寺本信嗣，山本寛,	II 呼吸器疾患「肺炎」	井藤英喜	日常診療に活かす老年病ガイドブック6 高齢者に多い疾患の診療の実際	MEDICAL VIEW	東京	2006	pp12－17
松瀬健、寺本信嗣.	第3章、老年症候群. 嚥下障害.	日野原重明、井村裕夫.	「看護のための最新老年医学講座」[第2版]、第17巻 老人の医療.	中山書店	東京	2005	pp203 －207
寺本信嗣	呼吸器疾患	日本老年医学会	高齢者の安全な薬物療法ガイドライン2005	MEDICAL VIEW	東京	2005	Pp67－77
寺本信嗣	インフルエンザ、ノイラミニダーゼ阻害薬	長瀬隆英、大石 展也.	臨床に直結する呼吸器疾患治療のエビデンス	文光堂	東京	2005	Pp109-111
寺本信嗣	第4章到達目標の解説（14）感染症、③結核	水嶋春朔	新医師臨床研修制度における指導ガイドライン 試	厚生労働省	東京	2006	4－390
寺本信嗣	第4章到達目標の解説（6）呼吸器系疾患、③閉塞性・拘束性肺疾患（気管支喘息・気管支拡張症）	水嶋春朔	新医師臨床研修制度における指導ガイドライン 試	厚生労働省	東京	2006	4－238

寺本信嗣	第4章到達目標の解説（6）呼吸器系疾患、⑦肺癌	水嶋春朔	新医師臨床研修制度における指導ガイドライン試	厚生労働省	東京	2006	4−252

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Teramoto S, Ishii T, Yamamoto H, Yamaguchi Y, Matsuse T	Xenobiotic enzymes and genetics of COPD.	Chest	127(1)	408-9	2005
Teramoto S, Yamamoto H, Yamaguchi Y, Namba R, Ouchi Y	Obstructive sleep apnea causes systemic inflammation and metabolic syndrome.	Chest	127(3)	1074-5	2005
Teramoto S, Ishii T, Yamamoto H, Yamaguchi Y, Ouchi Y	Apoptosis of circulating neutrophils and alveolar macrophages in COPD.	Chest	127(3)	1079-80	2005

Teramoto S, Ishii T, Yamamoto H, Yamaguchi Y, Namba R, Hanaoka Y, Takizawa M, Okada T, Ishii M, Ouchi Y	Significance of chronic cough as a defense mechanism or a symptom in elderly patients with aspiration and aspiration pneumonia.	Eur Respir J.	25(1)	210-1	2005
Teramoto S, Ishii T, Yamamoto H, Yamaguchi, Ouchi Y	Nasogastric tube feeding is a significant cause of aspiration pneumonia in mechanically ventilated patients.	Eur Respir J.	27(2)	436-7	2006
Kume H, Teramoto S, Tomita K, Nishimatsu H, Takahashi S, Takeuchi T, Ota N, Kitamura T	Bladder recurrence of upper urinary tract cancer after laparoscopic surgery.	J Surg Oncol.	93(4)	318-322	2006
馬場幸，寺本信嗣，長谷川浩，町田綾子，秋下雅弘，鳥羽研二	痴呆高齢者に対する嚥下障害のスクリーニング方法の検討 簡易嚥下誘発試験と反復唾液嚥下テストの比較	日本老年医学会雑誌	42(3)	323-327	2005

寺本信嗣，山本寛，山口泰弘，南波亮一，石井正紀，大内尉義	【気管支喘息におけるプロピオン酸ベクロメタゾン(HFA-134a-BDP: キュバール TM)の使用経験】肺機能低下症例に対する使用経験	アレルギー・免疫	12(3)	490-494	2005
寺本信嗣，山本寛，山口泰弘，南波亮一，岡田徹，花岡陽子，石井正紀，大内尉義	【高齢者総合的機能評価】 Case Report 高齢者総合的機能評価が高齢者肺炎の治療戦略,予後改善に有効であった 2 例	Geriatric Medicine 老年医学	43(4)	603-608	2005
寺本信嗣	COPD 研究と診療の進歩 1)リスク因子からみたCOPDの成因—COPD 発症の危険因子—	日本内科学会雑誌	94	1775—1779	2005
寺本信嗣	慢性呼吸器疾患と骨粗鬆症との関係について	The Lung Perspective	13	89 - 91	2005
寺本信嗣	【日常診療にすぐ役立つ輸液と電解質異常】 私の処方．長期経口摂取不能患者の静脈栄養	臨床医	31	776-778	2005
寺本信嗣、山本寛	I． COPD とは 3.COPD 発症の危険因子	化学療法の領域	21(S－1)	21－26	2005
寺本信嗣、山本寛	全身性疾患としてのCOPD	Progress in Medicine	25(4)	960－964	2005

寺本信嗣、山本寛、山口泰弘	医学と医療の最前線 COPD における吸入療法の新展開 COPD の吸入	日本内科学会雑誌	94	788-793	2005
寺本信嗣、山本寛、山口泰弘	慢性閉塞性肺疾患（COPD）との境界例	診断と治療	93(11)	1992-1996	2005
寺本信嗣	呼吸器感染症と栄養障害	栄養、評価と治療	22(1)	52-54	2005
寺本信嗣	老化モデル肺にみる COPD	COPD FRONTIER	3(4)	325-329	2005

山田思鶴

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Akishita M, Yamada S, Nishiya H, Sonohara K, Nakai R, Toba K	Effects of physical exercise on plasma concentrations of sex hormones in elderly women with dementia.	J Am Geriatr Soc	53	1076-7	2005
鳥羽研二，大河内二郎，高橋泰，松林公蔵，西永正典，山田思鶴，高橋龍太郎，西島令子，小林義雄，町田綾子，秋下雅弘，佐々木英忠	転倒リスク予測のための「転倒スコア」の開発と妥当性の検証	日老医誌	42	346-352	2005

若槻明彦

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Oguri H, Maeda N, Yamamoto Y, Wakatsuki A, Fukaya T	Non-puerperal uterine inversion associated with endometrial carcinoma--a case report.	Gynecol Oncol	97	973-975	2005

柳瀬敏彦

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Wu Y, Ghosh S, Nishi Y, YANASE T, NAGAWA H, Hu Y	The orphan nuclear receptor NURR1 and NGFI-B modulate aromatase gene expression in ovarian granulose cells: A possible mechanism for repression of aromatase expression upon luteinizing hormone surge	Endocrinology	146	237-46	2005
Nishi Y, Hosoda H, Mori K, Kaiya H, Sato T, Fukue Y, Fukushima N, Yanase T, Nawata H, Kangawa K, Kojima M	Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghreln.	Endocrinology	146	2255-64	2005
Fan W, Yanase T, Wei L, Nomura M, Okabe T, Goto K, Harada N, Nawata H	Activation of peroxisome proliferator activated receptor and retinoid X receptor inhibits CYP19 transcription through NF-B in ovarian granulosa cells.	Endocrinology	146	85-92	2005

Fan W, Yanase T, Nomura M, Okabe T, Goto K, Sato T, Kawano H, Kato S, Nawata H	Androgen receptor null male mice develop late-onset obesity due to decreased energy expenditure and lypolytic activity but show normal insulin sensitivity with high adiponectin secretion.	Diabetes	54(4)	1000-1008	2005
Ashida K, Goto K, Zhao Y, Okabe T, Yanase T, Takayanagi R, Nomura M, Nawata H	Dehydroepiandrosterone negatively regulates the p38 mitogen-activated protein kinase pathway by a novel PTPN7 locus-derived transcript	Biochim Biophys Acta	1728	84-94	2005
Taniyama M, Tanabe M, Saito H, Ban Y, Nawata H, Yanase T	Subtle 17α-hydroxylase/17,20-lyase deficiency with homozygous Y201N mutation in an infertile women.	J Clin Endocrinol Metab	90	2508-2511	2005
Nagasawa E, Abe Y, Nishimura J, Yanase T, Nawata H, Muta K	Pivotal role of proxisome proliferators-activated receptor gamma (PPAR gamma) in regulation of erythroid progenitor cell proliferation and differentiation.	Experimental Hematology	33	857-64	2005
Kawate H, Wu Y, Ohnaka K, Tao RH, Nakamura K, Okabe T, Yanase T, Nawata H, Takayanagi R.	Impaired nuclear translocation, nuclear matrix targeting, and intranuclear mobility of mutant androgen receptors carrying amino Acid substitutions in the deoxyribonucleic Acid-binding domain derived from androgen insensitivity syndrome patients.	J Clin Endocrinol Metab.	90	6162-9	2005
Chen G, Nomura M, Morinaga H, Matsubara E, Okabe T, Goto K, Yanase T, Zheng H, Lu J, Nawata H	Modulation of androgen receptor transactivation by FoxH1. A newly identified androgen receptor corepressor.	J Biol Chem.	280	36355-63	2005
Harris SE, Chand AL, Winship IM, Gersak K, Nishi Y, Yanase T, Nawata H, Shelling A	INHA promoter polymorphisms areassociated with premature ovarian failure.	Mol Hum Reprod	11	779-784	2005

Fan S, Goto K, Chen G, Morinaga H, Nomura M, Okabe T, Nawata H, Yanase T	Identification of the functional domains of ANT-1, a novel coactivator of the androgen receptor.	Biochem Biophys Res Commun.	341	192-201	2006
Liu W, Liu M, Fan W, Nawata H, Yanase T	The Gly146Ala variation in human SF-1 gene: its association with insulin resistance and type 2 Diabetes in Chinese	Diabetes Research and Clinical Practice			2006 in press
柳瀬　敏彦, 名和田　新	Aging male における肥満（visceral obesity を中心に）.	Geriatric Medicine	43	197-201	2005
柳瀬　敏彦	医学と医療の最前線：DHEA補充療法の現状と展望.	日本内科学会雑誌	94	2195-2199	2005
柳瀬　敏彦, 范 呉強, 名和田　新	アンドロゲン受容体ノックアウトマウスの肥満機序の解明.	Therapeutic Research	26	52-58	2005
柳瀬　敏彦	特集；長寿医学と生活習慣病の克服:長寿とホルモン補充療法.	成人病と生活習慣病	35[7]	724-729	2005

keeping with the harsher American climate and influence of the Protestant ethic, cold-water wraps were the rule in the New World.

