHC). IHC staining for LMP2, IRF1, ER, PR, TP53, and Ki-67 was performed on serial human Ut-LMS sections. Antibodies for ER (ER1D5), PR (PR10A), TP53 (DO-1), and Ki-67(MIB-1) were purchased from Immunotech (Marseille,

Table I. *Biological properties of the primaey MEFs and mUt-SMCs.*

Cell line (clone)	Molphology	PDT[a]	SoftAgarColony Efficiency(%)[b]	Size[c]	Protein Expression[d]								DNA binding[g]	
					pSTAT1	STAT1	LMP2	LMP3	LMP7	LMP10	IRF1	IRF2	IRF1	IRF2
MEF-wt	[f]Epithelial	19.0	<0.5	3-5	H	H	H	M	H	H	H	M	Posi	Posi
MEF-LMP2–/+	Epithelial	19.2	<0.5	3-6	H	H	M	M	H	H	H	M	Posi	Posi
MEF-LMP2–/+	Epithelial	19.2	<0.5	4-5	H	H	M	M	H	H	H	M	Posi	Posi
MEF-LMP2–/–	Epithelial	19.4	<0.5	3-6	H	H		M	H	H	W	M	Neg	Posi
MEF-LMP2–/–	Epithelial	19.8	<0.5	4-5	H	H		M	H	H	W	M	Neg	Posi
mUtSMC-wt1	Epithelial	19.3	<0.5	4-6	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC-wt2	Epithelial	19.4	<0.5	3-5	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC-wt3	Epithelial	19.5	<0.5	3-6	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC-wt4	Epithelial	19.3	<0.5	5-7	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC –/+1	Epithelial	19.6	<0.5	3-5	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC –/+2	Epithelial	19.5	<0.5	4-5	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC –/+3	Epithelial	19.8	<0.5	3-6	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC –/+4	Epithelial	19.3	<0.5	3-5	H	H	H	M	H	H	H	M	Posi	Posi
mUtSMC –/–1	Epithelial	18.7	<0.5	4-5	H	H		M	H	H	–	M	Neg	Posi
mUtSMC –/–2	Epithelial	18.8	<0.5	5-7	H	H		M	H	H	–	M	Neg	Posi
mUtSMC –/–3	Epithelial	18.6	<0.5	5-6	H	H		M	H	H	–	M	Neg	Posi
mUtSMC –/–4	Epithelial	18.5	<0.5	4-6	H	H		M	H	H	–	M	Neg	Posi
CryoUTSMC[e]	Epithelial	19.4	<0.5	4-7	H	H	H	M	M	M	M	M	Posi	Posi
mUt-LMS–/–	Transform	16.7	6.2	10-19	M	M		M	M	M	–	M	Neg	Posi

[a]PDT: Population doubling time. [b]Ratio(%) of soft agar colonies/number of cells plated that have an ability to form colonies on plastic substrate. [c]Diameter of colonies as expressed by the number of cells lined up across the colonies; determined on day 21. The experiments were repeated three times with similar results. [d]Estimated by immunoblot analysis. W; weak expression, L; low expression, M; medium expression, H; high expression, –; no evidence of expression. [e]Cryo UtSMC: normal human uterine liomyosarcoma cell line (Cambrex BioScience Walkersville, Inc. MD, USA). [f]Morphology. After 2 to 3 weeks, when most of the colonies outgrew and detached from the substrate, some colonies consisting of very flat cells were found at frequencies of around a few percent of the total number of G418-resistant colonies initially observed in Table S4. Microscopic characteristics such as flat cell morphology and transformed cell morphology were analyzed. The number of flat cells or transformed cells in 10 consecutive high-power-fields was counted. Transformed; no evidence about appearance of flat cells, Flatrevertant; transformed cell number is less than 10% of total cell number; P.Flat.; Partially Flatrevertant; transformed cell number is less than 30% of total cell number, P.Tras.; Partially Transformed, transformed cell number is higher than 30% of total cell number. [g]Estimated by EMSA, Posi.; positive, Neg.; Negative.

