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The Requirement of p53 in Oral MSC differentiation Which Leads to Kaposi's Sarcoma

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Abstract

P53 is considered one of the most important defense proteins against cancer. Although the p53 mutation is associated with many cancers, its mutation is never found in Kaposi's sarcoma (KS), raising a possibility that functional p53 may play a role in KS development. To assess the role of p53 in Kaposi's sarcoma tumor, shRNA-mediated gene silencing was used to knockdown p53 expression. We examined Kaposi's sarcoma-associated herpesvirus (KSHV)-infected periodontal ligaments stem cells (PDLSC) for osteogenic differentiation and endothelial angiogenesis, and we observed noticeable decreases in osteogenic differentiation and angiogenesis when p53 expression was knocked down in KSHV-infected PDLSCs by p53 specific shRNA. The results suggest that p53 plays an essential role in sarcoma development of KSHV infected mesenchymal stem cells.

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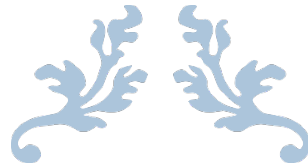MSOB (Master of Science in Oral Biology)

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The requirement of p53 in oral MSC differentiation which leads to Kaposi's Sarcoma

Master's Thesis

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Abstract.

P53 is considered one of the most important defense proteins against cancer. Although the p53 mutation is associated with many cancers, its mutation is never found in Kaposi's sarcoma (KS), raising a possibility that functional p53 may play a role in KS development. To assess the role of p53 in Kaposi's sarcoma tumor, shRNA-mediated gene silencing was used to knockdown p53 expression. We examined Kaposi's sarcoma-associated herpesvirus (KSHV)-infected periodontal ligaments stem cells (PDLSC) for osteogenic differentiation and endothelial angiogenesis, and we observed noticeable decreases in osteogenic differentiation and angiogenesis when p53 expression was knocked down in KSHV-infected PDLSCs by p53 specific shRNA. The results suggest that p53 plays an essential role in sarcoma development of KSHV infected mesenchymal stem cells.

Acknowledgment.

Introduction.

Kaposi's sarcoma (KS) is the most common malignancy associated with HIV infection. About 20% of AIDS patients develop KS, and most cases (60%) manifest as oral lesions. Oral KS is often the first presenting sign of AIDS and the most common intraoral KS sites are palate and gingiva[1]. With the development of AIDS-KS, KS lesions progress to the skin and internal organs, including the lungs and gastrointestinal tract. KS has proven to be a malignant, progressive and fatal disease, which contributes greatly to the morbidity and mortality of AIDS. In addition, it was found that patients with KS in their oral mucosa had a higher risk of death than those with KS appearing only on the skin. Patients with oral KS generally have a less than 10% 5-year survival rate [2]. Despite its dramatic decrease in frequency since the advent of highly active antiretroviral therapy (HAART), KS remains the most common AIDS-associated cancer in the United States. In addition to this AIDS-associated (epidemic) form, other epidemiological forms of KS include the classic (sporadic), African (endemic), and immunosuppression-associated (iatrogenic) forms [3]. Microscopically, KS Tumors comprise proliferating spindle-shaped KS cells with abnormal neoangiogenesis and abundant inflammatory infiltrate. The origin of the spindle-shaped KS cells lineage remains elusive. Based on initial immunohistochemistry studies as well as recent gene expression profiles, the most widely accepted theory is that KS cells may derive from the endothelial cell lineage [4]. KS cells express panendothelial marker CD31, CD34 and Factor VIII. However, KS cells are poorly differentiated and also express other markers such as smooth muscle markers, macrophage markers and mesenchymal markers, suggesting that KS cells do not faithfully represent endothelial cell lineage [5]. There is currently no definitive cure for KS. For classic KS, classic cancer therapies are generally used to treat

patients, which include surgical excision and radiation therapy for patients with a few lesions in a limited area and chemotherapy for patients with extensive or recurrent KS [1].