Almost 100 years after Alzheimer's death, we seem to have come full circle in our attempts to address humanely with the behavioral manifestations of dementia. Clearly, one hopes that articles such as these will prevent us from throwing out this important form of treatment along with the bath water.

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REFERENCES

1. Sloane PD, Hoeffer B, Mitchell M et al. Effects of person-centered showering and the towel bath on bathing-associated aggression, agitation, and discomfort in nursing home residents with dementia: A randomized, controlled trial. J Am Geriatr Soc 2004;52:1795–1804.
2. Naik AD, Concato J, Gill TM. Bathing disability in community-living older persons. Common, consequential, and complex. J Am Geriatr Soc 2004;52:1805–1810.
3. Evans L. The bath! Reassessing a familiar elixir in old age. J Am Geriatr Soc 2004;52:1957–1958.
4. Mauer K, Mauer U. Alzheimer: The Life of a Physician and the Career of a Disease. New York: Columbia University Press, 2004.
5. Whorton JC. Nature Cures. The History of Alternative Medicine in America. Oxford: Oxford University Press, 2002.

EFFECTS OF PHYSICAL EXERCISE ON PLASMA CONCENTRATIONS OF SEX HORMONES IN ELDERLY WOMEN WITH DEMENTIA

To the Editor: Physical exercise may slow the functional decline in elderly people and has been associated with a low incidence of dementia.[1] Physical activities have shown favorable effects on cognitive function as well as on neuropsychiatric symptoms and behavioral disturbance in demented subjects,[1,2] the mechanism of which is currently unknown. Because low plasma levels of sex hormones have been implicated in dementia,[3] it is reasonable to hypothesize that physical exercise could elevate plasma sex hormone levels. Here, we report a preliminary study in which daily physical exercise for 3 months increased the plasma levels of sex hormones, including dehydroepiandrosterone (DHEA) and testosterone, in elderly women with dementia. Thirteen women (aged 74–91, mean age ± standard deviation 84 ± 5) living in group homes for the elderly (small-scale facilities providing communal living) located in Nagano Prefecture, Japan, were enrolled. They were diagnosed as having Alzheimer's disease according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, but did not have malnutrition, malignancy, or endocrine disease. Blood sampling and functional assessment were performed at baseline, at the end of a 3-month exercise program, and at the end of a 3-month follow-up period, during which the subjects returned to ordinary sedentary living. The exercise program consisted of stretching and mild resistance training using a chair and a 0.5-kg weight. The exercise was performed as a group, with training for 30 minutes daily under the instruction of a physical therapist twice a week and by other caregiver staff five times a week. Care other than exercise was comparable throughout the study. Fasting blood samples were collected early in the morning before exercise. A commercial laboratory determined plasma levels of estradiol, testosterone, DHEA, DHEA sulfate, and sex hormone–binding globulin, in addition to blood cell counts and blood chemical parameters. Basic activities of daily living (ADLs) were assessed using the Barthel Index and cognitive function using the Mini-Mental State Examination.

At baseline, the subjects showed moderate cognitive impairment and dependency and relatively low sex hormone levels (Table 1). After 3 months of exercise, significant increases were found in plasma levels of testosterone of 18%, estradiol of 38%, and DHEA of 37%, all of which returned to the baseline levels 3 months after cessation of the exercise program. A similar alteration was found in plasma DHEA sulfate level, but the increase by exercise was not statistically significant (mean ± standard error 452 ± 62 ng/mL at baseline, 508 ± 72 ng/mL after exercise, and 464 ± 77 ng/mL after discontinuation). Sex hormone–binding globulin, albumin, and other blood parameters did not change throughout the study (Table 1 and data not shown). Despite the increases in sex hormones after the exercise program, neither Barthel Index nor Mini-Mental State Examination scores changed significantly during the study.

Table 1. Effects of Daily Physical Exercise on Plasma Concentrations of Sex Hormones in Elderly Women with Dementia (N = 13)

Measurement	Baseline	Exercise (3 Months)	Discontinuation (3 Months)
	Mean ± Standard Error of the Mean		
Testosterone, ng/dL	51.4 ± 3.3	60.8 ± 3.3†	47.9 ± 3.9
Estradiol, pg/mL	15.2 ± 1.2	21.0 ± 1.2†	19.4 ± 2.9
Dehydroepiandrosterone, ng/mL	1.84 ± 0.29	2.52 ± 0.41*	1.95 ± 0.27
Sex hormone–binding globulin, nmol/L	75.0 ± 6.1	69.1 ± 8.1	68.3 ± 8.3
Barthel Index	75.0 ± 5.4	70.0 ± 7.1	66.5 ± 9.4
Mini-Mental State Examination score	13.9 ± 1.9	13.8 ± 2.0	12.4 ± 2.5

$P <$ *.05; †.01 versus baseline using paired t test.

Previous studies[4,5] have shown stimulatory effects of endurance or resistance exercise on circulating hormones in healthy postmenopausal women; metabolic alterations and increased blood flow of endocrine organs via nitric oxide and cyclic adenosine monophosphate production may play a causal role, but hormonal responses in frail or demented women have not been examined. In the present study, plasma levels of estradiol, testosterone, and DHEA were higher after 3 months of physical exercise in elderly women with dementia, whereas cognitive function and basic ADLs did not improve. Given the protective effect of exercise and sex hormones on cognitive impairment, a control sedentary group should be included to examine whether this exercise program might delay cognitive decline. Nevertheless, the finding that exercise can increase plasma sex hormone levels in demented women provides a mechanistic insight into the effect of exercise or physical activities on cognitive impairment. The results of this preliminary study need to be confirmed using larger randomized, controlled trials with longer follow-up periods.

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REFERENCES

1. Colcombe S, Kramer AF. Fitness effects on the cognitive function of older adults: A meta-analytic study. Psychol Sci 2003;14:125–130.
2. Cummings JL. Alzheimer's disease. N Engl J Med 2004;351:56–67.
3. Almeida OP, Barclay L. Sex hormones and their impact on dementia and depression: A clinical perspective. Expert Opin Pharmacother 2001;2:527–535.
4. Copeland JL, Consitt LA, Tremblay MS. Hormonal responses to endurance and resistance exercise in females aged 19 to 69 years. J Gerontol A Biol Sci Med Sci 2002;57A:B158–B165.
5. Kemmler W, Wildt L, Engelke K et al. Acute hormonal responses of a high impact physical exercise session in early postmenopausal women. Eur J Appl Physiol 2003;90:199–209.

RACIAL DIFFERENCES IN PRESSURE ULCER PREVALENCE IN NURSING HOMES

To the Editor: We read with great interest the recent article regarding black/white differences in the rate of nursing home–acquired pressure ulcers. Indeed, the findings reported were similar to what was found using the Health Care Financing Administration's Multi-State, Case-Mix and Quality Demonstration Project, which involved all Medicare/Medicaid certified nursing homes (n = 1,492) in five U.S. states (Kansas, Maine, Mississippi, New York, South Dakota). We identified 223,448 entrants to nursing homes in these five states over a 4-year period (1992–96). Patients were evaluated using the federally mandated Resident Assessment Instrument, which includes a 300-item Minimum Data Set (MDS). At least 100 residents of each racial/ethnic category were required. A nested linear model provided estimates of state- and sex-stratified differences in pressure ulcer prevalence after adjustment for pressure ulcer risk factors. Across all state/sex strata, blacks were substantially less likely than whites to have a Grade I pressure ulcer recorded. Higher-grade ulcers (II–IV) were, alternatively, consistently higher in blacks than whites, and even greater disparities were seen when only the highest-grade (IV) ulcers were compared. These findings were slightly adjusted when physical mobility (as measured by activities of daily living) was controlled for, although not so much as to change the basic interpretation. Control by additional clinical, diagnostic, behavioral, social, facility, and area-level characteristics failed to reveal any further confounding, demonstrating that this analysis was robust to adjustment for a wide-ranging set of factors identified or hypothesized as risk factors for pressure ulcer development. Moreover, it also observed that pressure ulcer prevalence in Native Americans demonstrated a similar pattern in South Dakota and Mississippi to that of whites; rates of Grade I ulcers were generally lower, whereas higher-grade ulcers were more common in Native Americans than in non-Hispanic whites.

Underdiagnosis of low-grade pressure ulcers in racial minorities, with subsequent progression to open lesions of higher grade, is largely consistent with these findings, as well as the findings of another study.[1] It has been long noted that the definition of low-grade pressure ulcers (persistent nonblanchable erythema) could result in the underdiagnosis of these lesions on dark skin. Because detection of low-grade pressure ulcers is an important factor in preventing their progression to higher stages, underdiagnosis likely contributes to the higher rates of high-grade ulcers found in older blacks and Hispanics. The relationship between contextual factors (such as resources and staffing) related to the types of nursing homes serving predominantly people of color and the underdiagnosis of pressure ulcers needs to be explored. Facilities serving primarily African Americans may have fewer funds and consequently offer fewer services and provide less staff training and amenities than other facilities.[2,3]

Although attempts have been made to enhance the detection of low-grade ulcers in blacks and Hispanics,[4] the extent to which public reporting of quality indicators focused on pressure ulcers and other quality improvement initiatives[5] is likely to reduce or exacerbate[3] racial/ethnic differences in pressure ulcer occurrence in nursing homes remain to be evaluated.