France). The antibody for α-SMA was purchased from Covance Research Products, Inc. (Princeton, NJ, USA). Anti-LMP2 and anti-IRF1 were produced by SIGMA-Aldrich Israel Ltd. (Rehovot, Israel). IHC was performed using the avidin-biotin complex method as described previously. (14). Briefly, one representative 5-μm tissue section was cut from a paraffin-embedded sample of a radical hysterectomy specimen from each patients with Ut-LMS patient. Sections were deparaffinized and rehydrated in graded concentrations of alcohol, incubated with normal mouse serum for 20 min, and then incubated at room temperature for 1 h with a primary antibody. Sections were then incubated with a biotinylated secondary antibody (DAKO Japan, Minoto-ku, Tokyo, Japan) (Dako, CA, USA) and exposed to a streptavidin complex (Dako). The completed reaction was revealed by 3,3'-diaminobenzidine, and the slide was counterstained with hematoxylin. Normal myometrium portions in each specimen were used as positive controls. To examine the expression levels of target molecules, we performed

Table II. *Biological activity of IRF1 in mUt-LMS.*

Morphology	Number of colonies[a]	
	pcDNA1	pIRF1
$G418^R$ (total)	42.0	40.5
Partially flat[b]	1.0 (2%)	2.0 (4%)
Transformed[c]	41.0 (98%)	39.0 (96%)
Flatrevertant[c]	0.0	0.0

Five micrograms of pcDNA1 or pIRF1 was transfected into 5×10^5 mUt-LMS cells and selected in a growth medium containing 1.0 mg/ml of G418 respectively. [a]Total number of colonies observed in the experiment is shown. Number in parentheses indicates percent of total G418-resistant (G418) colonies. [b]Observed after 1 week of selection with G418. [c]Observed after 1 week of selection with G418. The experiments were repeated three times with similar results.

Table III. *Biological properties of the mUt-LMS-transfectants with IRF1.*

Clone no.[a] or Vector DNA[b]	shRNA[c]	Molphology[d]	PDT	Protein Expression			Tumori-genecity[f]	Soft Agar Colony Efficiency(%)[g]	Size[h]
				IRF1	IRF2	αSMA			
mUt-LMS#1	pcDNA1	Transformed	15.6	–	+	+	+++	6.2	10-19
mUt-LMS#2	pcDNA1	Transformed	15.7	–	+	+	+++	6.6	11-13
mUt-LMS#3	pcDNA1	Transformed	15.9	–	+	+	+++	6.3	10-18
mUt-LMS#4	pcDNA1	Transformed	15.7	–	+	+	++	6.2	11-16
mUt-LMS#5	pcDNA1	Transformed	15.8	–	+	+	+++	6.8	12-20
mUt-LMS#6	pcDNA1	Transformed	16.0	–	+	+	++	6.5	10-17
mUt-LMS#7	pcDNA1	Transformed	15.7	–	+	+	+++	6.7	11-17
mUt-LMS#8	pcDNA1	Transformed	15.8	–	+	+	+++	6.3	10-18
mUt-LMS-IRF1#1[b]	pIRF1	Transformed	16.8	+	+	+	+	6.5	7-10
mUt-LMS-IRF1#2	pIRF1	Transformed	17.1	+	+	+	+	5.6	7-12
mUt-LMS-IRF1#3	pIRF1	Transformed	16.9	+	+	+	+	5.9	7-12
mUt-LMS-IRF1#4	pIRF1	Transformed	16.8	+	+	+	+	5.8	6-10
mUt-LMS-IRF1#5	pIRF1	Transformed	16.8	+	+	+	–	6.2	6-12
mUt-LMS-IRF1#6	pIRF1	Transformed	16.9	+	+	+	+	6.1	5-10
mUt-LMS-IRF1#7	pIRF1	Transformed	16.6	+	+	+	+	5.9	6-11
mUt-LMS-IRF1#8	pIRF1	Transformed	16.8	+	+	+	–	5.8	7-11