Kaposi's sarcoma-associated herpesvirus (KSHV), also named human herpesvirus 8 (HHV-8), has been proven to be an etiologic agent of Kaposi's sarcoma. Irrespective of the source or clinical subtype (i.e., classic, AIDS-associated, African endemic, and iatrogenic KS), almost 100% of KS lesions are found to carry KSHV. KSHV is also unequivocally associated with two B-cell-associated lymphoproliferative disorders, namely, primary effusion lymphoma (PEL) and the plasma cell variant of multicentric Castleman's disease (MCD)[1,3].

Oral Mesenchymal stem cells (MSCs) have been identified as a population of hierarchical postnatal stem cells with the potential to self-renew and differentiate into osteoblast, chondrocytes, adipocytes, cardiomyocytes, myoblasts and neural cells[6,7]. MSCs are capable of generating mineralized tissues in vivo[8] and organizing host tissue to collaboratively form bone/marrow organ-like structures and to re-establish normal tissue homeostasis[9]. MSC-mediated tissue regeneration is a promising approach for developing new clinical treatments. In addition to their tissue regeneration capacity, MSCs interplay with various immune cells and possess an immunomodulatory function that leads to successful therapies for a variety of immune diseases[10,11]. The oral cavity contains a variety of distinctive MSC populations, including dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), apical papilla stem cells, dental follicle stem cells, and gingiva/mucosa-derived mesenchymal stem cells (GMSCs)[12,13,14,15,16,17]. These MSCs show significantly increased proliferation and self-renewal capacities compared to bone marrow MSCs, which may be associated with their neural crest origin[18,19,20,21]. Among

these MSCs, only GMSCs in gingiva and PDLCs in periodontal ligaments have the potential to directly interact with oral cavity saliva, microbiota, and virus. There is increased chance that KHSV contacts and transfects GMSCs and PDLSCs in the oral cavity[22]. MSCs are found in solid tumors stroma which migrate to the tumor site, then they form part of the microenvironment affecting tumor survival and angiogenesis mechanism of tumor development [23]. Recently, Yuan Lab and others showed that oral MSCs can be efficiently infected by KSHV and latent infection can be established in the cells. KSHV infection of oral MSCs promoted cell differentiation that led to morphological changes and enhanced capacities of adipogenesis, osteogenesis and angiogenesis. Further study provided evidences supporting the hypothesis that KSHV–infected oral MSCs can be the progenitor of KS malignant cells and mesenchymal-to-endothelial transition (MEndT) driven by KSHV infection contributes to the development of KS [24](Yuan lab unpublished data).

p53 or so called "guardian of the genome" plays a pivotal role in maintaining the genetic stability and in regulating cell differentiation particularly cell proliferation and apoptosis [25] [26]. p53 also called "tumor suppressor gene", is activated upon certain signals such as DNA damage and stresses, and p53 subsequently acts by arresting cell cycle or inducing apoptosis to help preserving cellular integrity and function. In about 30% of human cancer [27], the p53 gene is frequently mutated or inactivated[28] such as in melanoma, lymphoma and leukemia [29]. Whereas in KSHV tumors the opposite has been reported "tumors express functional p53" supported by the hypothesis that KHSV has encoded proteins. These proteins have an inhibitory function against p53[30] like ORF50, K10, K10.5 ORF22, ORF25, ORF37, ORF64, ORF68, ORF72, ORF74 and K14 [31].

Recent studies indicate diversity in the p53 functions and it is mostly related to the cell type. Some suggest that p53 induce cell differentiation and in others suppression. In MSCs, p53 acts as a master regulator of proliferation and differentiation [28], and its absence or malfunction will increase the proliferation and differentiation rate to osteocyte in case of osteogenesis [28]. Additionally, p53 effectively limits angiogenesis differentiation in many different ways such as inhibition of proangiogenic factors, increase the production of endogenous angiogenesis inhibitors and interference with angiogenesis mediators. Thus, inactivation of p53 will switch to angiogenic phenotype which represents in aggressive vascular tumors[32][33].