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ORIGINAL ARTICLE

Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras–MAPK signaling in human cancer cells

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The induction of senescence-like growth arrest has emerged as a putative contributor to the anticancer effects of chemotherapeutic agents. Clinical trials are underway to evaluate the efficacy of inhibitors for class I and II histone deacetylases to treat malignancies. However, a potential antiproliferative effect of inhibitor for Sirt1, which is an NAD^+-dependent deacetylase and belongs to class III histone deacetylases, has not yet been explored. Here, we show that Sirt1 inhibitor, Sirtinol, induced senescence-like growth arrest characterized by induction of senescence-associated β-galactosidase activity and increased expression of plasminogen activator inhibitor 1 in human breast cancer MCF-7 cells and lung cancer H1299 cells. Sirtinol-induced senescence-like growth arrest was accompanied by impaired activation of mitogen-activated protein kinase (MAPK) pathways, namely, extracellular-regulated protein kinase, c-*jun* N-terminal kinase and p38 MAPK, in response to epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I). Active Ras was reduced in Sirtinol-treated senescent cells compared with untreated cells. However, tyrosine phosphorylation of the receptors for EGF and IGF-I and Akt/PKB activation were unaltered by Sirtinol treatment. These results suggest that inhibitors for Sirt1 may have anticancer potential, and that impaired activation of Ras–MAPK pathway might take part in a senescence-like growth arrest program induced by Sirtinol.
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Keywords: Sirt1; Sirtinol; cellular senescence; Ras; MAPK; Akt/PKB

Introduction

Cellular senescence is a state with permanent loss of replicative capability even upon mitogenic stimuli. Cellular senescence is characterized by phenotypic alterations including induction of senescence-associated β-galactosidase (SA-β-gal), a large and flat cell morphology and increased expression of plasminogen activator inhibitor 1 (PAI-1) (Goldstein *et al.*, 1994; Dimri *et al.*, 1995).

Immortalization, an escape from the replicative senescence program, is a necessary step for neoplastic transformation of cells. Hence, transformed cells can bypass the replicative senescence program. However, cancer and leukemia cells still retain the capacity to undergo premature senescence in response to various stimuli. Senescent normal human fibroblasts usually exhibit G1 cell cycle arrest. However, polyploidy and multinucleation are also associated with replicative senescence and premature senescence in various cell types, including human normal endothelial cells (Aviv *et al.*, 2001; Wagner *et al.*, 2001) and breast cancer MCF-7 cells (Kim *et al.*, 2003).

Anticancer chemotherapeutic agents and ionizing radiation have been shown to cause senescence-like growth arrest in human cancer cells *in vitro* and *in vivo* (Han *et al.*, 2002; Schmitt *et al.*, 2002; te Poele *et al.*, 2002; Shay and Roninson, 2004). Induction of SA-β-gal staining by chemotherapy was observed *in vivo* in cancer and lymphoma cells in rodents (Elmore *et al.*, 2002; Roninson, 2002; Schmitt *et al.*, 2002; Christov *et al.*, 2003) and in patients with breast cancer (te Poele *et al.*, 2002). Thus, senescence-like growth arrest has been proposed to be a putative determinant of *in vivo* tumor response to chemotherapeutic agents and ionizing radiation (Mathon and Lloyd, 2001; Wang *et al.*, 2003; Ben-Porath and Weinberg, 2004; Kahlem *et al.*, 2004; Pelicci, 2004; Sharpless and DePinho, 2004; Shay and Roninson, 2004).

Sirt1 is a mammalian NAD^+-dependent deacetylase that belongs to class III histone deacetylases (HDACs) (Imai *et al.*, 2000; Landry *et al.*, 2000; Blander and Guarente, 2004). Sir2, yeast homologue of Sirt1, is involved in a number of cellular processes including gene silencing at telomere and mating loci, DNA repair, recombination and aging. Recent studies demonstrated that Sirt1 plays an important role in the regulation of cell fate and stress response in mammalian cells. Sirt1 promotes cell survival by inhibiting apoptosis or cellular senescence induced by stresses including DNA damage

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and oxidative stress. An increasing number of proteins have been identified as substrates of Sirt1, including p53 (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002), FOXO transcription factors (Brunet *et al.*, 2004; Daitoku *et al.*, 2004; Motta *et al.*, 2004; van der Horst *et al.*, 2004), peroxisome proliferator-activated receptor-γ (Picard *et al.*, 2004) and Ku70 (Cohen *et al.*, 2004).

Sirt1 deacetylase, a member of the class III HDAC family, exhibits distinct biochemical characteristics from conventional class I and class II HDACs. Inhibitors for class I and class II HDACs, such as trichostatin A and its derivatives, do not inhibit the deacetylating activity of Sirt1 (Imai *et al.*, 2000). Conversely, specific inhibitors for Sirt1 such as Sirtinol do not inhibit class I and class II HDACs, either (Bedalov *et al.*, 2001; Grozinger *et al.*, 2001).

Class I and class II HDAC inhibitors exhibit antiproliferative effects in human cancer cells (Rosato and Grant, 2004; Vanhaecke *et al.*, 2004; Vigushin and Coombes, 2004). The efficacy of class I and class II HDAC inhibitors for treatment of patients with cancer or leukemia has been examined in phase I and II clinical trials (Piekarz *et al.*, 2001; Sandor *et al.*, 2002; Kelly *et al.*, 2003; McLaughlin and La Thangue, 2004; Piekarz and Bates, 2004). However, the effects of inhibitor for Sirt1 or class III HDACs on cell growth have not yet been investigated. Here, we show that Sirtinol, a specific inhibitor for Sirt1, induced senescence-like growth arrest in human breast cancer MCF-7 and lung cancer H1299 cells, and that Sirtinol-induced senescence-like growth arrest was accompanied by blunted activation of Ras–mitogen-activated protein kinase (MAPK) pathways in response to growth factors.

Results

Sirtinol, Sirt1 inhibitor, induced senescence-like growth arrest in human MCF-7 and H1299 cells

MCF-7 and H1299 cells were exposed to Sirtinol (100 μM) for 24 h; then Sirtinol was removed from the culture media. Treatment with Sirtinol inhibited cell growth in both MCF-7 and H1299 cells (Figure 1a and b). The inhibition of cell growth was persistent and observed up to 9 days after Sirtinol withdrawal. These results suggest that Sirtinol caused a sustained growth arrest. This was supported by reduced incorporation of BrdU in Sirtinol-treated MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, as compared with untreated cells (Figure 1c and d).

We examined the effects of Sirtinol treatment on SA-β-gal activity and the expression of PAI-1, characteristic features of senescence-like growth arrest. Sirtinol treatment increased SA-β-gal-positive cells in a dose-dependent manner 10 days after the addition of Sirtinol in both MCF-7 and H1299 cells (Figures 2 and 3a), but the extent of SA-β-gal induction was relatively smaller in H1299 than in MCF-7 cells. Only a small number of MCF-7 and H1299 cells were SA-β-gal-positive when untreated. Enlarged, flattened morphology was

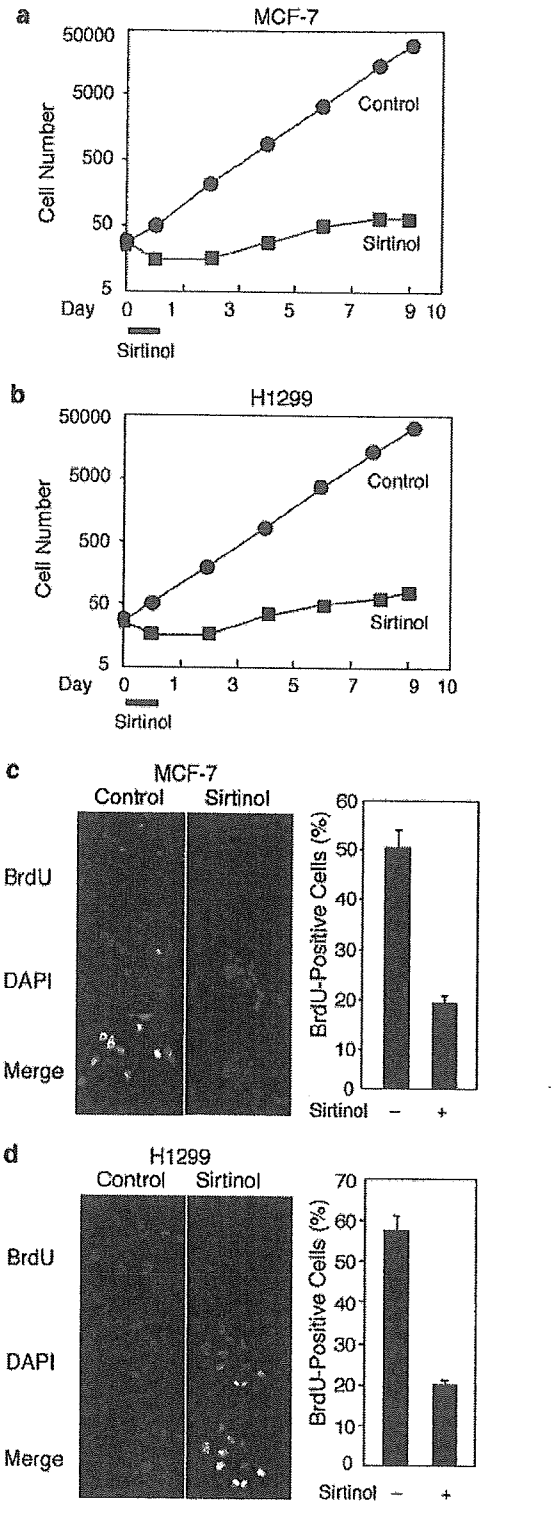

Figure 1 Effects of Sirtinol on cell growth and BrdU incorporation. MCF-7 (**a**) and H1299 (**b**) cells were treated with Sirtinol (100 μM) for 24 h. At 24 h after the addition of Sirtinol, Sirtinol was removed from the media, and then the cells were cultured in inhibitor-free complete media. (**c**, **d**) BrdU incorporation was evaluated at 10 days after the addition of Sirtinol (100 μM). BrdU incorporation was decreased in Sirtinol-treated MCF-7 (**c**) and H1299 (**d**) cells compared with untreated cells (Control).

observed in Sirtinol-treated MCF-7 cells and, to a lesser extent, in Sirtinol-treated H1299 cells, as compared with untreated cells. Sirtinol treatment also resulted in increased expression of PAI-1 in both MCF-7 and H1299 cells (Figure 3b). β-Actin expression, however, was not affected by Sirtinol.

