

The Regulation of Exosome Secretion: a Novel Function of the p53 Protein

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Abstract

The p53 protein responds to stress signals by regulating the transcription of a variety of genes. Some of these genes encode secreted proteins that may be involved in the communication between adjacent cells. In this study, a proteomics approach was employed to identify proteins secreted by cells in a p53-dependent manner after DNA damage. In addition to the known transcriptional targets of p53, a set of proteins encoded by genes that are not transcriptional targets of p53 were found to increase in the culture medium after p53 activation. These proteins exit the cell via small, secreted vesicles called exosomes and exosome production by cells was found to be regulated by the p53 response. A p53-regulated gene product, TSAP6, was shown to enhance exosome production in cells undergoing a p53 response to stress. Thus, the p53 pathway regulates the production of exosomes into the medium and these vesicles can communicate with adjacent cells and even cells of the immune system. (Cancer Res 2006; 66(9): 4795-802)

Introduction

The p53 protein and its signal transduction pathway respond to a wide variety of stress signals including genotoxic stress, hypoxia, and even the expression of activated oncogenes (1). After DNA damage, the p53 protein is activated to become a transcription factor and it enhances or represses the transcription of a number of genes (1, 2). This results in the creation of a cellular program for one of several responses to the DNA damage: cell cycle arrest, senescence, or apoptosis (2). Among the genes which are positively regulated by the p53 protein are a number of genes encoding secreted proteins which may act to regulate growth factors (insulin-like growth factor binding protein-3 or IGFBP-3), inhibit proteases that may alter the extracellular matrix (maspin, plasminogen activator inhibitor or PAI-1), or even change the extracellular matrix to block angiogenesis or cell migration (thrombospondin; ref. 3). Thus, part of the p53 response to stress is to produce secreted proteins that can communicate with adjacent cells, which may not themselves be damaged, but may then produce a more coordinated response to stress by a group of cells or a tissue.

This phenomenon has been called the "bystander effect," which has been documented in a wide variety of studies (reviewed in refs. 4–6). For example, exposure to a very low dose of α particles with only 2% of the cell nuclei damaged in a culture can change the gene expression in bystander cells (p53, p21, p34^{CDC2}, cyclin B1) through intercellular communication (7, 8). Even conditioned medium from cells treated with α particles had the ability to alter gene expression

patterns in nonirradiated cells treated with this conditioned medium (9, 10). Several studies have suggested various mechanisms that mediate this communication between cells, including the transfer of information through gap junctions, the alteration of the extracellular matrix, or via soluble proteins in the medium (9–11). A manifestation of this phenomenon *in vivo* has been suggested to occur after p53-mediated gene therapy of tumors in which an adenovirus carrying a p53 cDNA infects a cancer cell and the surrounding cells are affected by this event (12–14).

Classical secretion is mediated by a signal sequence at the NH₂ terminus of a secreted protein that directs the protein through the endoplasmic reticulum/Golgi pathway followed by transport to the plasma membrane via vesicles and release of the protein into the extracellular space in a soluble form. In addition to these soluble proteins, secretion can be affected by vesicles termed exosomes (15). Based on observations made by electron microscopy, intracellular vesicles may be generated by "inward" budding from the limiting membrane into the lumen of endosomes and engulfing cytoplasmic components, and these vesicles are packaged into multivesicular bodies. The multivesicular bodies may then fuse with the lysosome, resulting in protein degradation, or alternatively, multivesicular bodies may fuse with the plasma membrane, thus, releasing cargo-loaded small vesicles into the extracellular space (16). These vesicles are termed exosomes. It has been suggested that exosomes may play a role in several different physiologic processes (17).

Recently, Telerman and colleagues showed that overexpression of TSAP6, a multi-pass transmembrane protein, could facilitate the secretion of the histamine-releasing factor (also called TCTP) via exosomes and suggested a role for TSAP6 in either the selective transport of proteins to the exosome or a more general role in regulating exosome production (18). *TSAP6* is a p53-regulated gene and is transcribed in response to stress (19). Thus, the requirement for p53 in exosome production might be through the ability of p53 to up-regulate *TSAP6* transcription in response to DNA damage.

The experiments reported here were designed to examine both the secretion of soluble proteins and proteins in exosomes after a p53-mediated stress response of cells in culture. Two human non-small cell lung cancer cell lines, one containing a wild-type *p53* gene (H460) and the other a mutated *p53* allele (H1299) were treated with γ radiation to induce p53 and apoptosis. By examining both the soluble proteins in the medium and the exosomal particles in this medium, generated by these two cell lines untreated or treated with γ radiation, a novel p53-regulated mechanism emerged. After the activation of the p53 protein in the H460 cells, there was an increased level of secretion of p53-regulated gene products as soluble proteins and a dramatic increase in exosomal vesicles in the medium. This increased exosome production was not observed in untreated cells (no γ radiation) nor in γ irradiated cells that contained a mutant *p53* allele. The cells with short interfering RNA (siRNA) to knock down the *p53* gene produced less exosomes. Mouse embryo fibroblasts (MEFs) with a wild-type *p53*

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gene produced exosomes after DNA damage and G₁ arrest but isogenic MEFs with no *p53* genes (from knockout mice) failed to produce exosomes after DNA damage. Expression of a wild-type *p53* gene allows the *p53*-null cells to produce exosomes. Finally, expression of the *TSAP6* gene allows the cells with or without functional *p53* to produce exosomes, suggesting *TSAP6* involvement in the *p53*-regulated exosome secretion pathway. The secretion of both soluble proteins and exosome vesicles occurred after γ radiation of cells and *p53* activation even when apoptosis was blocked and no cell death occurred. The exosomal vesicles may well communicate with the surrounding cells via phagocytosis, which occurs after apoptosis, or they might communicate with the cells of the immune system which are known to be activated by apoptotic bodies. A *p53*-regulated exosome production suggests additional functions of the *p53* response to stress.