In this work, we analyzed the role of p53 in the osteogenesis and angiogenesis differentiation of Kaposi's sarcoma herpesvirus infected mesenchymal cells by comparing KSHV infected MSC with wild type p53 (WT) or p53 knockdown (KD). We observed that the knocking down of p53 in MSCs will inhibit the osteogenesis rate and angiogenesis ability. Therefore, we hypothesized that p53 plays a pivotal role in the differentiation of KHSV-infected MSC, a crucial step toward KS development.

Methods and materials

Cells.

The periodontal ligament stem cell line (PDLSC) were kindly provided by professor Song tao Shi [Investigation of multipotent postnatal stem cells from human periodontal ligament]. The cells were cultured in alpha Minimum Essential Medium (αMEM) (Invitrogen) containing 10% FBS (BD Clontech), 100 U/ml penicillin/100 μ g/ml streptomycin (Invitrogen), 2 mM L –

glutamine, 100 mM nonessential amino acid, and 550 μM 2-ME (Sigma-Aldrich), and cultured

at 37°C in a humidified tissue culture incubator with 5% CO2 and 95% O2. For maintenance

cultures, cells were passaged every 2-3 days with 0.05% Trypsin /4mM EDTA and seeded at 3-

4.5 x 10^3 per cm^2 in 100-mm T75 flasks.

Human embryonic kidney (HEK) 293T cells were obtained from ATCC. 293T was cultured in

Dulbecco modified Eagle medium(DMEM) supplemented with 10% FBS, 2mM L-glutamine,

and antibiotics. Ecoli stain GS1783 containing BAC16 which carries the entire KSHV genome,

was obtained from Shou-Jiang Gao at the University of Texas at San Antonio.

 iSLK cells known for their ability to harbor rKSHV.iSLK cells were cultured in the presence of

1 μg/ml puromycin and 250 μg/ml G418. BAC16 and its derivatives were introduced into iSLK

cells via Fugene HD transfection. GFP confirms iSLK-BAC transfection success[31]. iSLK cells

induced using 1ug/ml doxycycline and 1 mM sodium butyrate, were then harvested 5 days.

KSHV genomic DNA was quantified by real-time PCR on a Roche LightCycler instrument [34].

shRNA-mediated gene silencing technique.

Mission shRNA gene sets against human TP53 were purchased from Sigma-Aldrich. This

shRNA system is a lentiviral vector-based RNA interference library against annotated human

genes, which generates siRNAs in cells and mediates gene-specific RNA interference for

extended periods of time. The TP53 set consists of two individual shRNA lentiviral vectors in

pLKO.1-puro plasmids against different target sites of TP53 mRNA (with clone ID NM_000546.

for convenience sake referred as p53 shRNA clone #s 55). Each of the shRNA vectors and the

control vector were used to prepare lentiviral stocks by cotransfecting HEK 293T cells with the

shRNA vector and two packaging vectors (pHR=8.2DR and pCMV-VSV-G) at a ratio of 4:2:1, respectively. Three days post-transfection, the culture media that contained shRNA retroviruses were harvested, centrifuged (500 g for 10 min at 4°C), and filtered through a 0.45-m-pore-size filter to ensure removal of any nonadherent cells. PDL cells were transduced with the shRNA-encoding lentivirus stocks in the presence of Polybrene (8 microgram/ml). Transduced cells were selected with puromycin (2 microgram/ml) for a week. Efficacies of these shRNAs in knockdown of the respective protein were assayed by Western blotting with specific antibodies.

Western blotting.

Cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 10% glycerol, 40 mM glycerophosphate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1% Nonidet P-40, 1 mM sodium orthovanadate) supplemented with protease inhibitor cocktail (Roche). The cell lysates were homogenized and centrifuged at 13,000 rpm for 5 min at 4°C. The whole-cell extract was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 5% dried milk in 1 phosphate-buffered saline (PBS) and then incubated with diluted primary p53 rabbit antibodies (Cell Signaling Technology) and left overnight at 4°C. Anti-rabbit immunoglobulin G antibodies conjugated to horseradish peroxidase (Cell Signaling Technology) were used as the secondary antibodies. An enhanced chemiluminescence system (Cell Signaling Technology) was used for detection of antibody-antigen complexes. Following this, we repeated some steps for beta actin incubating the membrane with primary anti-beta actin antibody (genetex) overnight and secondary goat anti mouse antibody (scbt).

