Table 2. Relationship between serum NM23-H1 protein levels and clinicopathological findings in 86 patients with neuroblastoma found clinically

Characteristics	No. of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	*P*-value (analysis)
All patients	86	239 ± 357	
Age			
< 12 months	27	282 ± 471	
≥ 12 months	59	219 ± 294	0.7694 (MW)
Stage			
1 + 2 + 4s	21	154 ± 187	
3 + 4	65	266 ± 394	0.3900 (MW)
Primary site			
Mediastinal	11	124 ± 207	
Adrenal	46	285 ± 383	
Abdominal	26	220 ± 375	0.0982 (KW)
Others	3		
MYCN copy number			
1	59	157 ± 193	
> 3	27	418 ± 534	0.0028 (MW)
TrkA expression	63		
Medium + high	28	154 ± 189	
0 + low	35	296 ± 422	0.1865 (MW)
Ploidy	66		
Diploid	37	255 ± 436	
Hyperdiploid	27	234 ± 352	0.4304 (MW)

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test.

indicate that the serum NM23-H1 level serves as a useful prognostic factor for neuroblastoma, as well as the other well-known prognostic factors.

Subsequently, we classified the 86 patients into two groups according to the age of the patients, stage of the disease, or copy numbers of *MYCN*, and evaluated the influence of the serum NM23-H1 levels on the overall survival in each one of the six groups (Fig. 3). Of the 29 patients younger than 12 months of age, the seven patients with higher levels of NM23-H1 had a worse outcome than the 22 patients with the lower levels ($P = 0.0401$ according to the generalized Wilcoxon test and $P = 0.0273$ according to the log-rank test; Fig. 3a). The seven patients with higher levels of NM23-H1 had the following attributes: stage 1 + 2 + 4S ($n = 3$); stage 3 + 4 ($n = 4$); with non-amplified *MYCN* ($n = 4$); with more than three *MYCN* ($n = 3$). Likewise, of the 19 patients with a stage 3 tumor, four patients with higher levels had a worse outcome than the 15 patients with lower levels ($P = 0.0005$ and $P < 0.0001$; Fig. 3c). The four patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 0$); > 12 months of age ($n = 4$); with non-amplified *MYCN* ($n = 1$); with more than three *MYCN* ($n = 3$). Of the 59 patients with a single copy of *MYCN*, the 11 patients with higher levels had a worse outcome than the 48 patients with lower levels of serum NM23-H1 ($P = 0.0301$ and $P < 0.0366$; Fig. 3e). The 11 patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 4$); > 12 months of age ($n = 7$); stage 1 + 2 + 4S ($n = 2$); stage 3 + 4 ($n = 9$). In contrast, a higher serum NM23-H1 level did not influence overall survival in the 57 patients 12 months old or older, in the 46 patients with stage 4 disease, or in the 27 patients with *MYCN* amplification (Fig. 3b,d,f).

Four prognostic factors, including the age of the patients, stage of the disease, *MYCN* copy number, and the serum NM23-H1 level, were available for multivariate analysis in the 217 patients (Table 3a) and 86 patients (Table 3b). According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients (Table 3).

Discussion

The *NM23-H1* gene is overexpressed in various hematological malignancies and other neoplasms including neuroblastoma. Overexpression of *NM23-H1* mRNA is indicative of a poor prognosis in patients with neuroblastoma, and mutations and increased copy numbers of *NM23-H1* have been reported in advanced neuroblastoma.[6,24] In the present study, we found that the serum NM23-H1 level was significantly higher in patients with neuroblastoma than in the control children (Fig. 1), and that the serum NM23-H1 level predicted a poor outcome for patients with tumors (Fig. 2a). Furthermore, the higher level of NM23-H1 was correlated with a worse outcome in patients younger than 12 months of age, in those with stage 3 disease, or in those with a single *MYCN* copy (Fig. 3). In contrast, a higher serum NM23-H1 level did not influence overall survival in patients who were 12 months old or older, in those with stage 4 disease, or in those with *MYCN*

Table 3. Univariate and multivariate analysis for predictors of survival in neuroblastoma

Prognostic factors	Univariate (χ^2, log-rank)	*P*-value	Multivariate (relative risk & 95% CI)	*P*-value
Patients found by mass-screening or clinically ($n = 217$)				
Serum NM23-H1(< 250/> 250 ng/mL)	11.211	0.0008	1.7294 (0.7997–3.7398)	0.1639
Age (< 12/≥ 12 months)	32.353	< 0.00001	3.8979 (1.3818–10.996)	0.0101
Stage (1, 2, 4s/3, 4)	33.142	< 0.00001	8.2514 (1.8173–37.466)	0.0063
NMYC amplification (–/+)	43.997	< 0.00001	2.3253 (1.0541–5.1297)	0.0366
Patients found clinically ($n = 86$)				
Serum NM23-H1(< 250/> 250 ng/mL)	4.493	0.0340	1.6143 (0.7386–3.5282)	0.2299
Age (< 12/≥ 12 months)	5.825	0.0158	1.4742 (0.4877–4.4563)	0.4916
Stage (1, 2, 4s/3, 4)	6.994	0.0082	3.5721 (0.7158–17.826)	0.1206
NMYC amplification (–/+)	7.749	0.0054	1.9682 (0.9016–4.2967)	0.0892

CI, confidence interval.

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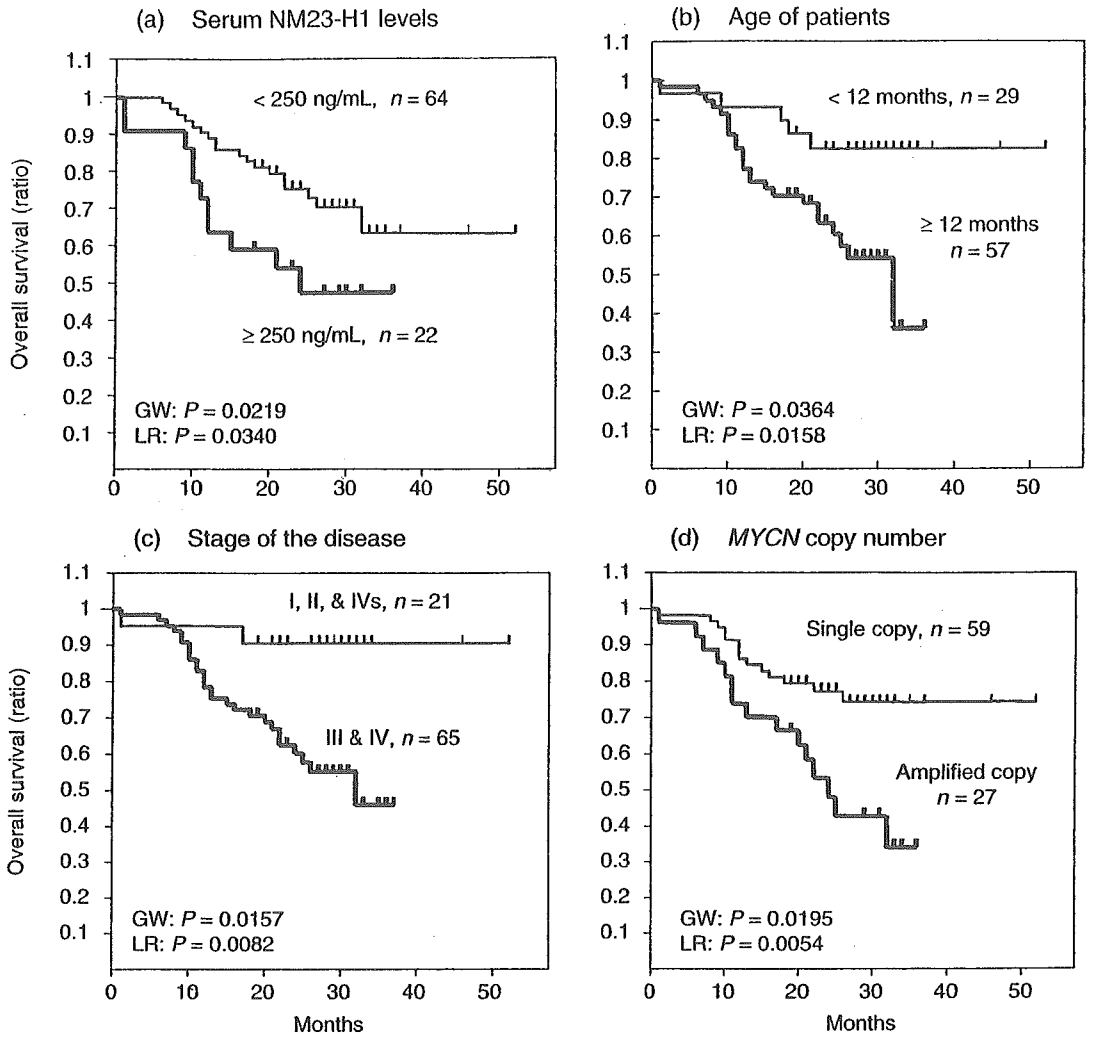

Fig. 2. Overall survival curves for 86 patients with neuroblastoma who were found clinically. (a) Overall survival curves for 22 patients with a serum NM23-H1 level ≥ 250 ng/mL, and for 64 patients with a level < 250 ng/mL. (b) Overall survival curves for 57 patients 12 months of age or older, and for 29 patients younger than 12 months. (c) Overall survival curves for 65 patients at stages 3 and 4 of the disease, and for 21 patients at stages 1, 2 and 4s. (d) Overall survival curves for 27 patients with *MYCN* amplification, and for 59 patients with a single copy of *MYCN*. GW, generalized Wilcoxon's test; LR, log-rank test.

amplification (Fig. 3). These findings suggest that the NM23-H1 level may be an important factor for predicting the outcome of patients in these low or intermediate risk groups (i.e. patients younger than 12 months of age, with stage 3 disease, or with a single copy of *MYCN*). In addition, the serum NM23-H1 level may be a clinically useful prognostic factor, because the measurement of serum NM23-H1 protein is easily and quickly carried out prior to treatment.

According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients shown in Table 3. These results might be due to the short observation time, the small number of cases, or the strong correlation between *MYCN* amplification and the elevated serum NM23-H1 level.

Although all the 131 patients found by MS were alive at the last follow-up (18–51 months) and were excluded from survival analysis, they contained 15 patients (the last follow-up: 19–37 months) with higher levels than 250 ng/mL of serum NM23-H1. It might be interesting to follow up these patients to clarify the clinical significance of serum NM23-H1 in the MS group.