[a]Clone no, Details of mUt-LMS transtectant clones are in Table I and Table II. [b]Vector DNA, An Inf1 expression vector was transfected into mUt-LMS cells with shRNA vector. [c]Transfected Vector, pcDNA1 or pIRF1 was transfected into mUt-LMS cells according to the manufacturer's recomendations (Ref14,39, Hoffmann-La Roche Ltd., Grenzacherstrasse, Basel Schweiz). [d]Morphology, Microscopic characteristics such as flat cell morphology and transformed cell morphology were analyzed. The number of flat cells or transformed cells in 10 consecutive high-power-fields was counted. Transformed, no-evidence regarding the appearance of flat cells; Flatrevertant, transformed cell number was less than 10% of the total cell number; P.Flat.; Partially Flatrevertant, the transformed cell number was less than 30% of the total cell number; P.Trans., Partially Transformed, the flatrevertant cell number was less than 30% of the total cell number. [e]Estimated by immunoblot analysis, +, high expression; –, no evidence of expression; MD, markedly down-expression. [f]Cells (1×10^7) were inoculated subcutaneously into 7-8-weeks old nude mice, and the mice were periodically examined for evidence of tumors: –, no evidence of tumor; +/–, tumor of <0.5 cm diameter: +, tumor of 0.5-1.0 cm diameter: ++, tumor of 1.0-1.5 cm diameter: +++, tumor of 1.5-2.0 cm diameter; in two inoculated mice. Experiments were terminated at 5 weeks after inoculation. [g]Ratio(%) of soft agar colonies/number of cells plated that have an ability to form colonies on plastic substrate. [h]Diameter of colonies as expressed by the number of cells lined up across the colonies; determined on day 21. The experiments were repeated three times with similar results.

LMP2 and IRF1 immunofluorescence experiments on paraffin-embedded human myometrium or Ut-LMS. These sections were then incubated with the appropriate antibodies at 4˚C overnight. Regarding primary antibodies, we used a rabbit polyclonal antibody to LMP2 (1:200;SIGMA-Aldrich Israel Ltd.) or a mouse monoclonal antibody to IRF1 (1:200; SIGMA-Aldrich Israel Ltd.). After incubation with secondary antibody of Alexa Fluor 488- or 546-conjugated anti-goat, anti-rabbit, or anti-mouse IgG (1:200; Santa Cruz Biotechnology), respectively, the sections were coverslipped with mounting medium and DAPI (VECTASHILD, Vector Laboratory, Burlingame, CA, USA)(VECTASHILD; Vector Laboratory, CA) and visualized using a confocal microscope (Carl Zeiss, Thornwood, NY, USA). Negative controls consisted of tissue sections incubated with normal rabbit IgG instead of the primary antibody. These experiments were conducted at Shinshu University in accordance with institutional guidelines (approval no. M192).

Results

Inactivation of the Irf1 tumor-suppressor gene under the Lmp2-deficient condition. Murine Ut-LMS was demonstrated to spontaneously develop at a high frequency in 6-month-old Lmp2-deficient mice (13). In the present study, we performed experiments with mice and human uterine tissues to elucidate the molecular mechanisms underlying sarcomagenesis in murine Ut-LMS involving the defective expression of Lmp2. To increase the tumor incidence and better assess the role of the systemic expression of tumor-related protein p53 (Trp53) in response to the initiation of murine Ut-LMS tumorigenesis (19), *Lmp2*-deficient mice were bred with *Trp53*-deficient mice to create $Lmp2^{-/-}Trp53^{-/-}$ mice and closely matched control $Lmp2^{-/-}Trp53^{+/+}$ mice. However, no significant differences were observed regarding tumor incidence between these three genetically-modified mouse groups (Figure 1). The relationship between the onset of human Ut-LMS and TP53 was not clarified from the clinical data or experimental results obtained from these mice.

The expression of *Lmp2* was previously shown to be significantly induced by IFN-γ, as was the expression of other subunits of immunoproteasome (20). Accordingly, to demonstrate whether the cell-cycle regulators and each subunit