Transfection of KSHV and PDL cells.

PDL Cells were seeded in 24-well plates about 4-6 ×10^4 cells per well, we made sure the cells were 90% cell confluency in the infection day. The next day, final 4 µg/mL polybrene added to the virus/medium and incubate for 5 min. Remove the medium of the cells, add the virus/medium to the cells (500ul/each well). Spin the cells at 2,500rpm for 1h at room temperature. Then the plate placed in 37 ℃ incubator for 1h. Replaced the virus/medium with virus-free medium and took the plates into 37 ℃ incubator.

2-3 days following we changed the medium. We used 3 plates at different time point 1-2-3 weeks for evaluation purpose.

Osteogenic Differentiation.

MSCs were cultured under osteogenic culture condition, containing 2 mM beta-glycerophosphate (sigma-Aldrich), 100 microM L-ascorbic acid 2-phosphate (Wako),and 10 nM dexamethasone (Sigma-Aldrich) in the growth medium .After 2-3 weeks induction , 1% Alizarin Red S (Sigma-Aldrich) staining was performed to detect matrix mineralization .

Matrigel tube formation assay.

48-well plates were coated with Matrigel (BD) (100 µl/well) and incubated at 37ºC for 1h to allow gelation to occur. KSHV-infected PDLSCs suspended in 200µl a-MEM were added to the top of the gel in the presence or absence of p53 inhibitor. Then the cells were incubated at 37°C with 5% CO2 for 4-8 h, and images of tube formation were captured using a ZEISS fluorescence microscope.

Results.

<u>Evaluation of the role of p53 in KSHV infected-MSC osteogenesis differentiation</u>

Our study identified the need of P53 in the KSHV infected periodontal ligament mesenchymal cells to differentiate and develop Kaposi's sarcoma. Recent evidences suggested that the initial target cells for KSHV infection could be progenitor cells such as mesenchymal stem cells (MSCs) and mesenchymal-to-endothelial transition (MEndT) may account for sarcomagensis of KS. Therefore, we explored the role of p53 in oral periodontal ligament mesenchymal stem cells as target by KSHV and tested the potentials of KSHV-infected oral MSCs in two different lineage differentiation. It was demonstrated in Yuan Lab that KSHV-infection efficiently promote differentiation of several lineages including osteogenic, adipogenic and endothelial differentiation (Yuan Lab unpublished data). Using p53 specific shRNA-mediated knock down procedure, we first examined the effect of p53 on the ability of oral PDLSCs to differentiate into osteoblasts.

To assess whether this P53 is essentially required for KSHV-PDLSCs differentiation, we attempted to knockdown P53 expression in PDLSCs through a short-hairpin RNA (shRNA)-based approach and examined the effects on PDLSCs differentiation and evaluate KHSV DNA replication. Mission shRNA gene set against P53 were purchased from Sigma-Aldrich. After introduction into PDLSCs by lentiviral transduction, the expression of the target protein was evaluated by Western blotting (Fig. 1).

We used UV light at dose 20 J/m for 2 seconds to effectively induce p53 before Western blot. P53 shRNA was found to successfully downregulate the expression of p53 in comparison to the control as shown in Figure.1. After confirming that p53 was effectively knocked down (KD), the p53 shRNA-expressed PDLSCs and control PDLSCS were infected with KHSV. Mock-infected PDLSCs were also prepared as controls. KSHV- and mock-infected PDLSCs were grown in the induced medium MSCOIM for two weeks. Starting from the 3rd day after infection, the medium was changed every 2-3 days using induction medium MSCOIM and some other plates with the regular medium for comparison purpose.

Osteogenesis of MSCs was assayed by Alzarin Red staining and results showed positive nodule formation (indicating calcium accumulation in vitro) in PDLSCs was greatly enhanced with KHSV infected PDLSCs that has functional p53 in the presence of osteo-inductive condition whereas in the KHSV infected PDLSCs with knocked down P53 shows low rate osteogenesis and was detachable from the surface within 3 weeks. We used a quantification measure Image J to compare the relative amount as ratio of calcium deposition between the samples which also indicate high rate of osteogenesis seen in KSHV infected PDLSCs with wild type p53. Furthermore, calcium deposition was significantly reduced in both KSHV-infected and mock-infected PDLSCs where p53 expression was silenced by shRNAs, suggesting that p53 is required for osteogenic differentiation of oral MSCs.