Prognostic factors in neuroblastoma have been thoroughly investigated and include *MYCN* copy number, *TRKA* expression level, chromosomal ploidy, 1p loss, and 17q gain in tumor cells. Laborious and time-consuming work is required to examine these biological factors in tumor tissues. Therefore, serum markers that are easily measurable and can predict a clinical outcome are desired. Serum levels of lactate dehydrogenase (LDH) and ferritin are high in advanced stage neuroblastomas, but both may reflect a rapid cellular turnover or a large tumor burden.[25,26] Neuron-specific enolase (NSE) is a cytoplasmic protein that is associated with neural cells,

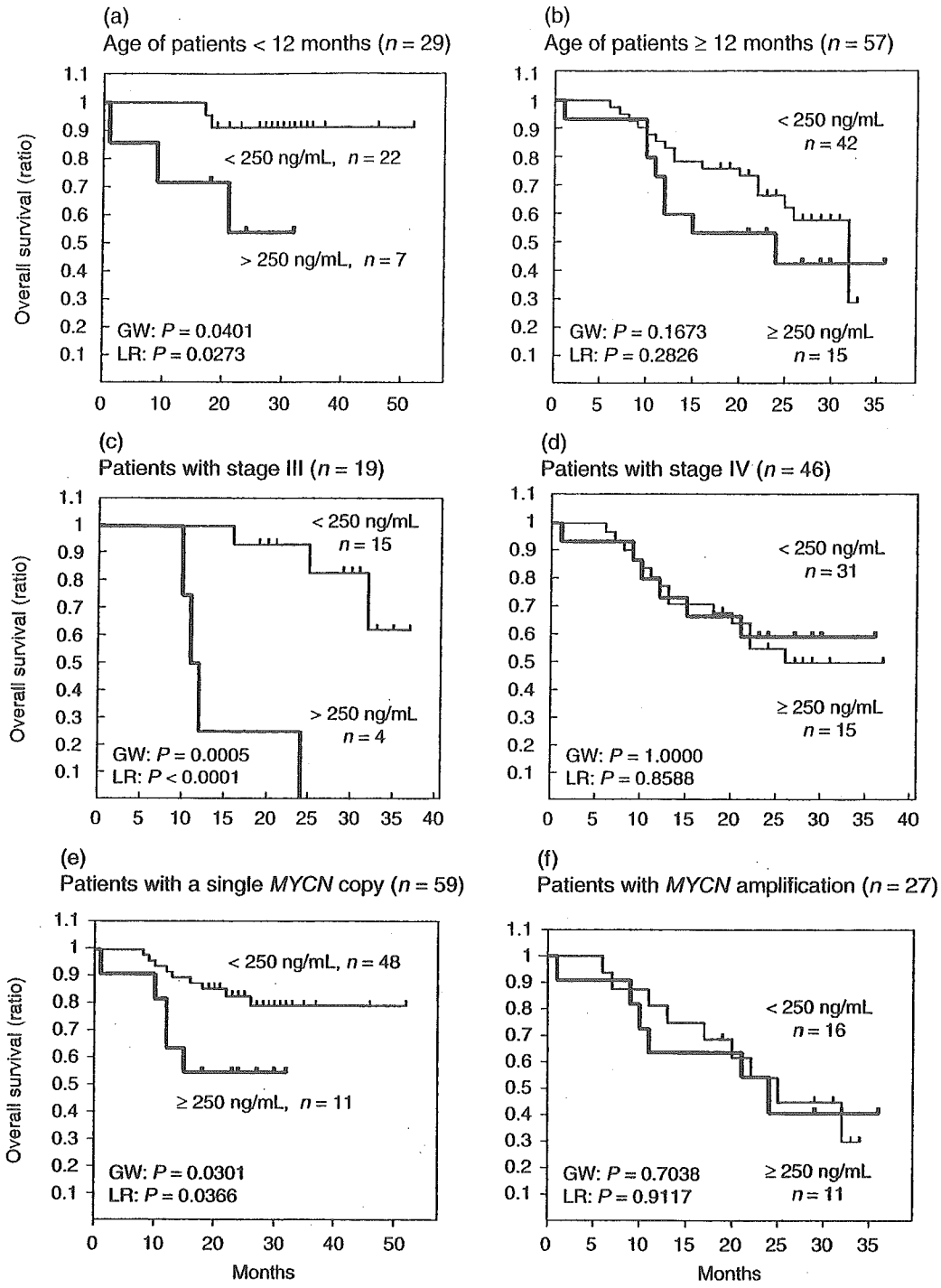

Fig. 3. Clinical significance of the serum NM23-H1 levels in the groups classified according to the age of the patients, or stage of the disease, or copy number of *MYCN*. (a) Survival curves for seven patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 22 patients with a level < 250 ng/mL. Both groups of patients were younger than 12 months of age. (b) Survival curves for 15 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 42 patients with a level < 250 ng/mL. Both groups of patients were 12 months old or older. (c) Survival curves for four patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 15 patients with a level < 250 ng/mL. Both groups of patients were at stage 3 of the disease. (d) Survival curves for 15 patients with the serum NM23-H1 level ≥ 250 ng/mL, and for 31 patients with the level < 250 ng/mL. Both groups of patients were at stage 4 of the disease. (e) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 48 patients with a level < 250 ng/mL. Both groups of patients had a single copy of *MYCN*. (f) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 16 patients with a level < 250 ng/mL. Both groups of patients had *MYCN* amplification in the tumor. GW, generalized Wilcoxon's test; LR, log-rank test.

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and serum NSE is a useful marker for patients with advanced neuroblastoma in whom the elevated levels are associated with a poor outcome.[27] The disialoganglioside GD2 is found on the surface of most neuroblastoma cells, and elevated plasma levels have been found in patients.[28] Nevertheless, none of these markers is used at present to predict clinical outcomes or to choose treatment protocols. Therefore, serum NM23-H1 levels might be useful for clinical purposes.

The elevated serum level of NM23-H1 was correlated with a poor prognostic feature, namely, *MYCN* amplification (Table 1). Godfrid *et al.* identified genes that are part of the *MYCN* downstream pathway using SAGE libraries of *MYCN* transfected and control neuroblastoma cell lines.[13] The chromosome 17q genes *NM23-H1* and *NM23-H2* were strongly induced in *MYCN*-expressing cells. A striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes was found in the cell lines. The present multivariate analysis showed no influence of serum NM23-H1 level on overall survival, and this finding might be caused by the overlap of patients with *MYCN* amplification with those with a high serum level of NM23-H1. However, within the group of patients with a single copy of *MYCN*, patients with a higher level of NM23-H1 had a worse outcome (Fig. 3e). The findings suggest that *NYCN* amplification may influence serum NM23-H1 levels as well as clinical outcome, and that neuroblastomas with a single copy of *MYCN* and a higher serum NM23-H1 level may have had a mutation or an increased copy number of the *NM23-H1* gene.[6,24,29] *MYCN* overexpression in some neuroblastomas with a single copy of *MYCN* may have resulted in higher serum NM23-H1 levels and a poor outcome; however, a recent study showed that *MYCN* overexpression did not affect the prognosis of advanced-stage neuroblastomas with a single *MYCN* copy.[30]

In patients with NHL and AML, it is thought that serum NM23-H1 protein is produced directly by the tumor cells, and its serum level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14] High concentrations of NM23 protein were found in the serum and body fluid of patients with lung cancer overexpressing the *NM23* genes.[31] Tumor cells may secrete this protein through some unknown mechanism, because there is no signal peptide sequence for secretion in the NM23 molecule. Serum NM23-H1 in patients with neuroblastoma might be derived from tumor cells and might be induced by *MYCN* amplification/overexpression or by *NM23-H1* overexpression independent of *MYCN*.

The serum level of NM23-H1 protein is clinically useful as an important prognostic factor in NHL or AML, and the present study showed that the protein could be a factor predicting an outcome of patients with neuroblastoma. It would be interesting to examine whether the serum NM23-H1 level generally predicts a poor outcome for patients with other tumors. The mechanisms by which the NM23-H1 protein is secreted into the serum and how it affects patient outcome are unclear. We are now studying the possibility that a high concentration of serum NM23-H1 may positively affect tumor cell growth or negatively affect normal cells.

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Review Article

p73, a sophisticated p53 family member in the cancer world

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p73 belongs to a family of p53-related nuclear transcription factors that includes p53, p73 and p63. The overall structure and sequence homology indicates that a *p63/p73*-like protogene is the ancestral gene, whereas *p53* evolved later in higher organisms. In accordance with their structural similarity, p73 functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in the cell cycle checkpoint control through transactivating an overlapping set of p53/p73-target genes. In sharp contrast to p53, however, p73 is expressed as two NH_2-terminally distinct isoforms including transcriptionally active (TA) and transcriptionally inactive (ΔN) forms. ΔNp73, which has oncogenic potential, acts in a dominant negative manner against TAp73 as well as p53. p73 is induced to be stabilized in response to a subset of DNA-damaging agents in a way that is distinct from that of p53, and exerts its pro-apoptotic activity. Several lines of evidence suggest that p73 can induce tumor cell apoptosis in a p53-dependent and p53-independent manner. Some tumors exhibit resistance to the p53-dependent apoptotic program, therefore p73, which can induce apoptotic cell death by p53-independent mechanisms, is particularly useful. In this review, we discuss the regulatory mechanisms of p73 activity, and also the functional significance of p73 in the regulation of cellular processes including tumorigenesis, apoptosis and neurogenesis. (*Cancer Sci* 2005; 96: 729–737)

Until recently, the tumor suppressor p53 has been believed to be encoded by a single gene which lacks any structural or functional homologs. The identification of two p53-related proteins, termed p73 and p63, revealed that p53 belongs to a small family of sequence-specific nuclear transcription factors.[1–3] p53 family members share three major functional domains: the NH_2-terminal transactivation domain; the central core sequence-specific DNA-binding domain; and the COOH-terminal oligomerization domain. Of these, the central DNA-binding domain is highly conserved across the family. As expected from their structural similarities, p73 can bind to the p53-responsive elements, and transactivate an overlapping set of p53-target genes implicated in G1/S cell cycle arrest and apoptotic cell death.[1,4] Recent studies demonstrated that p73 is required for p53-dependent apoptosis.[5] Unlike p53, p73 is expressed as at least six variants with different COOH-terminal ends, arising from the alternative splicing at the 3′ portion of the primary transcript.[1,6,7] Each of these splicing variants (TAp73) contains an intact NH_2-terminal transactivation domain, and exerts its transcriptional activity to various degrees. Additionally, *p73* contains a second transcriptional start site within intron 3, giving rise to the NH_2-terminally truncated form of p73 (ΔNp73) which has little transcriptional activity.[8] Similar to p73, ΔNp63 is also generated by an alternative promoter (Fig. 1a,b).[2] ΔNp73 displays dominant negative behavior toward p73 as well as wild-type p53, and has oncogenic potential.[9,10] Of note, we and others found that ΔNp73 is a direct transcriptional target of p73, suggesting that there exists a negative feedback regulation of p73 by ΔNp73, to modulate cell survival and death.[11–13]

Steady-state expression levels of endogenous p73 are kept extremely low under physiological conditions. Similar to p53, p73 is induced to be stabilized at the protein level in response to a subset of DNA-damaging agents, and exerts its pro-apoptotic activity.[14] Accumulating evidence suggests that p73 turnover is regulated through a ubiquitination-dependent and ubiquitination-independent degradation pathway. MDM2 acts as an E3 ubiquitin protein ligase for p53, and promotes the proteasome-mediated proteolytic degradation of p53.[15–17] On the other hand, MDM2 increases the stability of p73,[18] indicating that p73 stability is regulated through a pathway distinct from that of p53. Alternatively, Ohtsuka *et al.* reported that cyclin G binds to p73 and stimulates its proteolytic degradation in a ubiquitination-independent manner, however, the precise molecular mechanism of cyclin G-mediated degradation of p73 remains unknown.[19]

Considering that p73 has a p53-like property and is mapped to the human chromosome 1p36.2-3, a region which is frequently lost in a wide variety of human tumors including neuroblastoma, it is likely that p73 could be one of the classic Knudson-type tumor suppressors.[1] In spite of extensive mutation searches, *p73* was rarely mutated in primary tumors.[20] Additionally, initial genetic studies demonstrated that p73-deficient mice exhibit severe developmental defects, however, they do not develop spontaneous tumors, suggesting that p73 might participate in the regulation of normal development *in vivo*, and that p73 does not link directly to tumor suppression.[8] Indeed, p73 has the ability to induce neuronal differentiation of undifferentiated neuroblastoma cells.[21] However, this viewpoint has been challenged by the observation that mice mutant for p73 and p63 develop spontaneous tumors, and their spectrum is quite different from that of p53-deficient mice.[22] Thus, it is likely that p73 and

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Abbreviations: EEC, ectrodactyly, ectodermal dysplasia, and facial clefts (syndrome); OPC, oligodendrocyte precursor cell; SAM, sterile α motif; YAP, Yes-associated protein.

