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Polyclonal antibody to porcine p53 protein: A new tool for studying the p53 pathway in a porcine model

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ABSTRACT

Although the tumor suppressor protein p53 is important in the control of various cellular activities, the analysis of p53 in the porcine model has been hampered by a lack of a suitable antibody that is specific for porcine p53. Using a recombinant porcine p53, we generated a rabbit polyclonal antibody (designated SH0797) that is directed against porcine p53. The results of the study show that the antibody is capable of detecting recombinant p53 protein expressed in *Escherichia coli*, as well as FLAG-tagged p53 that is expressed in the transfected cells. This demonstrates that the antibody is specific for the porcine p53 protein. The antibody also showed the ability to immunoprecipitate p53 protein from extracts of porcine cells and to cross-react with human p53 protein. In addition, expression of porcine p53 could be induced readily in porcine cells and detected using this new tool. This antibody is a useful tool for use in studies of the cellular pathways that involve p53 in the porcine model.

The tumor suppressor p53 is one of the most studied of the proteins that are involved in cellular stress response pathways. These pathways, which lead to cell cycle arrest, DNA repair, cellular senescence and apoptosis, serve to protect the organism from stimuli that lead to DNA damage [1]. One of the major mechanisms by which p53 functions is as a transcription factor that regulates, both positively and negatively, the expression of a large and disparate group of responsive genes. These genes have an important role in mediating cell-cycle arrest, senescence and apoptosis [2]. As a component of the response to acute stress, p53 has a well established role in protecting against the development of cancer. However, recent studies have shown that p53 has a much broader role in a variety of cellular activities, such as the regulation of glycolysis [3,4], autophagy [5], oxidative stress [6], invasion and motility [7], angiogenesis [8], differentiation [9], bone remodeling [10], and antiviral responses [11].

Because of its close physiological and anatomical similarities with humans, when compared to other non-rodent species, the pig, and especially the miniature pig, has increasingly been used as a model in a variety of biomedical studies [12]. It has been used in studies of surgical techniques, and in research on the cardiovascular system, the digestive system, transplantation and xenografts, and syndromes such as ataxia–telangiectasia [13–17]. Despite the importance of the p53 molecule in the control of the cellular activities involved in such research models, the analysis of porcine p53 has been hampered by the lack of a suitable antibody directed against it. The commercial anti-p53 antibodies are raised against either human or mouse p53 proteins and have been shown to be poorly reactive with porcine p53 ([18]; our unpublished observations). The availability of suitable antibodies specific for the porcine p53 protein would, therefore, offer a powerful tool for use in studies of the p53 signaling pathway in the porcine model. In the present report, we describe the production and characterization of a polyclonal antibody directed against porcine p53.

Materials and methods

Cells and antibodies. Cells of the porcine kidney epithelial cell line (PK15), porcine testis cell line (ST), porcine vascular endothelial cell line (PIEC), human osteosarcoma cell line (Saos-2), human cervical carcinoma cell line (Hela) and murine fibroblast cell line (NIH3T3) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) in an atmosphere containing 5% CO_2. The commercial antibodies employed were an anti-FLAG monoclonal antibody (M2, Sigma, St. Louis, MO, USA), an anti-β-actin monoclonal antibody (AC-15, Sigma), an anti-p53 monoclonal antibody (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-p53 monoclonal antibody (Ab-1, Calbiochem, Cambridge, MA, USA).

Expression and purification of porcine p53 fusion protein. A sequence encoding the full length of porcine p53 was amplified by reverse transcription polymerase chain reaction (RT-PCR) using the forward primer 5′-ATATGGATGAGGCGCAGTCCGAGC-3′, and the reverse primer 5′-GCGTCAGTCTGAGTCAGGTCCTTCTCTC-3′.

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The RT-PCR product was inserted into the BamHI-HindIII cloning site of the expression vector pET-28a (Novagen, Madison, WI, USA) in order to express the histidine (His)-tagged porcine p53 protein (His-p53) heterologously in *Eshcerichia coli* strain BL21. The expression was induced by treatment with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 5 h, and the expression product was purified using a His Band kit (Novagen), according to the manufacturer's instructions. The relative mass of His-p53 and the purity of the preparation were assessed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie Blue.

Immunization. To produce polyclonal antibody against porcine p53, four New Zealand white rabbits were immunized with 1 mg of the purified His-p53 emulsified in complete Freund's adjuvant (Sigma). Subsequent booster immunizations of 1 mg of the purified His-p53 in incomplete Freund's adjuvant (Sigma) were given to each rabbit on days 14, 28, and 42. The rabbits were bled 7 days after the final immunization, following the procedures set down by the Guidelines for Animal Experimentation of the Shanghai Veterinary Research Institute. The titer of the antiserum was tested by enzyme-linked immunosorbent assay (ELISA).

Plasmids and transfection. Plasmids engineered to express FLAG-tagged porcine p53 protein (FLAG-p53) in Saos-2 cells were constructed by inserting the full-length porcine p53 cDNA into the HindIII-BamHI cloning site of the p3xFLAG-CMV-7.1 vector (Sigma). Saos-2 cells were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol, and were assayed 24 h post-transfection.

ELISA. A microtiter plate was coated with 2 μg/ml of the purified His-p53 in carbonate-buffered saline and incubated for 16 h at 4 °C. The plate was then washed with phosphate-buffered saline (PBS), and excess binding sites were blocked with PBS that contained 1% bovine serum albumin (Sigma). After incubation, 100 μl aliquots of antiserum at different dilutions were added to the appropriate wells and incubated for 1 h at 37 °C. The plate was washed and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Santa Cruz) at 37 °C. The reaction was initiated by adding *o*-phenylenediamine (Sigma) substrate solution, and the absorbance at 490 nm was determined using a bichromatic microplate reader.

Western blot. Cells were lysed in Nonidet P-40 (NP-40) buffer (1% SDS, 1% NP-40, 50 mM Tris (pH8.0), 150 mM NaCl, 4 mM Pefabloc SC, 2 mg/ml leupeptin, 2 mg/ml aprotinin). The lysates were briefly sonicated, boiled for 5 min, and then cleared by centrifugation for 10 min at 20,000*g* and 4 °C. The concentration of protein in the lysates was determined with the BCA protein assay reagent (Pierce Biotechnology, Rockford, IL, USA). The lysates were further denatured by incubation for 5 min at 95 °C in sample buffer (2% SDS, 10% glycerol, 60 mM Tris (pH 6.8), 5% β-mercaptoethanol, 0.01% bromophenol blue). Thereafter, the samples were subjected to SDS–PAGE. The samples were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA), which were incubated first in blocking buffer (5% non-fat milk powder in Tris-buffered saline containing 0.2% Tween 20 (TBS-T)) for 1 h at room temperature, and subsequently in a solution of primary antibody for 16 h at 4 °C. Finally, they were washed in TBS-T to removed unbound primary antibody, and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. After a further wash in TBS-T, the antibody–antigen complex was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Immunofluorescence. Cells grown on coverslips were washed in PBS, and fixed in 10% formalin/10% methanol for 20 min at room temperature. The cells were then incubated with blocking solution (10% normal goat serum in PBS) for 1 h, and probed with a primary antibody for 1 h. The antibody–antigen complexes were detected with either Alexa Fluor 488- or Alexa Fluor 594-conjugated goat secondary antibody (Molecular Probes, Eugene, OR, USA) following incubation for 1 h at room temperature. The cells were washed three times with PBS after each incubation, and then counterstained for DNA with 4′,6′-diamidino-2-phenylindole (DAPI). Cells were examined under a fluorescence microscope. The fluorescence images were captured with a SPOT CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Immunoprecipitation. Cells were lysed in NP-40 lysis buffer (1% NP-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 100 mM Na_3VO_4). The lysates were precleared by incubation with Recombinant Protein G Agarose beads (Invitrogen) for 1 h at 4 °C. The lysates were then incubated with antibody on a rotating wheel for 6 h at 4 °C. Protein G agarose beads were then added and the mixture was further incubated on a rotating wheel for 2 h at 4 °C. The agarose beads were pelleted and washed three times in the NP-40 lysis buffer. Antibody–antigen complexes bound to the beads were eluted in the sample buffer by boiling, resolved by SDS–PAGE, and analyzed by Western blot with appropriate antibodies.