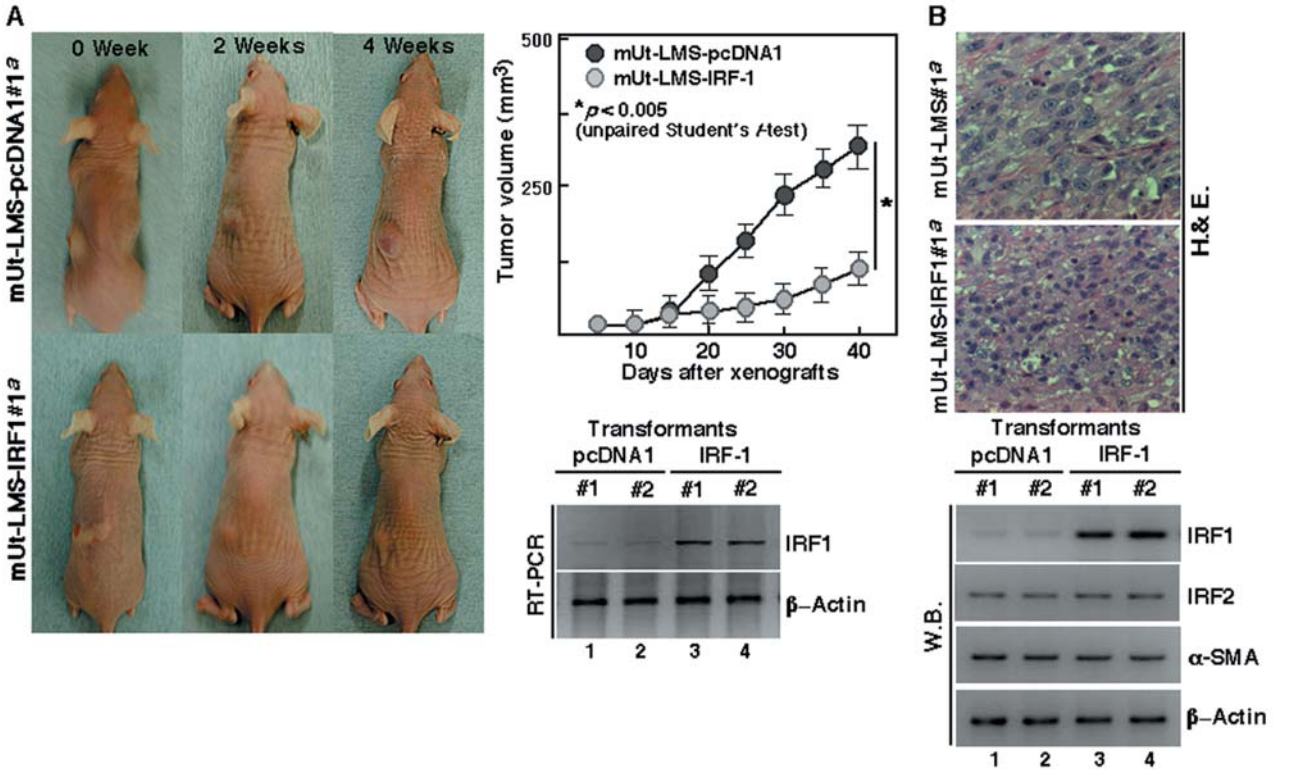
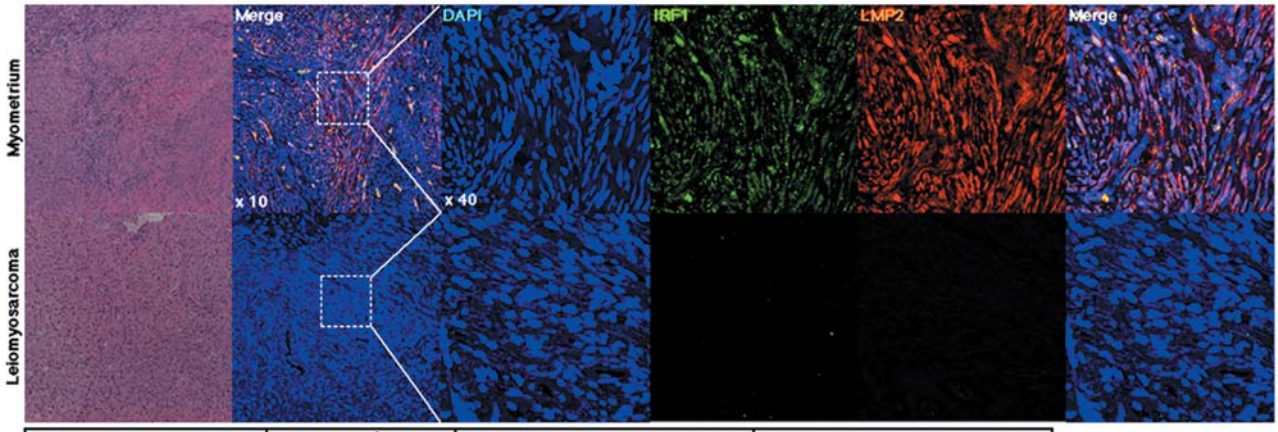