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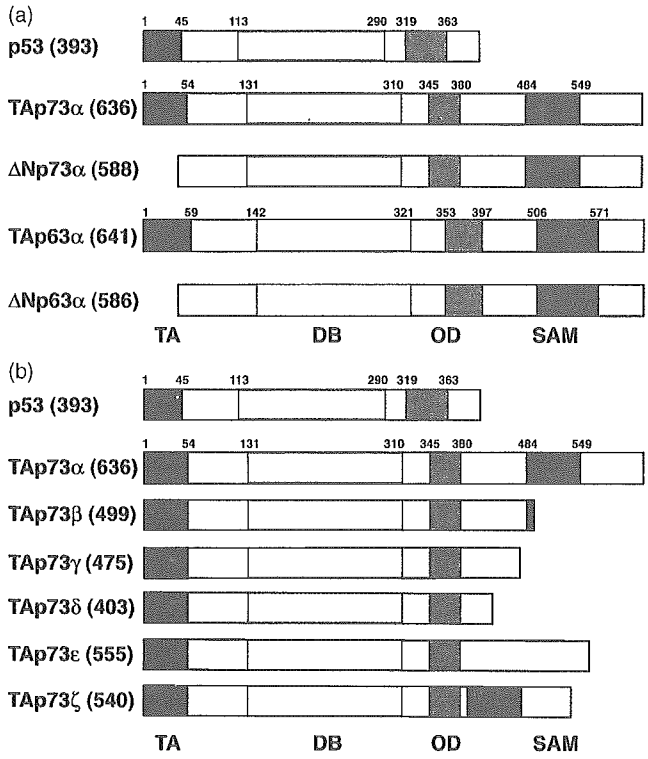

Fig. 1. (a) Structural comparison between TA and ΔN variants. The transactivation domain (TA), DNA-binding domain (DB), oligomerization domain (OD) and sterile α motif domain (SAM) are indicated. ΔN variant, which lacks the NH_2-terminal transactivation domain, is generated by alternative promoter usage. The numbers in parentheses are the amino acid lengths of each protein. (b) Schematic drawing of the splicing variants of p73. p73 is expressed as multiple variants with different COOH-terminal ends arising from an alternative splicing at the 3′ portion of the primary transcript. The numbers in parentheses are the amino acid lengths of each protein.

p63 exert their tumor suppressor activity in specific tissues. In this review, we will discuss the functional significance of p73 in the regulation of cellular processes such as tumorigenesis, apoptotic cell death and neuronal differentiation.

Splicing variants of p73

The overall genomic organization of *p73* is quite similar to that of *p53*. The *p53* gene is 20 kb in length and contains 11 exons. The *p73* gene is larger than 60 kb in length and contains 14 exons.[23] In sharp contrast to p53, p73 is expressed as multiple variants arising from an alternative splicing of the primary *p73* transcript including p73α, p73β, p73γ, p73δ, p73ε, and p73ζ.[1,6,7] Among them, p73α is the longest form, which contains a sterile α motif domain (SAM domain) and an extreme COOH-terminal region, whereas p73β lacks the extreme COOH-terminal tail and most of the SAM domain. We and others revealed that these COOH-terminal splicing variants display different transcriptional and biological properties.[6,7,24] Indeed, the ability of p73β to transactivate a variety of p53/p73 target genes and to induce apoptotic cell death in certain cancerous cells was stronger than those of the full-length p73α.[7,25] This indicated that the COOH-terminal region of p73 might possess a regulatory role, which modulates its transcriptional and pro-apoptotic activity[24,25] These splicing variants with different COOH-terminal extensions were expressed differentially among normal human tissues and cell lines, suggesting that they have distinct physiological functions.[6,7]

TAp73 and ΔNp73

In addition to the differential splicing variants of p73, there exist the ΔN variant forms of p73 (ΔNp73α and ΔNp73β) which are transcribed from an internal promoter located within an extra exon (exon 3′) of the full-length *p73* gene, and lacks the NH_2-terminal transactivation domain in TAp73.[8] Like p73, ΔNp63 is also generated using an alternative promoter.[2] As expected, ΔNp73 has little transactivation activity. Furthermore, ΔNp73 displays dominant negative behavior toward TAp73 as well as wild-type p53,[9] and also has oncogenic potential.[10] ΔNp73-mediated inhibition of TAp73 and p53 occurs at the oligomerization level or by the competition for binding to the same p53/p73-responsive element, with ΔNp73 displacing TAp73 and p53 from the DNA binding site.[26,27] For example, ΔNp73 was expressed predominantly in sympathetic neurons, and inhibited p53-dependent neuronal apoptosis.[9] ΔNp73-dependent repression of apoptosis induced by p53 is critical for the normal development of the neural system. In addition, the endogenous expression levels of ΔNp73 were significantly associated with poor prognosis in human cancers such as neuroblastoma.[28] Thus, it is likely that a balance between the intracellular expression levels of pro-apoptotic TAp73 or p53 and anti-apoptotic ΔNp73 plays an important role in regulating cell fate determination. Intriguingly, we and others demonstrated that there exists a functional p53/p73-responsive element within the *ΔNp73* promoter region, and indeed the expression of ΔNp73 is directly transactivated by TAp73 and/or wild-type p53, creating a dominant negative feedback loop which regulates the pro-apoptotic activities of both TAp73 and wild-type p53[11–13] (Fig. 2).

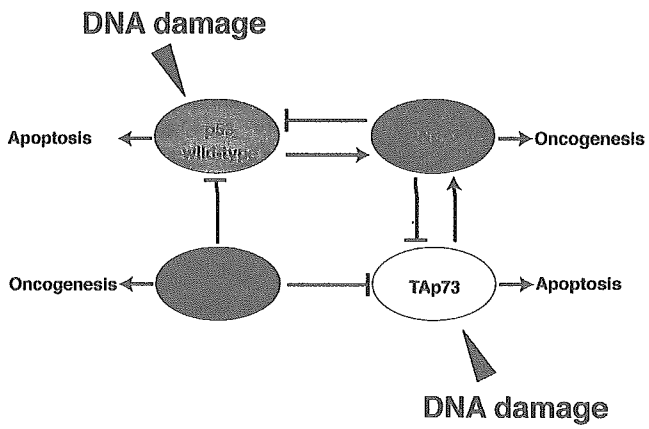

Fig. 2. Functional interactions between TAp73, ΔNp73, wild-type p53 and a mutant form of p53. DNA damage induces TAp73 and wild-type p53 through distinct pathways. The mutant form of p53 inhibits the pro-apoptotic activity of TAp73 and wild-type p53. ΔNp73, which is directly transactivated by TAp73 and wild-type p53, displays dominant negative behavior toward TAp73 and wild-type p53.

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As described above, ΔNp73 is transcriptionally inactive due to a lack of the NH_2-terminal transactivation domain in TAp73. However, this viewpoint has been challenged by the recent finding of Liu *et al.* showing that ΔNp73β has weak but distinct transcriptional activity, thereby inducing cell cycle arrest and/or apoptosis.[29] In contrast to ΔNp73β, ΔNp73α failed to induce cell cycle arrest and/or apoptosis under their experimental conditions. According to their results, the NH_2-terminal 13 unique amino acid residues as well as PXXP motifs of ΔNp73β might be a novel activation domain. Thus, it is possible that ΔNp73β might exert a distinct function under certain cellular processes.

Transcriptional regulation of the main promoter of p73

E2F1 transcription factor plays an important role in the regulation of cell cycle progression by inducing the transcription of genes whose products are directly or indirectly required for entry into the S phase.[30] In addition to the proliferative effect of deregulated E2F1 activity, unscheduled E2F1 activation leads to apoptosis to protect cells from cellular transformation.[31] Consistent with this notion, E2F1-deficient mice exhibited a high incidence of unusual tumors.[32,33] E2F1-induced apoptosis is regulated in a p53-dependent or p53-independent manner. It is interesting that the *p73* promoter region contains a TATA-like box and at least three E2F1-binding sites, and indeed the enforced expression of E2F1 strongly stimulates the transcription of *p73* through the direct binding to the E2F1-responsive elements in the *p73* promoter.[34,35] The E2F1-mediated up-regulation of p73 results in a significant induction of apoptosis. Other studies demonstrated that T cell receptor-mediated apoptosis is dependent on both E2F1 and p73.[36] Thus, E2F1-mediated apoptosis requires p73, at least in part. Alternatively, E2F1 might also contribute to the up-regulation of *p73* mRNA levels during muscle and neuronal differentiation of murine C2C12 myoblasts and P19 cells, respectively.[37] It is worth noting that Chk1 and Chk2 are required for the induction of p73 in response to DNA damage, and E2F1 contributes to the Chk kinase-dependent transcriptional regulation of p73.[38] In addition to E2F1, cellular and viral oncogene products such as c-Myc and E1A indirectly activated the transcription of p73.[39]

Recently, Fontemaggi *et al.* identified a 1 kb negative regulatory fragment within the first intron of *p73* gene.[37] Under their experimental conditions, this intronic fragment significantly reduced the activity of the *p73* promoter upon E2F1 overexpression. Of note, the *p73* intronic fragment contained six consensus binding sites for transcriptional repressor ZEB. Ectopic expression of ZEB in C2C12 myoblasts attenuated myotube formation, and repressed the transcription of *p73*. In accordance with these results, the dominant negative form of ZEB had an ability to restore the expression levels of p73 in proliferating cells.

Because DNA hypermethylation contributes to the alteration of the entry of transcription factors into the regulatory region, the epigenetic modification of the *p73* promoter region through aberrant hypermethylation could be an alternative molecular mechanism for silencing the *p73* gene. Corn *et al.* described the aberrant promoter methylation of *p73* as occurring frequently in primary acute lymphoblastic leukemias and Burkitt's lymphomas, whereas the *p73* promoter methylation was not detected in normal lymphocytes or bone marrow.[40] Similar results were also reported by Kawano *et al.*[41] In contrast, hypermethylation of the *p73* promoter region was not observed in solid tumors including breast, renal, colon cancers or neuroblastomas,[42] suggesting that the methylation-dependent silencing of *p73* transcription might be specific to hematological malignancies.

p73 is rarely mutated in human cancers

The *p73* gene has been mapped to human chromosome 1p36.2-3, a region which exhibits frequent loss of heterozygosity in a wide variety of human cancers including neuroblastoma, and its gene product has an ability to promote G1/S cell cycle arrest and/or cell death through apoptosis in certain cancerous cells. Therefore, p73 could act as a tumor suppressor.[1] In spite of an extensive search of the *p73* status in human primary tumors, *p73* was infrequently mutated in many human tumors.[20,43–48] *p73* mutations were detected in fewer than 0.5% of human cancers, whereas over 50% of cancers carry *p53* mutations. For example, only two types of *p73* mutations with amino acid substitution (P405R and P425L) were found in primary neuroblastoma and lung cancer.[20] In addition to the NH_2-terminal transactivation domain, Takada *et al.* found a potential second transactivation domain within the COOH-terminal portion of p73α (amino acid residues 380–513), albeit to a lesser extent than the NH_2-terminal transactivation domain.[49] This region is rich in glutamine and proline residues. Among the two types of p73 mutations, the P425L substitution significantly reduced the transcriptional and growth-suppressive activity of p73α, whereas the P405R substitution had a negligible effect on p73α.[50]

In sharp contrast to p53-deficient mice, which develop tumors with high frequency,[51] p73-deficient mice were viable, but the loss of p73 did not predispose mice to cancer, suggesting that p73 does not function as a classic Knudson-type tumor suppressor,[8] and its possible contribution to tumor suppression is still unclear. Instead, mice lacking p73 displayed severe developmental defects, including hydrocephalus, hippocampal dysgenesis, and abnormalities in the pheromone sensory pathways. These observations strongly suggest that p73 and p53 have distinct biological functions, and p73 plays an important role in normal development, especially in neural development and apoptosis. Because those p73-deficient mice lacked both TAp73 and ΔNp73 variants, further studies of variant-specific knockout mice might provide an insight into the unique role of each variant in tumorigenesis.