Materials and Methods

Cell culture, DNA damage treatment, and transfection. H460 was purchased from the American Type Culture Collection (Rockville, MD) (ATCC HTB-177). It was cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum. H460 is able to grow in serum-free medium (Life Technologies) supplemented with glutamine (20). H1299 was purchased from American Type Culture Collection. It was cultured in DMEM (Life Technologies) with 10% fetal bovine serum. The wild-type and *p53* MEFs were generated as described previously (21). Before being treated with γ -irradiation, the cells were seeded to 25 cm² flasks and settled for 24 hours. Culture medium was replaced with fresh serum-free medium immediately prior to γ -irradiation. Cell cultures were irradiated with 5 Gy (CIS BioInternational IBL 437C ¹³⁷Cs γ -irradiation source; dose rate, 0.49 Gy/min). Post-irradiation incubation time varied (see below for details) from 0 to 48 hours. All cells were grown at 37°C with 5% CO₂. The pRC/CMV-wt *p53* expression plasmid was generated as described before (22). The plasmids of pcDNA-3.1-HA-*TSAP6* and the vector were gifts from Dr. A. Telerman (Molecular Engines Laboratories, France). siRNA for *p53* and *TSAP6* were purchased from Dharmacon (Chicago, IL). The siRNA for lamin A/C and a nonspecific siRNA were purchased from Qiagen (Chatsworth, CA). The plasmids were transfected with LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and the siRNA was transfected with OligofectAMINE (Invitrogen).

Culture medium collection and cell lysis. Culture media were collected at different time points. Any cell debris was removed by shortly spinning at 300 × *g*. The conditioned medium was then concentrated using Amicon Ultra-4 Centrifugal filter device (Millipore, Bedford, MA). Cell lysis was done by incubating the cells in the cell lysis buffer [50 mmol/L Tris (pH 7.5), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 5 mmol/L EDTA] and a protease inhibitor cocktail (Complete Mini; Roche Applied Science, Germany) for 30 minutes with gentle shaking followed by centrifugation at 14,000 rpm for 20 minutes at 4°C. The supernatant was the cell lysate. Protein concentration was measured using Bio-Rad assay of the Bradford method. Extracts were stored at -80°C until use.

Isolation of the components of the culture medium and identification using mass spectrometry. Concentrated medium was separated on SDS-PAGE (4-20% gradient; Bio-Rad, Hercules, CA) followed by silver staining (Invitrogen). Concentrated medium at 16-hour and 24-hour time points (post-irradiation incubation times) were then separated on SDS-PAGE (4-20%, gradient; Invitrogen), followed by SimplyBlue staining (Invitrogen) for mass spectrometry analysis. Mass spectrometry analysis was done using MALDI-TOF MS protein identification based on peptide mass database searching by the facility at the Center for Advanced Proteomics Research at University of Medicine and Dentistry of New Jersey (UMDNJ)-Newark (Newark, NJ).

Western blot. The concentrated medium was run on SDS-PAGE (4-20%) at different time points and transferred to Immobilon-P membranes (Millipore). The membranes were blotted with the following antibodies, heat shock protein 90- β (Hsp90 β ; D-19, sc-1057), maspin (G167-70, 554292; BD Biosciences Pharmingen, San Diego, CA), PAI-1 (C-9, sc-5297), cyclophilin A

(CyPA; 07-313, Upstate, Charlottesville, VA), peroxiredoxin 1 (PRDX-1; C-18, sc-23970), cathepsin L (CTSL; S-20, sc-6500), IGFBP-2 (C-18, sc-6001), phosphoglycerate kinase 1 (PGK1; Y-12, sc-17943), Enolase 1 (Eno1; C-19, sc-7455), and elongation factor-1 α (EF-1 α ; EF-Tu; CBP-KK1, sc-21758). Other antibodies used in this study include *p53* (DO-1, sc-126), *p53* (FL-393, sc-6243), p21 (Ab-1, OP64; Oncogene Research Products, San Diego, CA), HLA-DR α (FL-254, sc-25614), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; FL-335, sc-25778). All antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, except where indicated. The transfer and blotting procedure were according to the manufacturer's instructions for Immobilon-P membranes (Millipore) and enhanced chemiluminescence detection reagents (Amersham Biosciences Corp., Piscataway, NJ). Cell lysates were also used for Western blot to check the intracellular protein level.

Real-time PCR. Total RNA was isolated at different time points using Trizol (Invitrogen) followed by one-step reverse transcription (TaqMan reverse transcription reagents, Applied Biosystems, Foster City, CA) and real-time PCR (ABI Prism 7000 sequence detection system, Applied Biosystems). GAPDH is used as an internal control. Probes and primer sets were purchased as predeveloped assays from Applied Biosystems. Each sample was done in triplicate and each experiment was repeated. The relative induction/repression level was calculated by the ratio of the value of the gene to that of GAPDH and then to the 0-hour time point.

Exosome isolation. To isolate exosomes, the conditioned medium was collected, and subjected to differential centrifugations: 300 × *g* for 5 minutes, and 1,200 × *g* for 20 minutes to remove cell debris. Exosomes were then pelleted at 70,000 × *g* for 1 hour and washed with PBS at 100,000 × *g* for 1 hour using Swinging-bucket rotor AH-650 (Kendro, Asheville, NC), all at 4°C. Exosomes were resuspended in PBS and aliquoted. For electron microscopy, exosome isolates were stored at 4°C, and the other aliquots were stored at -80°C.

Electron microscopy. Exosomes isolated from the culture medium using differential centrifugation were loaded onto Formvar carbon-coated grids. Negative staining was done with 0.5% uranyl acetate. After removing the excess fluid, the grid was air-dried and then analyzed with a JEOL 1200 EX electron microscope operated at 80 kV (Electron Microscopy Facility at UMDNJ-Piscataway, NJ).