Induction of p53 Response. To induce the p53 response, cells were treated with the p53-activating drug doxorubicin (Dox). Doxorubicin (Sigma) was added at 0.1, 0.5, 1.0, and 1.5 μg/ml to the medium, and incubated for 24 h at 37 °C. The p53 responses were investigated using Western blot and immunohistochemical analysis with the antibody produced by the method described above.

Results

Production of polyclonal antibody against the porcine p53 protein

To generate the recombinant antigen for immunization, porcine p53 cDNA was amplified by RT-PCR and heterologously expressed with a histidine tag in *E. coli*. As shown in Fig. 1, induction of *E. coli* using IPTG resulted in a high level of expression of a 48 kDa protein with a molecular mass corresponding to that of His-p53 (lane 2). The expressed product was purified by Ni^{2+} affinity chromatography using Ni-NTA beads. Peak A_{280} fractions eluted from the column were pooled and showed a predominant single His-p53 band (lane 4). The purified His-p53 was concentrated and injected into four rabbits for production of antibodies. The titer of the antiserum was determined by ELISA and the highest dilution was 1:1,000,000,000 (data not shown). The antiserum with the highest ELISA titer was designated SH0797 and used for subsequent experiments.

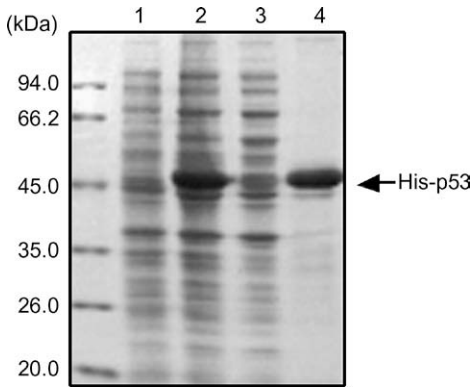

Fig. 1. Analysis of the expression and purification of His-tagged recombinant porcine p53 protein (His-p53). Bacterial lysates and purified His-p53 were separated on a 15% SDS–PAGE gel and stained with Coomassie blue R250. Lane 1, bacterial cells transformed with empty expression vector; Lane 2, bacterial cells induced with isopropyl-β-D-thiogalactopyranoside (IPTG); Lane3, uninduced bacterial cells; Lane 4, purified His-p53 protein.

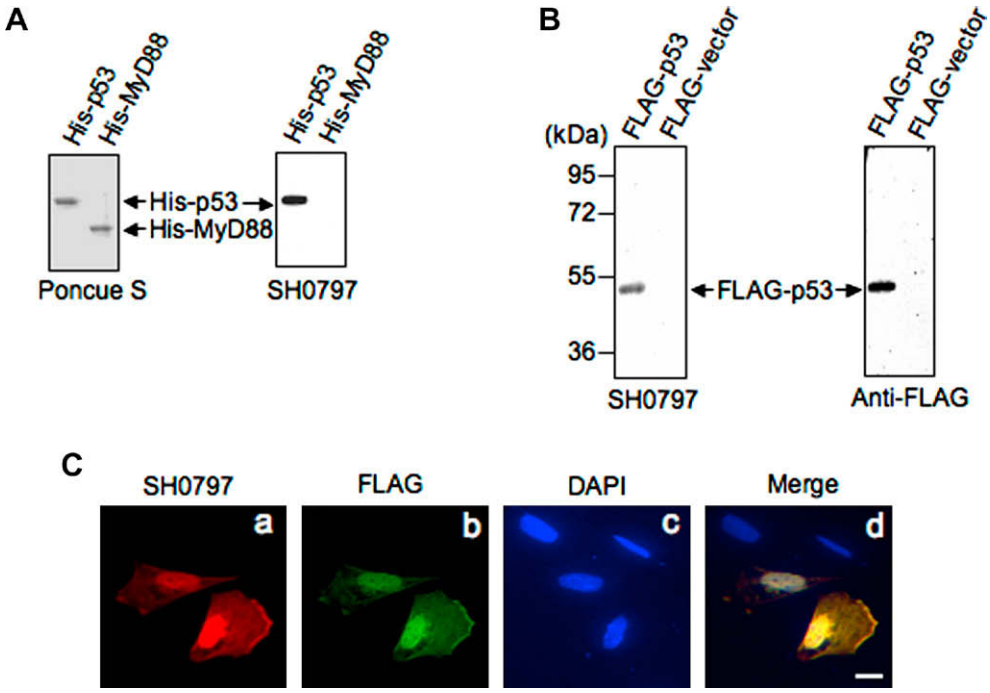

Fig. 2. Analysis of the specificity of the antibody produced, SH0797. (A) The antibody SH0797 reacted with recombinant porcine p53 protein. 400 ng of purified His-p53 and His-MyD88 were electrotransferred to an Immobilon-P membrane after SDS–PAGE, and stained with Poncue S (Sigma) (left panel) and the antibody SH0797 (right panel). (B) Western blot analysis of FLAG-p53 expressed in p53-null Saos-2 cells. Cell lysates (20 μg) from Saos-2 cells transfected with plasmid expressing FLAG-p53 or FLAG-vector were probed with the antibody SH0797 (left panel) and then re-probed with anti-FLAG antibody (right panel). (C) Immunofluorescence analysis of FLAG-p53 expressed in Saos-2 cells. Saos-2 cells transfected with plasmid expressing FLAG-p53 were double-immunostained with the antibody SH0797 (panel a, red) and anti-FLAG antibody (panel b, green). The cells were also stained for DNA with 4′,6′-diamidino-2-phenylindole (DAPI) (panel c, blue). Panel d shows the superimposed image. Bar: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Antibody SH0797 specifically recognizes the porcine p53 protein

The antibody SH0797 was prepared using the recombinant porcine p53 protein tagged with six His residues, and therefore it is possible that it could react with the His tag rather than with the p53 protein. Western blot analysis of the His-tagged proteins revealed specific reactivity with His-p53, and no reactivity with His-MyD88 [19] (Fig. 2A), indicating that antibody SH0797 detects the p53 protein rather than the His tag. As an independent test to analyze the specificity of the antibody, we transfected p53-null Saos-2 cells with a plasmid expressing FLAG-p53 or with the FLAG empty vector. The transfectants were then subjected to Western blot and immunofluorescence analysis using the antibody produced in this study as a probe. The Western blot analysis revealed that the antibody SH0797 specifically reacted with the FLAG-p53 protein expressed in Saos-2 cells (Fig. 2B). The immunofluorescence analysis demonstrated the staining of cells transfected with the plasmid expressing FLAG-p53 (Fig. 2C). Taken together, these results indicate that the antibody SH0797 is highly specific and can detect the porcine p53 protein expressed in mammalian cells as well as that expressed in *E. coli*.

Cross-reactivity

The cross-reactivity of the antibody SH0797 was analyzed by Western blot. The lysates from Hela, NIH3T3 and PIEC cells were immunoblotted with the antibody SH0797, and then re-probed with either the anti-p53 antibody DO-1 (for human p53) or the anti-p53 antibody Ab-1 (for murine p53). The antibody SH0797 recognized the human p53 protein (Fig. 3A, left panel), despite the presence of two nonspecific bands, but did not react with the murine p53 protein (data not shown). The antibody Ab-1 did not

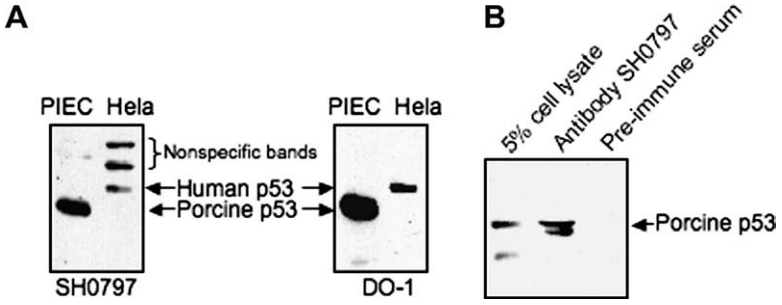

Fig. 3. (A) Western blot analysis of the cross-reactivity of the antibody SH0797 with human p53 protein. Cell lysates (20 μg) from PIEC and Hela cells were probed with the antibody SH0797 (left panel) and then re-probed with the anti-p53 antibody DO-1 (right panel). (B) Immunoprecipitation of porcine p53 protein with the antibody SH0797. The PIEC cell lysates were immunoprecipitated with the antibody SH0797 or the pre-immune serum. The immunprecipitates were immunoblotted with the antibody DO-1. Five per cent of the amount used for immunoprecipitation was used as a reference.

recognize porcine p53 (data not shown), which is consistent with the observations previously described by Burr et al. [18]. Unexpectedly, the antibody DO-1, which is supposed to be specific for human p53 protein, showed an ability to react with porcine p53 (Fig. 3A, right panel).

Immunoprecipitation of p53 protein from porcine cells

The ability of the antibody SH0797 to immunoprecipitate the p53 protein from porcine cells was tested. The PIEC cell lysates were immunoprecipitated with the antibody, and the pre-immune serum was used as a control. The immunoprecipitates were immunoblotted with the antibody DO-1, which has been shown to recognize porcine p53, as described in the "cross-reactivity" section. As shown in Fig. 3B, a distinct 46 kDa protein corresponding to porcine p53 was immunoprecipitated with the antibody SH0797. An equivalent band was not detected in the immunoprecipitates pulled down with the pre-immune serum, suggesting that the antibody was able to immunoprecipitate porcine p53 protein. A protein band that was slightly lower than the 46 kDa p53 band was also detected by the antibody DO-1, which presumably represented p53 protein that had decayed during the immunoprecipitation process.

Antibody SH0797 as a reagent for studying the p53 pathway in pigs

Given that the antibody SH0797 showed specificity for porcine p53, it should serve as a suitable reagent for use in studies of the p53 pathway in pigs. Porcine cells were treated with Dox, a drug that stabilizes and activates p53 protein [20], at the indicated concentrations (Fig. 4A). The intracellular levels of p53 were monitored by Western blot analysis using the antibody SH0797 as a probe. After exposure to Dox, an increase in the level of the p53 protein was detected in both PK15 and ST cells, but not in PIEC cells, compared with the untreated cells (Fig. 4A). It is known that p53 accumulates in the nucleus in response to many stresses, therefore we next visualized the subcellular localization of p53 in the Dox-treated cells by immunofluorescence analysis with the antibody SH0797. The untreated ST cells showed only weak staining of p53 in the nucleus (Fig. 4B, panel a). When the cells were exposed to 1 μg/ml Dox, however, strong nuclear staining was observed (Fig. 4B, panel d). In the untreated PIEC cells, p53 was distributed in both the nucleus and the cytoplasm (Fig. 4B, panel g). In the treated PIEC cells, the p53 signal did not increase significantly compared with that in the untreated cells, but accumulation of p53 protein in the nucleus was observed (Fig. 4B, panel j). These data demonstrate that the antibody SH0797 could be used for studying the p53 pathway in porcine models of human diseases.

Discussion

The objective of our study was to produce and characterize a polyclonal antibody specific for the porcine p53 protein. There are a few commercial antibodies that cross-react with porcine p53, but their reactivity and specificity is inadequate (our unpublished observations). Therefore, we decided to produce a polyclonal antibody that was specific for porcine p53. For production of the antibody, recombinant porcine p53 was heterologously expressed and highly purified for immunization of rabbits (Fig. 1). The antibody SH0797 that was produced from the immunized rabbits specifically recognized the recombinant porcine p53 protein expressed in *E. coli* (Fig. 2A), which indicated that the antibody is capable of recognizing the porcine p53 protein. Its specificity was further tested by analysis of Saos-2 cells that had been transfected with FLAG-tagged p53, using Western blot and immunofluorescence analysis. The antibody SH0797 specifically reacted with the FLAG-p53 that was expressed in Saos-2 cells (Fig. 2B and C). Taken together, these results indicate that the antibody SH0797 is highly specific and recognizes the porcine p53 protein in the denatured as well as the native form. The antibody SH0797 is also capable of immunoprecipitating p53 protein from porcine cells (Fig. 3B).

The anti-human p53 antibody DO-1 was produced to recognize human p53 specifically. Unexpectedly, we found that the antibody DO-1 reacted with porcine p53. This may therefore provide an alternative reagent to use in studies of porcine p53. For instance, we used the antibody DO-1 to detect porcine p53 in the immunoprecipitates pulled down with the antibody SH0797 (Fig. 3B).

Burr et al. [18] reported that porcine p53 is a protein of approximately 50 kDa when identified by SDS–PAGE, whereas we found that the molecular weight of porcine p53 was approximately 46 kDa. This is probably due to the different methods used for the

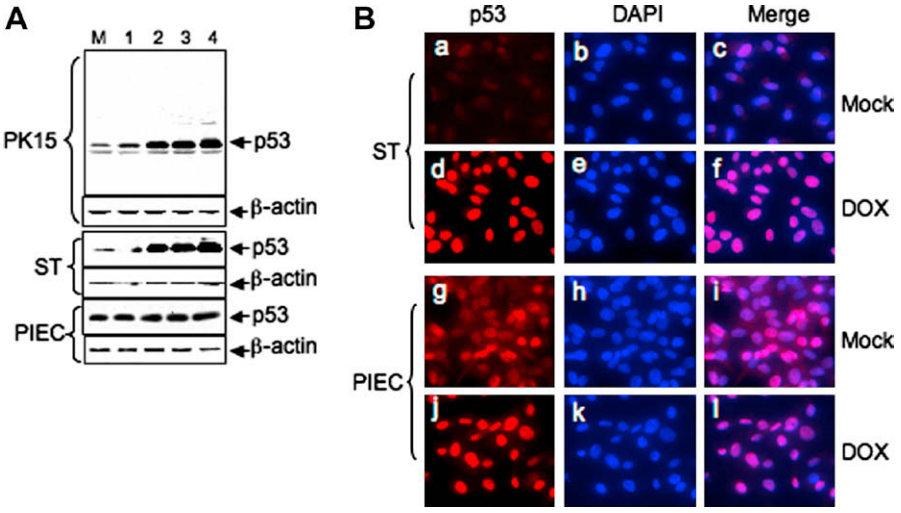

Fig. 4. Analysis of p53 responses in cells treated with doxorubicin (Dox). (A) Western blot analysis of p53 responses in Dox-treated cells. The lysates from cells treated with Dox were immunoblotted with the antibody SH0797 and re-probed with anti-β-actin antibody. The β-actin was used as a protein loading control, and untreated cells were used as negative controls. M, untreated cells; Lane 1, cells treated with 0.1 μg/ml Dox; Lane 2, cells treated with 0.5 μg/ml Dox; Lane 3, cells treated with 1.0 μg/ml Dox; Lane 4, cells treated with 1.5 μg/ml Dox. (B) Immunofluorescence analysis of the subcellular localization of p53 in response to Dox treatment. Cells treated with 1 μg/ml Dox and the untreated cells (Mock) were immunostained with the antibody SH0797 (panels a, d, g, and j, red). The cells were also stained for DNA with DAPI (panels b, e, h, and k, blue). Panels c, f, i, and l show the superimposed image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

preparation of the porcine p53 protein. We prepared the p53 protein by expression in either *E. coli* or mammalian cells, which both contain a post-translational modification system that will modify the newly synthesized protein. In contrast, Burr et al. [18] prepared the p53 protein using an in vitro transcription/translation system, which did not modify the newly synthesized protein.

In conclusion, a rabbit polyclonal antibody, SH0797, was generated against porcine p53 using recombinant porcine p53 protein as the immunogen. The antibody produced is highly specific and recognizes the porcine p53 protein in the denatured as well as the native form. The antibody is capable of immunoprecipitating p53 protein from porcine cells. In addition, the porcine p53 protein can be induced readily by administration of a p53-activating drug and detected using this new antibody. This antibody is a useful tool for use in studies of the p53 pathways in porcine models of human disease.

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