As mentioned above, initial genetic studies revealed that p73-deficient mice do not display an increased susceptibility to spontaneous tumorigenesis. More recently, Flores *et al.* examined whether synergistic effects of p73 and p63 could exist, alone or in combination with p53, in tumor suppression.[22] Strikingly, they found that p73 and p63 heterozygous mice ($p73^{+/-}$ and $p63^{+/-}$) developed malignant tumors at high frequency including various tumor types not observed in $p53^{+/-}$ mice, and $p53^{+/-}$; $p73^{+/-}$ and $p53^{+/-}$; $p63^{+/-}$ mice

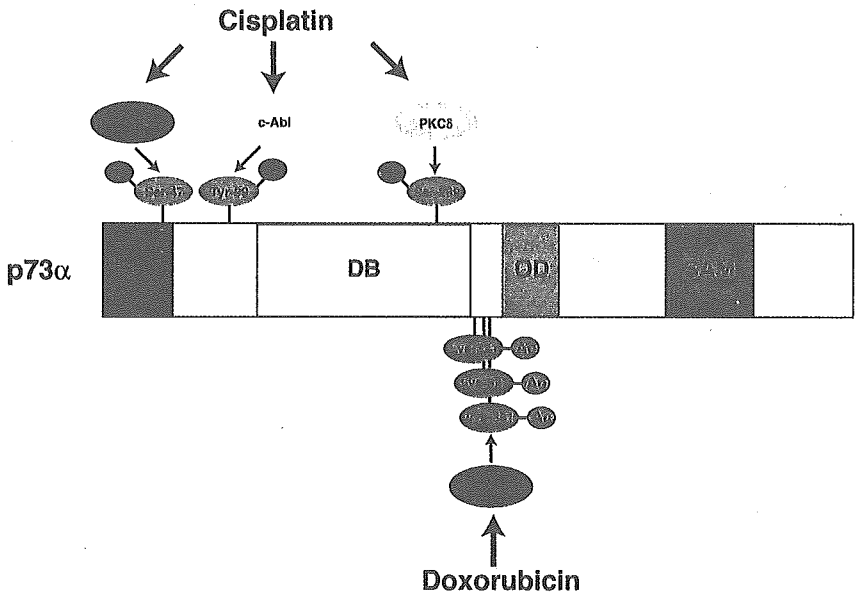

Fig. 3. DNA damage-induced activation of p73 is mediated by post-translational modifications including phosphorylation and acetylation. During cisplatin-mediated apoptosis, p73 is induced to be phosphorylated at Ser-47, Tyr-99 and Ser-289 by Chk1, c-Abl and PKCδ, respectively. In response to doxorubicin, p300 acetylates p73 at Lys-321, Lys-327 and Lys-331.

developed a more aggressive tumor phenotype.[22] In addition, tumors derived from $p73^{+/-}$ and $p63^{+/-}$ mice exhibited loss of the remaining wild-type allele at high frequency. These results strongly suggest that loss of p73 and/or p63 function causes tumor development, and their tumor suppressor activities play a pivotal role in specific tissues distinct from those of p53.

Activation of p73 at protein level by genotoxic stresses

Under normal physiological conditions, the expression levels of the p73 protein are maintained at an extremely low level, keeping this pro-apoptotic protein in an inactive state. The initial studies demonstrated that, unlike p53, p73 is not induced at the protein level in response to DNA damage.[1] Indeed, the exposure to either actinomycin D or ultraviolet radiation had no significant effects on p73 protein levels, whereas p53 and one of its target $p21^{WAF1}$ levels were markedly elevated in response to actinomycin D or ultraviolet radiation. However, recent studies revealed that p73 is induced to be accumulated in response to a subset of DNA-damaging agents, including cisplatin, adriamycin, camptothecin and etoposide.[14] p73 is predominantly regulated at the post-translational level, and the stabilization of p73 results in either G1/S cell cycle arrest or cell death through apoptosis. Therefore, the stabilization of p73 is directly linked with its activity.

Accumulating evidence strongly suggests that chemical modifications of p73, such as phosphorylation and acetylation, prolong its half-life, which, in turn, enhance its transcriptional and pro-apoptotic activity. During the cisplatin-mediated apoptotic process, p73 is phosphorylated at Tyr-99 and stabilized in a pathway dependent on nuclear non-receptor tyrosine kinase c-Abl.[52–54] In addition to c-Abl, exposure to cisplatin promoted a complex formation between p73 and a protein kinase Cδ catalytic fragment, which phosphorylated p73 at Ser-289 and increased its stability and transcriptional activity.[55] Recently, it has been shown that cisplatin-induced apoptosis is associated with p73 phosphorylation at Ser-47 mediated by Chk1.[56] Chk1-dependent phosphorylation resulted in an increase in the transcriptional activity of p73 (Fig. 3). In contrast, CDK-mediated phosphorylation of p73 led to significant inhibition of its transcriptional activity,[57] indicating that the phosphorylation of p73 might not always convert a latent form of p73 to an active one.

Alternatively, p73 is regulated by acetylation. p73 was previously found to be associated with p300 histone acetyltransferase through its NH_2-terminal transactivation domain, and this interaction resulted in a significant enhancement of p73-mediated transcriptional activation as well as apoptosis.[58] Costanzo *et al.* reported that p300 acetylates p73 at Lys-321, Lys-327 and Lys-331 in response to doxorubicin in a c-Abl-dependent manner, and the acetylated forms of p73 have pro-apoptotic activity[59] (Fig. 3). Intriguingly, the p300-mediated acetylation of p73 was stimulated by prolyl isomerase Pin1, thereby stabilizing p73.[60] It is likely that p73 acetylation catalyzed by p300 reduces its ubiquitination levels by competition between acetylation and ubiquitination.

Regulation of p73 turnover

Lee and La Thangue described p73 as being stabilized in cells treated with proteasome inhibitor such as LLnL.[25] They also showed that p73β is much more stable than p73α, indicating that the COOH-terminal extension of p73α might be involved in the stability control of p73. In support of this notion, we have found that RanBPM binds to the extreme COOH-terminal region of p73α, and prolongs the half-life of p73α.[61] Subsequently, several lines of evidence suggest that p73 turnover is regulated by a ubiquitination-dependent and a ubiquitination-independent proteasome pathway. MDM2, which is transcriptionally activated by p53, acts as an E3 ubiquitin protein ligase for p53. MDM2 promotes the ubiquitination of p53 through physical interaction with its

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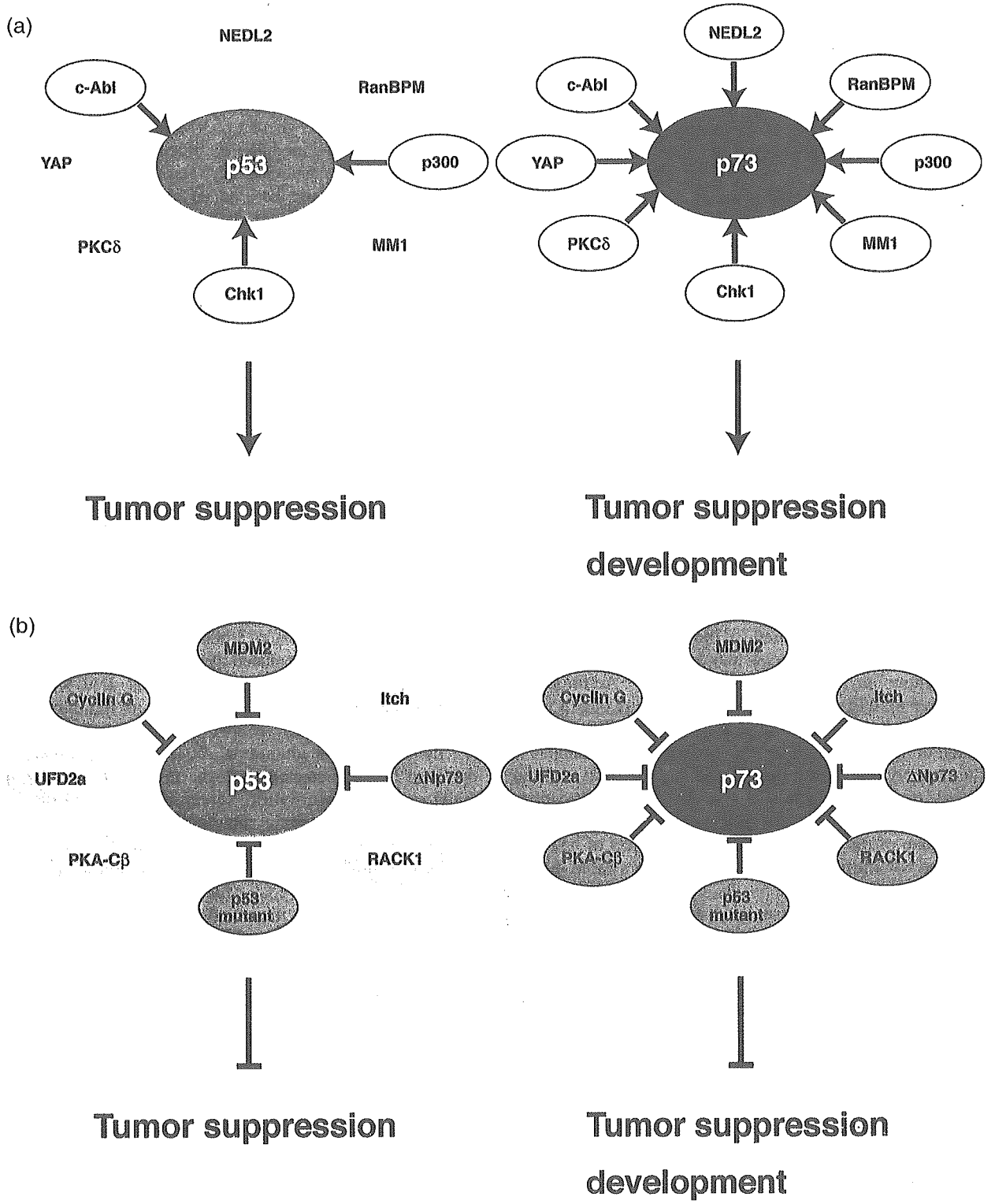

Fig. 4. Positive (a) and negative (b) regulation of p73 or p53 activity through the physical and functional interaction with various cellular proteins. Uncircled proteins have an undetectable effect on p53.

NH_2-terminal transactivation domain, and subsequent proteolytic degradation of p53.[15–17] Similar to p53, MDM2 was also a direct transcriptional target of p73, bound to its NH_2-terminal transactivation domain and thereby inhibiting p73-mediated transcriptional activation and apoptosis. However, MDM2 failed to ubiquitinate p73, and this interaction resulted in an increase in p73 protein stability.[18] Additionally, a newly identified p53-induced E3 ubiquitin

protein ligase Pirh2, which stimulates the ubiquitination-dependent proteolytic degradation of p53, had a negligible effect on p73.[62,63] These observations strongly indicate that p73 turnover is regulated by an as yet unidentified E3 ubiquitin protein ligase(s) distinct from that used for p53.