Treatment with Splitomicin, another specific inhibitor for Sirt1, for 24 h also led to the induction of SA-β-gal staining in a dose-dependent manner (Figure 3a). However, greater concentrations of Splitomicin appeared to be required to induce SA-β-gal, as compared with Sirtinol.

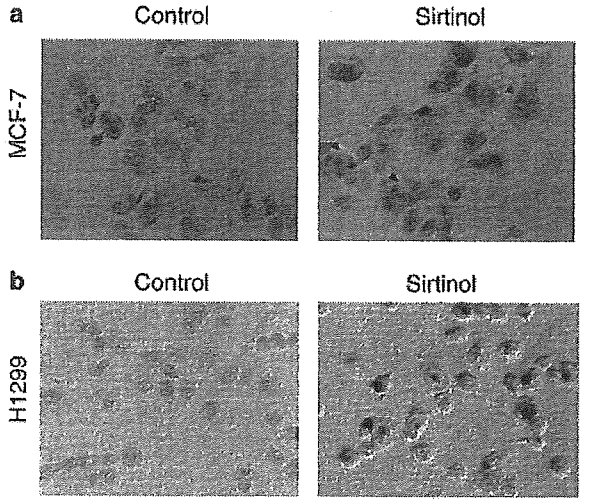

Figure 2 SA-β-gal staining in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), MCF-7 (**a**) and H1299 (**b**) cells were stained for SA-β-gal. Sirtinol treatment increased SA-β-gal-positive cells in MCF-7 and H1299 cells. In addition, the number of multinucleated cells was increased in Sirtinol-treated MCF-7 cells, but not in Sirtinol-treated H1299 cells, compared with untreated (Control) cells. Arrowheads denote multinucleated cells in Sirtinol-treated MCF-7 cells.

Colony formation assay also revealed that both Sirtinol and Splitomicin elicited antiproliferative effects in MCF-7 and H1299 cells in a dose-dependent manner (Figure 4). Sirtinol inhibited colony formation at concentrations of 33 μM and higher in MCF-7 and H1299 cells. On the other hand, 33 μM Splitomicin failed to decrease the number of colonies, but Splitomicin at 100 and 333 μM effectively inhibited colony formation in MCF-7 and H1299 cells.

Senescence-like growth arrest by Sirt1 inhibition was further corroborated by experiments using short interfering RNA (siRNA). Gene knockdown of Sirt1 by siRNA resulted in induction of SA-β-gal staining, large and flat cell morphology, decreased BrdU incorporation and increased PAI-1 expression in both MCF-7 and H1299 cells, as compared with control siRNA (Figure 5). Moreover, Sirt1 inhibition by Sirtinol, Splitomicin or siRNA also induced senescence-like phenotype in human diploid fibroblasts, WI-38 and IMR-90 cells, reflected by induction of SA-β-gal staining, and enlarged and flattened cell morphology (Supplementary Figure 1).

In Sirtinol-treated MCF-7 cells, the number of multinucleated cells was increased compared with untreated cells (Figure 2), but multinucleated cells were not found in H1299 cells regardless of whether treated with or without Sirtinol. Consistent with these observations, flow cytometric analysis revealed that the substantial cell population of Sirtinol-treated MCF-7 cells exhibited DNA content over 4*N*, indicative of polyploidy (Figure 6a). Polyploidy fraction estimated by the ratio

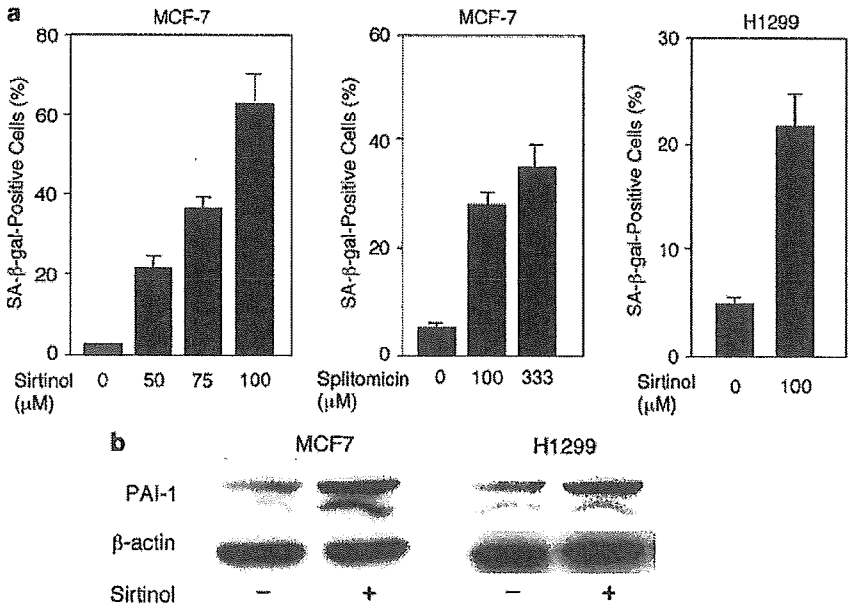

Figure 3 Effects of Sirtinol and Splitomicin on SA-β-gal activity and PAI-1 expression. (a) SA-β-gal-positive cells were counted 10 days after the addition of indicated concentrations of Sirtinol or Splitomicin in MCF-7 and H1299 cells. Treatment with Sirtinol and Splitomicin increased SA-β-gal-positive cells in a dose-dependent manner. (**b**) Sirtinol treatment resulted in the increased expression of PAI-1 as compared with untreated cells.

of cell population with DNA content of over 4*N* to that with over 2*N* was greater in Sirtinol-treated MCF-7 cells than in untreated cells (Figure 6a, right panel). In contrast, Sirtinol-treated H1299 cells were cell cycle arrested at G1 (Figure 6b). There was little, if any, increase in polyploidy fraction by Sirtinol in H1299 cells (control: 0.6%; Sirtinol: 1.6%).

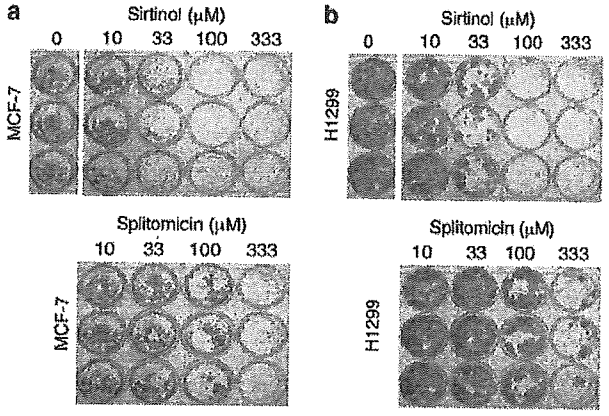

Figure 4 The effects of Sirtinol and Splitomicin on colony formation. MCF-7 (a) and H1299 (b) cells were inoculated onto 12-well plates at the density of 500 cells/well, and treated with the indicated concentrations of Sirtinol or Splitomicin for 24 h. After withdrawal of the Sirt1 inhibitors, the cells were cultured for 14 days. Both Sirtinol and Splitomicin inhibited colony formation in a dose-dependent manner.