Figure 6. *Biological significance of IRF1 in sarcomagenesis in mouse uterine leiomyosarcoma (mUt-LMS). A: Changes in mUt-LMS clone, mUt-LMS-pcDNA1#1 clone, and mUt-LMS-Irf1#1 clone xenograft volumes in mice (n=8). Representative photographs of xenografts in mice (left panel). The tumor growth of mUt-LMS-Irf1#1 was markedly less than that of the control transfectant mUt-LMS-pcDNA1#1 clone. Tumor growth kinetics after a subcutaneous injection of the mUt-LMS-pcDNA1#1 clone and mUt-LMS-Irf1#1 clone (right panel). RT-PCR experiments revealed the expression of IRF1 and β-actin mRNA in tumors. B: A pathological analysis revealed no significant differences between the mUt-LMS-pcDNA1 tumor and mUt-LMS-Irf1 tumor (upper panel). Western blotting revealed IRF1, IRF2, α-smooth muscle actin (α-SMA), and β-actin in mUt-LMS-transfectant clones (lower panel). The details of mUt-LMSc-pcDNA1 and mUt-LMS-Irf1 clones are indicated in Tables I and II.*

of immunoproteasome, which are controlled by the IFN-γ signal cascade, abnormally expressed in *Lmp2*-deficient mUt-SMCs, molecular biological and histopathological analyses were performed with human and mouse uterine tissues. STAT1, having been activated by IFN-γ, significantly induced the expression of tumor suppressors such as IRF1 (14, 21) (Figures 2 and 3). IRF1as a transcriptional activator reportedly up-regulated the expression of *Lmp2* gene (14, 21). We then examined whether the IFN-γ signal cascade induced the expression of each subunit of the immunoproteasome, STAT1, IRF1, and IRF2 in murine Ut-SMCs derived from the myometria of *Lmp2*-deficient mice and its parental strain, C57BL/6. No significant difference was observed in the expression of STAT1 or the subunits LMP7, LMP10, LMP3, and IRF2 (Figure 4, Table I). Although the IFN-γ-induced phosphorylation of STAT1 was not influenced by the lack of *Lmp2*, the expression of the *Irf1* tumor-suppressor gene was significantly lower in murine Ut-SMCs derived from the myometria of *Lmp2*-deficient mice than from heterozygous and wild-type mice (Figure 1, Table I). The expression of IRF1 was induced by the treatment with IFN-γ in murine Ut-SMCs derived from the myometria of wild-type or heterozygous mice (Figure 4, Table I). In addition, MEFs derived from wild-type embryos were treated with the proteasome inhibitor MG115 or MG-132, the experimental results showed the loss of *Ifn-γ*-inducibility, reproducing the phenotype of the *Lmp2*-deficient mouse (Figure 5). It is likely that the transcription of *Irf1* mRNA depended on the function of the immunoproteasome and is considered to involve the formation of a STAT1 homodimer. Recent studies have suggested that immunoproteasomal function may contribute to transcriptional activation of *Irf1* mRNA (22, 23).

Irf1 plays role as tumor-suppressor in murine Ut-LMS. Primary cultured murine Ut-LMS clones were established from the Ut-LMS of *Lmp2*-deficient mice, and *Irf1*-stable-expressing



	Age, years	n	LMP2 expression				IRF1 expression			
			-	-/+	focal+	+++	-	-/+	focal+	+++
Myometrium	32-83	35				35				35
Leiomyoma	33-83	23				23				23
Bizarre leiomyoma	44,49	2				2			1	1
Leiomyosarcoma	32-83	32	29	1	1	1	31		1	

-/+: partially positive 5% to 10% of cells stained, focal+: focal or sporadic staining with < 5% of cells stained, +++: homogeneous distribution with more than 90% of cells stained, -: no stained cells.

Figure 7. *Defect in LMP2/β1i expression and IRF1 expression in human uterine leiomyosarcoma (Ut-LMS) tissue. Immunohistochemistry (IHC) for LMP2/β1 and IRF1 in human myometrium and Ut-LMS tissues located in the same tissue section. In all samples, 5-μm-thick sections of tissue were stained with anti-LMP2/β1 and anti-IRF1 and the reaction revealed by second antibody conjugated-Alex488 or Alex548 (magnification ×40). IHC experiments were performed with tissue sections from patient #3. Details regarding the myometriuma and leiomyosarcomab are indicated in Table IV.*

murine Ut-LMS clones were then further established by genetic engineering (Table II). The murine Ut-LMS *Irf1*-expressing clones were intracutaneously transplanted into BALB/c *nu/nu* mice, and the preventive effects of Irf-1 on tumor cell proliferation were observed (Figure 6, Table III). However, a histopathological analysis revealed no significant differences between the murine Ut-LMS-pcDNA1 tumor and murine Ut-LMS-*Irf1* tumor (Figure 6). The expression of the IRF1 molecule in the murine Ut-LMS-*Irf1* tumor was re-confirmed by RT-PCR and western blotting (Figure 6).