Recently, Rossi *et al.* found that a HECT-type E3 ubiquitin protein ligase Itch interacts with p73 through the WW protein–protein interaction domains of Itch and the p73 region containing the PY motif, and p53 which does not contain the PY motif fails to interact with Itch.[64] According to these results, Itch had an ability to ubiquitinate and degrade p73. Upon DNA damage induced by chemotherapeutic drugs including cisplatin, doxorubicin or etoposide, the endogenous expression levels of Itch were significantly down-regulated through an unknown mechanism, thereby increasing the stability and activity of p73. On the other hand, we have found that a novel HECT-type E3 ubiquitin protein ligase NEDL2 directly binds to p73, and this interaction is mediated by the WW domains of NEDL2 and the COOH-terminal region of p73 containing the PY motif.[65] Unexpectedly, NEDL2 promoted the ubiquitination of p73 in cells, however, NEDL2-mediated ubiquitination increased the stability of p73 and enhanced the p73-dependent transcriptional activation, indicating that there exists a non-proteolytic regulatory role of ubiquitination. Other studies demonstrated that the NH_2-terminally truncated form of p73 (ΔNp73) is much more stable than TAp73, suggesting that p73-mediated transcriptional activation is required for the rapid turnover of p73, and that, like p53, one or more transcriptional targets of p73 might promote its proteolytic degradation.[63] Additionally, Toh *et al.* reported that c-Jun increases the stability of p73 without direct interaction, and c-Jun-mediated stabilization of p73 is regulated in its transactivation function-dependent manner.[66]

Alternatively, several lines of evidence suggest that the proteolytic degradation of p73 is regulated in a ubiquitination-independent manner. For example, Ohtsuka *et al.* found that cyclin G, one of the direct transcriptional targets of p53 and p73, interacts with p73 and induces the latter's rapid degradation.[19] According to these results, cyclin G-mediated degradation of p73 was not associated with an increase in its ubiquitination levels. Recently, we have demonstrated that a U-box-type E3/E4 ubiquitin protein ligase UFD2a interacts with p73 through its COOH-terminal SAM domain, and induces the proteasomal turnover of p73.[67] Intrinsic E3/E4 ubiquitin protein ligase activity was not necessary for the UFD2a-mediated proteolytic degradation of p73, and UFD2a failed to increase the ubiquitination levels of p73. Similar to Itch, the intracellular expression levels of UFD2a were significantly down-regulated at protein levels in response to cisplatin, thereby leading to a dissociation of free active p73 from the p73/UFD2a complex. Although the precise molecular mechanisms underlying the proteasome-dependent degradation of p73 mediated by UFD2a are not yet known, it is likely that p73 might be recruited to the proteasome through its interaction with UFD2a.

Molecules interacting with p73

In addition to post-translational modifications including phosphorylation and acetylation, the activity of p73 is regulated by physical interaction with several viral and cellular proteins. As p53 and p73 share the same domain organization, consisting of the NH_2-terminal transactivation domain, the central sequence-specific DNA-binding domain and the COOH-terminal oligomelization domain, initial studies were performed to examine whether p53-binding proteins could also interact with p73 and modulate its function. Like p53, p73 was associated with the adenovirus E1A and the T-cell lymphotropic virus I-derived Tax, and these interactions inhibited the activity of p73.[23] On the other hand, the viral proteins which can bind to and inactivate p53, including the adenovirus E1B, papilomavirus E6 and simian virus 40 T antigen, failed to interact with p73.[68–70] For cellular proteins, MDM2 interacted with both p53 and p73, and inactivated their activities.[18,71]

Recently, several experimental approaches have been employed to identify the specific binding partners of p73. We and others focussed attention on the PY motif of p73 not found in p53, and identified the Yes-associated protein (YAP), NEDL2 and Itch.[64,65,72] As mentioned above, NEDL2 ubiquitinated p73 but extended its half-life, thereby enhancing its transcriptional activation. Itch promoted the ubiquitination-mediated proteasomal turnover of p73. YAP interacted with the PY motif of p73 through its WW domain, and stimulated p73-mediated transcriptional activation. We performed a conventional yeast-based two-hybrid screening using the extreme COOH-terminal tail of p73 not found in p53. Finally, we identified the c-Myc-binding protein (MM1), RACK1 and RanBPM as p73-binding proteins.[61,73,74] Based on our results, MM1 attenuated the c-Myc-mediated inhibition of transcriptional activity of p73, whereas RACK1 significantly inhibited the function of p73 and its inhibitory effect was counteracted by pRB. RanBPM increased the stability of p73 by reducing its ubiquitination levels. The proteins we identified had no detectable effects on p53. By using a new CytoTrap yeast two-hybrid screening, we identified the protein kinase A catalytic subunit β (PKA-Cβ) as a novel binding partner of p73.[75] PKA-Cβ bound to both the NH_2- and COOH-terminal regions of p73, and inhibited its transcriptional activity. PKA-Cβ efficiently phosphorylated p73, and PKA-Cβ-mediated inhibition of p73 was dependent on the kinase activity of PKA-Cβ (Fig. 4a,b). These observations strongly suggest that the regulatory mechanisms of p73 are distinct from those of p53.

Mutual regulation between p73 and p53

Previously, it has been shown that tumor-derived p53 mutants but not wild-type p53 interact with p73, and abrogate its function.[76] Subsequent studies demonstrated that the ability of p53 mutants to interact with p73 depends on the nature of the p53 mutations as well as the polymorphism at codon 72 (Pro-72 or Arg-72) of p53 mutants.[77] According to their results, p53 mutants carrying Arg-72 bound to p73 better than p53 mutants with Pro-72. Consistent with this notion, p53 mutants carrying Arg-72 act as more potent inhibitors of chemotherapy-induced apoptosis than the p53 mutants with Pro-72.[78] Other studies focused on the functional interaction between wild-type p53 and p73. Miro-Mur *et al.* reported that p73 induces both accumulation and activation of wild-type p53 by preventing MDM2-mediated degradation

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through MDM2 titration.[79] In addition, Goldschneider *et al.* found that p73 promotes the nuclear localization of wild-type p53 in neuroblastoma cells in which p53 is predominantly expressed in cytoplasm.[80] These results suggest that p73 has an ability to enhance the activity of wild-type p53. In contrast, Vikhanskaya *et al.* described that p73 reduces the p53-mediated transcriptional activation through the competition of the same DNA-binding site.[81] These controversial results regarding the effects of p73 on wild-type p53 might be at least in part due to the different cell systems used in those studies.

Recently, it has been shown that p53-dependent apoptosis requires the indirect contribution of at least one other p53 family member, p73 or p63.[5] Thus, it is likely that p73 cooperates with p53 to promote apoptotic cell death. These findings emphasize the functional importance of p73 in the regulation of the DNA damage-induced apoptotic response.

Role of p73 in neuronal differentiation

Considering that p73-deficient mice in which both TAp73 and ΔNp73 have been deleted, displayed profound developmental defects in their nervous and immune systems including a severe distortion of the hippocampal formation, it is likely that p73 contributes to normal neural development.[8] Indeed, ΔNp73 was expressed predominantly in the developing brain and sympathetic neurons, and p53-dependent neuronal apoptosis was inhibited by ΔNp73.[9] Consistent with this notion, De Laurenzi *et al.* demonstrated that p73 is induced to be accumulated during retinoic acid-mediated neuronal differentiation in neuroblastoma cell lines, whereas p53 levels remained unchanged in response to retinoic acid.[21] Under their experimental conditions, ectopic overexpression of p73 in undifferentiated neuroblastoma cell lines resulted in the induction of neurite extension as well as the expression of neuronal differentiation markers. In contrast, the transcriptionally inactive mutant form of p73 had undetectable effects on the neuronal differentiation. Similar results were also observed during neuronal differentiation in P19 cells exposed to retinoic acid.[46] Of note, Billon *et al.* described that the ectopic expression of p73 induces oligodendrocyte precursor cell (OPC) differentiation, and that ΔNp73 inhibits OPC differentiation in culture.[82] These observations strongly suggest that, in addition to its apoptosis-inducing activity upon DNA damage, p73 plays a pivotal role in the regulation of proper neuronal differentiation.

Role of p73 in the p53-independent cellular pathway

p53 plays a central role in the regulation of apoptotic cell death in response to DNA damaging agents. p53 function is lost by various mechanisms, including loss of function mutations within the *p53* gene itself or defects in upstream and/or downstream mediators of p53. Recent findings clearly demonstrated that p53-dependent apoptosis in response to DNA damage is impaired in cells lacking both p73 and p63, indicating that p73 and p63 are critical components of the apoptotic response to DNA damage.[5] Accumulating evidence strongly suggests that the pro-apoptotic activity of p73 is regulated through a pathway distinct from that used for p53. As certain cancerous cells were resistant to p53-dependent apoptotic cell death, p73 could be one of several candidate tumor suppressor proteins which can promote apoptosis by p53-independent mechanisms. Previous studies revealed that the exogenous expression of p73 in p53-deficient cells results in significant cell death through apoptosis in a p53-independent manner.[4] It is worth noting that p73 has an ability to promote apoptotic cell death in various pancreatic cells lacking functional p53, which are resistant to wild-type p53 gene transfer.[83] Thus, it is possible that p73 could be particularly useful in treating cancerous cells with non-functional p53.

What is the difference between p73 and p63?

The overall genomic organization of *p63* is quite similar to that of *p73*; the *p63* gene contains 15 exons.[23] Although *p63* is mapped to human chromosome 3q27–29, a region which is altered in a variety of cancers derived from lung, cervix or ovary, *p63* was infrequently mutated in primary tumors.[2,20] Like p73, p63 gives rise to at least six splicing variants as well as an NH_2-terminally truncated form of p63 (ΔNp63) arising from the alternative promoter usage. ΔNp63, a direct transcriptional target of p53, had a dominant negative effect on TAp63.[84] As expected from its structural similarity to p73, p63 can bind to the p53-responsive element and transactivate an overlapping set of p53-regulated genes, thereby inducing cell cycle arrest and/or apoptosis.[2] In contrast to p73, E2F1 did not stimulate the transcription of *p63*, although the putative E2F1-binding sites were found within the *p63* promoter region.[84]

At a sequence level, *p63* is much more similar to *p73* than *p53*, raising the possibility that *p73* or *p63* might be the original *p53* family gene, and that *p53* might be phylogenetically younger.[85] In support of this notion, p73 and p63 contribute to normal development, and p53 holds additional biological properties such as strong tumor suppressor activity. Despite the structural and functional similarities between p73 and p63, knockout phenotypes and the expression patterns of p63 were quite different from those of p73. In sharp contrast to *p73*, whose expression was restricted to the epidermis, sinuses, inner ear and brain, *p63* was predominantly expressed in the epidermis, cervix, urothelium and prostate.[23] Unlike p73-deficient mice, p63-deficient mice exhibited severe defects in limb, cranio-facial and epithelial development.[86,87] For example, p63-deficient mice lacked all squamous epithelia, and displayed severe forelimb truncations. Consistent with these developmental defects, *p63* mutations were detected in children affected by EEC (ectrodactyly, ectodermal dysplasia, and facial clefts) syndrome.[88] Thus, it is likely that p73 and p63 have overlapping and distinct biological activities, and they express their specific functions depending on their unique sites of action.

Conclusion

p53 and p73 share extensive structural and functional similarities. They have overlapping as well as distinct biological functions. In addition to its potent tumor suppressor function, at least in specific tissues, p73 plays a pivotal role in normal neurogenesis *in vivo*. Similar to

p53, p73 is induced to be accumulated in response to a subset of DNA-damaging agents, however, the regulatory mechanisms of the pro-apoptotic activity of p73 are distinct from those used for p53. p73 has the ability to induce apoptotic cell death in a p53-independent manner. Indeed, p73 can promote apoptotic cell death in tumor cells that lack functional p53. In addition, p73 might enhance the chemosensitivity of tumor cells. Therefore, p73 alone or in combination with the other p53 family members might provide a clue to overcoming chemoresistance in tumor cells.