In cellular senescence, induction of p53, dephosphorylation of Rb and increased expression of cyclin-dependent kinase inhibitors such as p16, p21 and p27 have been shown to be involved (Serrano *et al*., 1997; Collado *et al*., 2000; Alexander and Hinds, 2001; Beausejour *et al*., 2003; Jirawatnotai *et al*., 2003; Mallette *et al*., 2004). We found that phosphorylated Rb was decreased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells, while the protein expression of Rb was unaltered (Figure 7a and b). p27 expression was induced in Sirtinol-treated MCF-7 and H1299 cells (Figure 7f and g), while β-actin expression was unaltered. However, the mRNA level of p27 was not increased by Sirtinol treatment in MCF-7 and H1299 cells at both 3 and 10 days after the addition of Sirtinol, while tamoxifen and serum starvation upregulated the p27 mRNA level in MCF-7 and H1299 cells, respectively (Supplementary Figure 2). In contrast, p21 was not increased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells. p16 was not induced in Sirtinol-treated H1299 cells (Figure 7g). MCF-7 and H1299 cells are deficient in p16 and p53, respectively. On the other hand, treatment with tamoxifen (1 μM) for 24 h increased protein expression of p21 and p27 in MCF-7 cells, and serum starvation for 24 h induced p16, p21 and p27 expression in H1299 cells. Neither expression nor acetylation of p53 was upregulated by Sirtinol

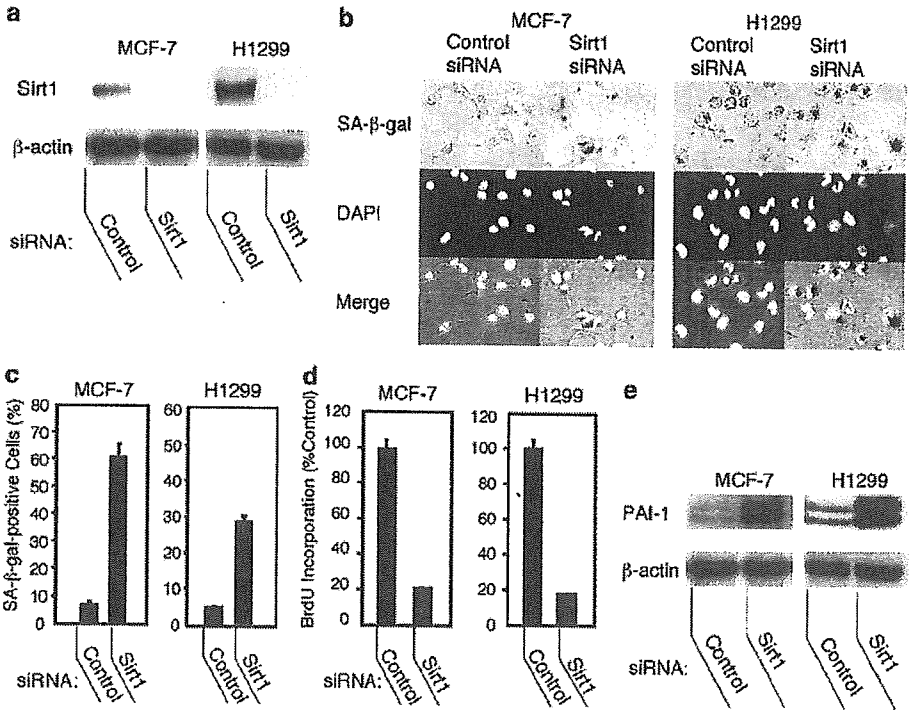

Figure 5 Gene knockdown of Sirt1 by siRNA induced senescence-like phenotype. MCF-7 and H1299 cells were treated with siRNA for Sirt1 or control siRNA. (**a**) At 3 days after the transfection, immunoblot analysis revealed that Sirt1 siRNA effectively reduced Sirt1 expression in both MCF-7 and H1299 cells. (**b**, **c**) At 10 days after the transfection, SA-β-gal-positive cells were significantly increased in Sirt1 siRNA-treated cells compared with control siRNA-treated cells. (**d**) BrdU incorporation was decreased in Sirt1 siRNA-treated cells compared with control siRNA-treated cells at 10 days after the transfection. (**e**) Sirt1 siRNA increased PAI-1 protein expression compared with control siRNA at 10 days after the transfection.

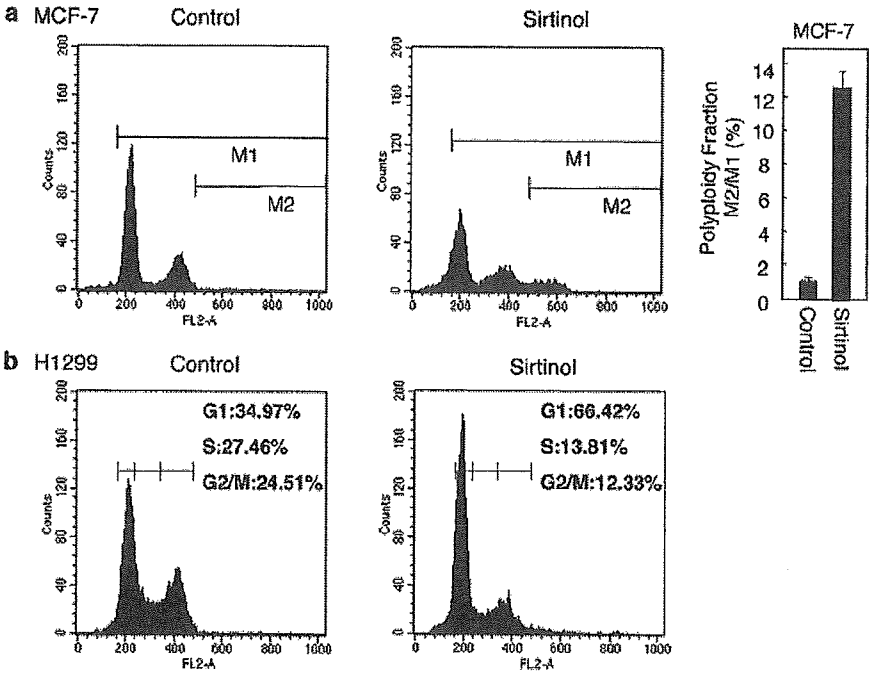

Figure 6 Flow cytometric analysis of Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), the cell cycle of MCF-7 (**a**) and H1299 (**b**) cells was analysed by flow cytometry. In Sirtinol-treated MCF-7 cells, polyploidy fraction (M2/M1; cell population with DNA content of over 4*N* normalized to that with DNA content of over 2*N*) was increased compared with untreated MCF-7 cells (Control). Sirtinol-treated H1299 cells exhibited G1 cell cycle arrest (**b**).

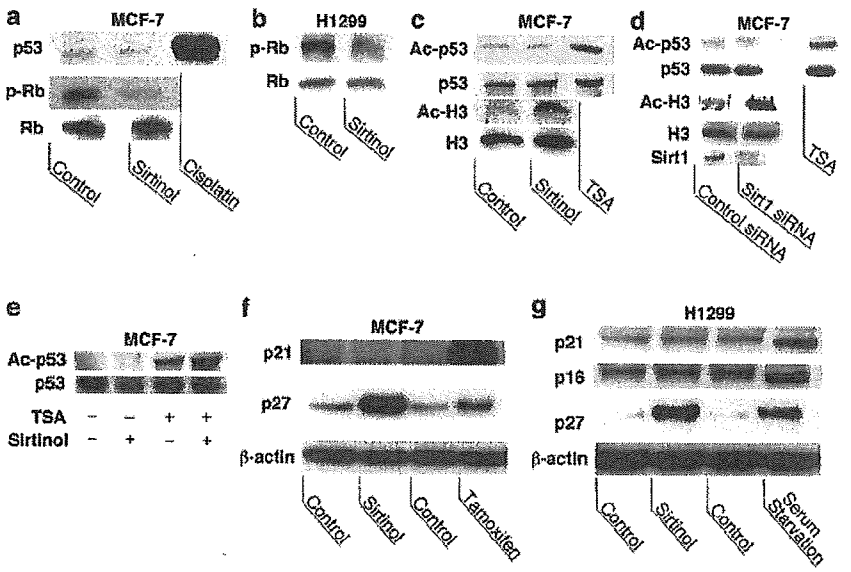

Figure 7 p53, Rb and cyclin-dependent kinase inhibitors in Sirtinol-treated cells. (**a**, **b**) At 10 days after the addition of Sirtinol (100 μM), phosphorylated Rb (p-Rb) was decreased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells. (**a**, **c**, **d**) The expression and acetylation of p53 were not increased by Sirtinol (**a**, **c**) or siRNA for Sirt1 (**d**) in MCF-7 cells. However, a robust increase in expression and acetylation of p53 (Ac-p53) were found in MCF-7 cells when treated with cisplatin (40 μM) for 18 h and trichostatin A (TSA, 5 μM) for 4 h, respectively. In contrast, Sirtinol (100 μM) treatment and siRNA for Sirt1 increased acetylated histone H3 (Ac-H3), while the abundance of histone H3 was unaltered (**c**, **d**). (**e**) MCF-7 cells were treated with or without Sirtinol (100 μM) in the presence or absence of trichostatin A (TSA, 0.5 μM) for 24 h. Sirtinol enhanced trichostatin A-induced acetylation of p53. p53 expression was not altered by Sirtinol or trichostatin A. (**f**, **g**) p21 expression was not increased by Sirtinol treatment in MCF-7 and H1299 cells. p16 expression was not induced by Sirtinol treatment in H1299 cells. In contrast, the expression of p27 was increased in Sirtinol-treated MCF-7 and H1299 cells compared with untreated cells.

or siRNA for Sirt1 in MCF-7 cells that harbor wild-type p53 (Figure 7c and d), while acetylation of histone H3 was increased by Sirtinol and siRNA for Sirt1. However, cisplatin and trichostatin A, class I and class II HDAC inhibitor, caused robust induction of p53 and acetylation of p53 in MCF-7 cells, respectively (Figure 7a and c–e). Although Sirtinol alone did not increase p53 acetylation, Sirtinol enhanced p53 acetylation in the presence of trichostatin A (Figure 7e). These results are consistent with previous observations that inhibition of Sirt1 by itself did not induce p53 acetylation in the absence of other stimulus, while DNA damage- or oxidative stress-induced p53 acetylation was accentuated by Sirt1 inhibition (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002; Cheng *et al.*, 2003).