Furthermore, IHC revealed a pronounced loss in the ability to induce IRF1 expression in human Ut-LMS tissue in comparison with normal myometrium located in the same section (Figure 7). Out of the 32 cases of human Ut-LMS examined, 29 were negative for the expression of LMP2, one was focally positive, one was partially positive, and one was positive (Figure 7). Defects in the expression of IRF1 in human Ut-LMS were similar to the findings in *Lmp2*-deficient mice (Figure 7, Table IV). Thus, lower levels of IRF1 due to a deficiency in *Lmp2* appeared to be a risk factor for mUt-LMS in *Lmp2*-deficient mice.

The effects of Irf-1 on tumor cell proliferation were achieved through the expression of $p21^{CLIP1}$ cell-cycle inhibitors (inhibiting transition from the G_1 to S stage) (14, 24, 25). We further examined whether the expression or activation of $p21^{CLIP1}$ was affected in *Lmp2*-deficient mice, and observed the defective expression of $p21^{CIP1}$ in mUt-SMCs derived from *Lmp2*-deficient mice (Figure 8). The tumor suppressor, RB was previously shown to be phosphorylated by a complex of cyclin D/CDK2 and then inactivated (26, 27). Furthermore, the activity of CDK2 was negatively regulated *via* degradation of cyclin D by the proteasome (26-28).

Phosphorylated-RB was detected in Lmp2-lacking murine Ut-SMCs, and the activity of CDK2 in this phosphorylation was stronger than that in normal murine Ut-SMCs (Figure 7). However, experiments using gene-deficient mouse models and clinical research suggested that the defective expression of RB may not be involved in the onset of human Ut-LMS (29, 30-31). In the case of murine Ut-LMS in *Lmp2*-deficient mice, defective IRF1 was considered to play a role in cellular transformation and cell proliferation.

Table IV. *Expression of ER, PR, Ki-67, TP53, LMP2, and IRF1 in human uterine leiomyosarcoma (Ut-LMS).*

Patient No.	Age in yrs	Immunohistochemical staining									Somatic mutations					Follow-up (months)
		TMN stage	MF	CCN	ER	PR	Ki-67	TP53	LMP2	IRF1	TP53	JAK1	JAK2	STAT1	LMP2 pro	
1	37	T4N1M0	97	+	–	–	3000	+++	–	–	SM	ND	ND	ND	ND	D(1)
2	58	T3N0M0	24	+	–	–	3500	+	–/+	–	SM	ND	ND	ND	SM	D(23)
3	45	T2N1M0	32	+	–/+	–/+	2150	+++	–	–	SM	M	ND	SM	SM	D(24)
4	65	T1N0M0	30	+	–/+	–/+	1700	+++	–	–	SM	M	ND	ND	ND	D(20)
5	52	T1N1M0	107	+	–	+	2600	++	+	–	ND	M	ND	ND	ND	D(13)
6	49	T1N0M0	46	+	–	–	4300	+	–	–	ND	ND	ND	ND	ND	D(24)
7	55	T1N1M0	75	+	–	–	4000	+++	–	–	ND	ND	ND	SM	SM	D(18)
8	43	T3N0M0	57	+	+	–	2000	–	–/+	–/+	ND	ND	ND	ND	ND	D(10)
9	67	T1N1M0	13	+	–	–/+	1430	–	–	–	ND	M	ND	ND	ND	A(34)
10	67	T1N0M0	37	+	–	–	2100	–	–	–	ND	ND	ND	SM	SM	A(15)
11	51	T1N1M0	93	+	–	–	4500	–	–	–	ND	ND	ND	SM	ND	A(94)
12	48	T1N0M0	14	+	–	–	900	+++	+	+	ND	ND	ND	ND	ND	A(58)
13	51	T1N1M0	22	+	–/+	+	450	+	–	–	ND	M	ND	ND	SM	A(34)
14	67	T1N0M0	64	+	–	+	1450	++	–	–	ND	ND	ND	ND	ND	A(15)
15	52	T1N1M0	65	+	–	–	1780	++	–	–	ND	M	ND	SM	ND	D(23)
16	42	T3N0M0	73	+	–	–	2130	++	–	–	ND	ND	ND	ND	SM	A(21)
17	80	T1N1M0	98	+	–	–	1980	+++	–	–	ND	M	ND	ND	ND	D(19)
18	56	T1N0M0	78	+	–	–	1860	++	–	–	ND	ND	ND	ND	ND	A(11)
19	58	T1N0M0	40	+	–	–	1750	++	–	–	ND	ND	ND	ND	ND	A(10)
20	65	T2N1M0	67	+	–	–	780	+++	–	–	ND	M	ND	ND	SM	A(12)
21	45	T1N0M0	52	+	–	–	1045	++	–	–	SM	ND	ND	SM	ND	A(13)
22	57	T2N0M0	62	+	–	–	980	++	–	–	SM	ND	ND	SM	ND	A(11)