P53 and KSHV-PDLC proliferation

Moreover, during the process of growing KSHV- and mock-infected PDLSCs, we noticed that there was an increased and accelerated proliferation of the KHSV-infected PDLSCs in comparison to mock-infected cells, confirming the fact that KHSV infection enhances differentiation of oral MSCS. In addition, PDLSCs with control shRNA (wild type P53) show the most intense differentiation among all the samples , suggesting that KSHV promotes stronger osteogenic differentiation of oral MSCs in the presence of p53 (table 1).

KSHV infected PDLSC	Mock infected PDLSC
Control p53 +++++ Osteogenesis	+++ Osteogenesis
Kd p53 ++ Osteogenesis	+ Osteogenesis

Table 1: Comparison between KHSV- and mock infected PDLSCS once with p53 and other with knockdown the expression of p53, showing that KSHV promotes stronger osteogenic differentiation of oral MSCs in the presence of p53.

Evaluation of the role of p53 in KSHV infected-MSC angiogenesis differentiation

KS is a vascular tumor and abnormal angiogenesis is the hallmark of the malignancy. Angiogenesis is a multi-step process involving endothelial cell activation, proliferation, differentiation, migration, and formation of vascular structure. It have been shown that KSHV infection of oral mesenchymal stem cells (MSCs) confers the cells with certain KS features including angiogenic, invasive and transformation phenotypes [32]. In our lab, we also demonstrated that KSHV-infected PDLSCs exhibit highly increased angiogenesis activity as showed in an *in vitro* Matrigel tubulogenesis assay (unpublished data of Yuan and Shi Labs). Thus, we would investigate whether p53 is crucial for KSHV-induced angiogenesis in PDLSCs and if knockdown of p53 expression or inhibition of p53 activity block the KSHV-mediated angiogenesis of PDLSCs. P53 shRNA was introduced into PDLSCs by lentiviral transduction. After one week drug selection, transduced cells were infected with KSHV at multiplicity of infection (MOI) of 50 (viral genomic DNA equivalent). Ninety-six hours post-infection, an *in vitro* Matrigel tubulogenesis assay was performed to assess the ability of KSHV-infected oral MSCs in formation of capillary-like tubules and the effect of p53 knockdown on this process. As shown in Fig. 3A, KSHV-infected PDLSCs can form capillary-like tubules that represent the later stage of angiogenesis, while tight cell junction was not formed in the p53-knowndown PDLSCs. The results suggest that a differentiation from KSHV-infected MSCs to endothelial lineage (such as angioblast) take place and this process is p53-dependent.

In addition, we also examined the importance of p53 in angiogenesis activity of KSHV-infected MSCs by using a pharmacological inhibitor of p53. A wide range of concentration of

Pifithrin was added in the Matrigel tubulogenesis assay and the effect of the p53 inhibitor on the ability of KSHV-infected oral MSCs in formation of capillary-like tubules was examined. As shown in Fig. 3B, the tubule formation was obviously blocked starting at 10 ϔ M. The result showed that in consistent with shRNA knockdown experiment, inhibition of p53 by its specific inhibitor dramatically block KSHV-induced angiogenesis. Taken together, our result suggests that KSHV infection promotes differentiation of oral MSCs that may lead to KS development and this process is p53-dependent.

Discussion.

Kaposi's sarcoma (KS) is a serious disease, especially for HIV-infected population. Currently there is no cure for KS and other KSHV-associated malignancies. Little is known about the nature of the target cells of KSHV infection in oropharynx. The multifocal nature of KS tumor suggests that KSHV infects progenitor cells with proliferation and differentiation potentials and drives differentiation of the cells to KS spindle cells. The proliferation and self-renewal nature of MSCs and the observation that KSHV infection of oral MSCs promoted cell differentiation that led to morphological changes and enhanced capacities of adipogenesis, osteogenesis and angiogenesis provide a theoretic support for the hypothesis that KSHV-infected oral MSCs could be the cellular origin of KS in oral cavity. The transformation of KS progenitors, such as KSHV-infected MSCs, to KS malignant cells undergoes a mesenchymal-to-endothelial transition (MEndT) process . A study on gene expression profiling of KSHV-infected oral MSCs revealed how KSHV infection reprograms the infected MSCs which includes

activation of a number of genes that contribute to MEndT and have been identified as KS

expression signature genes previously (Yuan Lab unpublished data).

The role of p53 as the "guardian of the genome" in regulation of cell proliferation,

apoptosis and tumor suppression has been well established, but its involvement in MSC

differentiation has not been extensively explored. The role of p53 in MSC differentiation and

tumorigenesis is elusive and contentious. A study by Molchadsky et al. suggests that as a general

regulator, p53 facilitates differentiation of MSC in cell fate dependent manner[37]. Huang and

colleagues isolated bone marrow-derived mesenchymal stem cells (BMSCs) from p53 wild type

(WT) and knock out (KO) mice and demonstarted that loss of p53 pushes BMSCs toward pre-

osteoblast differentiation. The best explanation as indicated by many study is that p53 indirectly

represses the expression of Runx2 by activating the microRNA-34 family, which suppresses the

translation of Runx2. Higher levels of Runx2 account for faster osteogenic differentiation[40]. Our

study indicated that in KSHV-infected oral MSCs, p53 is required for multi-lineage

differentiation, suggesting its role in MEndT that leads to KS development. This finding may

provide an explanation that although p53 loss or mutation are frequently seen in many forms of

tumors, but is never seen in KS lesion.

Formation of abundant irregular blood vessels is a hallmark of KS. Previous studies have

reported that KSHV infection increased the angiogenic property in oral MSCs. KSHV infection

can drive oral MSC into endothelial lineage differentiation and promote neoangiogenesis, a

crucial pathogenic feature of KS. Our work has shown that KSHV-infected PDLSCs are capable

to well form blood vessels which represent angiogenic differentiation in the presence of p53

protein, while the differentiation is remarkably inhibited in the p53-knowndown PDLSCs. This

suggests that the differentiation from KSHV-infected MSCs to endothelial lineage is p53-dependent.

Given that p53 functions as an tumor suppressor, how does KSHV-infected KS precursor cells use p53 to promote differentiation, but avoid its anti-oncogenic activity in order to develop malignancy. It has been reported that KSHV encodes several proteins that show to inhibit p53 activity and apoptosis [31] [38] [39]. Accordingly, this fact confirms that wild p53 express in KSHV associated tumors. It is assumed that p53 is initially needed by KSHV for cell differentiation but gradually inhibits p53 mediated apoptosis function allowing production of virus particles and thereby facilitate viral oncogenesis. This notion is interesting but needs to be further studied for better understanding.

Future studies are required to understand in details the role of p53 in KHSV infected MSCs differentiation and its contribution to Kaposi sarcoma development. Furthermore, the studies will help in developing new strategy in molecular target therapy for KS.
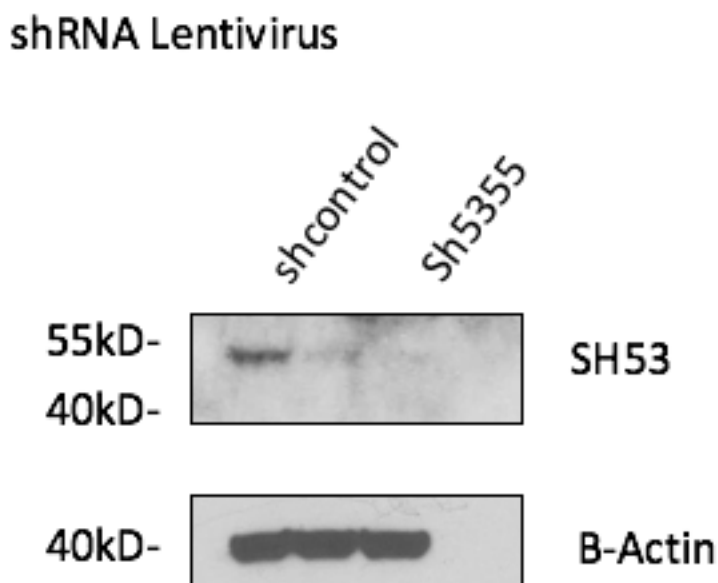
Figures