Senescence-like growth arrest was accompanied by attenuated activation of MAPK pathways in response to growth factors

We examined the activation status of signaling pathways of MAPKs and Akt/PKB in response to growth factors, epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I). When untreated with Sirtinol, upon exposure to EGF or IGF-I, robust phosphorylation of extracellular-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK/SAPK, also termed stress-activated protein kinase) and p38 MAPK was observed in MCF-7 and H1299 cells. By contrast, in Sirtinol-treated senescent MCF-7 and H1299 cells at 10 days after the addition of Sirtinol (100 μM), basal (unstimulated) phosphorylation of ERK, JNK/SAPK and p38 MAPK was reduced compared with untreated cells (Figure 8). In addition, EGF- or IGF-I-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was attenuated in MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, compared to untreated cells.

Reduced activation of ERK, JNK/SAPK and p38 MAPK was corroborated by the phosphorylation status of the endogenous substrates of these MAPKs. Basal (unstimulated) as well as EGF- or IGF-I-stimulated phosphorylation of Elk-1, c-Jun and ATF-2 was also decreased in Sirtinol-treated senescent MCF-7 and H1299 cells at 10 days after the addition of Sirtinol, compared with untreated cells (Figure 9). The protein expression of ERK, JNK/SAPK, p38 MAPK, Elk-1, c-Jun and ATF-2 did not differ between Sirtinol-treated and untreated MCF-7 and H1299 cells.

However, at 3 days after the addition of Sirtinol, unlike at 10 days after the inhibitor addition, EGF-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was not attenuated in MCF-7 cells, compared with untreated cells (Figure 8d). These results suggest that attenuated MAPK pathways may be a consequence, rather than a cause, of Sirtinol-induced commitment of senescence-like growth arrest.

In contrast, tyrosine phosphorylation of the receptors for EGF and IGF-I by their ligands was not altered by Sirtinol treatment in MCF-7 and H1299 cells at 10 days after the addition of Sirtinol (Figure 10). The expression of EGF receptor and IGF-I receptor did not differ between Sirtinol-treated and untreated cells. These findings suggest that the defects responsible for impaired activation of MAPKs may exist at the level(s) of postreceptor signaling cascades.

Ras plays a critical role in growth factor-stimulated activation of MAPK pathways. Active Ras was markedly increased by EGF in untreated MCF-7 and H1299 cells. In Sirtinol-treated senescent MCF-7 and H1299 cells, however, the basal (unstimulated) level of active Ras was reduced compared with untreated cells, and EGF failed to increase active Ras (Figure 11a). Consistent with defective activation of Ras, basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Raf-1, MEK, SEK1/MKK4 and MKK7 was attenuated in Sirtinol-treated cells relative to untreated cells (Figure 11b). However, no difference was found between Sirtinol-treated and untreated MCF-7 and H1299 cells in the protein expression of Ras, Raf-1, MEK, SEK1/MKK4 and MKK7.

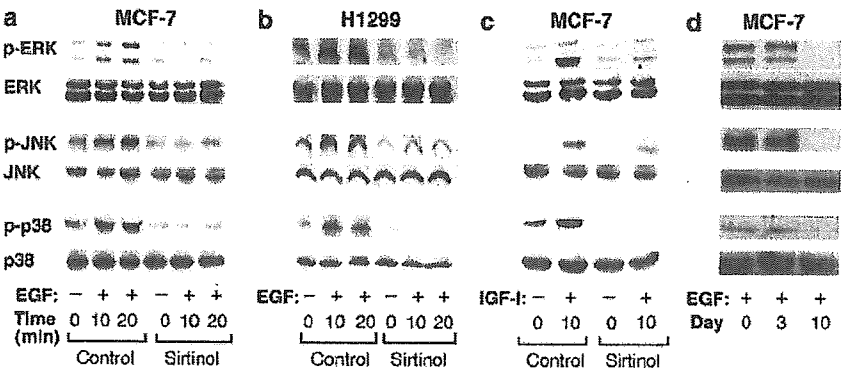

Figure 8 Growth factor-stimulated phosphorylation of MAPKs in Sirtinol-treated cells. (a–c) At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**), or to IGF-I (100 ng/ml) for 10 min (**c**). In untreated (Control) MCF-7 and H1299 cells, marked phosphorylation of ERK, JNK/SAPK and p38 MAPK was induced by EGF or IGF-I. In Sirtinol-treated MCF-7 and H1299 cells, basal (unstimulated) as well as EGF- and IGF-I-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was decreased compared with untreated cells. (**d**) At 0, 3 and 10 days after the addition of Sirtinol (100 μM), MCF-7 cells were stimulated with EGF (50 ng/ml) for 20 min. EGF-stimulated phosphorylation of ERK, JNK/SAPK and p38 MAPK was markedly impaired at 10 days, but preserved at 3 days after the addition of Sirtinol.

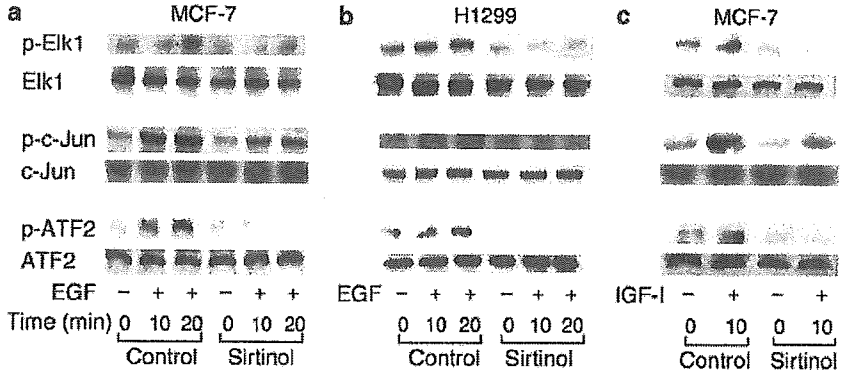

Figure 9 Growth factor-stimulated phosphorylation of Elk1, c-Jun and ATF2. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**) or to IGF-I (100 ng/ml) for 10 min (**c**). In untreated (Control) MCF-7 and H1299 cells, marked phosphorylation of Elk1, c-Jun and ATF2 was induced by EGF and IGF-I. In Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Elk1, c-Jun and ATF2 were decreased compared with untreated cells.

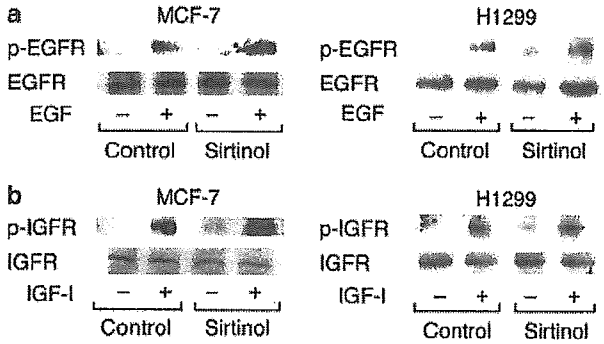

Figure 10 Growth factor-stimulated phosphorylation of EGF receptor and IGF-I receptor in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) (**a**) or IGF-I (100 ng/ml) for 2 min (**b**). There was no difference in tyrosine phosphorylation and protein expression of EGF receptor (EGFR) and IGF-I receptor (IGFR) between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

In contrast to the Ras–MAPK pathway, EGF- or IGF-I-induced as well as basal (unstimulated) phosphorylation of Akt/PKB was not decreased in Sirtinol-treated senescent MCF-7 and H1299 cells compared with untreated cells at 10 days after Sirtinol addition (Figure 12). The expression of Akt/PKB was not altered by Sirtinol treatment, either.

Discussion

We found that Sirt1 inhibition by specific inhibitors, Sirtinol and Splitomicin, and siRNA caused senescence-like growth arrest in human cancer MCF-7 and H1299 cells, as judged by SA-β-gal staining, PAI-1 expression, BrdU incorporation, flattened and enlarged morphology of the cells and flow cytometric analysis (Figures 1–6). Sirtinol-induced senescence-like growth arrest was accompanied by attenuated responses to growth factors in terms of activation of Ras–MAPKs (Figures 8, 9 and 11). By contrast, phosphorylation (activation) of EGF receptor, IGF-I receptor and Akt/PKB by growth factors was not affected in Sirtinol-treated senescent MCF-7 and H1299 cells (Figures 10 and 12).

Consistent with impaired activation of MAPKs, EGF- and IGF-I-stimulated phosphorylation of downstream targets, Elk-1, c-Jun and ATF-2, was also reduced (Figure 9). A hallmark feature of senescent cells is unresponsiveness to mitogenic stimuli in terms of induction of c-*fos* as well as cell proliferation. Previous studies in senescent human diploid fibroblasts showed that induction of c-*fos* (Seshadri and Campisi, 1990) and activation of Elk-1 (Tresini *et al*., 2001) and MEK–ERK (Torres *et al*., 2003) in response to growth factors are impaired. Transcriptional activity of Elk-1 regulates the induction of c-*fos*, an immediate early gene. Thus, our results of attenuated activation of Ras–MEK–ERK–Elk-1 in Sirtinol-treated senescent cancer cells are in agreement with previous findings in senescent human fibroblasts.

Our results indicate that the signaling defect in Sirtinol-treated cells is specific for MAPK pathways and that the PI3-K–Akt/PKB pathway is preserved. Ras is a key regulator of MAPK pathways (Lange-Carter and Johnson, 1994). However, Ras does not play a major role in activation of the PI3-K–Akt/PKB pathway (Sakaue *et al*., 1995; Gnudi *et al*., 1997; Klesse *et al*., 1999). Our results showed that active, GTP-bound Ras was reduced in Sirtinol-treated cancer cells compared with untreated cells (Figure 11a). The present data, therefore, suggest that reduced activation of Ras might be involved in a specific attenuation in MAPK pathways in Sirtinol-treated senescent MCF-7 and H1299 cells.