ER; Estrogen receptor, PR; progesterone receptor, TP53; tumor protein p53, LMP2; low-molecular mass protein 2, IRF1; interferon regulatory factor 1, ki-67; positive cell number/10 high power fields, SM; somatic mutation, ND;not detected, D; died of disease, A; alive, MF; mitotic figure/10 high power fields, CCN; conagulative cell necrosis.

Discussion

Ut-LMS mainly develops in the myometrium or endometrial stroma, and menstrual anomalies, such as hypermenorrhea and prolonged menstruation, and symptoms, such as abnormal hemorrhage, hypogastric pain, lumbar pain, and abdominal strain, have been reported previously (4). In the case of gynecological cancer, such as breast cancer, a female hormonal imbalance is often a risk factor for the development of tumors. However, as is also the case for uterine LMA, the relationship between the development of human Ut-LMS and female hormones and their receptors has yet to be elucidated (32, 33). A recent study showed the expression of *Lmp2* mRNA and protein in luminal and glandular epithelia, placenta villi, trophoblastic shells, and arterial endothelial cells (34). These findings implicated LMP2 in the invasion of placental villi, degradation of the extracellular matrix, immune tolerance, glandular secretion, and angiogenesis (34). Unfortunately, it currently remains unclear whether the estrous cycle with the defective expression of LMP2 is involved in the onset of Ut-LMS. Human Ut-LMS often appears to develop in individuals exposed to radiation in the pelvis. Risk factors for the development of human Ut-LMS have not yet been identified because of the absence of a suitable animal model. The *Lmp2*-deficient mouse was established as the first mouse model of spontaneous Ut-LMS (13). To establish whether LMP2 can be used as a potential biomarker to distinguish human Ut-LMS from LMA, we are now investigating the reliability and characteristics of LMP2 as a diagnostic biomarker with several clinical research facilities. This research is ongoing and large-scale clinical studies must also be conducted. Studies using gene-expression profiling revealed differential expression of several known pro-oncogenic factors and previously reported factors *i.e.* brain-specific polypeptide PEP19, CALPONIN h1, and the transmembrane tyrosine kinase receptor, c-KIT, which were associated with the pathogenesis of human Ut-LMS (35-40). Since the spontaneous development of murine Ut-LMS has not been reported in *Irf1-, calponin h1*-deficient mice or heterozygous Rb mice, it is likely that the cellular condition of lack of LMP2 results in induction of the expression of other known or unknown cell-cycle regulatory factors. Further studies are needed to demonstrate the correlative

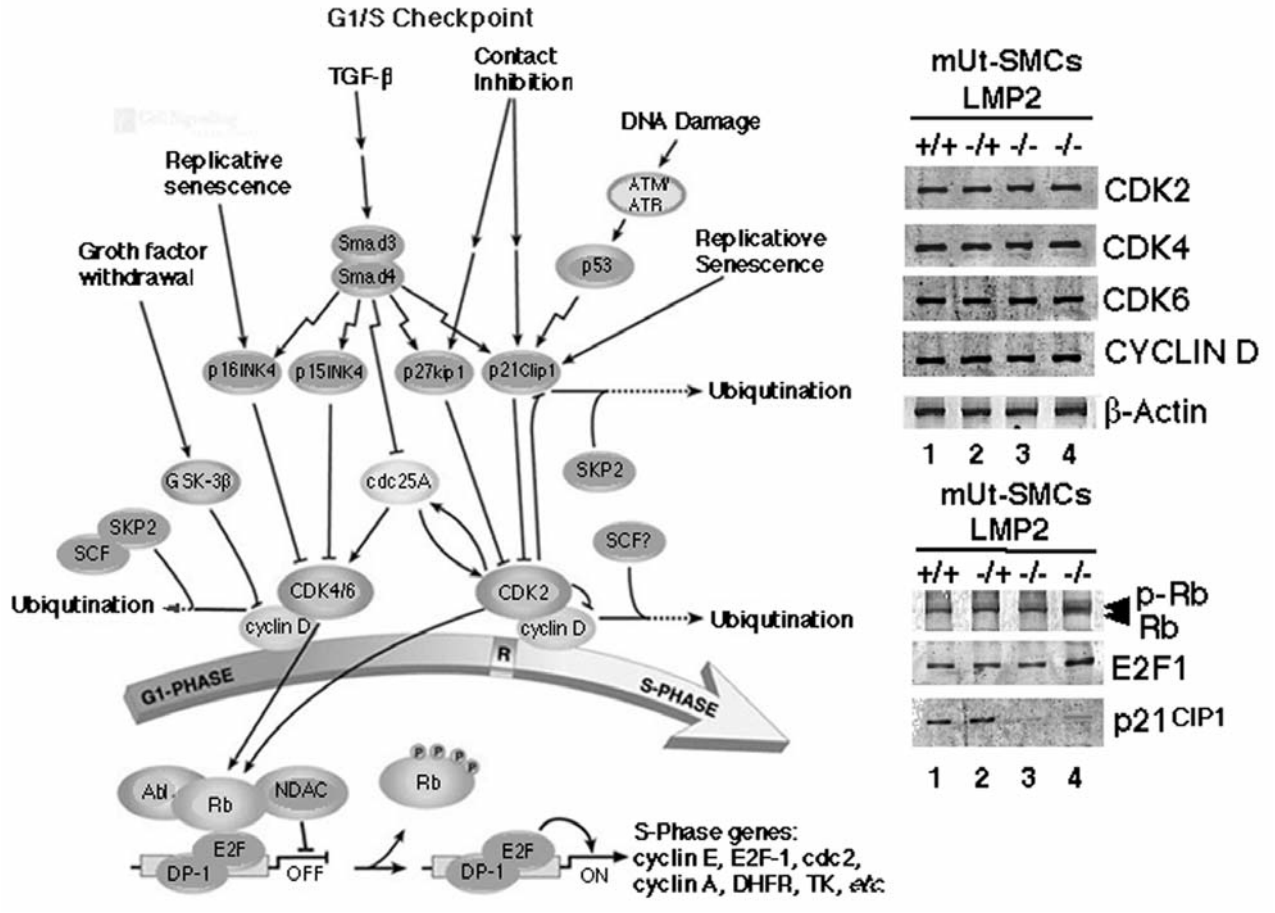


Figure 8. *Differential expression of cell-cycle regulating factors in mouse uterine smooth muscle cells. Expression of cell-cycle regulating factors in mouse uterine smooth muscle cells (mUt-SMCs), which were isolated from the uteri of homozygous or heterozygous mice deficient for Lmp2, and parental mice (wild-type). Cytosolic extracts were prepared from mUt-SMCs, and 50 μg of cytosolic extracts was resolved using 10% SDS-PAGE. The expression levels of CDK2, CDK4, CDK6, cyclin D, p-RB, RB, E2F1, $p21^{CLIP1}$, and β-actin were examined by western blotting analysis with appropriate antibodies.*

functions of LMP2 and other anti-oncogenic factors with CALPONIN h1 and IRF1 in human Ut-LMS sarcomagenesis. Clarifying the relationship between these factors and the development of human Ut-LMS, and the identification of specific risk factors may lead to the development of new treatment methods against this disease. Human Ut-LMS is refractory to chemotherapy and has a poor prognosis. Molecular biological and cytological information obtained from *Lmp2*-deficient mice will markedly contribute to the development of preventative methods against, potential diagnostic biomarkers for, and new therapeutic approaches to treat human Ut-LMS.

Conflicts of Interest

We do not have any conflicts of interest.

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