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ORIGINAL ARTICLE

Hypoxia selects for high-metastatic Lewis lung carcinoma cells overexpressing Mcl-1 and exhibiting reduced apoptotic potential in solid tumors

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Low oxygen tension (hypoxia) is a common feature of solid tumors and stimulates the expressions of a variety of genes including those related to angiogenesis, apoptosis and endoplasmic reticulum (ER) stress response. Here we show a close correlation between metastatic potential and the resistance to hypoxia- and ER stress-induced apoptosis among the cell lines with differing metastatic potential derived from Lewis lung carcinoma. An apoptosis-specific expression profiling and immunoblot analyses revealed that the expression of antiapoptotic Mcl-1 increased as the resistance to apoptosis increased. Downregulation of the Mcl-1 expression in the high-metastatic cells by Mcl-1 small interfering RNA increased the sensitivity to hypoxia-induced apoptosis and decreased the metastatic ability. The hypoxia-induced apoptosis was not associated with p53 accumulation, although at present it is not possible to conclude that apoptosis-induced apoptosis is p53-independent. There was no correlation between the expression levels of ER stress-response proteins GADD153, GRP78 and ORP150 and the resistance to hypoxia or ER stresses. *In vitro*, small numbers of the high-metastatic cells overtook the low-metastatic cells after exposure to several rounds of hypoxia and reoxygenation. In solid tumors initially established from equal mixtures, the proportion of the high-metastatic cells to low-metastatic cells was significantly higher in hypoxic areas. Moreover, the high-metastatic cells were overtaking the low-metastatic cells in some of the tumors. Thus, tumor hypoxia and ER stress may provide a physiological selective pressure for the expansion of the high-metastatic cells overexpressing Mcl-1 and exhibiting reduced apoptotic potential in solid tumors.
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Introduction

Response to low oxygen tension (hypoxia) is a fundamental biological phenomenon and therefore hypoxia gives rise to a variety of physiological responses at cellular, local and systemic levels. The cells placed under hypoxic conditions activate many genes including those related to cell survival, glycolysis, angiogenesis, erythrocyte production and iron metabolism to adapt the environment (Semenza, 2000, 2002; Harris, 2002). The oxygen sensing mechanisms have been intensively studied and found to involve hypoxia-inducible factors (HIFs) as key regulatory transcription factors that are composed of HIF-α subunit and HIF-β/aryl hydrocarbon receptor nuclear translocator subunit (Semenza, 2000, 2002; Harris, 2002). HIF binds to the hypoxia-responsive element of hypoxia-responsive genes such as vascular endothelial growth factor (VEGF) and proapoptotic Bnip3, a member of the Bcl-2 family (Semenza, 2000, 2002; Harris, 2002).

Most solid tumors harbor areas of hypoxia, both acute and chronic, due to aberrant vasculature formation and high interstitial pressure (Chaplin and Hill, 1995; Brown and Giaccia, 1998). Although most of the tumor cells die in chronic hypoxia, some of them actually can survive for more than several days in a quiescent or the so-called dormant state (Durand and Sham, 1998) and restart to divide once closed vessels reopen or new vasculatures reach the hypoxic areas. It has been shown that hypoxia induces genetic instability, DNA over-replication and gene amplification in a variety of cultured cells (Rice *et al.*, 1986; Russo *et al.*, 1995; Coquelle *et al.*, 1998). A short-term hypoxia followed by reoxygenation transiently enhances invasive and metastatic potential of some tumor cells (Young and Hill, 1990; Graham *et al.*, 1999; Cairns *et al.*, 2001). Tumor hypoxia selects $p53^{-/-}$ transformed cells and thereby expands cells with diminished apoptotic potential *in vitro* (Graeber *et al.*, 1996). These mechanisms all together are likely to influence the malignant progression of tumor cells (Hill, 1990; Russo *et al.*, 1995; Graeber *et al.*, 1996; Coquelle *et al.*, 1998; Dachs and Chaplin, 1998). Besides, since hypoxic tumor cells cease to divide, they are resistant to conventional radiotherapy and chemotherapy (Rice *et al.*, 1986; Young and Hill, 1990; Teicher, 1994).

Physiological endoplasmic reticulum (ER) stress such as glucose starvation is also present in solid tumors. Hypoxia has been shown to upregulate ER stress-response genes including growth arrest/DNA damage-inducible protein 153 (GADD153/CHOP), which is a proapoptotic transcription factor (Friedman, 1996) and ER chaperones such as glucose-regulated protein (GRP)78/BIP (Munro and Pelham, 1986) and oxygen-regulated protein (ORP)150, which are antiapoptotic proteins (Kuwabara *et al.*, 1996). Upregulation of these ER stress proteins is HIF-independent.

There is accumulating evidence that developing resistance to common apoptotic stimuli is one of the factors that confer high metastatic capability to tumor cells (Glinsky and Glinsky, 1996; McConkey *et al.*, 1996; Bufalo *et al.*, 1997; Glinsky, 1997; Inbal *et al.*, 1997; Shtivelman, 1997; Takaoka *et al.*, 1997; Fernandez *et al.*, 2000; Lowe and Lin, 2000; Wong *et al.*, 2001). The apoptosis-resistant phenotype may be advantageous for tumor cells to survive in the metastatic process. We reported that the high-metastatic clone (A11 cells) established from Lewis lung carcinoma is more resistant to apoptosis induced by serum starvation, hypoxia and glucose deprivation than the low-metastatic clone (P29 cells) (Takasu *et al.*, 1999). However, it remained to be examined whether there is a correlation between metastatic ability and resistance to apoptosis induced by various stresses among various clones with differing metastatic potential. In addition, molecular mechanisms of the apoptosis resistance of the high-metastatic cells remained obscure. We addressed here these points and, furthermore, if hypoxia could act as a physiological selective pressure in solid tumors for the expansion of high-metastatic tumor cells that possess diminished apoptotic potential. The results showed that the high-metastatic Lewis lung carcinoma cell lines are more resistant to hypoxia- and ER stress-induced apoptosis than the low-metastatic cell lines, that the high-metastatic cells overexpress antiapoptotic Mcl-1, and that hypoxia selects for the high-metastatic cells in solid tumors.

Results

Correlation between metastatic potential and resistance to hypoxia- and ER stress-induced apoptosis in the low- and high-metastatic cell lines

To investigate the correlation between susceptibility to hypoxia-induced cell death and metastatic potential, we exposed the five cell lines with differing metastatic potential derived from a mouse Lewis lung carcinoma (metastatic capability; P29 = P34 < C2 < D6 < A11) to hypoxia (~0.1% O_2), corresponding to oxygen concentrations commonly found in solid tumors. Cell death was monitored after culturing the cell lines for 72 h under hypoxia. The results showed that only less than 8% of P29 and P34 cells were viable while about 20% of C2 cells and over 45% of D6 and A11 cells remained viable (Figure 1a). Thus, we observed a tendency where the resistance to hypoxia-induced cell death is correlated with the metastatic ability. The time course showed that hypoxia induced cell death more rapidly in P29 cells than in A11 cells (Figure 1b). Clonogenic assays in which the cells were exposed to hypoxia for 3 or 4 days and then reoxygenated to form colonies also demonstrated that A11 cells survived longer than P29 cells under hypoxic conditions (Figure 1c). The cells positive for annexin V and TUNEL staining increased in hypoxic P29 cells (Figure 1d). An increase in the number of cells exhibiting chromatin condensation and fragmentation as assessed by DAPI staining was also observed in hypoxic P29 cells (0.1 and 26.1% for normoxic and hypoxic P29 cells, respectively) (Figure 1d). In addition, flow cytometric analysis revealed an increase in the percentage of sub-G1 population in these cells (0.7 and 20.6% for normoxic and hypoxic cells, respectively) (Figure 1e). Thus, these data indicate that hypoxic P29 cells were dying through apoptosis. We confirmed that hypoxic A11 cells died of apoptosis based on the same criteria.

To test whether the high-metastatic cell lines are also resistant to ER stresses compared with the low-metastatic cell lines, we treated P29, P34, D6 and A11 cells with chemical ER stress inducers for 2 days and examined their viability. As shown in Figure 2, compared to P29 and P34 cells, D6 and A11 cells were much more resistant to apoptosis induced by tunicamycin (5 μg/ml), brefeldin A (5 μg/ml), thapsigargin (250 nM) and A23187 (1 μM).

Mcl-1 is overexpressed in the high-metastatic cell lines

To find out the genes responsible for the susceptibility to hypoxia-induced apoptosis, we compared the expression profile of apoptosis-related genes among normoxic and hypoxic P29 and A11 cells using a cDNA expression microarray cumulated apoptosis-related genes. The data showed that A11 cells expressed antiapoptotic *Mcl-1* gene at higher levels than P29 cells (not shown). Immunoblot analysis confirmed a higher expression of Mcl-1 in A11 cells than in P29 cells under both normoxic and hypoxic conditions (Figure 3A). We detected two close bands (40 and 37 kDa) on the blots. Since the expressions of the bands were decreased by treatment with Mcl-1 siRNA (see below), the 37 kDa band may be a degradation product of Mcl-1 or, though less likely, a splicing variant of *Mcl-1* gene. It is of note that the cell lines expressed Mcl-1 (40 kDa) at the levels according to the resistance to hypoxia- and other stress-induced apoptosis (Figure 3A and B). Consistent with the recent report that hypoxia enhances Mcl-1 expression in hepatoma HepG2 cells through HIF-1 (Piret *et al.*, 2005), the amount of Mcl-1 was increased by hypoxia in C2, D6 and A11 cells (Figure 3B). Immunohistochemistry for Mcl-1 on the sections prepared from paraffin-embedded P29 and A11 tumors showed a higher expression of Mcl-1 in A11 cells than in P29 cells, indicating that Mcl-1 overexpression is persistent even *in vivo* (Figure 3C).

The expression profiling also showed that hypoxia induced proapoptotic *Bnip3* gene expression in both P29