Growth factor-initiated mitogenic signals are conveyed mainly by two major signaling cascades: Ras–ERK and PI3-K–Akt/PKB. Senescent cells remain viable and metabolically active, in spite of irreversible loss of replication capability (Roninson, 2003; Shay and Roninson, 2004). One can reasonably speculate, therefore, that the preserved PI3-K–Akt/PKB pathway might

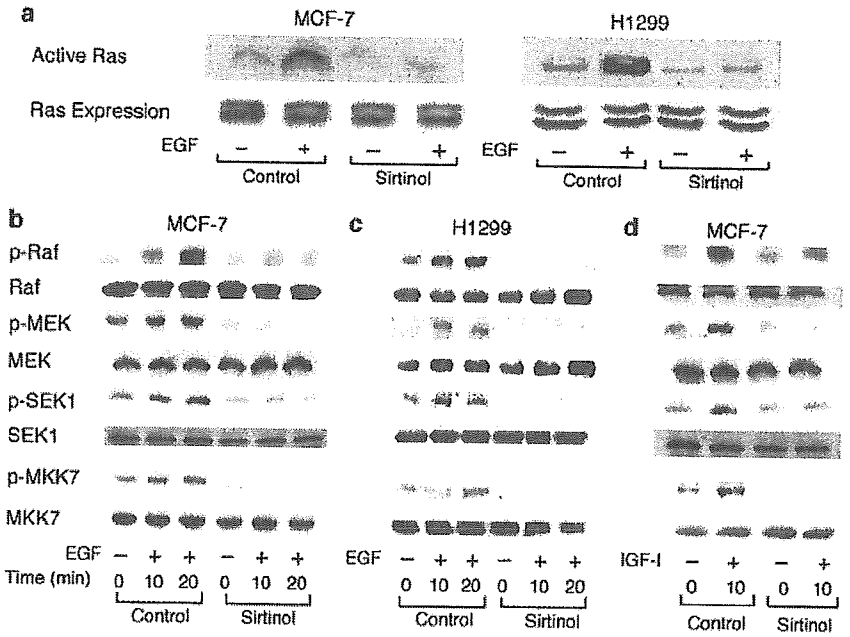

Figure 11 Activation status of Ras and its downstream signaling molecules in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min, or IGF-I (100 ng/ml) for 10 min. (**a**) Active Ras was evaluated as described in Materials and methods. In untreated (Control) MCF-7 and H1299 cells, active Ras was markedly increased by EGF treatment for 20 min. In Sirtinol-treated MCF-7 and H1299 cells, basal (unstimulated) level of active Ras was decreased compared with untreated cells, and EGF failed to increase active Ras. (**b–d**) In untreated (Control) MCF-7 and H1299 cells, EGF and IGF-I induced robust phosphorylation of Raf, MEK, SEK1/MKK4 and MKK7. However, in Sirtinol-treated MCF-7 and H1299 cells, both basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of these molecules were decreased compared with untreated (Control) cells.

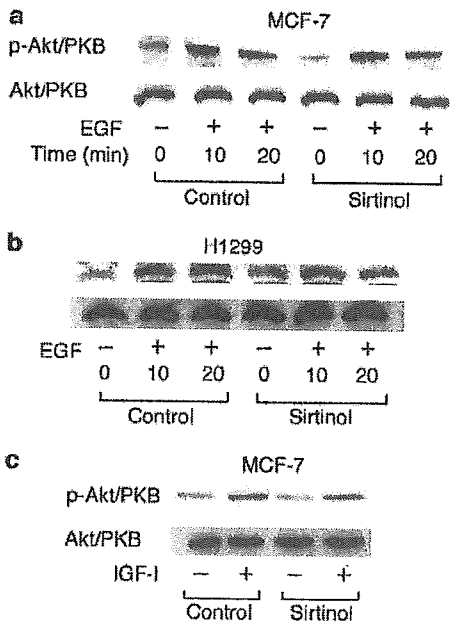

Figure 12 Growth factor-stimulated Akt/PKB phosphorylation in Sirtinol-treated cells. At 10 days after the addition of Sirtinol (100 μM), following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 10 or 20 min (**a**, **b**) or to IGF-I (100 ng/ml) for 10 min (**c**). No difference was found in basal (unstimulated) and EGF- or IGF-I-stimulated phosphorylation of Akt/PKB between Sirtinol-treated and untreated (Control) MCF-7 and H1299 cells.

contribute to cell viability and metabolic activities in Sirtinol-treated cells, because the PI3-K–Akt/PKB pathway plays critical roles in cell survival and regulation of metabolism.

p53, Rb and cyclin-dependent kinase inhibitors such as p16, p21 and p27 have been recognized as key mediators of cellular senescence (Serrano *et al.*, 1997; Collado *et al.*, 2000; Alexander and Hinds, 2001; Beausejour *et al.*, 2003; Jirawatnotai *et al.*, 2003; Mallette *et al.*, 2004). Our results showed that hypophosphorylation of Rb and increased expression of p27 were associated with Sirtinol-induced senescence-like growth arrest in MCF-7 and H1299 cells (Figure 7). In addition to regulation at transcriptional level, increased p27 expression may result from reduced protein degradation through a ubiquitin–proteasome system (Carrano *et al.*, 1999). Since Sirt1 is an HDAC, it is possible that Sirt1 inhibition may directly modulate p27 transcription. However, our finding of unaltered p27 mRNA in Sirtinol-treated cells (Supplementary Figure 2) suggests that decreased protein degradation of p27, rather than increased transcription, may contribute to increased p27 protein expression in Sirtinol-treated cells. Thus, our data argue against a direct effect of Sirtinol on transcription of p27.

On the other hand, we did not find increased expression of p53, p21 and p16 in Sirtinol-treated MCF-7 and H1299 cells. It is important to note, however, that previous studies showed that premature senescence can be readily induced independent of p53,

p21 or p16 in cancer cells (Zhu *et al.*, 1997; Chang *et al.*, 1999a, b; Collado *et al.*, 2000; Wainwright *et al.*, 2001; Wright and Shay, 2001; te Poele *et al.*, 2002; Beausejour *et al.*, 2003; Mallette *et al.*, 2004; Munro *et al.*, 2004). p27 was shown to be required for premature senescence mediated by Rb (Alexander and Hinds, 2001) or PI3-K inhibitor (Collado *et al.*, 2000). In accordance with previous findings (Chang *et al.*, 1999a; Collado *et al.*, 2000; Wainwright *et al.*, 2001; Mallette *et al.*, 2004), Sirtinol induced senescence-like growth arrest in p16-deficient MCF-7 and p53-deficient H1299 cells. These results indicate that p53 and p16 are not required for Sirtinol-induced senescence-like growth arrest in H1299 and MCF-7 cells, respectively.

Recently, senescence-like growth arrest has been proposed as a new target of cancer therapy. Since p53 and p16 are not expressed or mutated in many types of malignancies, the effectiveness of Sirtinol to induce senescence-like growth arrest in p53- and p16-deficient cancer cells may be of clinical significance for the treatment of patients with malignancy. Taken together, the present study highlights Sirt1 inhibitor as an antitumor drug candidate.

Materials and methods

Materials
Sirtinol, Splitomicin, IGF-I, EGF (Calbiochem, La Jolla, CA, USA), trichostatin A, cisplatin, propidium iodide, RNase, tamoxifen (Sigma, St Louis, MO, USA), anti-phospho-ERK (Thr202/Tyr204), ERK, phospho-JNK/SAPK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), p38, phospho-Raf (Ser259), Raf-1, phospho-MEK1/2 (Ser217/221), MEK1/2, phospho-SEK1/MKK4 (Ser80), SEK1/MKK4, MKK7, phospho-Elk1 (Ser383), Elk1, phospho-ATF2 (Thr71), ATF2, phospho-Rb (Ser795), Rb, phospho-Akt (Ser473), Akt, p53 (Cell Signaling, Beverly, MA, USA), acetylated p53, EGFR, phospho-MKK7 (Thr275/Ser277) (Upstate, Lake Placid, NY, USA), phospho-EGFR (Tyr1173), p16, p21, phospho-c-Jun (Ser63), c-Jun, JNK1 (Santa Cruz, Santa Cruz, CA, USA), p27 (BD Transduction Laboratories, Lexington, KY, USA), PAI-1 (Molecular Innovations, Southfield, MI, USA), acetylated histone H3, histone H3 (Upstate, Charlottesville, VA, USA), phospho-IGF-I receptor (Tyr1162/1163), IGF-I receptor (Biosource, Camarillo, CA, USA) and pan-Ras antibodies (Oncogene, San Diego, CA, USA) were purchased commercially.

Cell culture
Human breast cancer MCF-7 cells and non-small lung cancer H1299 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Sigma), respectively. Logarithmically growing cells were treated with the indicated concentrations of Sirtinol or Splitomicin for 24 h. After exposure to Sirtinol or Splitomicin for 24 h, the cells were washed three times with inhibitor-free medium and cultured for an additional 9 days with the complete media in the absence of inhibitor. Cell viability was determined by the Trypan blue exclusion test, and viable cells were counted. At 9 days after the addition of Sirtinol to the culture media, the cells were serum-deprived for overnight, and then treated with EGF (50 ng/ml) or IGF-I (100 ng/ml) for 2, 10 or 20 min.

SA-β-gal staining
SA-β-gal staining was performed as previously described (Dimri *et al.*, 1995) (see Supplementary section).

BrdU incorporation assay
At 10 days after the addition of Sirtinol, BrdU incorporation was assayed as previously described (Takahashi *et al.*, 1992). In cells treated with siRNA, at 10 days after the transfection of siRNA, BrdU incorporation was evaluated using a commercial kit (Roche, Indianapolis, IN, USA).