Sucrose gradient fractionation. The sucrose gradient was prepared as 1 mL each of 60%, 40%, 30%, and 20% sucrose and 0.5 mL of 10% sucrose. Using differential centrifugation, 250 μ L of exosome isolates were loaded onto the top of the gradient, followed by immediate centrifugation at 270,000 × *g* for 18 hours in rotor AH-650 (Kendro). After centrifugation, 1 mL fractions were collected from the bottom of the gradient [designated fraction number 1 (bottom) through 7 (top)]. The fractions were then diluted in 3 mL PBS and concentrated with centrifugation at 348,000 × *g* for 1 hour in fixed angle rotor S100-AT6 in Discovery M150 SE Micro-ultracentrifuge (Kendro). The pellets were resuspended in 40 μ L 1× SDS-PAGE loading buffer and boiled. Twenty microliters of each sample was separated on SDS-PAGE followed by Western blot.

Results

The kinetics of the secretion of proteins after a *p53*-dependent response to γ -radiation. Two human non-small cell lung cancer cell lines, H460, with a wild-type *p53* allele, and H1299, with a partially deleted *p53* allele, were incubated in serum-free medium after no treatment or after treatment with 5 Gy of γ radiation. At various time points, the culture medium was collected and concentrated, and the proteins were separated by SDS-PAGE and detected by silver staining (Fig. 1A). In H1299 cells, with no functional *p53* protein, the proteins in the medium did not detectably change with γ radiation. By contrast, within 12 to 16 hours after γ radiation the protein composition of the medium from H460 cells was altered and the abundance of several proteins increased, whereas others declined. To identify those proteins, the protein bands were excised from a larger-scale gel and the digested peptides were analyzed using a MALDI-TOF mass spectrometer.

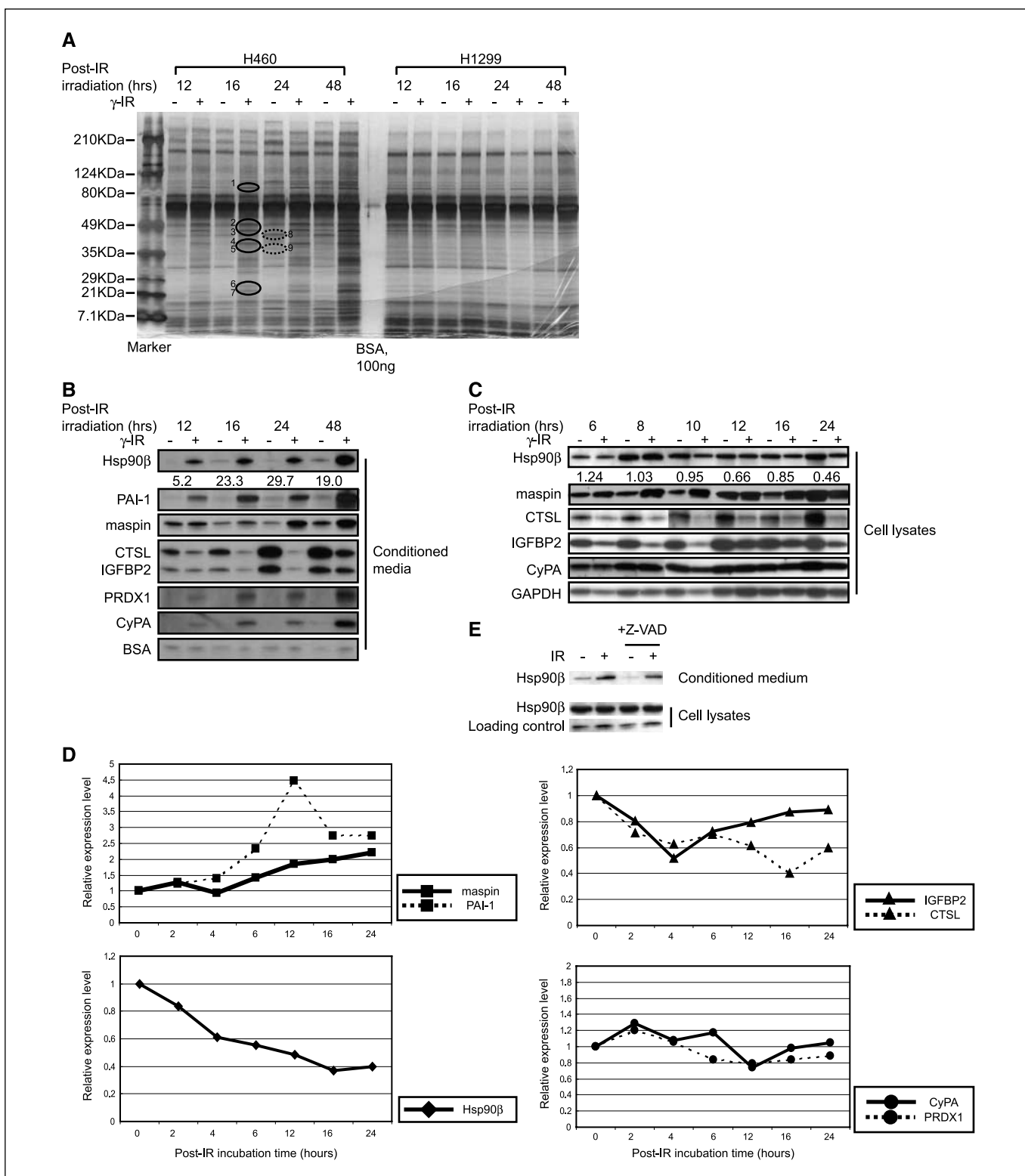


Figure 1. Different secretion patterns upon p53-regulated response to γ -irradiation. **A**, silver staining analysis of the conditioned medium from H460 (wild-type p53) and H1299 (p53-null) after γ -irradiation (5 Gy) for varied time periods. The proteins were identified by MALDI-TOF mass spectrometry. 1, Hsp90 β ; 2, EF1 α ; 3, Eno1; 4, γ -actin; 5, PGK1; 6, PRDX1; 7, CyPA; 8, CTSL; 9, IGFBP2. **B**, confirmation of the protein components in culture medium from H460 cells by Western blot. The goat-derived antibody can recognize bovine serum albumin while probing the other proteins. The relative levels of Hsp90 β are quantified by comparing the protein levels in the conditioned medium from irradiated and nonirradiated cells. **C**, Western blot analysis of the protein expression in H460 cells. The protein level of Hsp90 β at each time point is quantified by comparing the amount of protein in the cell lysates from irradiated and nonirradiated cells, after being normalized with GAPDH. **D**, transcriptional regulation of the genes identified in the assay in H460 cells. Maspin and PAI-1 are induced by p53. Hsp90 β transcription is repressed by p53. IGFBP2 and CTSL are repressed by p53. Expressions of CyPA and PRDX1 are not modified. **E**, secretion of Hsp90 β in the conditioned medium from H460 cells is not the result of apoptosis. H460 cells were either treated with Z-VAD-Fmk 9 (+Z-VAD) or without treatment followed by irradiation. The collected conditioned medium and cells after 24 hours of incubation were analyzed by Western blot.

This process identified the nine proteins indicated in the legend to Fig. 1A. The protein identity was then confirmed by Western blots employing specific antibodies (Fig. 1B; data not shown). In addition, Fig. 1B shows that the concentration of PAI-1 and maspin, both known secreted proteins encoded by p53-inducible genes, are increased in the conditioned medium after stress. Together, these results confirmed that the concentration of several proteins, Hsp90 β , PAI-1, maspin, PRDX-1, and CyPA, increased in the culture medium after DNA damage, whereas others, CTSL and IGFBP-2, decreased. The simultaneous increase and decrease of specific proteins in the medium suggests the active regulation of this process rather than a nonspecific leakage of all proteins from the cells.

The regulation of protein expression in the cell. Western blot analysis was used to determine if the extracellular level of the secreted proteins is reflected in altered intracellular protein concentrations (Fig. 1C). To examine the regulation of these genes at the mRNA level after irradiation of cells in culture, the RNA was isolated from H460 cells at various times after irradiation (0, 2, 4, 6, 12, 16, and 24 hours) and the levels of RNA were determined using real-time PCR for seven of these genes (Fig. 1D). As expected, because maspin is a p53 target gene (23), the concentration of maspin protein increases in both the intracellular and extracellular compartments after radiation. Treatment of H460 cells with radiation results in increased transcription of both the maspin (a 2-fold increase) and PAI-1 (a 5-fold increase) genes (Fig. 1D). The concentration of CTSL and IGFBP-2 proteins both declined with time in the intracellular and extracellular compartments after irradiation (Fig. 1B and C) and the expression of mRNA from both genes showed a small decline (Fig. 1D). Thus, for maspin, PAI-1, CTSL, and IGFBP-2 there is a positive correlation between the amount of gene expression, intracellular protein expression, and extracellular concentration after DNA damage. Interestingly, several other proteins do not show this correlation. For example, the gene expression and intracellular protein concentrations of CyPA and PRDX1 remained constant after irradiation, whereas the concentration of both proteins in the medium increased. For Hsp90 β , gene transcription and intracellular protein concentration declined by approximately 2-fold, whereas the extracellular concentration increased almost 30-fold in response to irradiation (Fig. 1A-D). Together, these results suggest that in addition to the ability of p53 to respond to stress by inducing the transcription of genes that encode secreted proteins (PAI-1 and maspin), DNA damage induces a change in the profile of secreted proteins that is not dependent on changes in their intracellular concentrations.

Once again, these data argue that irradiation and the p53 response (this did not happen in cells with a mutant *p53* gene) actively regulates the secretion of proteins whether or not the levels of these proteins in the cell change. These results also strongly argue against the idea that cell lysis or leakage is giving rise to these observations.

Secretion is not the result of apoptosis. At the time proteins began appearing in the medium (16-24 hours post-radiation), 10% to 20% of the H460 cells were undergoing apoptosis. By contrast, the H1299 cells, with a mutant *p53* gene, fail to undergo apoptosis after DNA damage. Thus, the proteins detected in the medium could arise as a result of a p53 response, apoptosis, or both. To examine these variables, the H460 cells were irradiated following treatment with Z-VAD-Fmk 9 [carbobenzoxy-valyl-alanyl-aspartyl-(*O*-methyl)-fluoromethylketone] a pan-caspase inhibitor that blocks the caspase activation (and therefore apoptosis) downstream of p53 activation. Under these conditions, the apoptosis of H460 cells was reduced to <1%. The concentration of the Hsp90 β

protein in the medium was identical to the H460 cells irradiated and p53 activated but not treated with Z-VAD-Fmk 9 (Fig. 1E), demonstrating that p53-mediated events upstream from caspase activation are responsible for the pattern of secretion from irradiated cells and that apoptosis is not required.

Distinct secretory pathways regulated by the p53-dependent response to DNA damage. Classical secretion is mediated by a signal sequence for a secreted protein. The PAI-1, IGFBP-2, and CTSL proteins each contain a signal sequence that can be identified by employing the SignalP software (24). Maspin can be identified as a secreted protein without a signal sequence (25) and has been shown to have an uncleaved facultative secretion signal that allows small amounts of the protein to be secreted via the endoplasmic reticulum/Golgi pathway (26), however, the CyPA, PRDX-1, PGKI, Eno1, translation EF-1 α , and Hsp90 β proteins are not predicted by any criteria to be secreted proteins. This led to a test of a hypothesis that these proteins are transported outside the cell in vesicles, and if so, vesicle transport might be regulated by the p53 protein.

To examine this idea, the culture media from nonirradiated and irradiated H460 cells were subjected to a differential centrifugation procedure that has been used to isolate vesicles (named exosomes) from several cell types (for example, refs. 27, 28). The vesicular nature of these fractions was confirmed by electron microscopy as shown in Fig. 2A (*bottom*) in which vesicles of the characteristic size and shape of exosomes (cup-shaped, 50-150 nm in diameter) are visualized. These vesicles were below the level of detection in the medium of nonirradiated H460 cells (Fig. 2A, *top*). Vesicles were only detected in the culture medium from cells containing wild-type p53 that were exposed to γ radiation, suggesting that the enhanced production of these vesicles, which have been previously characterized as exosomes, is regulated by p53. As a further characterization, the vesicles were sedimented through a sucrose gradient and were found to band at a density of 1.14 g/cm³, as shown by the presence of the marker protein Hsp90 β in Fig. 2B. This is the density previously reported for exosomes (28-32).

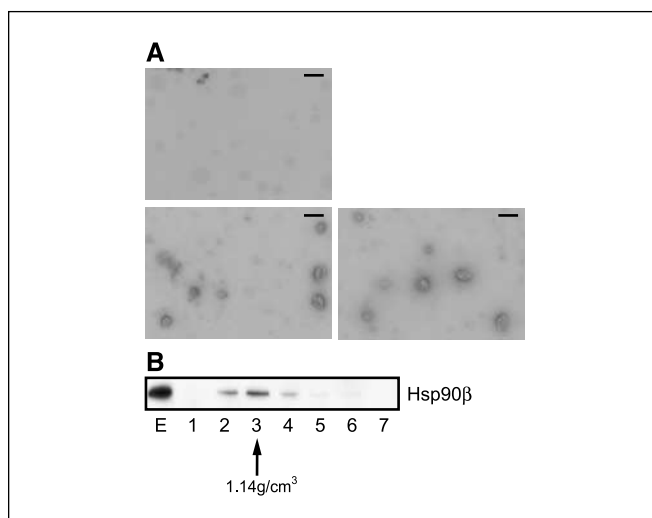


Figure 2. Characterization of the exosomes from the conditioned medium. A, electron microscopy of the exosome isolations. Exosomes are cup-shaped and 50 to 150 nm in size. *Top*, conditioned medium without irradiation; *bottom*, conditioned medium with irradiation; *bar*, 200 nm. B, Western blot of Hsp90 β in the sucrose gradient fractions of the exosome isolations. Fractions 1 to 7 were collected from the bottom to the top. E, the exosome isolation, as loading control.

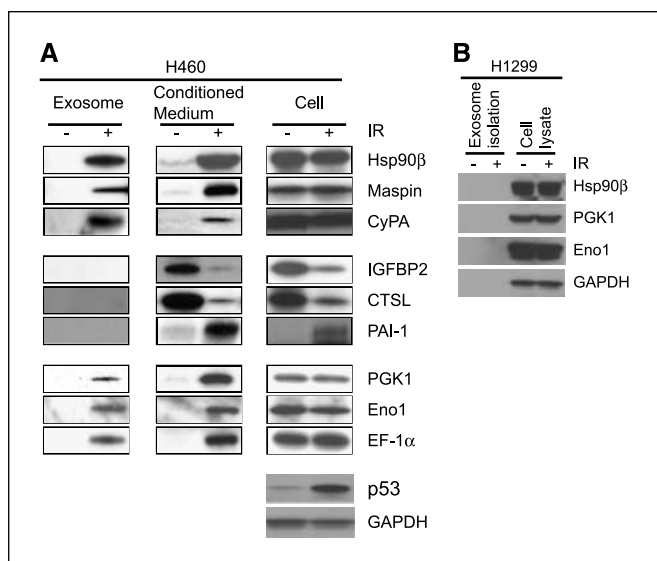


Figure 3. Secretion of proteins and exosomes in H460 and H1299 cells after γ -irradiation. *A*, in H460 cells, Hsp90 β , maspin, CyPA, IGFBP2, CTSL, PAI-1, PGK1, Eno1, and EF-1 α were detected in the exosome isolation by Western blot. IGFBP2, CTSL, and PAI-1 were not present in the exosome, as they are known secreted proteins with traditional signal peptides. p53 is induced by γ -irradiation in H460 cells. *B*, in H1299 cells, although the proteins Hsp90 β , PGK1, and Eno1 (as well as GAPDH) were expressed, they were not secreted in the exosomes, nor were they detectable in the conditioned medium (data not shown).

Proteins in the exosome. In a comparison of nonirradiated and irradiated H460 and H1299 cells, only the irradiated H460 cells produced the exosomes shown in the Western blot analysis of the proteins in the medium and the purified exosomes (Fig. 3*A* and *B*). This figure shows that, in addition to Hsp90 β , maspin, CyPA, PGK1, Eno1, and EF-1 α were detected in the exosome fraction from irradiated p53 wild-type cells but not p53-null H1299 cells. However, whereas IGFBP-2, CTSL, and PAI-1 were secreted into the medium from H460 cells, they were not detected in the exosome (Fig. 3*A*). All of the secreted proteins excluded from the exosome have a signal sequence. Thus, the exosome may sample the soluble cytoplasmic proteins but it does not capture the proteins in the vesicles of the secretory pathways of the cell. The presence of maspin in the exosome is interesting in that its secretion has been enigmatic. Maspin has been detected in the extracellular space in a number of systems, both *in vitro* and *in vivo*, and has shown extracellular activity, yet it has no NH₂-terminal signal sequence. Thus, it is possible that the majority of maspin reaches the extracellular space via exosomes.

The p53 requirement for enhanced production of exosomes after DNA damage. The experiments presented above all suggested that the activation of the p53 protein could regulate the enhanced production of exosomes. Three experiments were carried out to test this. Primary cultures of MEFs derived from wild-type mice or isogenic p53 knockout mice were irradiated. By 24 hours post-irradiation, neither culture had any detectable apoptotic cells. The MEF p53^{+/+} cells underwent G₁ arrest, whereas the p53-null cells grew. The medium from both cultures was collected and subjected to differential centrifugation to isolate exosomes. Western blots to detect PGK1, Eno1, and the exosome marker protein, HLA-DR α , were done (Fig. 4*A*, *left*), and in addition, an equal volume of aliquots of the total protein recovered from the each exosome isolation was shown by silver staining (Fig. 4*A*, *right*). Exosomes are produced in

wild-type MEFs treated with radiation but are absent or undetectable in the medium from p53-null cells treated with DNA damaging agents. Furthermore, transfection of the p53-null MEF cells with a plasmid that constitutively expresses p53 results in the production of exosomes as detected by silver staining of total exosomal protein and the presence of Eno1 and HLA-DR α by Western analysis (data not shown). Exosomal proteins are only present in the medium from MEFs expressing active p53, suggesting that exosome production is p53-dependent. Importantly, p53 wild-type MEF cells do not undergo cell death in response to DNA damage, and instead arrest in G₁ (33, 34). The finding of exosomes in the medium in the absence of cell death again argues that apoptosis or cell death is not a requirement for the formation of exosomes.

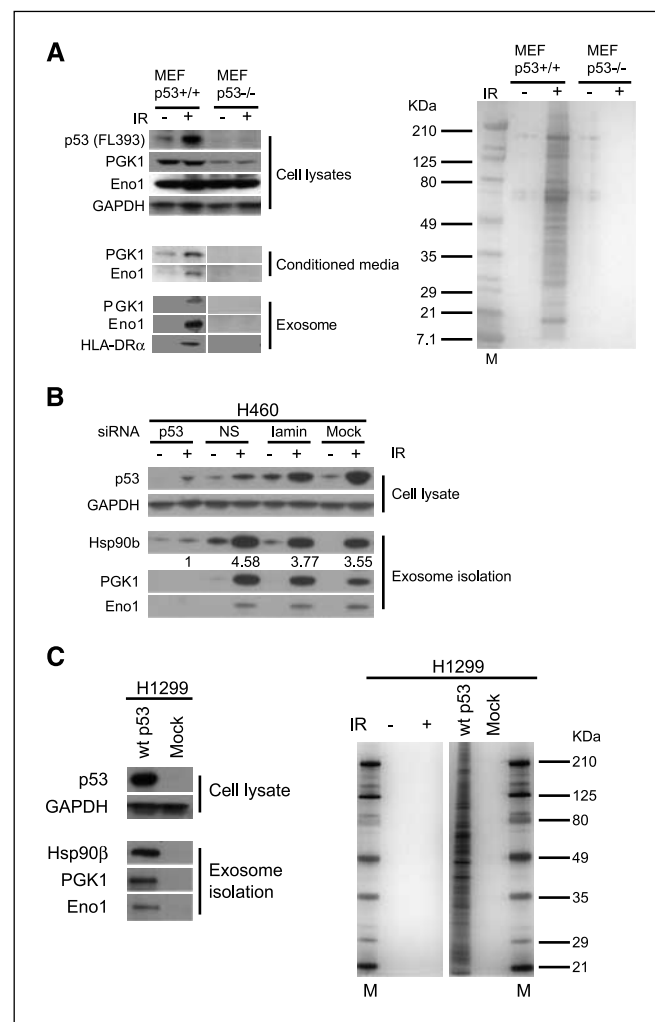


Figure 4. p53 dependency of secretion and exosome production. *A*, exosome production in MEF cells. *Left*, Western blot analysis. In MEF p53^{+/+} cells, p53 is induced by treatment with γ -irradiation, and the secretion of PGK1 and Eno1 through exosomes was induced by p53 activation, whereas the observation is absent in MEF p53^{-/-} cells; *right*, silver staining of the isolated exosomes on SDS-PAGE. *B*, decrease of exosome production with reduced p53 expression in H460 cells. H460 cells were transfected with siRNA to p53, nonspecific siRNA (NS), siRNA to lamin A/C and no siRNA (Mock). The cell lysates and the isolated exosomes from the nonirradiated and irradiated cells following transfection were analyzed using Western blot. The protein levels of Hsp90 β in the exosomes from the irradiated cells were quantified. The relative level was determined as the ratio of each exosome isolation from irradiated cells to that from the cells treated with siRNA to p53. *C*, wild-type p53 expression allows exosome production in H1299 (p53 null) cells. *Left*, Western blot analysis; *right*, silver staining of the isolated exosomes on SDS-PAGE. M, protein size marker.

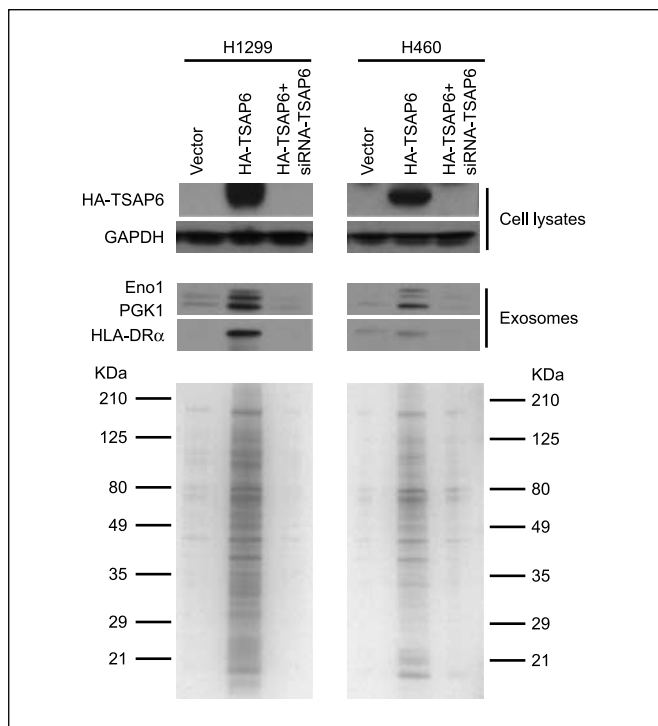


Figure 5. TSAP6 involvement in the p53-dependent exosome production. The cells were transfected with the vector alone, the construct of HA-TSAP6, or the construct of HA-TSAP6 and the siRNA to TSAP6. *Top*, Western blot analysis of the cell lysates and exosome isolations; *bottom*, silver staining of the isolated exosomes on SDS-PAGE; *left*, H1299 cells; *right*, H460 cells.

In a second experiment, p53 expression in H460 cells was reduced using siRNA. The induction of p53 was largely decreased in the cells, as well as the expression of the p53-transcribed gene, p21 (data not shown). Exosomes were isolated from the medium of cultured cells transfected with siRNAs followed by radiation. Western blot analysis shows the presence of Hsp90 β , PGK1, and Eno1 in the exosome fraction (Fig. 4B) from mock or control transfections. The reduction of p53 expression resulted in decreased exosome protein by 4-fold.

Finally, the p53-null cell line, H1299, previously shown to be deficient in exosome production, was transfected with a construct that allows constitutive expression of p53. Culture medium from H1299 cells expressing p53 was analyzed for the presence of exosomes by Western analysis and total protein composition of the exosome pellet (Fig. 4C). Exosome proteins are only detectable in the medium from cells expressing p53. In summary, these experiments show that the enhanced production of exosomes is regulated by the activation of the p53 protein.

TSAP6 facilitates the secretion of exosomes. The product of a p53-regulated gene, *TSAP6*, was recently found to facilitate the secretion of a protein, TCTP, via exosomes (18, 19). To test the hypothesis that the requirement for p53 in exosome production might be through the up-regulation of *TSAP6* transcription by activated p53 to increase exosome release, H1299 cells that lack p53 activity and do not produce exosomes were transfected with a construct that constitutively expresses HA-tagged TSAP6 (19). As can be seen in Fig. 5, the expression of HA-TSAP6 allows the production of exosomes by the H1299 cells. In addition, H460 cells that normally only produce exosomes after p53 activation could produce exosomes in the absence of stress stimuli if they express

HA-TSAP6. In both the H1299 and H460 cell lines, treatment of the HA-TSAP6-expressing cells with an siRNA specific for TSAP6 results in decreased exosome production (Fig. 5). Together, these results suggest that the role of p53 in exosome production is linked to the expression of the *TSAP6* gene.

Discussion

The activation of the p53 protein leads to an increase in the export of growth-suppressive factors by inducing the transcription of their corresponding genes (Fig. 6). Komarova et al. showed that the p53-dependent accumulation of the factors, thrombospondin and PAI-2, in the conditioned medium from established and primary cell cultures and in the urine of irradiated mice caused growth inhibition in a number of cell lines (35). Similarly, the transcription of the PAI-1 gene, whose product inhibits urokinase and tissue plasminogen activator, and has an inhibitory effect on angiogenesis (36), is increased upon p53 activation (this article; ref. 3). Maspin (mammary serine protease inhibitor) has inhibitory effects on angiogenesis, tumor invasion, and metastasis (37–40), and is also up-regulated by p53 in response to stress (this article; ref. 3). These results suggest that activation of p53 may cause a bystander effect by inducing the secretion of tumor-suppressive factors from damaged cells that may influence the behavior of neighboring cells.

In addition, we have shown that activation of p53 leads to an increase in the export of proteins whose genes are not transcribed by p53 and that these proteins are secreted into the medium via exosomes. Furthermore, evidence is presented that the role of p53 in this process is through the ability of p53 to transcribe the *TSAP6* gene whose product is sufficient to induce the secretion of exosomes *in vitro* (Fig. 6). TSAP6 protein has been reported to facilitate the secretion of TCTP as well as its presence in exosomes, suggesting its role in exosome production and/or sorting (18). Although exosome secretion occurs at a low rate in many cells, it is

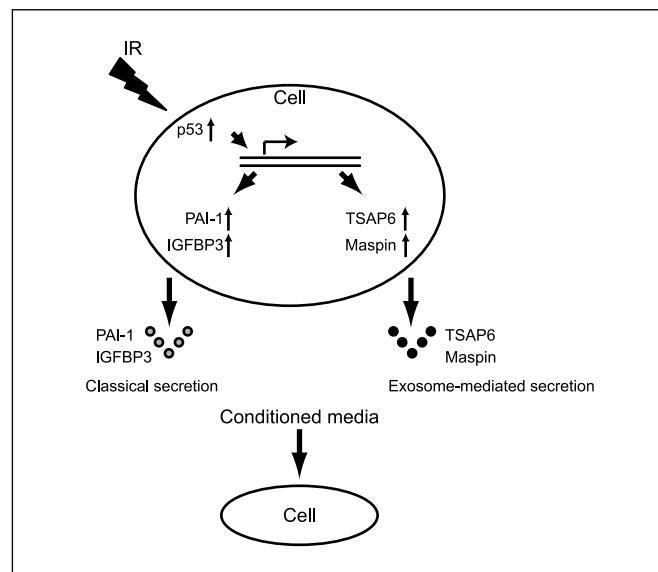


Figure 6. Secretion regulation by p53. Activation of p53 by DNA damage in the cells induces expression of genes and the gene products, which are secreted in two ways. The proteins with the signal peptides, for example, PAI-1 and IGFBP3, are secreted using the classical secretion pathway. The other p53-regulated proteins, such as TSAP6 and maspin, are induced by p53 and play a role in the exosome-mediated secretion. The secreted proteins may be involved in cell-cell communication.

clearly induced at a higher rate by p53 activation. This is the first report demonstrating a role for p53 in the regulation of this process.

The biological functions of exosomes remain unclear. However, the protein composition of exosomes secreted by a number of different cell types have been examined and shown to be somewhat cell type-specific. Hsp90 β , Eno1, EF-1 α , actin, PGK1, PRDX1, and CyPA have each been shown to be secreted via exosomes in at least one cell type (27, 28, 41, 42). The fact that the contents of exosomes vary by cell type may be related to their function. Exosomes isolated from the malignant effusions (ascites) of patients with cancer contain antigens specific to the tumor, including Her2/Neu from ovarian cancer ascites, and Mart1 from patients with melanoma (43). Exosomes containing the tumor antigens can then fuse with dendritic cells allowing cross-presentation to clones of CTLs specific to the tumor antigen (44). Wolfers et al. have proposed that exosomes may function as a tumor-antigen sampling compartment spread by tumor cells for immunosurveillance (44).

The role of the tumor suppressor p53 in inducing the secretion of exosomes from lung tumor cell lines in response to DNA damage is consistent with this hypothesis.

Moreover, it is possible that exosomes can fuse with cells adjacent to a cell undergoing a p53 response (a cancer cell or a stromal cell) and this might affect gene expression patterns in those cells. As such, this is a possible mechanism for cell-cell communication. These are all testable ideas.

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References

- Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323–31.
- Jin S, Levine AJ. The p53 functional circuit. *J Cell Sci* 2001;114:4139–40.
- Zhao R, Gish K, Murphy M, et al. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. *Genes Dev* 2000;14:981–93.
- Snyder AR. Review of radiation-induced bystander effects. *Hum Exp Toxicol* 2004;23:87–9.
- Azzam EI, Little JB. The radiation-induced bystander effect: evidence and significance. *Hum Exp Toxicol* 2004; 23:61–5.
- Goldberg Z, Lehnert BE. Radiation-induced effects in unirradiated cells: a review and implications in cancer. *Int J Oncol* 2002;21:337–49.
- Azzam EI, de Toledo SM, Gooding T, Little JB. Inter-cellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of α particles. *Radiat Res* 1998;150:497–504.
- Azzam EI, De Toledo SM, Spitz DR, Little JB. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from α -particle-irradiated normal human fibroblast cultures. *Cancer Res* 2002;62:5436–42.
- Lyng FM, Seymour CB, Mothersill C. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *Br J Cancer* 2000;83:1223–30.
- Mothersill C, Seymour CB. Cell-cell contact during γ irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiat Res* 1998;149:256–62.
- Little JB, Azzam EI, de Toledo SM, Nagasawa H. Bystander effects: intercellular transmission of radiation damage signals. *Radiat Prot Dosimetry* 2002;99:159–62.
- Waku T, Fujiwara T, Shao J, et al. Contribution of CD95 ligand-induced neutrophil infiltration to the bystander effect in p53 gene therapy for human cancer. *J Immunol* 2000;165:5884–90.
- Nishizaki M, Fujiwara T, Tanida T, et al. Recombinant adenovirus expressing wild-type p53 is antiangiogenic: a proposed mechanism for bystander effect. *Clin Cancer Res* 1999;5:1015–23.
- Qazilbash MH, Xiao X, Seth P, Cowan KH, Walsh CE. Cancer gene therapy using a novel adeno-associated virus vector expressing human wild-type p53. *Gene Ther* 1997;4:675–82.
- Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci* 2000;113:3365–74.
- Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G. The biogenesis and functions of exosomes. *Traffic* 2002;3: 321–30.
- Fevrier B, Raposo G. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol* 2004;16:415–21.
- Amzallag N, Passer BJ, Allanic D, et al. TSAP6 facilitates the secretion of translationally controlled tumor protein/histamine-releasing factor via a nonclassical pathway. *J Biol Chem* 2004;279:46104–12.
- Passer BJ, Nancy-Portebois V, Amzallag N, et al. The p53-inducible TSAP6 gene product regulates apoptosis and the cell cycle and interacts with Nix and the Myt1 kinase. *Proc Natl Acad Sci U S A* 2003; 100:2284–9.
- Brower M, Carney DN, Oie HK, Gazdar AF, Minna JD. Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res* 1986;46:798–806.
- Harvey DM, Levine AJ. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes Dev* 1991;5: 2375–85.
- Lin J, Chen J, Elenbaas B, Levine AJ. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev* 1994;8:1235–46.
- Zou Z, Gao C, Nagaich AK, et al. p53 regulates the expression of the tumor suppressor gene maspin. *J Biol Chem* 2000;275:6051–4.
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 2004;340:783–95.
- Bendtsen JD, Jensen LJ, Blom N, Von Heijne G, Brunak S. Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng Des Sel* 2004; 17:349–56.
- Pemberton PA, Tipton AR, Pavloff N, et al. Maspin is an intracellular serpin that partitions into secretory vesicles and is present at the cell surface. *J Histochem Cytochem* 1997;45:1697–706.
- Hegnans JR, Bard MP, Hemmes A, et al. Proteomic analysis of exosomes secreted by human mesothelioma cells. *Am J Pathol* 2004;164:1807–15.
- Thery C, Boussac M, Veron P, et al. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol* 2001;166:7309–18.
- Thery C, Regnault A, Garin J, et al. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J Cell Biol* 1999;147:599–610.
- Raposo G, Nijman HW, Stoorvogel W, et al. B. Lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996;183:1161–72.
- Escala JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* 1998;273:20121–7.
- Wubbolts R, Leckie RS, Veenhuizen PT, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem* 2003;278:10963–72.
- Harvey M, Sands AT, Weiss RS, et al. *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* 1993;8:2457–67.
- Matsui Y, Tsuchida Y, Keng PC. Effects of p53 mutations on cellular sensitivity to ionizing radiation. *Am J Clin Oncol* 2001;24:486–90.
- Komarova EA, Diatchenko L, Rokhlin OW, et al. Stress-induced secretion of growth inhibitors: a novel tumor suppressor function of p53. *Oncogene* 1998;17: 1089–96.
- Chorostowska-Wynimko J, Skrzypczak-Jankun E, Jankun J. Plasminogen activator inhibitor type-1: its structure, biological activity and role in tumorigenesis [review]. *Int J Mol Med* 2004;13:759–66.
- Shi HY, Zhang W, Liang R, et al. Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. *Cancer Res* 2001;61: 6945–51.
- Zou Z, Anisowicz A, Hendrix MJ, et al. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science* 1994;263:526–9.
- Sheng S, Carey J, Sefror EA, Dias L, Hendrix MJ, Sager R. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc Natl Acad Sci U S A* 1996;93:11669–74.
- Zhang M, Sheng S, Maass N, Sager R. mMaspin: the mouse homolog of a human tumor suppressor gene inhibits mammary tumor invasion and motility. *Mol Med* 1997;3:49–59.
- Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* 2004;101:13368–73.
- Mears R, Craven RA, Hanrahan S, et al. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics* 2004;4:4019–31.
- Andre F, Scharzt NE, Movassagh M, et al. Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* 2002;360:295–305.
- Wolfers J, Lozier A, Raposo G, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* 2001;7:297–303.