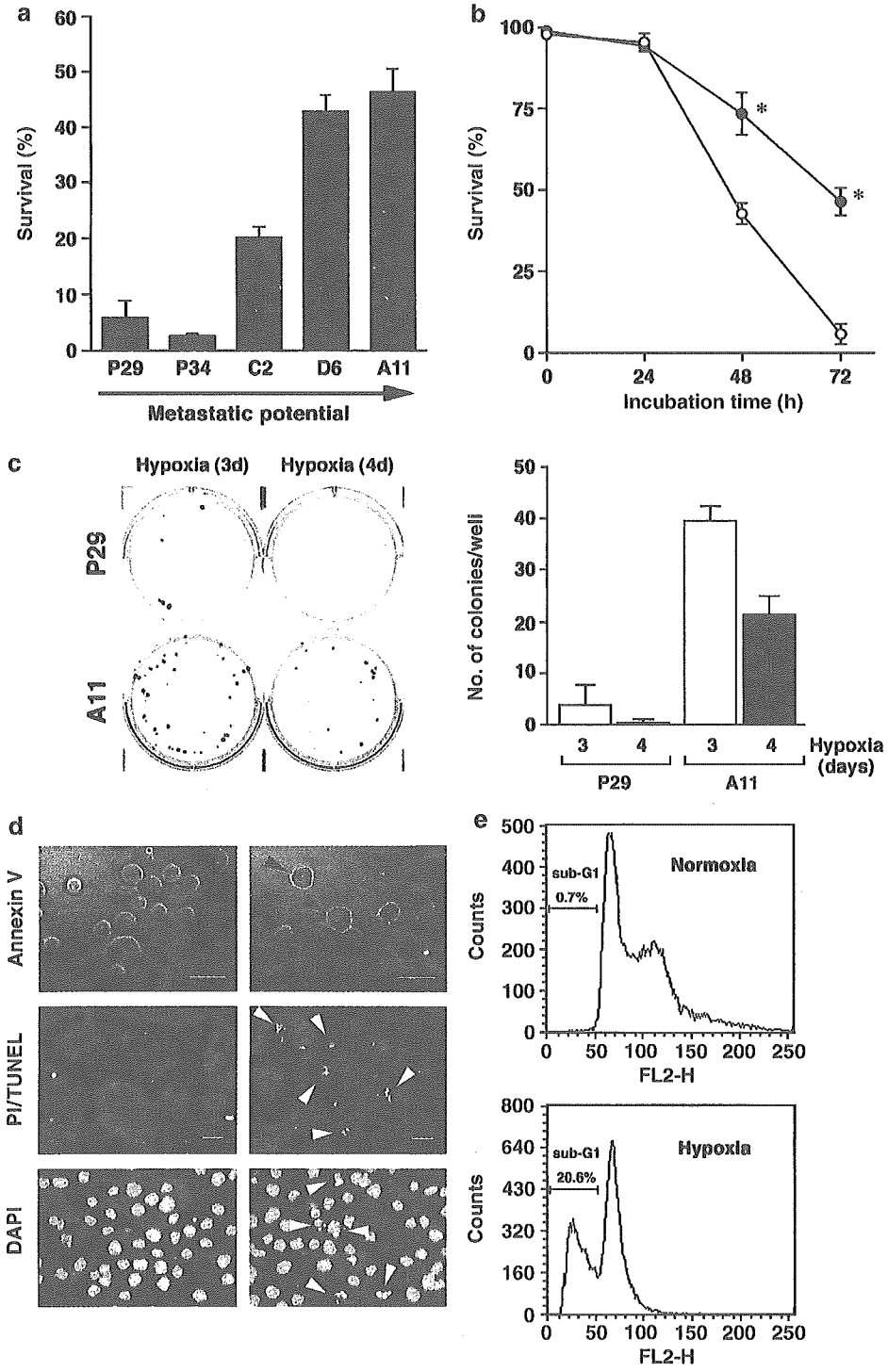

Figure 1 Sensitivity to hypoxia-induced apoptosis of the Lewis lung carcinoma cell lines. (**a**) Hypoxia-induced cell death of the cell lines with differing metastatic potential. The cell lines were exposed to hypoxia for 72 h. Percentage of living cells was determined on the basis of trypan blue exclusion. Bars; s.d. of triplicate determinations. (**b**) Time course of cell death induced by hypoxia. P29 (○) and A11 cells (●) were exposed to hypoxia for the indicated time period. Percentage of living cells was determined on the basis of trypan blue exclusion Bars; s.d. of triplicate determinations. *Significant at $P<0.002$. (**c**) Clonogenic assay of cell survival. P29 and A11 cells (100 cells/well) were cultured under hypoxic conditions for 3 or 4 days followed by culturing under normoxic conditions. Colonies were stained with crystal violet (left panel) and then counted (right panel). Bars; s.d. of triplicate determinations. (**d**) Annexin V, TUNEL and DAPI stainings of normoxic (left panels) and hypoxic P29 cells (right panels). P29 cells were cultured under hypoxic conditions for 18, 27 or 28 h, and then stained for annexin V-EGFP, TUNEL (green) and PI (red), or DAPI, respectively. Arrowheads show apoptotic cells. (**e**) Flow cytometric analysis of DNA fragmentation. P29 cells cultured under hypoxic conditions for 27 h were subjected to FACscan analysis. The percentage of sub-G1 fraction is also shown.

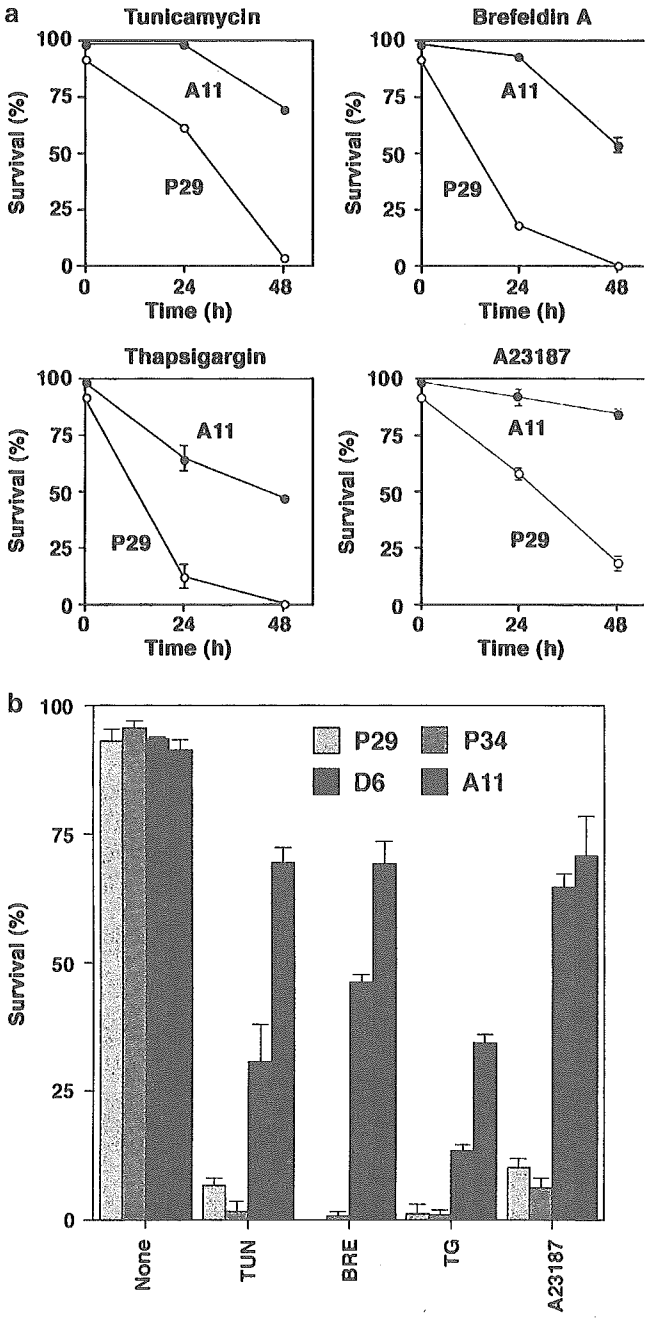

Figure 2 Sensitivity to ER stress-induced apoptosis of the Lewis lung carcinoma cell lines. (**a**) Time course of cell death of P29 (○) and A11 cells (●) exposed to ER stress-inducing agents. The cells were exposed to tunicamycin (5 μg/ml), brefeldin A (5 μg/ml), thaspigargin (250 nM), A23187 (1 μM)). (**b**) Sensitivity of the cell lines with differing metastatic potential to ER stress-inducing agents. P29, P34, D6 and A11 cells were exposed to tunicamycin (5 μg/ml), brefeldin A (5 μg/ml), thaspigargin (250 nM), A23187 (1 μM)) for 2 days. Percentage of living cells was determined on the basis of trypan blue exclusion. Bars; s.d. of triplicate determinations.

and A11 cells (data not shown). Actually, *Bnip3* mRNA expression was induced in all of the cell lines, but the expression level was not correlated with the susceptibility to hypoxia- and other stress-induced apoptosis (Figure 3D).

To investigate whether the hypoxia-induced apoptosis is associated with p53 accumulation, we examined the expression of p53 in hypoxia- and doxorubicin-treated P29, P34, D6 and A11 cells. Immunoblot analysis revealed that hypoxia reduced p53 expression (Figure 3E) and failed to induce endogenous downstream p53 effector proteins, Bax and $p21^{WAF1/CIP1}$, in these cell lines (not shown). By contrast, doxorubicin caused the accumulation of p53 (Figure 3E).

We next compared the expression levels of ER stress-response proteins, GADD153, GRP78 and ORP150, which are known to be induced by hypoxia, between P29 and A11 cells. As shown in Figure 3F and G, the expressions of these proteins were induced by tunicamycin and hypoxia, but there was no difference between 29 and A11 cells.

Effects of Mcl-1 siRNA on hypoxia-induced apoptosis and metastatic potential

To examine if the expression of Mcl-1 is responsible for the resistance to hypoxia-induced apoptosis, we transfected A11 cells with either Mcl-1 siRNA or control siRNA. As shown in Figure 4a and b, the expression of Mcl-1 was suppressed by Mcl-1 siRNA, but not by control siRNA. We then cultured these cells under hypoxic conditions for 60 h and monitored cell death. The results showed that Mcl-1 siRNA-treated A11 cells were more sensitive to hypoxia-induced apoptosis than mock and control siRNA-treated cells in both normal growth medium and serum-starved medium (Figure 4c). Importantly, Mcl-1 siRNA-treated A11 cells were less metastatic than control siRNA-treated cells, as assessed by lung weight and the number of metastatic nodules in the lung (Figure 4d). Thus, it appeared that Mcl-1 is at least in part involved in resistance to hypoxia-induced apoptosis and metastatic potential of A11 cells.

Apoptosis of the low- and high-metastatic cells in hypoxic areas of solid tumors

To examine whether the difference in the susceptibility to hypoxia-induced apoptosis can also be observed *in vivo*, we injected EF5, a nitroimidazole compound, into mice bearing subcutaneous P29 or A11 tumors of nearly equal size for detecting hypoxic areas and stained cryosections of the tumors first with TUNEL assay using fluorescein-labeled nucleotides, and then with Cy3-labeled antibodies against EF5-cellular macromolecule adducts (Figure 5a). EF5 binding occurs under low-oxygen conditions and only in viable cells (Lord *et al.*, 1993). The number of TUNEL-positive cells per 100 μm^2 in EF5-positive (hypoxic) and -negative (normoxic) areas was counted (Figure 5b). We omitted necrotic areas from the investigation. The results showed that the number of apoptotic cells in hypoxic areas of P29 tumors was fourfold larger than that in hypoxic areas of A11 tumors. In normoxic areas, the number of apoptotic cells was small but statistically larger in P29 tumors than in A11 tumors.

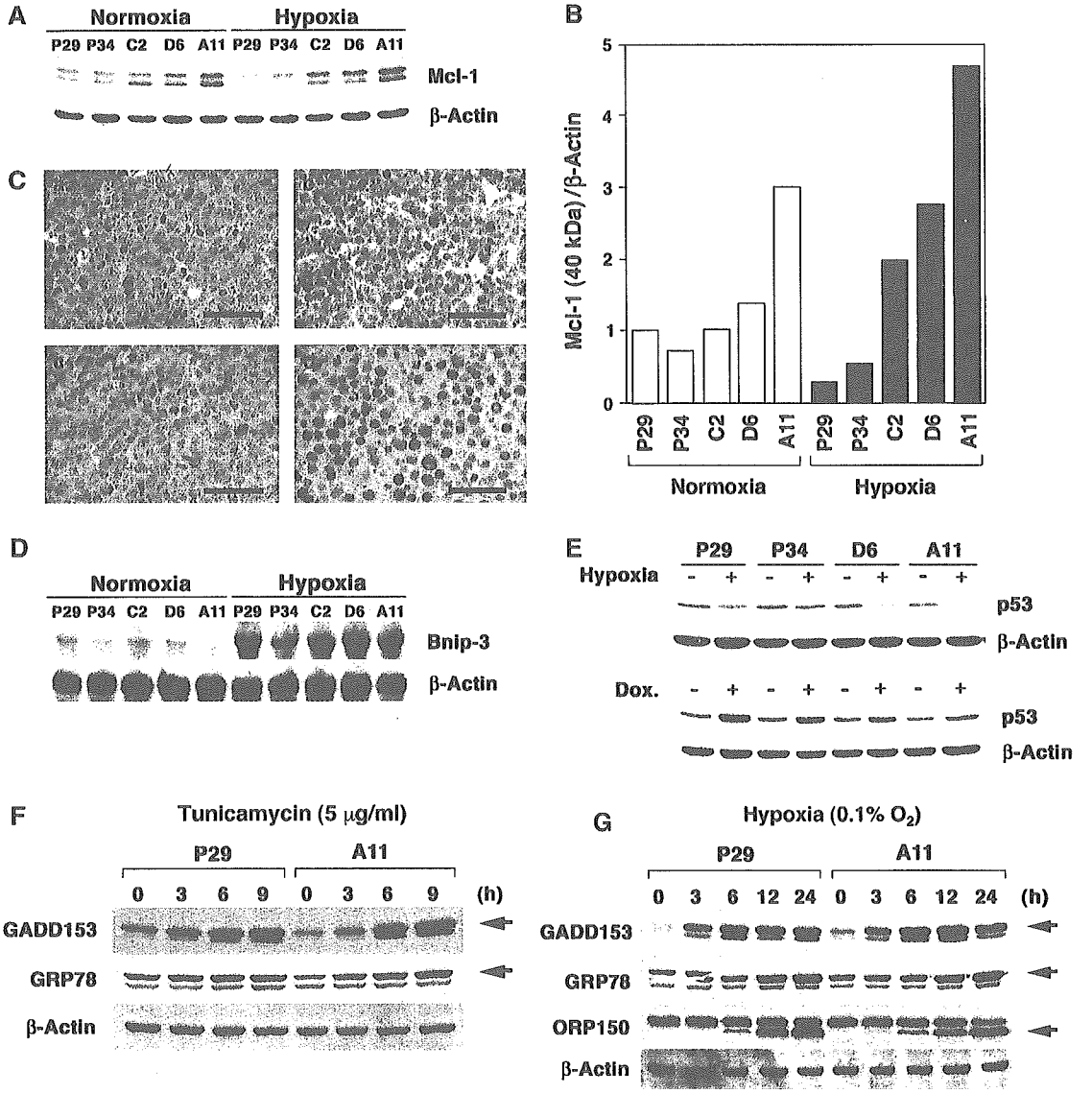

Figure 3 Expressions of apoptosis-related genes in Lewis lung carcinoma cell lines. (**A**) Western blot analysis of the effect of hypoxia on Mcl-1 expression. The cells exposed to hypoxia (~0.1% O_2) for 8 h were subjected to immunoblot analysis for Mcl-1 expression. β-actin served as loading controls. (**B**) Relative values for signal intensity of Mcl-1 (40 kDa) after normalization to the level of β-actin. Scanning densitometry of the gel was performed and the normalized values were represented by the white (under normoxia) and black (under hypoxia) bars. All values are shown as a percentage of the value for normoxic P29 cells. The results are representative of two separate experiments in which similar results were obtained. (**C**) Immunohistochemical analysis of Mcl-1 expression in P29 and A11 tumors. Sections from P29 tumors (a and c) and A11 tumors (b and d) were immunostained with anti-Mcl-1 antibody (a and b) and control IgG (c and d). Bars; 50 μm. (**D**) Effects of hypoxia on *Bnip3* mRNA expression. The cells exposed to hypoxia (0.1% O_2) for 8 h were subjected to Northern analysis for Bnip3 mRNA expression. β-Actin mRNA served as loading controls. (**E**) Western blot analysis of the effects of hypoxia and doxorubicin on the accumulation of p53 protein. The cells exposed to hypoxia (0.1% O_2) for 24 h or 5 μg/ml doxorubicin (Dox) for 20 h were subjected to immunoblot analysis for p53 expression. β-Actin served as loading controls. (**F**) Western blot analysis of the effects of tunicamycin on the expressions of GADD153 and GRP78. P29 and A11 cells were exposed to 5 μg/ml tunicamycin for the indicated periods of time. βActin served as loading controls. (**G**) Western blot analysis of the effects of hypoxia on the expressions of GADD153, GRP78 and ORP150. P29 and A11 cells were exposed to hypoxia (0.1% O_2) for the indicated periods of time. β-actin served as loading controls.

Survival advantage of the high-metastatic cells under hypoxic conditions

The above results prompted us to examine whether A11 cells have a survival advantage over P29 cells under hypoxic conditions. To this end, we established genetically labeled P29 ($P29^{EGFP}$ cells) and A11 cells ($A11^{IRES\text{-}EGFP}$ cells) after selecting P29 and A11 cells stably transfected with pEGFP-N1 and pIRES2-EGFP, respectively (Figure 6a), and characterized their properties. $P29^{EGFP}$ cells grew faster than $A11^{IRES\text{-}EGFP}$ cells *in vivo*, and at 17 days after tumor cell inoculation $P29^{EGFP}$ tumors were twice larger than $A11^{IRES\text{-}EGFP}$ tumors (Figure 6b). $P29^{EGFP}$ tumors contained large necrotic regions. $P29^{EGFP}$ and $A11^{IRES\text{-}EGFP}$ cells were low- and

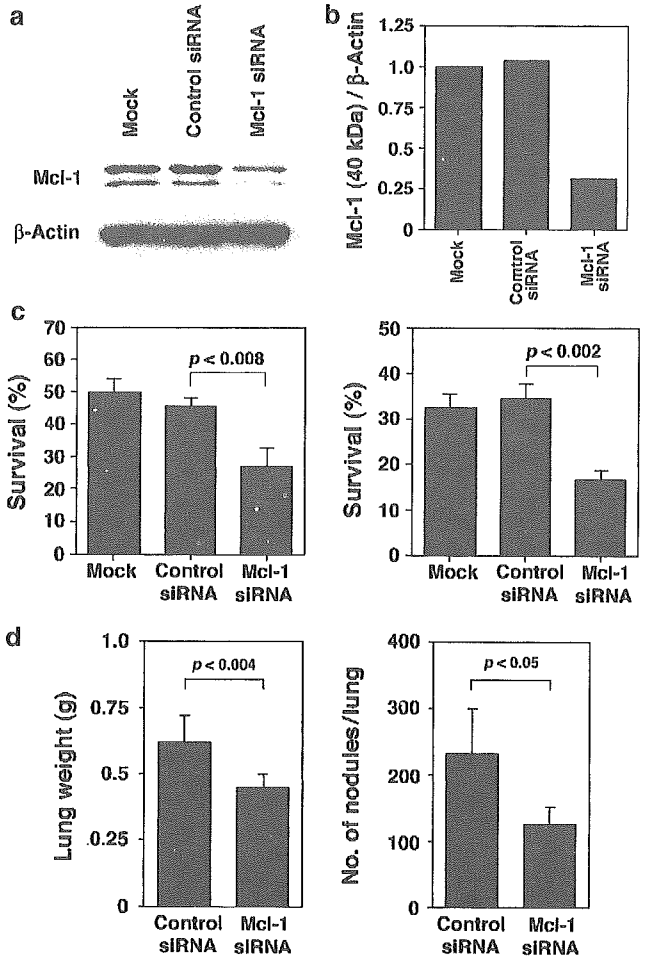

Figure 4 Effects of Mcl-1 siRNA on hypoxia-induced apoptosis and metastatic potential of A11 cells. (**a**) Expression of Mcl-1 in A11 cells treated with Mcl-1 siRNA. A11 cells pretreated with Lipofectamine 2000 alone (mock), 25 nM control siRNA or 25 nM Mcl-1 siRNA for 2 days were subjected to immunoblot analysis for Mcl-1 expression. β-Actin served as loading controls. (**b**) Relative values for signal intensity of Mcl-1 (40 kDa) after normalization to the level of β-actin. Scanning densitometry of the gel was performed and the relative values were represented. All values are shown as a percentage of the value for mock-transfected A11 cells. The results are representative of three separate experiments in which similar results were obtained. (**c**) Sensitivity of Mcl-1 siRNA-treated A11 cells to hypoxia-induced apoptosis. A11 cells pretreated with Lipofectamine 2000 alone (mock), 25 nM control siRNA or 25 nM Mcl-1 siRNA for 2 days were cultured under hypoxic conditions ($\sim$0.1% O_2) for 60 h in normal growth medium (left panel) or serum-starved (1% serum) medium (right panel). Cell death was examined by trypan blue staining. Bars; s.d. of triplicate determinations. (**d**) Metastatic potential of Mcl-1 siRNA-treated A11 cells. A11 cells pretreated with 25 nM control siRNA or 25 nM Mcl-1 siRNA for 2 days were injected intravenously into C57BL/6 mice (6 mice/group). At 17 days after the injection, the weight of the lungs (left panel) and the number of metastatic nodules (right panel) were measured. Bars; s.d.

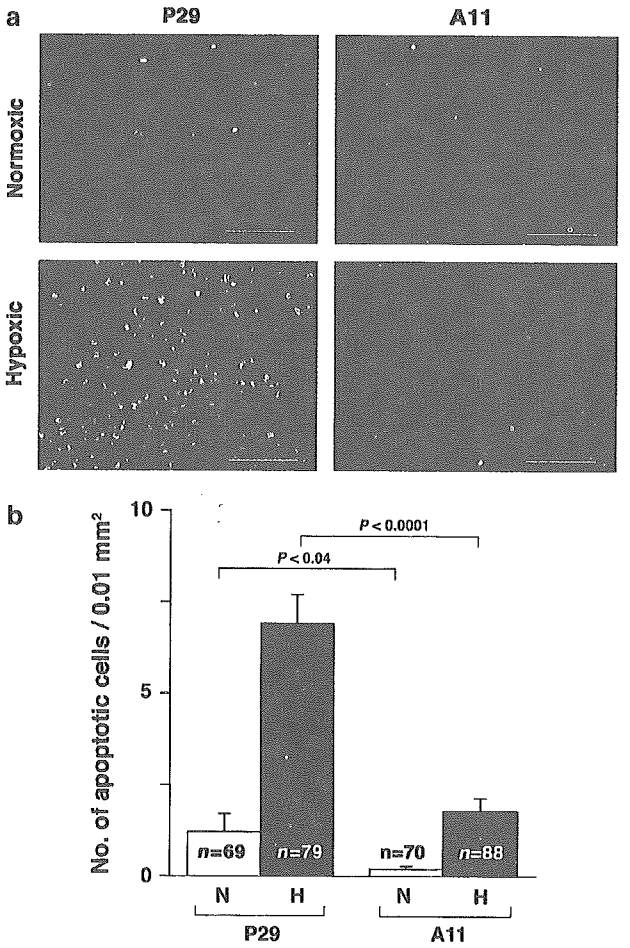

Figure 5 Apoptosis of P29 and A11 cells in tumor hypoxic areas. (**a**) TUNEL staining (green) and EF5 staining (red) of frozen sections of subcutaneous tumors established from P29 and A11 cells. (**b**) Frequency of apoptotic (TUNEL-positive) cells in normoxic (N) and hypoxic (H) areas. Bars; s.e.m.

high-metastatic, respectively, in both experimental and spontaneous metastasis assays (Figure 6c) and showed a similar apoptosis resistance to their parental cells (Figure 6d).

To obtain a standard curve by which the percentage of $A11^{IRES\text{-}EGFP}$ cells in mixtures of unknown proportions of $P29^{EGFP}$ and $A11^{IRES\text{-}EGFP}$ cells could be calculated, we extracted genomic DNA from mixtures of known proportions of the cells and performed PCR followed by Southern blot with an EGFP probe (Figure 6e). By plotting the relative intensities of the bands corresponding to EGFP and IRES-EGFP against the known proportion of $A11^{IRES\text{-}EGFP}$ cells, a standard curve, although slightly sigmoid, was obtained (Figure 6f). The value at each point did not significantly fluctuate even when we carried out PCR under various conditions (1–100 ng DNA, 20–35 PCR cycles) (not shown).

We then mixed $A11^{IRES\text{-}EGFP}$ and $P29^{EGFP}$ cells at a 1:1, 1:10 or 1:100 ratio and treated them with multiple rounds of hypoxia and reoxygenation (recovery in normoxia). The percentage of $A11^{IRES\text{-}EGFP}$ cells at the time of cell harvesting was determined from the standard curve after quantitation of radioactive intensity of the PCR bands. We found that the percentage of $A11^{IRES\text{-}EGFP}$ cells increased dramatically after several rounds of hypoxia-reoxygenation in every case (Figure 7a and b). The intensity of the band corresponding to EGFP and IRES-EGFP in $P29^{EGFP}$ and $A11^{IRES\text{-}EGFP}$