Gene knockdown with siRNA
Cells were plated in six-well plates at 20–30% confluency, and 24 h later transfected with 200 pmol of siRNA for Sirt1 ('5-GAT GAA GTT GAC CTC CTC A-3' (Picard *et al.*, 2004) and 5'-TGA AGT GCC TCA GAT ATT A-3') or control siRNA (Dharmacom, Chicago, IL, USA), using siIMPORTER (Upstate).

Flow cytometric analysis
At 10 days after the addition of Sirtinol, the cells were fixed with 70% ethanol and treated with 5 μg/ml (RNase) for 30 min. After staining with 50 μM propidium iodide, the cells were subjected to flow cytometric analysis with FACS Calibur and Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ, USA).

Immunoblot analysis
Immunoblot analysis was performed as previously described (Yasukawa *et al.*, 2005) (see Supplementary section).

Determination of activation status of Ras
Active, GTP-bound Ras was assayed using the Ras activation assay kit (Upstate) according to the manufacturer's instructions (see Supplementary section).

Colony formation assay
Colony formation assay was performed as previously described (Elegbede *et al.*, 2002) (see Supplementary section).

Northern blotting
The mRNA level of p27 was evaluated by Northern blotting as previously described (Sugita *et al.*, 2005), using cDNA probe for p27 that was kindly provided by Dr N Fujita (Fujita *et al.*, 2002).

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References

Alexander K, Hinds PW. (2001). *Mol Cell Biol* **21**: 3616–3631.

Aviv H, Khan MY, Skurnick J, Okuda K, Kimura M, Gardner J *et al.* (2001). *Atherosclerosis* **159**: 281–287.

Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P *et al.* (2003). *EMBO J* **22**: 4212–4222.
Bedalov A, Gatbonton T, Irvine WP, Gottschling DE, Simon JA. (2001). *Proc Natl Acad Sci USA* **98**: 15113–15118.
Ben-Porath I, Weinberg RA. (2004). *J Clin Invest* **113**: 8–13.
Blander G, Guarente L. (2004). *Annu Rev Biochem* **73**: 417–435.
Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y *et al.* (2004). *Science* **303**: 2011–2015.
Carrano AC, Eytan E, Hershko A, Pagano M. (1999). *Nat Cell Biol* **1**: 193–199.
Chang BD, Broude EV, Dokmanovic M, Zhu H, Ruth A, Xuan Y *et al.* (1999a). *Cancer Res* **59**: 3761–3767.
Chang BD, Xuan Y, Broude EV, Zhu H, Schott B, Fang J *et al.* (1999b). *Oncogene* **18**: 4808–4818.
Cheng HL, Mostoslavsky R, Saito S, Manis JP, Gu Y, Patel P *et al.* (2003). *Proc Natl Acad Sci USA* **100**: 10794–10799.
Christov KT, Shilkaitis AL, Kim ES, Steele VE, Lubet RA. (2003). *Eur J Cancer* **39**: 230–239.
Cohen HY, Lavu S, Bitterman KJ, Hekking B, Imahiyerobo TA, Miller C *et al.* (2004). *Mol Cell* **13**: 627–638.
Collado M, Medema RH, Garcia-Cao I, Dubuisson ML, Barradas M, Glassford J *et al.* (2000). *J Biol Chem* **275**: 21960–21968.
Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M *et al.* (2004). *Proc Natl Acad Sci USA* **101**: 10042–10047.
Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C *et al.* (1995). *Proc Natl Acad Sci USA* **92**: 9363–9367.
Elegbede JA, Hayes K, Schell K, Oberley TD, Verma AK. (2002). *Life Sci* **71**: 421–436.
Elmore LW, Rehder CW, Di X, McChesney PA, Jackson-Cook CK, Gewirtz DA *et al.* (2002). *J Biol Chem* **277**: 35509–35515.
Fujita N, Sato S, Katayama K, Tsuruo T. (2002). *J Biol Chem* **277**: 28706–28713.
Gnudi L, Frevert EU, Houseknecht KL, Erhardt P, Kahn BB. (1997). *Mol Endocrinol* **11**: 67–76.
Goldstein S, Moerman EJ, Fujii S, Sobel BE. (1994). *J Cell Physiol* **161**: 571–579.
Grozinger CM, Chao ED, Blackwell HE, Moazed D, Schreiber SL. (2001). *J Biol Chem* **276**: 38837–38843.
Han Z, Wei W, Dunaway S, Darnowski JW, Calabresi P, Sedivy J *et al.* (2002). *J Biol Chem* **277**: 17154–17160.
Imai S, Armstrong CM, Kaeberlein M, Guarente L. (2000). *Nature* **403**: 795–800.
Jirawatnotai S, Moons DS, Stocco CO, Franks R, Hales DB, Gibori G *et al.* (2003). *J Biol Chem* **278**: 17021–17027.
Kahlem P, Dorken B, Schmitt CA. (2004). *J Clin Invest* **113**: 169–174.
Kelly WK, Richon VM, O'Connor O, Curley T, MacGregor-Curtelli B, Tong W *et al.* (2003). *Clin Cancer Res* **9**: 3578–3588.
Kim JH, Lee GE, Kim SW, Chung IK. (2003). *Biochem J* **373**: 523–529.
Klesse LJ, Meyers KA, Marshall CJ, Parada LF. (1999). *Oncogene* **18**: 2055–2068.
Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L *et al.* (2000). *Proc Natl Acad Sci USA* **97**: 5807–5811.
Lange-Carter CA, Johnson GL. (1994). *Science* **265**: 1458–1461.
Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S *et al.* (2002). *EMBO J* **21**: 2383–2396.
Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A *et al.* (2001). *Cell* **107**: 137–148.
Mallette FA, Goumard S, Gaumont-Leclerc MF, Moiseeva O, Ferbeyre G. (2004). *Oncogene* **23**: 91–99.
Mathon NF, Lloyd AC. (2001). *Nat Rev Cancer* **1**: 203–213.
McLaughlin F, La Thangue NB. (2004). *Curr Drug Targets Inflamm Allergy* **3**: 213–219.
Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W *et al.* (2004). *Cell* **116**: 551–563.
Munro J, Barr NI, Ireland H, Morrison V, Parkinson EK. (2004). *Exp Cell Res* **295**: 525–538.
Pelicci PG. (2004). *J Clin Invest* **113**: 4–7.
Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R *et al.* (2004). *Nature* **429**: 771–776.
Piekarz R, Bates S. (2004). *Curr Pharm Des* **10**: 2289–2298.
Piekarz RL, Robey R, Sandor V, Bakke S, Wilson WH, Dahmoush L *et al.* (2001). *Blood* **98**: 2865–2868.
Roninson IB. (2002). *Drug Resist Updat* **5**: 204–208.
Roninson IB. (2003). *Cancer Res* **63**: 2705–2715.
Rosato RR, Grant S. (2004). *Expert Opin Investig Drugs* **13**: 21–38.
Sakaue H, Hara K, Noguchi T, Matozaki T, Kotani K, Ogawa W *et al.* (1995). *J Biol Chem* **270**: 11304–11309.
Sandor V, Bakke S, Robey RW, Kang MH, Blagosklonny MV, Bender J *et al.* (2002). *Clin Cancer Res* **8**: 718–728.
Schmitt CA, Fridman JS, Yang M, Lee S, Baranov E, Hoffman RM *et al.* (2002). *Cell* **109**: 335–346.
Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. (1997). *Cell* **88**: 593–602.
Seshadri T, Campisi J. (1990). *Science* **247**: 205–209.
Sharpless NE, DePinho RA. (2004). *J Clin Invest* **113**: 160–168.
Shay JW, Roninson IB. (2004). *Oncogene* **23**: 2919–2933.
Sugita H, Fujimoto M, Yasukawa T, Shimizu N, Sugita M, Yasuhara S *et al.* (2005). *J Biol Chem* **280**: 14203–14211.
Takahashi T, Nowakowski RS, Caviness Jr VS. (1992). *J Neurocytol* **21**: 185–197.
te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. (2002). *Cancer Res* **62**: 1876–1883.
Torres C, Francis MK, Lorenzini A, Tresini M, Cristofalo VJ. (2003). *Exp Cell Res* **290**: 195–206.
Tresini M, Lorenzini A, Frisoni L, Allen RG, Cristofalo VJ. (2001). *Exp Cell Res* **269**: 287–300.
van der Horst A, Tertoolen LG, de Vries-Smits LM, Frye RA, Medema RH, Burgering BM. (2004). *J Biol Chem* **279**: 28873–28879.
Vanhaecke T, Papeleu P, Elaut G, Rogiers V. (2004). *Curr Med Chem* **11**: 1629–1643.
Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK *et al.* (2001). *Cell* **107**: 149–159.
Vigushin DM, Coombes RC. (2004). *Curr Cancer Drug Targets* **4**: 205–218.
Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, Jansen-Durr P. (2001). *Exp Gerontol* **36**: 1327–1347.
Wainwright LJ, Lasorella A, Iavarone A. (2001). *Proc Natl Acad Sci USA* **98**: 9396–9400.
Wang X, Tsao SW, Wong YC, Cheung AL. (2003). *Curr Cancer Drug Targets* **3**: 153–159.
Wright WE, Shay JW. (2001). *Curr Opin Genet Dev* **11**: 98–103.
Yasukawa T, Tokunaga E, Ota H, Sugita H, Martyn JA, Kaneki M. (2005). *J Biol Chem* **280**: 7511–7518.
Zhu WY, Jones CS, Kiss A, Matsukuma K, Amin S, De Luca LM. (1997). *Exp Cell Res* **234**: 293–299.

Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc).