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Committee Report 17

Diagnosis of Atherosclerosis

Executive Summary of the Japan Atherosclerosis Society (JAS) Guidelines for the Diagnosis and Prevention of Atherosclerotic Cardiovascular Diseases in Japan—2012 Version

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Committee for Epidemiology and Clinical Management of Atherosclerosis

From the perspective of preventing atherosclerotic cardiovascular disease (CVD), it is essential to determine the presence or absence and degree of atherosclerosis before the development of clinical symptoms and to manage or treat risk factors in order to prevent progression or achieve regression of disease. It is necessary to diagnose whether atherosclerosis is present, and if so, to what extent. The diagnostic techniques for atherosclerosis employed in the primary prevention of CVD should be noninvasive. In secondary prevention, however, the use of invasive diagnostic techniques, including angiography, is necessary. Currently, morphological imaging tests are predominantly used to assess the presence and degree of atherosclerosis.

1. Ultrasonography

Noninvasive imaging tests include body surface ultrasonography (a high-frequency probe of ≥7 MHz), which enables observation of the degree of stenosis and plaque formation (localized atherosclerotic lesions) in the peripheral arteries, such as the carotid arteries and arteries of the lower extremities. In particular, in the carotid arteries, ultrasonography is used to determine the degree of stenosis (quantitatively) and detect vulnerable plaques that could cause cerebral embolism (qualitatively), thereby assessing the degree of systemic atherosclerosis and/or functioning as an alternative predictor of the presence or development of CVD (e.g., coronary artery disease (CAD), peripheral arterial disease (PAD) or cerebrovascular disease)[1, 2]. The existence of plaques and intima-media complex thickness (IMT) is often used as a measurement index on carotid ultrasonography[3]. Ultrasonography is also useful for making the diagnosis of atherosclerotic renal artery stenosis[4].

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2. Computed Tomography (CT)

Multidetector row CT (MDCT) offers superior imaging speed and spatial resolution and enables visualization of the coronary arteries following the injection of contrast medium into peripheral veins. This technique is starting to replace coronary angiography as a screening test for CAD. In particular, it is superior in specificity[5-8], and if no abnormalities are detected using this technique, the existence of organic coronary stenosis can be almost completely ruled out. In addition, this technique allows for visualization of coronary plaques, and the degree of calcification and fat and fiber content can also be estimated to some extent based on the CT number.

3. Magnetic Resonance Imaging (MRI) and MR Angiography (MRA)

MRA is used to visualize the cerebral/carotid arteries, aorta and renal arteries and enables the visualization of coronary stenotic lesions.

4. Angiography

Invasive diagnostic imaging techniques include angiographic evaluations of the degree of stenosis, which remains a central diagnostic technique for assessing arterial stenosis. The degree of arterial stenosis (the stenosis rate) is represented by the formula $(D-S)/D \times 100\%$, where D is the intravascular luminal diameter at the site proximal to the site of stenosis that appears to be normal and S is the luminal diameter at the site of stenosis. However, because intimal thickening is more or less observed even at sites that appear to be normal, the stenosis rate is underestimated consid-

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ering the amount of the plaque volume. Because plaques are usually eccentric and the intravascular luminal diameter is therefore not a precise circle, there are limitations in the ability to determine the stenosis rate based on one cross-section. If there is compensatory vascular remodeling, the blood vessel may not be considered to exhibit luminal stenosis even if the plaques are well-formed; thus, there are severe limitations in establishing the plaque volume using this technique.

5. Intravascular Ultrasound (IVUS)

IVUS is a technique used to observe the arterial wall from the arterial lumen using an ultrasound device. It enables the evaluation of both the plaque volume and the properties of the plaques.

6. Angioscopy

Angioscopy is a technique used to observe the color and estimate the properties of plaques.

7. Physiological Tests

Diagnostic techniques other than morphological tests include physiological tests, such as the brachial-ankle pulse wave velocity (baPWV) and cardio-ankle vascular index (CAVI). Although these parameters are easily determined by measuring the pulse wave in the extremities using a dedicated device, it should be noted that the values function as indices of artery stiffness and do not always reflect the presence of atherosclerosis. The ankle-brachial blood pressure index (ABI), which is measured simultaneously, can be used to diagnose PAD in the lower extremities (<0.9 or ≥ 1.3). The techniques used to measure the vascular endothelial function impaired in the early stage of atherosclerosis include flow-mediated vasodilation (FMD), which measures and calculates changes in the vascular diameter following ischemic reactive hyperemia of the extremities using ultrasound, and strain gauge plethysmography, which electrically observes and measures changes in the volume of the arterial blood flow in the extremities as changes in the circumference using a strain gauge. However, the use of these techniques is quite limited in general practice.

If a diagnosis of CAD, particularly effort angina, is suspected, the following noninvasive tests are useful.

8. Exercise Electrocardiography

Exercise electrocardiography has been shown to have a sensitivity of approximately 70% and a specificity of approximately 75% for detecting significant coronary stenosis[9], neither of which are superior; however, since the procedure can be easily performed at a low cost, it is widely used. Because myocardial ischemia can be induced, it is important to keep in mind the risk of possible cardiac events, including ventricular fibrillation and sudden death, when performing this technique.

9. Myocardial Perfusion Scintigraphy

This technique is widely used in the diagnosis of CAD to assess disease severity, myocardial viability and the prognosis and aids in decision making concerning therapeutic strategies. It is also used to screen for significant coronary stenosis, is relatively minimally invasive and may be a useful monitoring test for preventing atherosclerosis. Stressors include exercise stress, dipyridamole stress and adenosine stress. This technique has been shown to have a sensitivity of 80% to 90% and a specificity of 70% to 95% for detecting significant coronary stenosis[10].

At present, ultrasonography is a minimally invasive, simple and easy-to-use test for diagnosing atherosclerosis. Coronary CT, exercise electrocardiography and myocardial perfusion scintigraphy are noninvasive and useful diagnostic techniques in cases in which a diagnosis of CAD is suspected.

Footnotes

This is an English version of the guidelines of the Japan Atherosclerosis Society (Chapter 17) published in Japanese in June 2012.

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ORIGINAL REPORT

The increase in prescriptions of bisphosphonates and the incidence proportion of osteonecrosis of the jaw after risk communication activities in Japan: a hospital-based cohort study†

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ABSTRACT

Purpose The purpose of this study was to investigate the impact of risk communication about bisphosphonate (BP)-related osteonecrosis of the jaw (ONJ) on the number of reported cases to the Drug Adverse Reactions Reporting System and on the incidence proportion of ONJ in a hospital-based cohort study in Japan.

Method We conducted a survey of the safety information on BP-related ONJ available from regulatory authorities, pharmaceutical manufacturers and academic associations. We also performed a trend analysis of a dataset from the Drug Adverse Reactions Reporting System and a sub-analysis, using previously constructed data from a retrospective cohort study.

Results Risk communication from pharmaceutical manufacturers and academic associations began within 1 year after revisions were made to the package inserts, in October 2006. Twenty times more cases of ONJ have been reported to regulatory authority since 2007, compared with the period before 2007. In our cohort, the incidence proportion of ONJ during and after 2009 was four times greater than before 2009. During this period, BPs were frequently prescribed, whereas there was no increase in the use of alternative agents, such as selective estrogen receptor modulators.

Conclusion ONJ was increasingly diagnosed after risk communication efforts, but the impact of the communications was not clear. Safety notifications were diligently disseminated after the package insert was revised. However, there was no surveillance for ONJ before the revision. © 2014 The Authors. *Pharmacoepidemiology and Drug Safety* published by John Wiley & Sons, Ltd.

KEY WORDS—risk communication; osteonecrosis of the jaw; oral bisphosphonates; pharmacoepidemiology

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INTRODUCTION

Osteonecrosis of the jaw (ONJ), also called osteomyelitis of the jaw, is defined as the presence of exposed bone in the maxillofacial region that does not heal within 8 weeks.[1–3] ONJ has received increasing attention since case reports about patients exposed to bisphosphonates (BPs) were published in 2003.[4,5] In the United States of America (USA), regulatory authorities first indicated safety concerns about zoledronic acid and pamidronate with regard to osteonecrosis in 2003.[6] In 2004, the manufacturer of zoledronic acid revised the package insert in the USA and issued a "Dear Health Professional" letter.[7] Safety notifications regarding osteonecrosis were issued in other regions, such as Canada, Australia, New Zealand[7]

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and Japan, in 2004 and 2005. Early case reports were followed by the publication of epidemiological studies in 2005 and 2006.[8–10] Thereafter, position papers,[11] guidelines[12] and expert panel recommendations[3,13] were published in 2006 and 2007. Some of these papers cautioned patients receiving oral BPs.[3,11,13] The risk of ONJ for patients receiving oral BPs was considered much lower than the risk for patients receiving intravenous BPs.[11,13] However, the incidence proportion of an adverse reaction was not fully studied until later, when the risk associated with oral BPs was proved to be smaller than that for intravenous BPs.[14,15]

Although dissemination of safety information to health care professionals or patients is the most common method for minimizing risk when a novel safety concern is discovered, the impact of risk communication has remained unknown and cannot be guaranteed to result in the intended effect.[16,17] Few studies have addressed the long-term impact of risk communication on the incidence of adverse events and whether adverse events have been successfully reduced. Instead, the impact of risk communication is often assessed by measuring processes such as changes in drug use and by laboratory monitoring.[17] Because ONJ is uncommon in the general population and its background incidence rate is low, we attributed an increase in disease reports to greater recognition of the disease among BP-exposed patients after risk communication, if the characteristics of the patients and the use of BPs did not change substantially. We expected that the risk communication initiative would decrease the incidence proportion of ONJ among BP-exposed patients, after a temporary increase.

The purpose of this study was to investigate the impact of risk communication on oral BP-related ONJ in Japan; on the number of reported cases to the Japanese regulatory authority, the Drug Adverse Reactions Reporting System of the Pharmaceuticals and Medical Devices Agency (PMDA); and on the incidence proportion of ONJ in a hospital-based cohort study of 6923 osteoporosis patients at Kyoto University Hospital.

METHODS

We surveyed safety information about oral BP-related ONJ that was produced by the PMDA, pharmaceutical manufacturers and academic associations. We also conducted a trend analysis of a dataset from the Drug Adverse Reactions Reporting System of the PMDA and a sub-analysis, using the previously constructed data from a retrospective cohort study that was conducted at Kyoto University Hospital from February 2011 to July 2012.[18] The protocol was approved by the Ethical Committee of the Graduate School of Medicine, Kyoto University (E1445).

Risk communication regarding oral BP-related ONJ

First, we surveyed the safety information from the PMDA by searching the PMDA Web site for the words "jaw" or "BPs" (accessed June to July 2012). We extracted articles on periodic safety information and letters and guidance publications, and we listed the relevant information after removing duplicate information. Second, we surveyed the types of risk communication materials concerning oral BP-related ONJ that were released by manufacturers marketing oral BPs in Japan and how and when they were disseminated. Two pharmaceutical companies collected letters and guidelines from the 10 manufacturers marketing oral BPs in Japan between July 2012 and January 2013. Finally, we collected information on the risk communications materials (type, timing of dissemination and method of dissemination) that were released by two academic associations (the Japanese Society of Oral and Maxillofacial Surgeons and the Japanese Society for Bone and Mineral Research) between July and August 2012. One of the authors, a medical doctor, reviewed the collected communications materials and summarized the warnings and recommendations announced in the communications.

Reported cases of ONJ to the regulatory authority

A dataset containing the adverse drug reactions reported to the Drug Adverse Reactions Reporting System of the PMDA between April 2004 and December 2011 was downloaded, and the cases of ONJ suspected to be adverse reactions to osteoporosis medications (including oral BPs) were counted. We used the preferred terms in the Standardized Medical Dictionary for Regulatory Activities (MedDRA) Queries for "osteonecrosis," with the exception of anatomically irrelevant terms, to retrieve the cases of ONJ. The list of drugs included in this study is shown in Appendix 1.

Cohort study

We conducted a cohort study of outpatients and inpatients who were diagnosed with osteoporosis, using the International Classification of Diseases (ICD-10) code (Appendix 2), and who received at least one prescription for an osteoporosis medication at Kyoto University Hospital during a study period (November 2000 to October 2010).[18] The exclusion criteria were as follows: age younger than 20 years old; primary or

metastatic tumors in the maxillofacial region; history of trauma or radiation therapy in the maxillofacial region; and intravenous treatment with BPs.

We extracted the clinical data from the electronic medical records (EMRs) using an EMR retrieval system.[19] This system retrieves electronic data for outpatients and inpatients at Kyoto University Hospital, including demographic data, diagnoses and ICD-10 codes, medications and injections, laboratory tests and radiological and pathological studies. The median duration of oral BP administration, co-medications and comorbid conditions were also extracted using the EMR retrieval system.

The medications administered for osteoporosis between November 2000 and October 2010 in this cohort were collected by the retrieval system. The list of drugs included in the cohort study is shown in Appendix 3. The numbers of BP users, estrogen users and other osteoporosis drug(s) users in the cohort were calculated for each year, counting patients who were prescribed medications at least once during that year, regardless of the use of other osteoporosis medications.

To identify relevant ONJ cases, we reviewed the radiographic imaging and clinical records of the patients with a diagnosis of not only ONJ but also inflammatory conditions of the jaw that were possibly related to ONJ, as specified by the ICD-10 codes (Appendix 4). The diagnostic criteria were detailed in a previous report.[18] Briefly, ONJ was diagnosed independently by two oral and maxillofacial surgeons in accordance with the proposed criteria, using the findings from panoramic X-rays, technetium bone scans, computed tomography, histological images or surgery. We grouped the cases of osteomyelitis of the jaw with ONJ because we considered it difficult to distinguish between these two diseases. The radiographic findings for jawbone infections in patients treated with BPs are similar to those for ONJ related to BPs,[20–22] and the presence of osteonecrosis is a common histopathologic finding, both in ONJ and in osteomyelitis of the jaw related to BPs.[23]

The incidence proportion of confirmed ONJ was defined as the number of manually confirmed, newly developed ONJ cases in the cohort (e.g., BP group or non-BP group) in 2000–2002, 2003–2004, 2005–2006, 2007–2008 and 2009–2010, divided by the size of the cohort for each 2- or 3-year period. The BP group included the patients who were prescribed BPs at least once during the period and/or in the past, regardless of the use of other osteoporosis medications; the non-BP group included the patients who were prescribed osteoporosis medication(s) other than BPs and those who had never been prescribed BPs.

The distinction between BP users in the drug use survey and the BP group in the incidence proportion survey was as follows: we classified a patient who received both BPs and estrogen in the same year as one BP user and one estrogen user over the same time period in the drug use survey. However, we classified the patient into the BP group rather than the non-BP group in the incidence proportion survey. This distinction was made because the impact of osteoporosis medications other than BPs on the incidence proportion of ONJ was considered to be negligible.

We evaluated the proportions of the cases recorded as inflammatory conditions of the jaw and alveolitis of the jaw (specified by ICD-10 codes K10.2, K10.3 and K10.0 [Appendix 4] in the EMR); the proportions were defined as the number of newly recorded cases of the inflammatory condition of the jaw in the EMRs of the cohort (e.g., BP users or non-BP users) during each 2- or 3-year period, divided by the size of the cohort during the period.

RESULTS

Risk communication regarding oral BP-related ONJ

The risk communication materials regarding oral BP-related ONJ, released by the PMDA, pharmaceutical manufacturers and academic associations, are listed in Table 1. The pharmaceutical manufacturers revised the package inserts in October 2006. The case reports or epidemiological studies regarding ONJ were published after the package insert was revised. Six separate but overlapping guidance announcements, in addition to the package insert, were issued. An academic association held educational meetings for health professionals and patients during their annual meeting in April 2008.

Reported cases of ONJ to the regulatory authority

An increasing number of cases of ONJ that were suspected adverse reactions to oral BPs were reported to the PMDA after 2007, immediately after the safety information was disseminated (Figure 1). These cases included those with a past history of ONJ (that is, cases of ONJ that occurred earlier were reported as cases of ONJ after 2004 in the system). There were nearly 20 times more reported cases of ONJ during and after 2007, compared with the number of cases during and before 2006. Reported cases of ONJ that are suspected to have been adverse reactions to osteoporosis medications other than BPs have been rare. For reference, the estimated numbers of patients taking oral BPs in Japan were 2 082 928 in 2007 and 2 470 979 in 2008.[24]

Cohort study

The cohort consisted of 6923 osteoporosis patients; 4129 were prescribed oral BPs (59.6%; mean age,

Table 1. Risk communication about oral BP-related ONJ in Japan

Date*	Organization	Content
Oct. 2006	PMDA†, pharmaceutical manufacturers	Measure: revised package insert for alendronate and risedronate "ONJ has been reported in patients receiving bisphosphonates. The majority of reported cases have been associated with dental procedures, such as tooth extraction, or with local infection. Physicians should fully disclose the adverse reactions to their patients and observe them closely."
Jan. 2007	pharmaceutical manufacturers	Notices to hospitals and "Dear Health Professional" letters to inform them about the content of the revised package insert
June 2007	academic association	Publication of a case report[33] There was one case of osteoporosis diagnosed with oral BP-related ONJ; the other case, a case of multiple myeloma, was diagnosed with iv BP-related ONJ.
Sep. 2007	PMDA, pharmaceutical manufacturers	Measure: revised package insert for etidronate
Oct. 2007	academic association	Publication of an observational study[34] Questionnaires were sent to 239 institutions, and 30 patients with osteonecrosis were reported. Of them, 20 patients received iv BPs, eight received oral BPs and one received both.
Jan. 2008	academic association	News article entitled "osteonecrosis of the jaws induced by anti-osteoporosis treatment" "Patients on BP therapy requiring dental procedures should tell their dentists that they are being treated with BPs, and physicians should fully explain the adverse reactions to their patients when prescribing BPs."
Jan. 2008	academic association, pharmaceutical manufacturers	Announcement of a guidance publication, entitled "Bisphosphonates and osteonecrosis of the jaw" A 20-page pamphlet, with the diagnostic criteria, clinical manifestations, risk factors and epidemiology of iv and oral BP-related osteonecrosis of the jaw and instructions for physicians, pharmacists, dentists and oral surgeons
Mar. 2008	academic association	Announcement of guidance publication, entitled "management of patients on BP therapy" A four-page pamphlet with the diagnostic criteria, management, risk factors, epidemiology of iv and oral BP-related osteonecrosis of the jaw and instructions for physicians, dentists and oral surgeons
Apr. 2008	academic association	Public meeting for citizens: "The state of osteonecrosis of the jaw related to BPs"
Sep. 2008	academic association	A pamphlet, entitled "Bisphosphonates and osteonecrosis of the jaw: clinical manifestations and guidelines for management, 2008"
Feb. 2009	academic association	Training session for dentists, entitled "The state of osteonecrosis of the jaw related to BPs"
Feb. 2009	academic association	News article, entitled "Bisphosphonates and osteonecrosis of the jaws"
May 2009	PMDA, academic association	Announcement of a guidance publication, entitled "Bisphosphonate-Related Osteonecrosis of the Jaws"[35] This official therapeutic manual for severe adverse reactions included the diagnostic criteria, clinical manifestations, risk factors and management methods for iv and oral BP-related osteonecrosis of the jaw for citizens and health care professionals
June 2009	academic association	Public meeting for citizens, entitled "The state and the management of osteonecrosis of the jaws related to BPs"
July 2009	academic association	Training meeting regarding BP-related osteonecrosis of the jaw for health care professionals
Nov. 2009	academic association	Publication of an observational study[36] The follow-up survey showed that surgical treatment might be useful for BRONJ when performed at the appropriate time, and BRONJ was shown to be refractory because only nine of 17 cases were cured in these 2 years.
May 2010	academic association, pharmaceutical manufacturers	Publication of a position paper[37]
June 2010	PMDA	Measure: revised package inserts for alendronate, risedronate and etidronate "ONJ has been reported in patients receiving bisphosphonates, regardless of the route of administration. Treating physicians should advise their patients to undergo dental examinations and to finish any invasive dental procedures, such as tooth extraction, if necessary, prior to treatment with BPs. While on treatment with BPs, these patients should have regular dental consultations and avoid invasive dental procedures."
Sep. 2010	academic association	Publication of a book, entitled "The utility and osteonecrosis of the jaw of BPs"
Sep. 2010	PMDA	Release of safety measures ("The progress of assessments and measures regarding BP-related osteonecrosis of the jaw"), including a survey of the number of cases of BP-related osteonecrosis of the jaw and an outline of the individual case reports reported to PMDA

*The date indicates the first dissemination of safety information.
†PMDA: Pharmaceuticals and Medical Devices Agency.

65.0), and 2794 patients received other osteoporosis drugs (40.3%; mean age, 65.5). The median durations of administration were 364.0 days for BPs and 439.5 days for other osteoporosis drugs. For the BP group and the other osteoporosis drugs group, the numbers of patients using concomitant steroids were 2934 (71.0%) and 1508 (53.9%), respectively; the numbers of patients treated with anti-cancer drugs were 551 (13.3%) and 256 (9.1%), respectively; and the numbers of patients with diabetes were 707 (17.1%) and 442 (15.8%), respectively.[18]

The number of BP users has been increasing steadily since 2000 (Figure 2). The number of estrogen users, including users of selective estrogen receptor modulators, has been low. The number of users of other osteoporosis medications, including active vitamin D3 or calcium,

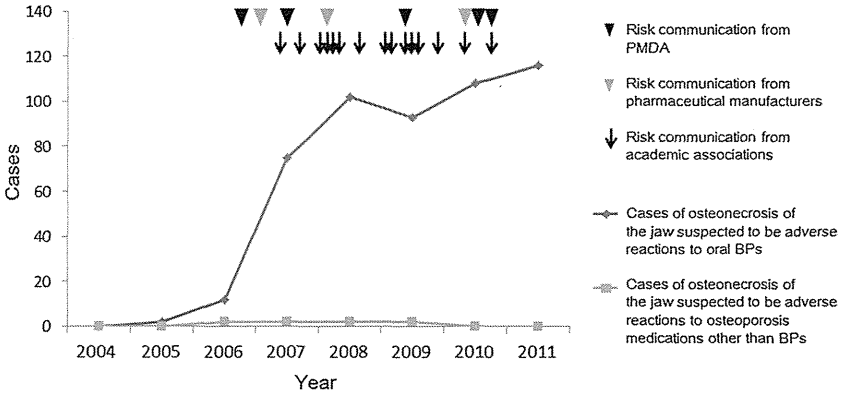

Figure 1. Trends in the number of ONJ cases per year reported to the Drug Adverse Reactions Reporting System of the PMDA and risk communication activities. Legend: The cases of ONJ that were suspected adverse reactions to oral bisphosphonates and those that were suspected adverse reactions to other agents for osteoporosis, reported to the Drug Adverse Reactions Reporting System of the PMDA, are shown as a dark gray line and a light gray line, respectively. Black arrowhead: risk communication from the PMDA; gray arrowhead: risk communication from pharmaceutical manufacturers; arrow: risk communication from academic associations

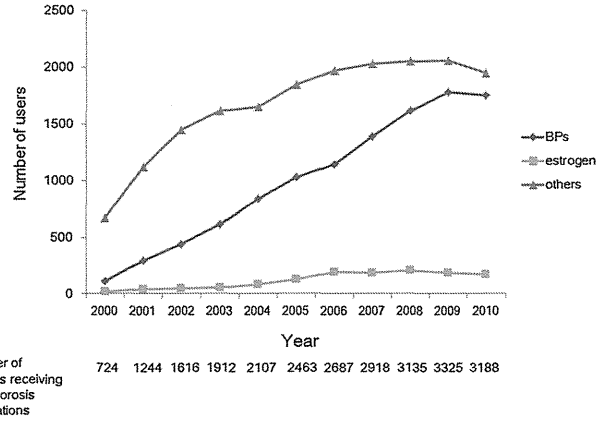

Figure 2. The number of patients prescribed each agent for osteoporosis in the cohort. Legend: The numbers of patients prescribed bisphosphonates, estrogen and a selective estrogen receptor modulator, as well as other agents for osteoporosis, each year in a cohort of 6293 osteoporosis patients are illustrated with a dark gray line of diamonds, a gray line of triangles and a light gray line of squares, respectively. The year 2000 contains 2 months, and the year 2010 contains 10 months. The numbers of patients receiving osteoporosis medications in each year are shown below the graph

increased before 2006 and since then has remained approximately constant.

The EMRs of a total of 1987 patients with records of ONJ or inflammatory conditions of the jaw that were possibly related to ONJ were manually reviewed, and 46 patients were confirmed to have ONJ.[18]

The incidence proportion of confirmed ONJ in the BP group increased approximately four-fold in 2009 and 2010, compared with the pre-2009 level. The incidence proportion of confirmed ONJ in the non-BP group remained low (Figure 3a). Both of the incidence proportion of confirmed ONJ cases and that of inflammatory conditions of the jaw increased after 2009; however, the increase in inflammatory conditions of the jaw was not as high as that of confirmed cases (Figure 3b). This measure was therefore not a good surrogate for confirmed ONJ in this study.

DISCUSSION

Risk communication efforts by pharmaceutical manufacturers and academic associations began within 1 year after the package insert was revised in October 2006, and ONJ was increasingly reported to the PMDA within 1 year. In our cohort, the incidence proportion of ONJ, diagnosed according to standardized criteria, increased in 2009 and in later years. During this period, BPs were frequently prescribed, and there were no increases in the use of alternative agents, such as selective estrogen receptor modulators.

Physicians' case reports regarding ONJ in 2003[4,5] in the USA led to revisions of package inserts in 2004 to 2005.[7,25,26] In Japan, the pharmaceutical manufacturers revised the package inserts for intravenous BPs in 2005 and for oral BPs in 2006 and 2007, but the revision was delayed for 2 years after the revision in the USA. The physicians' case reports regarding ONJ were first published in 2007, 4 years after their publication in the USA; thus, the physicians' reports in Japan did not contribute to the increased suspicion of ONJ related to BPs or to the revision of the package insert. Academic associations were rather active in risk communication in the later dissemination phase. Physicians and academic associations have been able to detect new safety concerns for marketed drugs and to conduct epidemiological studies effectively, and we should reconsider academic associations, as well as the regulatory authority

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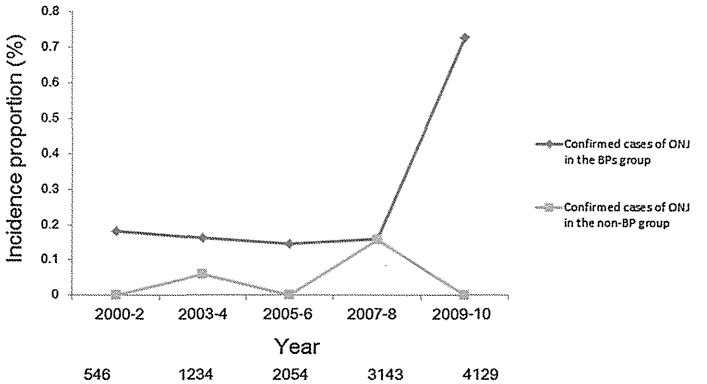
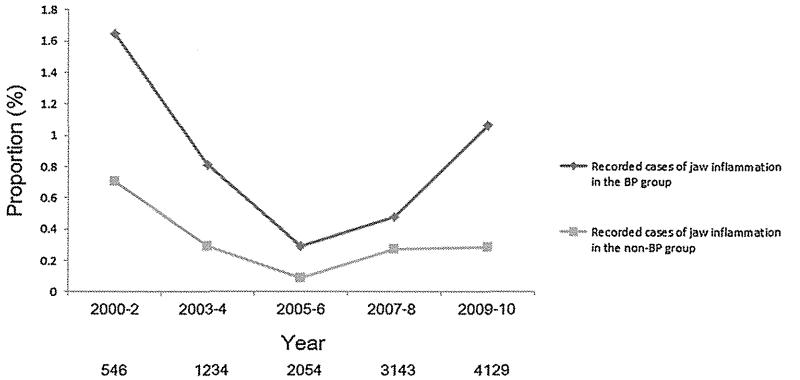
a



BP group	546	1234	2054	3143	4129
Confirmed cases of ONJ in the BP group	1	2	3	5	30
Non-BP group	1272	1702	2236	2564	2794
Confirmed cases of ONJ in the non-BP group	0	1	0	4	0

b



BP group	546	1234	2054	3143	4129
Recorded cases in the BP group	9	10	6	15	44
Non-BP group	1272	1702	2236	2564	2794
Recorded cases in the non-BP group	9	5	2	7	8

Figure 3. (a). The incidence proportion of confirmed cases of ONJ in the cohort. Legend: The incidence proportions of the confirmed ONJ cases in 100 BP-group patients in 2000–2002, 2003–2004, 2005–2006, 2007–2008 and 2009–2010 are indicated by a dark gray line of diamonds. The incidence proportions of confirmed ONJ cases per 100 non-BP-group patients in each 2- to 3-year period are indicated by a light gray line of squares. The number of patients in the BP group, the number of confirmed ONJ cases in the BP group, the number of patients in the non-BP group and the number of confirmed ONJ cases in the non-BP group are shown below the graph. (b). The proportions of recorded ONJ cases in the cohort. Legend: The proportions of recorded cases of inflammatory conditions of the jaw in 100 BP-group patients in 2000–2002, 2003–2004, 2005–2006, 2007–2008 and 2009–2010 are indicated by a dark gray line of diamonds. The proportions of recorded cases of inflammatory conditions of the jaw in 100 non-BP-group patients in each 2- to 3-year period are indicated by a light gray line of squares. The number of patients in the BP group, the number of recorded cases of inflammatory conditions of the jaw in the BP group, the number of patients in the non-BP group and the number of recorded cases of inflammatory conditions of the jaw in the non-BP group are shown below the graph

and pharmaceutical manufacturers, as resources for monitoring and minimization of the risks of medicines and for ensuring the accuracy of information.

We evaluated the impact of risk communications by analyzing the prescriptions of medications for osteoporosis and the incidence proportion of ONJ. The use of BPs increased steadily, but the prescriptions for BPs were not influenced by the risk communications in this study. BPs are among the most established drug types for the treatment of osteoporosis in postmenopausal women,[27] and the gradual increase in the use of BPs over the periods, before and after the dissemination of the safety information, was reasonable considering the risk–benefit balance. We could not determine whether the physicians prescribed BPs after considering the risk–benefit balance or simply did not receive the safety information. Many confounding factors can influence the prescription of BPs, such as the active participation of academic associations or the perceptions of physicians and patients toward adverse events. Physicians might hesitate to change prescribing habits because of known obstacles, such as the lack of time during outpatient care and the desire to maintain trust in the physician–patient relationship.[28]

The rapid increase in the cases of ONJ that were suspected adverse reactions to oral BPs reported to the regulatory authority after the risk communications efforts might indicate that the primary cause of the increase was awareness of the disease because the increase was quite sharp. The incidence proportion of ONJ in the BP group increased in our cohort, although the increase occurred 3 years after the risk communications began. There would have been few missed or misdiagnosed cases of ONJ in our cohort because the cases were diagnosed based on an extensive manual review of the EMRs, using well-established criteria. There might have been other causes for the increase in the incidence proportion of ONJ in our cohort in addition to risk communication; one possibility is the longer exposure to BPs[8,29] in the cohort. Longer exposure and risk communication occurred simultaneously; therefore, we could not distinguish the impact of risk communication from that of longer exposure. There was a time difference between the increase in the number of cases of ONJ reported in the Drug Adverse Reactions Reporting System and the increase in the incidence proportion of ONJ in the cohort. The cases of ONJ reported to the Drug Adverse Reactions Reporting System include past cases of ONJ: cases that occurred before 2006 might be reported as cases of ONJ after 2006. Moreover, the diagnosis of ONJ is not standardized and might include other inflammatory conditions of the jaw. However, the number of ONJ patients in the cohort reflects the number of active ONJ patients diagnosed in the hospital. The difference between the recording and the diagnosis of ONJ most likely resulted in the time difference.

Previous reviews have found it difficult to estimate the average effect of risk communication on clinical practice[16,17,30] because of heterogeneity in the study designs, analyses, outcome measurements, therapeutic areas and types of communication. ONJ can be reduced with preventive measures, including clinical oral examinations and good oral hygiene.[31,32] Unfortunately, we did not observe a decrease in the incidence proportion of ONJ in our cohort during this study period, which would have been the clinical outcome. Additional appropriately designed research is warranted to understand the effects of past communications strategies and to estimate the impact of future communication.

The limitations of our study are described below. First, factors other than safety information collected in our study, such as pharmaceutical use, could have simultaneously influenced the incidence proportion of ONJ. Second, we did not consider the scale, the duration or the content of the risk communication; it is therefore not possible to evaluate the impact of each risk communication material quantitatively. Third, the data on drug use and on the incidence proportion of ONJ in Kyoto University Hospital were limited to a single institution in Japan; thus, the generalizability of the results cannot be assured. The much higher incidence of ONJ in our study compared to the published literature might be explained by the inclusion of numerous steroid users, older patients and inpatients. Moreover, the cohort study was subject to a referral bias toward the selection of more severe cases, given that our department is the lead institution for oral and maxillofacial surgery in Kyoto City, as discussed in our previous report.[18] We could not account for BP exposure that occurred before consultation at Kyoto University Hospital, which might have affected the incidence proportion of ONJ. Finally, this study was retrospective, using a database derived from the EMRs, and the data were not as accurate and consistent as they would have been in a prospective study.

CONCLUSION

The use of oral BPs increased in osteoporosis patients, regardless of the safety notifications concerning ONJ related to BPs. ONJ was increasingly diagnosed after the dissemination of safety information about BP-related ONJ using repetitive and mixed communication methods; the impact of these communications materials was not clear. Our evaluation of the risk communication materials suggests that appropriate cooperation models involving the parties concerned with pharmacovigilance should be planned for the dissemination of safety information and for the delivery and evaluation of new safety concerns with marketed drugs.

CONFLICT OF INTEREST

Eriko Sumi collected information on when, how and what type of risk communications regarding osteonecrosis of the jaw were released from pharmaceutical companies.

KEY POINT

- The use of oral bisphosphonates (BPs) in osteoporosis patients has increased regardless of safety concerns about osteonecrosis related to BPs. Osteonecrosis of the jaw was increasingly diagnosed after risk communication; however, the impact of the risk communication was not clear. Safety notifications were disseminated diligently after the package insert was revised. However, there was no surveillance for osteonecrosis of the jaw before the revision.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix 1. List of drugs studied in cases of OMJ reported to the regulatory authority

Appendix 2. List of International Classification of Diseases (ICD-10) code for osteoporosis studied in the cohort study

Appendix 3. List of drugs studied in the cohort study

Appendix 4. List of International Classification of Diseases (ICD-10) code for inflammatory conditions of the jaw studied in the cohort study

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Senescence-inducing stress promotes proteolysis of phosphoglycerate mutase via ubiquitin ligase Mdm2

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Despite the well-documented clinical significance of the Warburg effect, it remains unclear how the aggressive glycolytic rates of tumor cells might contribute to other hallmarks of cancer, such as bypass of senescence. Here, we report that, during oncogene- or DNA damage–induced senescence, Pak1-mediated phosphorylation of phosphoglycerate mutase (PGAM) predisposes the glycolytic enzyme to ubiquitin-mediated degradation. We identify Mdm2 as a direct binding partner and ubiquitin ligase for PGAM in cultured cells and in vitro. Mutations in PGAM and Mdm2 that abrogate ubiquitination of PGAM restored the proliferative potential of primary cells under stress conditions and promoted neoplastic transformation. We propose that Mdm2, a downstream effector of p53, attenuates the Warburg effect via ubiquitination and degradation of PGAM.

Introduction

Enhanced glycolysis is a characteristic feature of cancerous cells and tissues, commonly referred to as the Warburg effect (Warburg, 1956). This property is used in clinical practice for the detection of metastatic tumor mass by positron-emission scanning of 2-[^{18}F]fluoro-2-deoxy-D-glucose. It has been widely assumed that cancer cells maintain up-regulated glycolytic metabolism to adapt to the hypoxic conditions in vivo, as solid aggressive tumors overgrow the blood supply of the feeding neovasculature.

Enhanced glycolysis under hypoxic conditions is mediated in part by activation of hypoxia-inducible transcription factor (HIF-1), which directly regulates most of the glycolytic enzymes (Iyer et al., 1998). In such a context, the glycolytic response represents a successful metabolic adaptation of cancer cells in vivo. However, the Warburg effect cannot be simply explained by cellular adaptation to hypoxia, as cancer cells maintain enhanced glycolysis even in standard tissue culture conditions (20% oxygen) and in circulating cancers (Koppenol et al., 2011). A more plausible rationalization is that it enables cancer cells to meet their requirements for both energy and metabolic precursors for biosynthesis (Vander Heiden et al., 2009).

We recently reported an intriguing relationship between the glycolytic pathway and cellular senescence (Kondoh et al., 2005). All primary somatic cells, with the exception of pluripotent stem cells, have a limited replicative capacity under standard tissue culture conditions and suffer a permanent cell cycle arrest, called replicative senescence (Hayflick, 1965). The senescent phenotype can also manifest prematurely, upon exposure to oncogenic mutation (Serrano et al., 1997), oxidative stress (Parrinello et al., 2003), DNA damage (Chen and Ames, 1994), and secreted cytokines (Acosta et al., 2008; Kuilman et al., 2008; for review see Campisi, 2013). Glycolytic flux declines during

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Abbreviations used in this paper: DKO, double knockout; MEF, mouse embryonic fibroblast; PGAM, phosphoglycerate mutase; SA-β-Gal, senescence-associated β-galactosidase; SIS, stress-induced senescence.

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senescence in mouse and human primary cells (Zwerschke et al., 2003; Kondoh et al., 2005), and inhibition of glycolytic flux provokes premature senescence (Kondoh et al., 2005).

A key finding in this regard was the identification of phosphoglycerate mutase (PGAM), the enzyme that converts 3-phosphoglycerate to 2-phosphoglycerate in the glycolytic pathway, in an unbiased genetic screen for bypass of senescence in mouse embryonic fibroblasts (MEFs; Kondoh et al., 2005). Conversely, MJE3, a compound identified in a chemical genomics screen for inhibitors of breast cancer cell proliferation, was shown to specifically target PGAM (Evans et al., 2005). PGAM activity is up-regulated in many cancerous tissues, including tumors of the lung, colon, liver, and breast (Durany et al., 1997, 2000; Ren et al., 2010). Indeed, a cancer-specific isoform of pyruvate kinase, designated M2, activates an alternative glycolytic pathway in cancer cells accompanied by dramatic enhancement of PGAM activity (Vander Heiden et al., 2010). As recent results suggest, the pivotal role of PGAM in coordinating glycolysis and biosynthesis make it an attractive target for therapeutic intervention (Hitosugi et al., 2012).

Despite our enhanced understanding of how PGAM regulates glycolysis, rather little is known about the regulation of PGAM. PGAM is the only glycolytic enzyme that is not transcriptionally controlled by HIF-1 (Iyer et al., 1998). Although the muscle-specific form of PGAM can be activated by p53 (Ruiz-Lozano et al., 1999), there is currently no evidence that PGAM is transcriptionally altered during tumorigenesis. Recent findings instead suggest that PGAM activity is regulated posttranscriptionally. For example, phosphorylation of PGAM by p21 (Cdc42/Rac1)-activated kinase1 (Pak1) results in loss of activity (Shalom-Barak and Knaus, 2002), but the precise mechanism and relevance remains unknown. Here we show that during oncogene-induced senescence or other forms of stress-induced senescence (SIS), Pak1-mediated phosphorylation of PGAM provokes its ubiquitination and turnover. The ubiquitin ligase MDM2, a transcriptional target of p53, binds to and ubiquitinates PGAM in a phosphorylation-dependent manner. Of particular note, ubiquitin site mutations in PGAM stabilize the protein and sustain cellular proliferation under stress conditions.

Results

Ubiquitin-dependent degradation of PGAM during SIS

Having previously shown that PGAM can bypass replicative senescence (Kondoh et al., 2005), we were interested to know what happens to PGAM levels and activity in MEFs undergoing oncogene-induced senescence or SIS. There are two reported isoforms of *PGAM—PGAM1* (brain form) and *PGAM2* (muscle form)—whose cDNA sequences are 79% and 81% identical in mouse and human cells, respectively. Although the respective homodimers predominate in specific cell types, most normal mouse tissues contain heterodimers of PGAM-1 and -2 (Zhang et al., 2001). Consistent with this study, we found that the levels of *PGAM2* mRNA are almost equivalent to or exceed those of *PGAM1* mRNA in some mouse tissues (skin, bone, and lung), whereas *PGAM1* mRNA is predominant in others (blood vessel, white adipose tissue, and liver; Fig. S1 A). Moreover, they have similar enzymatic activities and show similar effects on glycolytic flux when overexpressed in MEFs (Fig. S1 B). We generated polyclonal antiserum against recombinant PGAM2, and although it was affinity-purified using mouse PGAM2 protein, it was found to recognize both isoforms of the mouse and human PGAM proteins (Fig. S1, C–F).

To induce SIS, we exposed primary MEFs to low doses of etoposide (Chen and Ames, 1994) and confirmed that they acquired γ-H2AX foci and positive staining for senescence-associated β-galactosidase (SA-β-Gal) activity (Fig. S1 G). In this experimental setting, the total level of PGAM protein was greatly reduced, whereas $p21^{CIP1}$ increased as expected (Fig. 1 A). In contrast, the levels of other glycolytic enzymes, including PFK, PGK, enolase, and GAPDH, remained relatively constant (Fig. 1 A). As *PGAM1* and *PGAM2* mRNA levels also remained constant under these conditions (Fig. S1 H), the data suggested that PGAM protein levels might be regulated by proteolysis during SIS. In line with this idea, addition of the proteasome inhibitor MG132 restored the PGAM protein levels in stressed cells (Fig. 1 B). Similar results were obtained in MEFs undergoing Ras-G12V–induced senescence (Fig. 1 C). Stress-induced down-regulation of PGAM protein was also observed in human primary fibroblasts (WI-38, TIG3, and IMR90), but not in cancer cell lines (HeLa, 293T, and SW620) that evade SIS (Fig. S1, I and J).

We previously reported that the PGAM protein can be ubiquitinated (Kondoh et al., 2005), and, consistent with the MG132-sensitive turnover of endogenous PGAM during SIS, a His-tagged ubiquitin purification assay revealed heavy ubiquitination of FLAG-tagged PGAM1 and PGAM2 in primary MEFs exposed to etoposide (Fig. S1, K and L, left) or Ras-G12V (Fig. S1, K and L, right). Ubiquitination of endogenous PGAM could also be observed in these circumstances by immunoprecipitating PGAM and immunoblotting for ubiquitin (Fig. 1 D). Consistently, PGAM enzymatic activity, glycolytic flux, and lactate production were also substantially reduced in the cells exposed to etoposide (Fig. 1 E and Fig. S1 M) or RasG12V (Fig. 1 F and Fig. S1 N). Collectively, our data suggest that PGAM protein is subject to ubiquitin-mediated degradation during SIS.

Pak1 phosphorylates PGAM during SIS and provokes premature senescence in primary fibroblasts

As ubiquitination often requires prior modification of the target protein (Hagai et al., 2012), we investigated whether PGAM might be phosphorylated during SIS, specifically by Pak1. It was previously reported that Pak1 physically interacts with and phosphorylates PGAM in immortalized 293T cells, but that PGAM protein levels are not affected by Pak1 activation (Shalom-Barak and Knaus, 2002). However, in contrast to the situation in 293T cells, we found that Pak1 expression in normal WI-38 human fibroblasts caused a substantial reduction in PGAM protein levels (Fig. 2 A). A similar effect was seen in primary MEFs but only with wild-type Pak1 and not with a version in which the kinase domain was mutated (Fig. 2 B).

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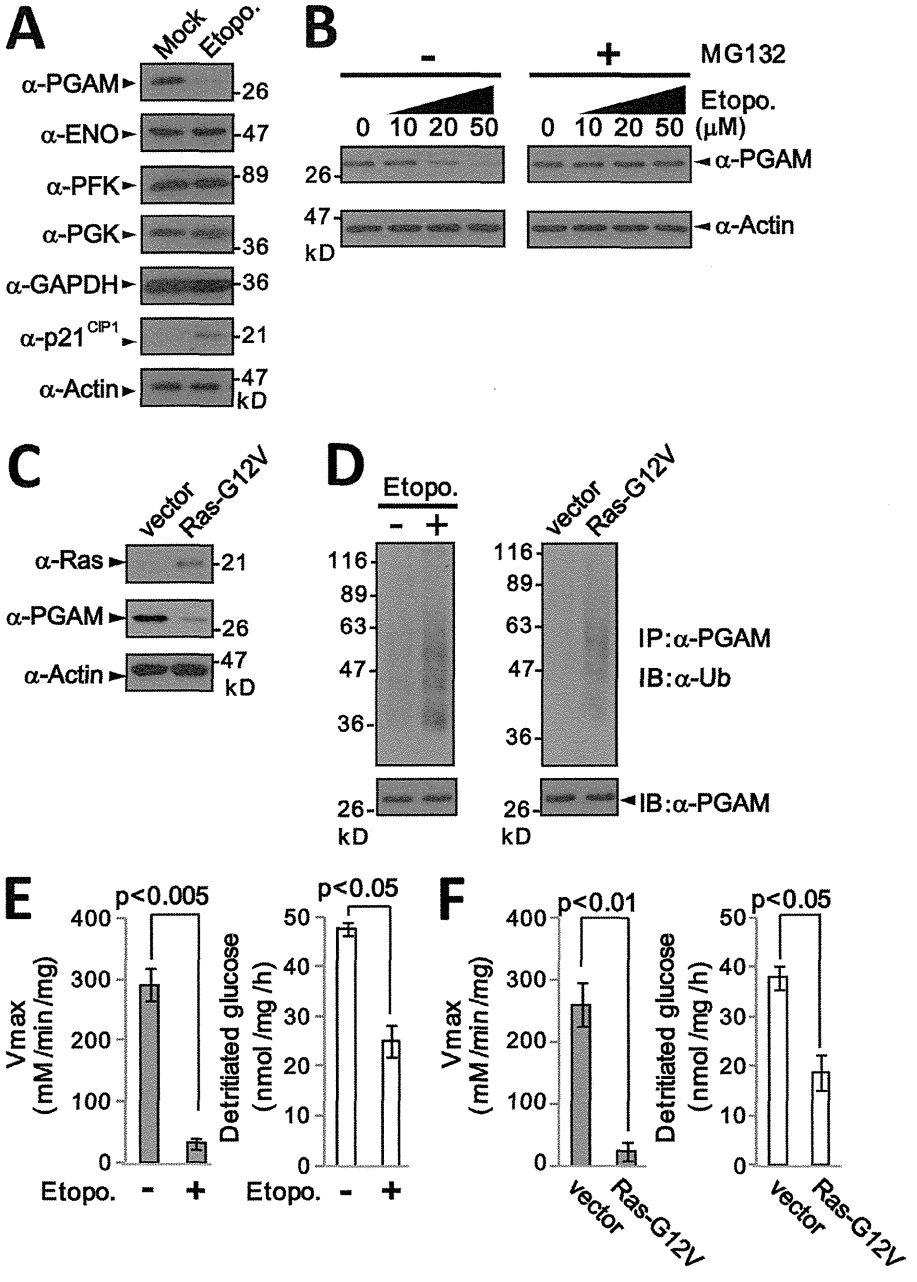

Figure 1. **Down-regulation of PGAM in primary fibroblasts after DNA damage or Ras-G12V expression.** (A) Extracts from primary MEFs (passage 2) treated without (Mock) or with 20 μM etoposide for 6 h were immunoblotted for the indicated proteins. PGAM, phosphoglycerate mutase; ENO, enolase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Actin was used as a loading control. (B) Proteasome-dependent down-regulation of PGAM protein after DNA damage. Extracts from primary MEFs treated for 6 h with increasing concentrations of etoposide (as indicated) in the presence or absence of the proteasome inhibitor MG132 (20 μM) were analyzed as in A. (C) Immunoblotting for Ras and PGAM in MEFs infected with Ras-G12V or empty vector. (D) Ubiquitination of endogenous PGAM in MEFs exposed to DNA damage (left) or to oncogenic Ras (right). Cell extracts were immunoprecipitated with anti-PGAM antibody and immunoblotted with a monoclonal anti-ubiquitin antibody. (E and F) Measurement of total cellular PGAM activity (left) and glycolytic flux (right) in primary MEFs exposed to DNA damage (E) or expressing Ras-G12V (F). Error bars indicate SEM ($n = 3$).

Importantly, the effects on PGAM protein levels were reflected in reduced PGAM activity, glycolytic flux, and lactate production (Fig. 2 B).

Concomitant with reduced PGAM activity, ectopic expression of Pak1 provoked features of senescence, including premature cessation of cell proliferation (Fig. 2 C), flattened and enlarged cell morphology, a single large nucleolus, and SA-β-Gal staining (Fig. 2 D). Importantly, the levels of Pak1 protein and mRNA were elevated during SIS resulting from DNA damage (Fig. 2 E and Fig. S2 A).

Previous work identified serines Ser23 and Ser118 as the residues in PGAM1 phosphorylated by Pak1 (Shalom-Barak and Knaus, 2002). As Ser118, but not Ser23, is conserved between PGAM1 and PGAM2, we raised a phospho-specific antibody against a 12–amino acid phosphopeptide centered on Ser118 (Fig. S2 B). Enhanced phosphorylation of Ser118 in PGAM was observed in primary MEFs after treatment with etoposide or expression of Ras-G12V in the presence of MG132 (Fig. 2 E and Fig. S2 C). To confirm that Ser118 is a direct target for Pak1 kinase, we prepared recombinant wild-type (PGAM2-WT) or mutant (PGAM-S118C) PGAM2 proteins and deployed them as substrates in an in vitro kinase assay using Pak1 immunopurified from primary MEFs. The ability of Pak1 to phosphorylate wild-type PGAM2 was increased after exposure of the cells to etoposide (Fig. 2 F). An equivalent increase was not observed using PGAM-S118C as a substrate, which suggests that stress-induced Pak1 mediates phosphorylation of PGAM at S118 (Fig. 2 F).

These data raised the possibility that phosphorylation of PGAM by Pak1 could promote the ubiquitination-mediated degradation of PGAM during SIS. In support of this notion, PGAM protein levels in Pak1-expressing primary MEFs were restored

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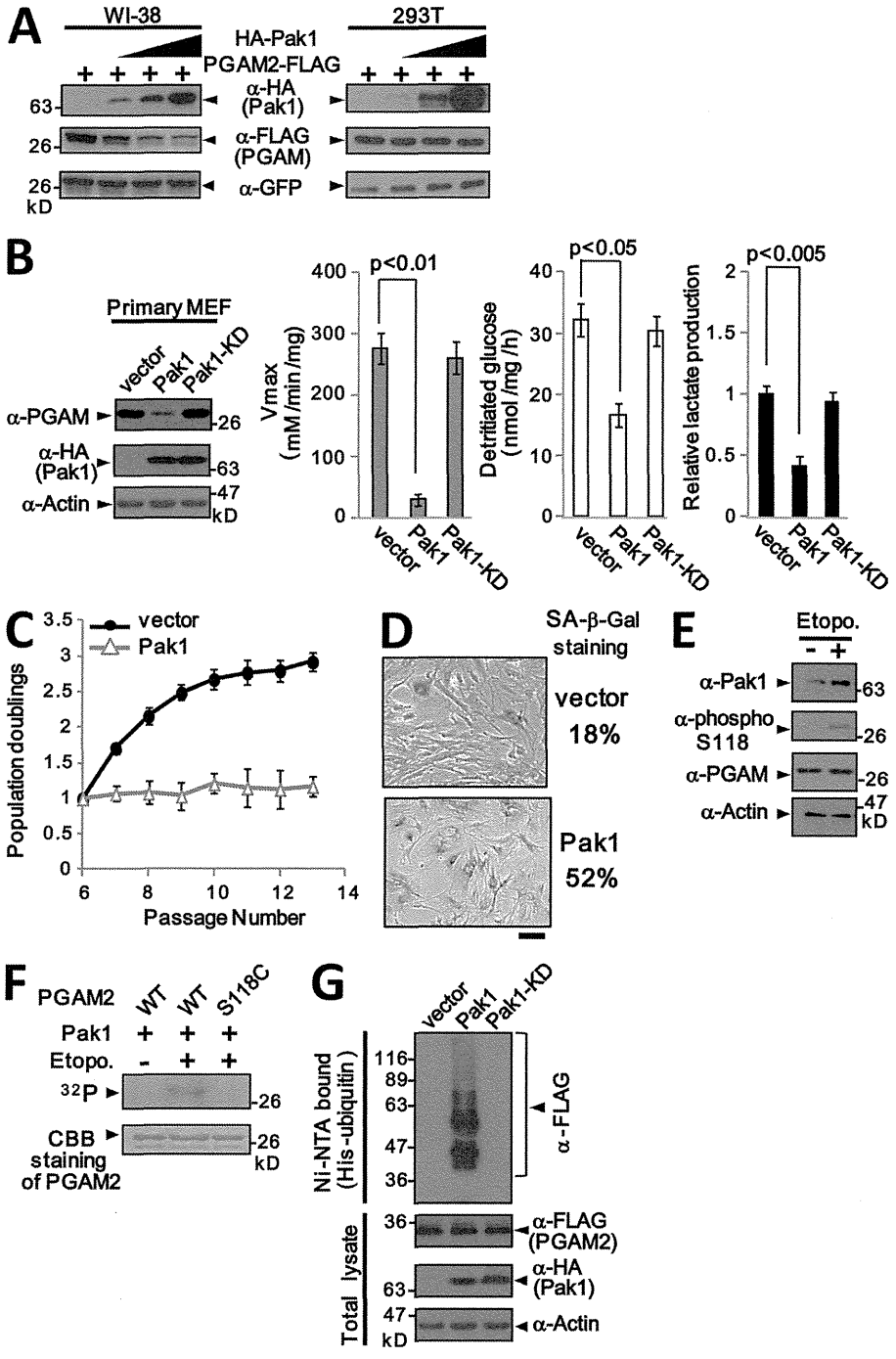

Figure 2. **Pak1 induces down-regulation of PGAM and premature senescence.** (A) Human primary fibroblasts (WI-38, left) or immortalized cells (293T, right) were cotransfected with plasmids encoding GFP, PGAM2-FLAG, and increasing amounts of a plasmid encoding HA-Pak1. Cell lysates were analyzed by SDS-PAGE and immunoblotted for the indicated proteins. (B) Primary MEFs were infected with vectors encoding HA-Pak1 or HA-Pak1-KD, in which kinase activity is inactivated by the K299R mutation. At passage 8, cell extracts were assessed for total PGAM protein levels (far left), PGAM enzymatic activity (second from the left), glycolytic flux (third from the left), and lactate production (far right). (C) Growth curves of primary MEFs infected with Pak1 or empty vector, passaged in a 3T3 protocol. (D) Example of SA-β-Gal staining in Pak1-expressing and control MEFs. Bar, 20 µm. (E) Primary MEFs at early passages were treated with 20 µM etoposide in the presence of 20 µM MG132, and cell lysates were immunoblotted with antibodies against Pak1 and phospho-Ser118 in PGAM as indicated. (F) Pak1 kinase phosphorylates PGAM2 at Ser118 in vitro. Pak1 proteins were immunoprecipitated from primary MEFs treated with 20 µM etoposide, as indicated. The precipitated proteins were co-incubated for 30 min with recombinant PGAM2 or the S118C mutant, and the incorporation of ^{32}P was detected by autoradiography. (G) Pak1 promotes ubiquitination of PGAM2. MEFs were transfected with PGAM2-FLAG, His_6-ubiquitin, and either HA-Pak1 or HA-Pak1-KD. Ubiquitinated proteins were recovered using Ni-NTA agarose and immunoblotted with FLAG antibody. In B and C, error bars indicate SEM ($n = 3$).

after MG132 treatment (Fig. S2 D), and polyubiquitinated forms of PGAM were detected in Pak1-expressing MEFs (Fig. 2 G and Fig. S2, E and F).

Phosphorylation by Pak1 is required for ubiquitination and degradation of PGAM

Next we examined whether phosphorylation by Pak1 is required for the degradation of PGAM, taking three different approaches. First, we used two different shRNAs against mouse Pak1 to knock down the level of endogenous Pak1 in primary MEFs, as assessed by real-time qRT-PCR and Western blotting (Fig. 3 A). Importantly, after ablation of Pak1, the level of endogenous PGAM protein was increased ~1.7–1.9-fold compared with that in control cells (Fig. 3 A). Also, the appearance of ubiquitinated forms of PGAM after DNA damage was reduced in Pak1-deficient MEFs (Fig. 3 B).

Second, we asked whether mutation of the Pak1 phosphorylation sites on PGAM restores its stability. Nonphosphorylatable mutants of PGAM2 in which Ser118 was changed to either Cys or Ala (PGAM2-S118C and PGAM2-S118A) were generated by site-directed PCR mutagenesis. Cotransfection experiments in MEFs revealed that the level of wild-type PGAM, but not of PGAM2-S118C or PGAM2-S118A, was reduced in parallel to the increase of Pak1 expression (Fig. 3 C and Fig. S2 G). We determined the stability of the PGAM2-FLAG protein in primary MEFs by blocking protein synthesis

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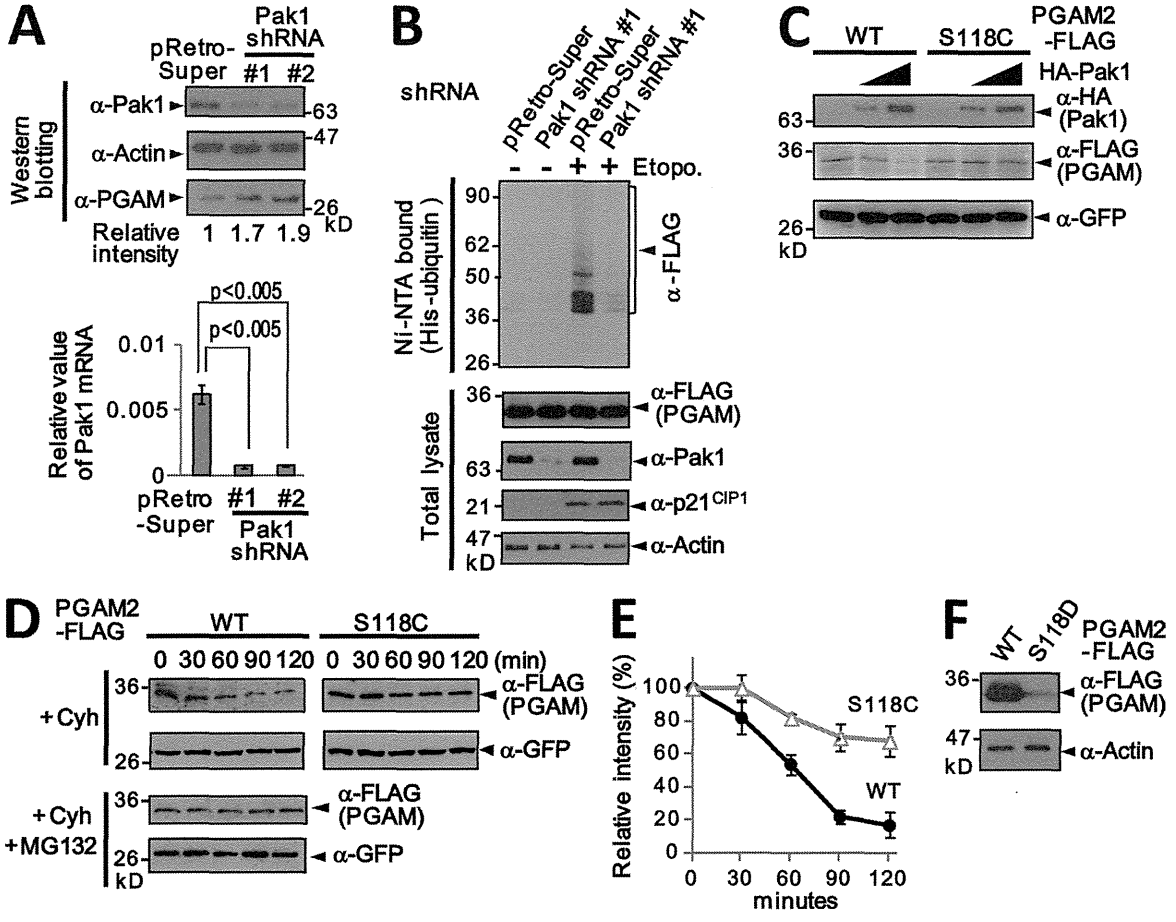

Figure 3. **Pak1-mediated phosphorylation of PGAM on serine 118 dictates PGAM turnover.** (A) Primary MEFs were infected with a retrovirus encoding shRNA against Pak1 (Pak1 shRNA Nos. 1 and 2) or vector control, and the effects on Pak1 mRNA and protein levels were determined by real-time qRT-PCR (bottom) or by immunoblotting (top). The levels of Pak1 mRNA were presented as relative values after normalization by those of RPL13. Probing the same samples with an anti-PGAM antibody showed that Pak1 knockdown by Pak1 shRNA Nos. 1 and 2 caused an ~1.7 and 1.9-fold increase in PGAM levels, respectively. (B) Pak1 knockdown impairs ubiquitination of PGAM2 in stressed cells. MEFs expressing PGAM2-FLAG and His_6-ubiquitin were infected with Pak1 shRNA or vector control, and the cells were then treated with or without 20 μM etoposide. Ubiquitinated proteins were recovered using Ni-NTA agarose and immunoblotted with FLAG antibody. (C) MEFs were cotransfected with plasmids encoding GFP, either the WT or S118C variant of PGAM2-FLAG, and increasing amounts of a plasmid encoding HA-Pak1. Cell extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. (D) Primary MEFs infected with retroviral plasmids encoding GFP and either PGAM2-WT-FLAG or PGAM2-S118C-FLAG were treated with cycloheximide, and samples taken at the indicated times were immunoblotted for PGAM2-FLAG and GFP. (E) The data in D were used to plot the relative band intensity of PGAM2-FLAG at different time points normalized to that of GFP. (F) Primary MEFs were infected with retroviral vectors encoding PGAM2-WT-FLAG or PGAM2-S118D-FLAG and cultured under normal conditions. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies against FLAG and actin. In A and E, error bars indicate SEM (n = 3).

with cycloheximide. The level of PGAM2-WT-FLAG rapidly decreased after cycloheximide treatment (Fig. 3 D, top left), but was restored by treatment with proteasome inhibitor (Fig. 3 D, bottom left). The S118C mutation restored PGAM2 stability by about twofold compared with that of the WT protein (Fig. 3, D and E).

Third, we changed the Ser118 residue to aspartic acid (PGAM-S118D) to mimic the phosphorylated form of PGAM, and examined the effects on stability. In transfected MEFs, under normal culture conditions, the expression of PGAM2-S118D-FLAG was much lower than that of PGAM2-WT-FLAG (Fig. 3 F). In this setting, we also noted that PGAM2-S118D-FLAG, but not PGAM2-S118A-FLAG, was highly ubiquitinated (Fig. S2 H). Collectively, our data indicate that Pak1-dependent phosphorylation of PGAM on Ser118 promotes its ubiquitin-dependent proteasomal degradation.

Mdm2 mediates PGAM ubiquitination

As ubiquitination generally requires a substrate-specific ubiquitin ligase (Hershko and Ciechanover, 1998), we next sought to identify the E3 ubiquitin ligase that targets PGAM. Having shown that PGAM is vulnerable to ubiquitin-mediated turnover in primary fibroblasts but not in 293T cells (Fig. 2 A), we extended these analyses to include additional examples of immortalized cells in order to uncover potentially relevant oncogenic determinants. Using a panel of colon cancer cell lines, we found that PGAM ubiquitination was induced by DNA damage in cells with wild-type p53 (HCT116 and RKO) but not in those with impaired p53 function (HT29 and Sw620; Fig. S3 A; Rodrigues et al., 1990). Consistent with the possibility that ubiquitination of PGAM might be p53 dependent, endogenous PGAM levels were down-regulated after tetracycline-induced expression of p53-GFP fusion protein in the TGP53-4 mouse cell line (Fig. S3 B,

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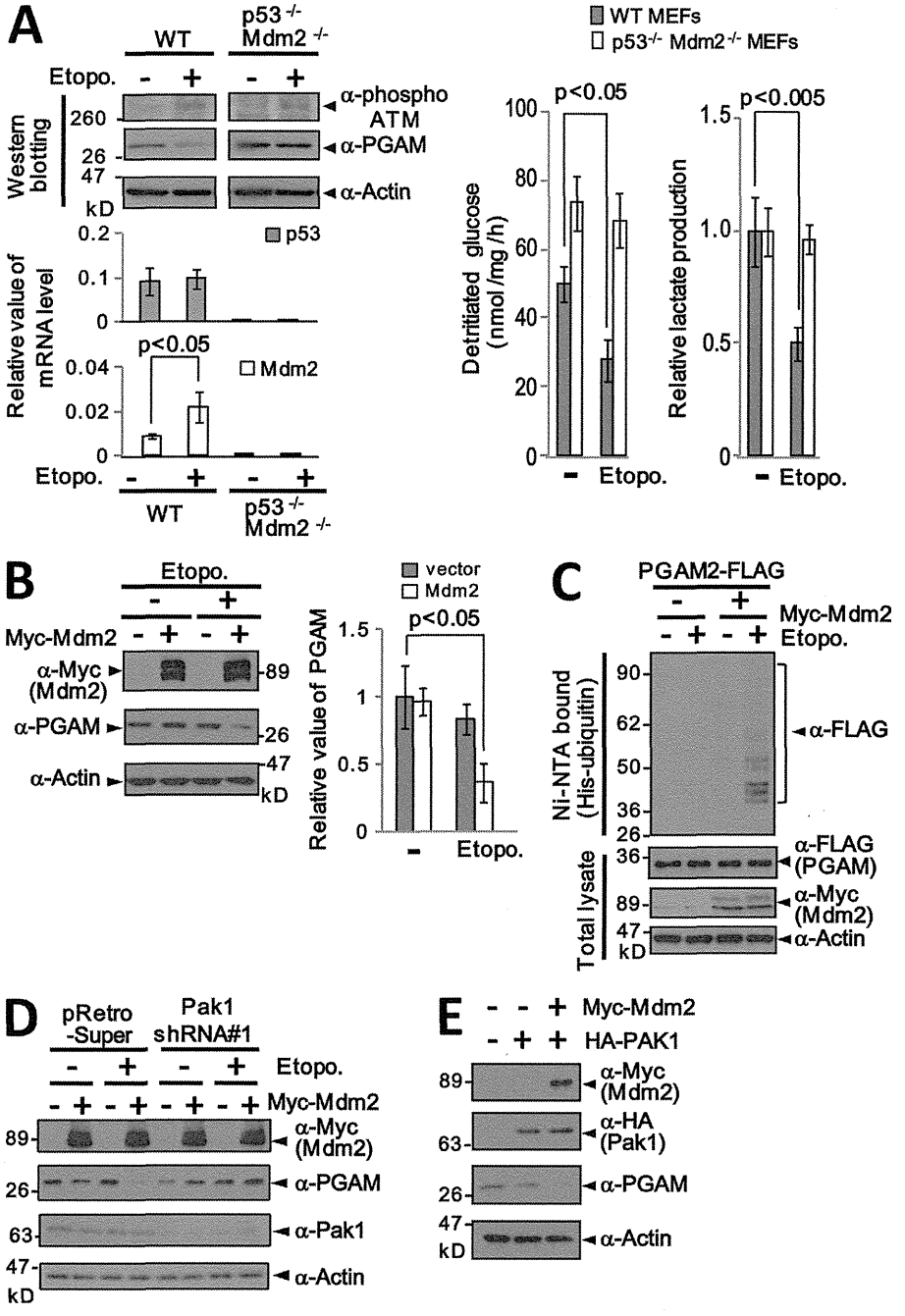

Figure 4. **Role of Mdm2 in PGAM ubiquitination.** (A) Effects of DNA damage (20 μM etoposide) on PGAM protein and RNA levels in wild-type and $p53^{-/-}Mdm2^{-/-}$ MEFs (left). The top panels show the results of immunoblotting; the bottom panels show real-time qRT-PCR for the indicated target genes. The levels were presented as relative values after normalization by those of RPL13. The effect of DNA damage on glycolytic flux (middle) and lactate production (right) in wild-type and $p53^{-/-}Mdm2^{-/-}$ MEFs is shown. (B) $p53^{-/-}Mdm2^{-/-}$ MEFs were transfected with control vector or a vector encoding Myc-Mdm2, and treated with or without etoposide. Extracts were immunoblotted for the indicated proteins (left), and band intensities were used to assess the levels of PGAM relative to those of actin in each sample (right). The results were normalized to the PGAM/actin ratio in untreated cells. (C) Myc-Mdm2 restores DNA-damage dependent ubiquitination of PGAM2-FLAG in $p53^{-/-}Mdm2^{-/-}$ MEFs. (D) Pak1 knockdown impairs Mdm2-mediated turnover of PGAM after DNA damage. $p53^{-/-}Mdm2^{-/-}$ MEFs infected with Pak1 shRNA or control retroviruses were transfected with Myc-Mdm2 and treated with etoposide as indicated. Cell extracts were analyzed by SDS-PAGE, and endogenous PGAM was detected by immunoblotting. (E) Assessment of endogenous PGAM protein levels in $p53^{-/-}Mdm2^{-/-}$ MEFs expressing HA-Pak1 and Myc-Mdm2 as indicated. In A and B, error bars indicate SEM ($n = 3$).

left), accompanied by a decline in glycolytic flux and lactate production (Fig. S3 B, middle and right). Conversely, the ability of Ras-G12V to induce down-regulation of PGAM in MEFs was abrogated by coexpression of HPV E6, which facilitates p53 degradation (Fig. S3 C).

As these data indicate that PGAM is ubiquitinated in a p53-dependent manner, we considered Mdm2 as a likely candidate for the relevant ubiquitin ligase. Mdm2 is both a transcriptional target for p53 and responsible for its ubiquitin-mediated degradation (Haupt et al., 1997; Kubbutat et al., 1997; Prives, 1998). Importantly, we did not observe down-regulation of PGAM, glycolytic flux, or lactate production after DNA damage in $p53^{-/-}$ or $p53^{-/-}Mdm2^{-/-}$ double knockout (DKO) MEFs (Fig. 4 A and Fig. S3 D). However, down-regulation and ubiquitination of PGAM1 and PGAM2 was reinstated after ectopic expression of Myc-Mdm2 in DKO MEFs (Fig. 4, B and C; and Fig. S3 E). This effect was Pak1 dependent, as shRNA-mediated knockdown of Pak1 reduced the turnover of PGAM in Myc-Mdm2–expressing DKO MEFs (Fig. 4 D and Fig. S3 F). Moreover, coexpression of Myc-Mdm2 and HA-Pak1 induced PGAM turnover in unstressed DKO MEFs (Fig. 4 E). The S118C or S118A mutation restored PGAM2 stability in DKO MEFs coexpressing Myc-Mdm2 and HA-Pak1 (Fig. S3, G and H). Collectively, the data imply that Mdm2 has a critical role in Pak1-dependent degradation of PGAM.

To investigate whether Mdm2 is directly responsible for ubiquitinating PGAM, we first confirmed that the proteins physically interact in the cell. Myc-Mdm2 can be coimmunoprecipitated

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with both PGAM1 and PGAM2 when the proteins are expressed in DKO MEFs (Fig. 5, A and B). To gain further insights, we constructed a series of deletions and point mutations in PGAM2-FLAG (Fig. 5 C, top) and showed that Myc-Mdm2 coprecipitated with full-length PGAM2-FLAG (T1) and with the central one-third of the protein (T4). However, the most robust interaction was with the N-terminally deleted variants of PGAM-FLAG (T2 and TC; Fig. 5 C).

Remarkably, the S118D mutation, which mimics S118 phosphorylation, enhanced the binding of full-length PGAM2 to Mdm2 (Fig. 5 D), which suggests that Pak1-dependent phosphorylation at S118 promotes the interaction. Moreover, DNA damage facilitated the association between endogenous PGAM and Mdm2 in primary MEFs (Fig. 5 E) and also of the ectopically expressed proteins in DKO MEFs (Fig. 5 F). Conversely, shRNA-mediated knockdown of Pak1 abrogated the physical interactions between Myc-Mdm2 and PGAM-FLAG (Fig. 5 G). Based on these results, we concluded that the physical interaction between PGAM and Mdm2 is facilitated by Pak1-dependent phosphorylation after DNA damage.

Mdm2 functions as an E3 ubiquitin ligase for PGAM in vitro

As further confirmation that Mdm2 functions directly as an E3 ubiquitin ligase for PGAM, we reconstituted the reaction in vitro using recombinant proteins. Ubiquitination is accomplished by the coordinated actions of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligase (E3). The assays comprised UBE1 as the E1, UbcH5b as the E2 protein, human Mdm2 as the E3 ligase, and PGAM2 as the substrate (Fig. S4 A), and functionality was confirmed using recombinant p53 protein as a control (Fig. 6 A). Although full-length PGAM2 did not appear to be ubiquitinated in vitro as a recombinant protein (Fig. 6 A), the central domain (T4) showed strong evidence for monoubiquitination and some degree of polyubiquitination (Fig. 6 B). Although the N- and C-terminal domains of PGAM2 (TN and TC, respectively) were not modified, polyubiquitination was clearly observed with the T2 construct, which contains both the central and the C-terminal domains of PGAM2 (Fig. 6, C and G), in line with its more avid binding to Mdm2 (Fig. 5 C). Importantly, a catalytically inactive mutant of Mdm2, C464A (Kubbutat et al., 1999), did not ubiquitinate PGAM2-T2 in vitro (Fig. 6 C), which supports the idea that Mdm2 directly ubiquitinates PGAM.

Full-length PGAM2 contains 20 lysine residues in total (Fig. 6 D), seven of which are located in the central domain that appears to be the major target for ubiquitination. Ser118 is also located in this central domain (Fig. S4 B). Mutated versions of PGAM2 were generated in which either four or seven of the lysine residues flanking Ser118 were changed to arginine (PGAM2-4R and -7R; Fig. 6 D and Fig. S4 B). These mutations did not affect the catalytic properties of PGAM2 as judged by its ability to stimulate glycolytic flux and lactate production in primary MEFs (Fig. S4 C). However, immunoblot analyses indicated that the down-regulation of PGAM by Pak1 in primary MEFs was partially alleviated by the 4R mutation, and completely alleviated by the 7R mutation (Fig. S4 D). The 7R mutation also restored PGAM catalytic activity, glycolytic flux, and lactate production in Pak1-expressing MEFs (Fig. S4 E). Strikingly, the 7R mutation rendered PGAM resistant to degradation by DNA damage (Fig. 6 E), and completely inhibited PGAM ubiquitination in cultured cells (Fig. 6 F and Fig. S4 F) and in vitro (Fig. 6 G).

Stabilization of PGAM restores the proliferative potential of primary fibroblasts under stress conditions

Although Mdm2 is generally considered to be an oncogene, its role is subtle, and ectopic expression of wild-type Mdm2 has been reported to cause cell cycle arrest (Brown et al., 1998). Mdm2 contains so-called growth inhibitory domains (ID1 and ID2; Fig. 7 A) that are aberrantly spliced out in some human cancers (Brown et al., 1998). These observations suggest that in some contexts Mdm2 can function as a tumor suppressor (Manfredi, 2010). Interestingly, we noticed that after DNA damage, wild-type MEFs expressing Mdm2 displayed reduced viability compared with control cells (Fig. 7 B). A plausible explanation would be that the negative effects of Mdm2 on cell proliferation and viability reflect destabilization of PGAM. In line with this idea, coexpression of PGAM-7R restored cell viability and PGAM activity in Mdm2-expressing wild-type MEFs (Fig. 7, C and D).

An obvious corollary would be that mutations in Mdm2 that abolish its negative effects on PGAM might be pro-tumorigenic. According to the Catalogue of somatic mutations in cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic/), three potentially oncogenic mutations in Mdm2 have been identified: Y281H in glioma and the W329G and M459I mutations in lung carcinoma (Cancer Genome Atlas Research Network, 2008; Ding et al., 2008). Y281H and W329G are located in the ID2 domain, whereas M459I is located in the RING finger motif that is essential for the ubiquitin ligase activity of Mdm2 (Fig. 7 A and Lipkowitz and Weissman, 2011).

We constructed mammalian expression vectors encoding the three cancer-associated variants of Mdm2, as well as an additional mutation in the catalytic RING finger domain (C464A), and evaluated their effects on both PGAM and p53. As Mdm2 can have an impact on p53 function both catalytically, by promoting its ubiquitination, or noncatalytically, by blocking its transcriptional activation domain (Kubbutat et al., 1999; Poyurovsky et al., 2003), we first assessed whether the variant forms of Mdm2 could suppress p53 activity in promoter assays. In the experiment, all four of the Mdm2 variants suppressed p53 activity on the $p21^{CIP1}$, Bax, and Mdm2 promoters to a degree similar to wild-type Mdm2 (Fig. S5 A). In contrast, only the mutations in RING domain (M459I and C464A) abolished the ability of Mdm2 to ubiquitinate p53 in cells and in vitro (Fig. 6 A and Fig. S5, B–D). Importantly, when assessed for their effects on PGAM2 stability, in conditions of DNA damage, all of the Mdm2 variants were at least partially impaired, and M459I fully so (Fig. 7, E–G). The impaired ubiquitination of PGAM2 shown by the cancer-associated Mdm2-M459I variant was comparable to that of the Mdm2-C464A mutant (Fig. S5 E). However, the M459I mutation did not abolish the physical interaction between Mdm2 and either PGAM or p53 (Fig. S5, F and G).

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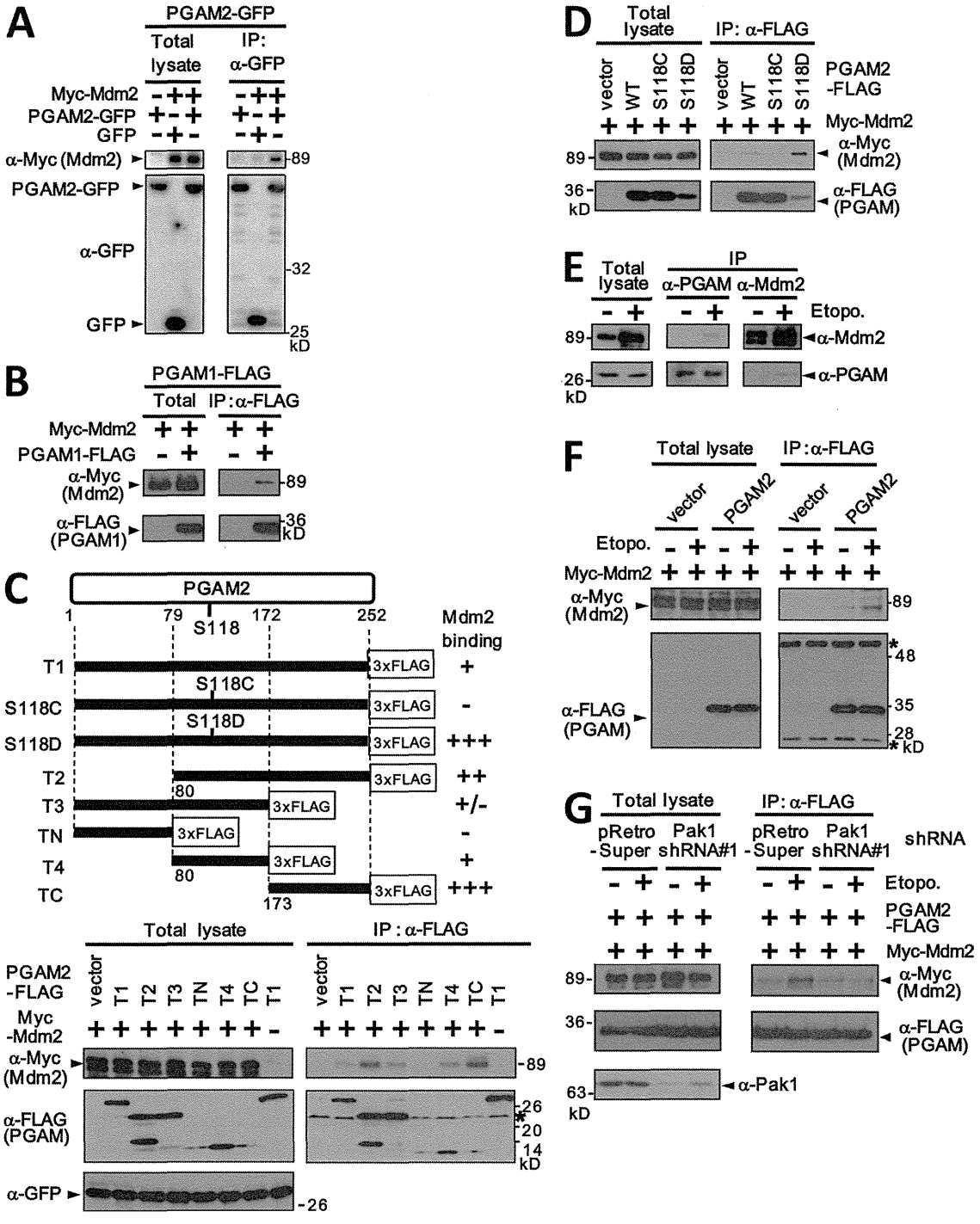

Figure 5. **Pak1-mediated phosphorylation of PGAM2 facilitates its interaction with Mdm2.** (A) *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were transfected with vectors encoding PGAM2-GFP, GFP, and Myc-Mdm2 as indicated. Cell lysates were either analyzed immediately (left) or after immunoprecipitation with an anti-GFP antibody (right), and immunoblotted with antibodies against Myc and GFP. (B) Coimmunoprecipitation of PGAM1-FLAG with Myc-Mdm2. *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were transfected with the indicated vectors and cell lysates were either analyzed directly (left) or after immunoprecipitation with an anti-FLAG antibody (right). (C) Schematic diagram of PGAM2 deletion mutants and the location of serine 118 (top). The relative binding of each mutant to Myc-Mdm2, shown on the right, was assessed by coimmunoprecipitation (bottom). Extracts from *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs transfected with the indicated plasmids were immunoprecipitated with anti-FLAG antibody and immunoblotted for Myc, FLAG, and GFP as indicated. Asterisks indicate the heavy and light chains of immunoglobulin. (D) Coimmunoprecipitation of Mdm2 and PGAM2-S118D under normal culture conditions. *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were transfected with Myc-Mdm2 and the indicated PGAM2-FLAG mutants. (E) Reciprocal coimmunoprecipitation of endogenous Mdm2 protein with endogenous PGAM protein. Extracts from primary MEFs, treated with or without etoposide in the presence of 20 µM MG132, were immunoprecipitated with either anti-PGAM or anti-Mdm2 antibodies and immunoblotted with the same reagents. (F) DNA damage facilitates the physical interaction between PGAM and Mdm2. *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs expressing Myc-Mdm2 and PGAM2-FLAG were treated with and without 20 µM etoposide, as indicated. Cell lysates were either analyzed immediately (left) or after immunoprecipitation with an anti-FLAG antibody (right). (G) The physical interaction between Mdm2 and PGAM2 is Pak1 dependent. *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were infected with Pak1 shRNA or control virus. The cells were then transfected with PGAM2-FLAG and Myc-Mdm2 and subjected to DNA damage with 20 µM etoposide. Cell extracts were immunoprecipitated with anti-FLAG antibody and immunoblotted for the FLAG and Myc epitopes.

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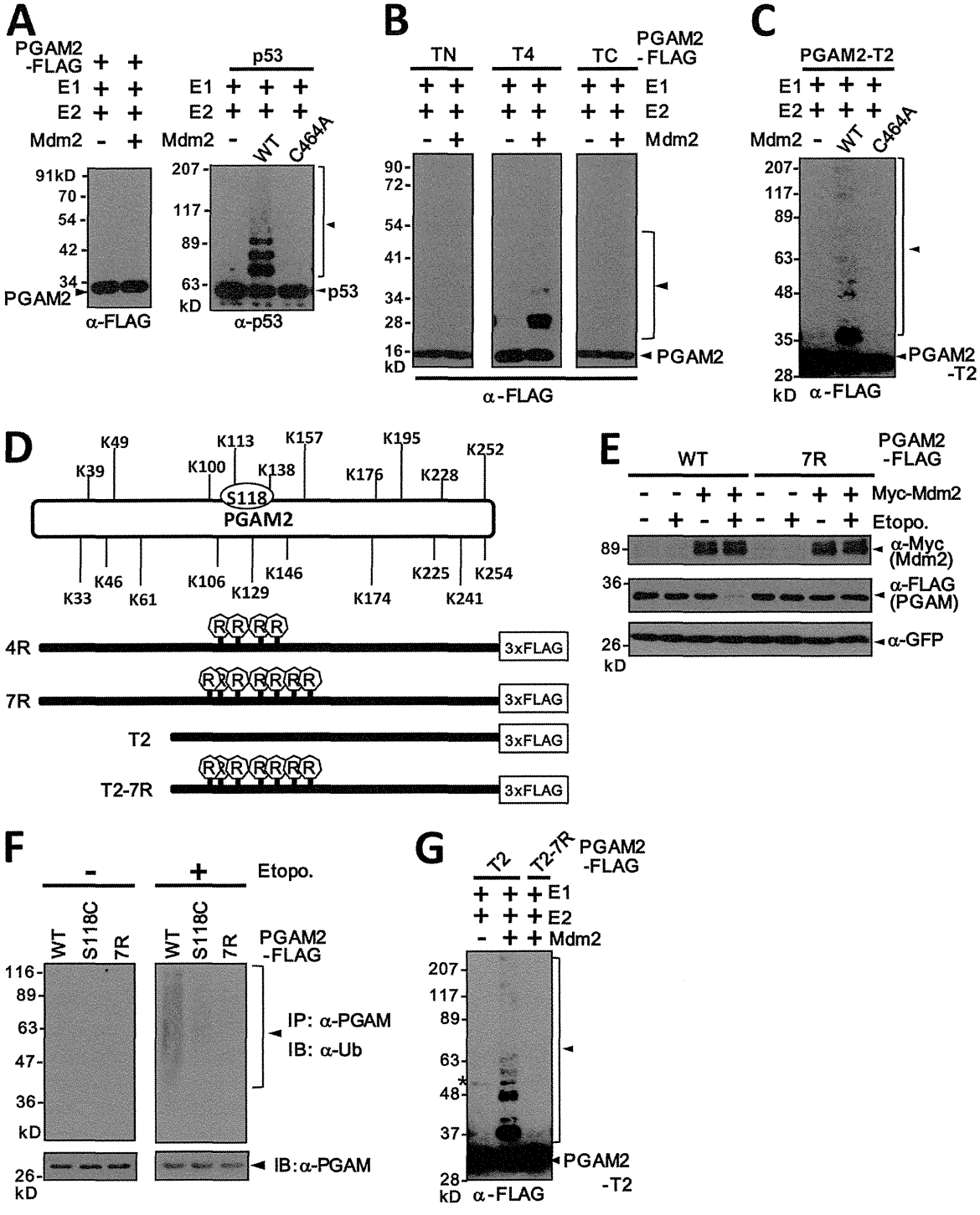

Figure 6. **Mdm2 ubiquitinates PGAM2 in vitro.** (A) An in vitro ubiquitination system comprising recombinant UBE1, UbcH5b-His, Mdm2, and His_6-ubiquitin was tested for its ability to ubiquitinate full-length recombinant PGAM2-FLAG (left) or p53 (right). Mdm2-C464A was used as a negative control. Ubiquitination of p53 resulted in the formation of a ladder of bands. (B) The same assay was performed on the deleted versions of PGAM2 described in Fig. 5 C. Only PGAM2-T4-FLAG, corresponding to residues 80–172 in PGAM2, was efficiently ubiquitinated under these conditions. (C) PGAM2-T2-FLAG is ubiquitinated in vitro by wild-type Mdm2, but not by a catalytically inactive mutant of Mdm2 (Mdm2-C464A). (D) Schematic representation of PGAM2, showing the location of lysine residues relative to Ser118. The 7R-FLAG and 4R-FLAG variants of PGAM2 were generated by site-directed mutagenesis of either seven or four lysine residues in the central domain as indicated. The mutated residues in the 4R mutant were K106, K113, K129, and K138, whereas three additional residues, K100, K146, and K157, were mutated in 7R. PGAM2-T2-7R-FLAG is an N-terminally truncated version of 7R. (E) *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were cotransfected with expression vectors encoding GFP, Myc-Mdm2, and either PGAM2-WT-FLAG or PGAM2-7R-FLAG. After treatment with or without 20 μM etoposide, cell extracts were analyzed by SDS-PAGE and immunoblotting. (F) Mutations in PGAM2 impair its ubiquitination. Primary MEFs expressing the indicated forms of PGAM2-FLAG were treated with or without etoposide, followed by MG132 treatment. Immunoprecipitated PGAM2-FLAG was analyzed by immunoblotting with anti-Ubiquitin antibody (α-Ub; top) and anti-FLAG antibody (bottom). (G) Mdm2 can ubiquitinate PGAM2-T2-FLAG, but not PGAM2-T2-7R-FLAG, in vitro. The indicated recombinant proteins were used in an in vitro ubiquitination assay as in A. The asterisk indicates a nonspecific band. The arrowhead indicates polyubiquitinated proteins.