Fig. 1. Effects of p53 knockdown by short-hairpin (shRNA)-mediated silencing. An shRNA

lentivirus which targets p53 shRNA (NM_000546.X-1095S1C1,-427s1c1-; Sigma-Aldrich),

along with a nontargeting control shRNA lentivirus, was transduced into PDL cells. Intracellular

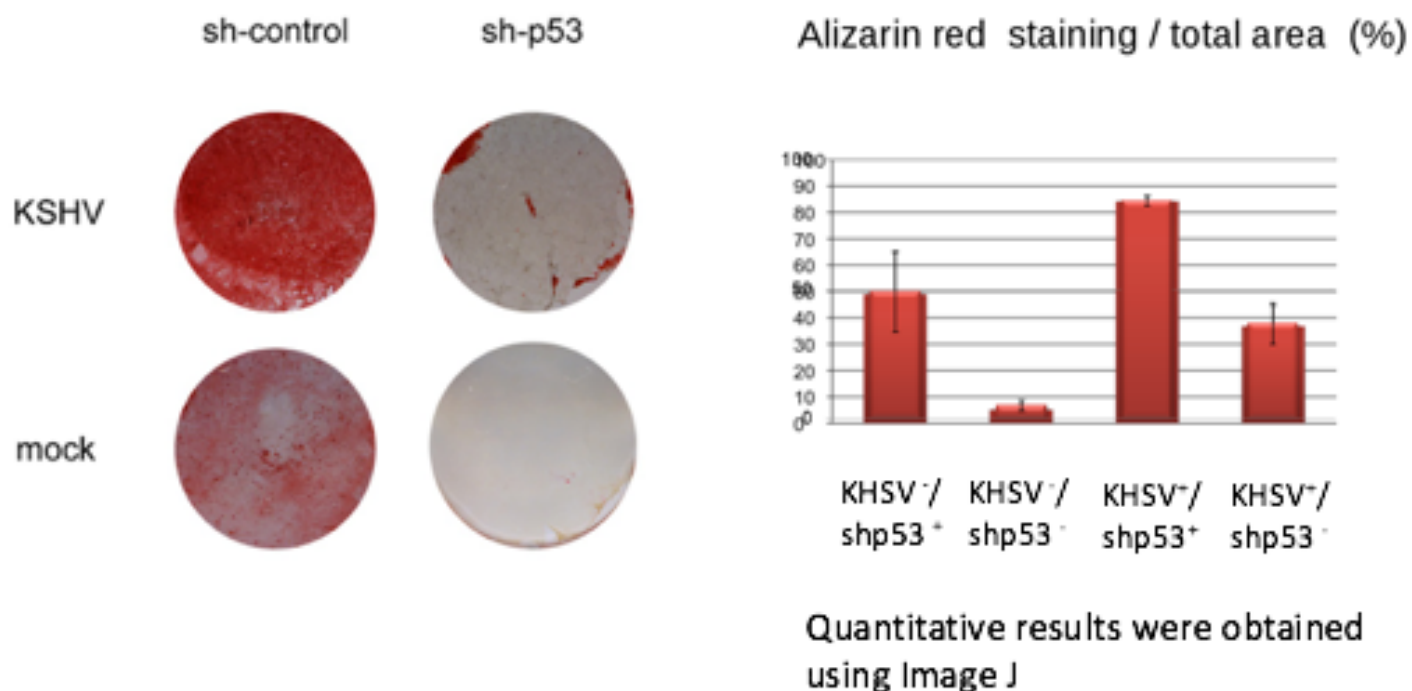levels of p53 (top), -actin (bottom) proteins were determined by Western blot analysis.

Fig. 2. Osteogenesis differentiation of KHSV-infected PDLSCs after 2 weeks of induction media. KSHV-PDLSCs with control shRNA (wild type p53) (top left) show higher osteogenic differentiation in compared to the cells with p53 knockdown (top right). The p53-dependent differentiation was also observed in the mock-infected PDLSCs (bottom left and right).
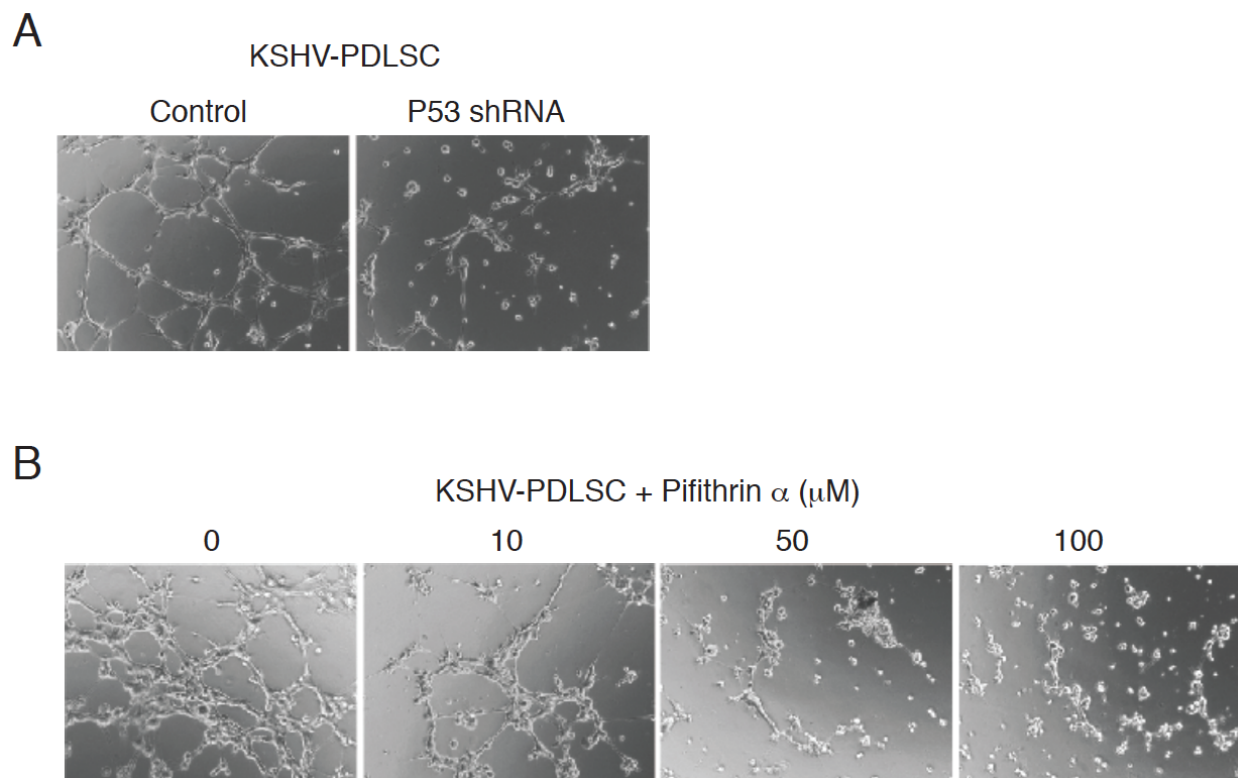
Fig. 3. p53-dependent angiogenesis of KSHV-infected PDLSCs demonstrated by Matrigel tube formation assays. (A) KSHV-infected PDLSCs were transduced by p53-specific shRNA or control shRNA lentiviruses. Cells were subjected to Matrigel tube formation assays as described in Material and Methods. (B) KSHV-infected PDLSCs were treated with p53 inhibitor Pifithrin ✔in a wide range of concentration. The effect of Pifithrin ✔ on angiogenesis of KSHV-PDLSCs were evaluated by a Matrigel tube formation assay.

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