Table 3 Correlation between ALDH1A1 expression and clinicopathologic characteristics

Variable	ALDH1A1 expression		*P*-value
	<5% positive cells	≥5% positive cells	
Overall	35	68	
Age (years, median 65)			
<65	18	32	
≥65	17	36	0.674
Gender			
Male	18	39	
Female	17	29	0.567
Smoking status			
Ever smoker	20	39	
Never smoker	15	29	0.984
p Stage			
I	24	54	
II	3	9	(I versus II–IV)
III	7	4	
IV	1	1	0.224
Tumor size			
≤3.0 cm	21	40	
>3.0 cm	14	28	0.908
Lymph node metastasis			
Absent	25	57	
Present	10	11	0.139
Differentiation			
Well or moderate	19	53	
Poor	16	15	0.013
IASLC/ATS/ERS classification			
AIS	1	2	
MIA	0	6	
Lepidic	4	4	
Papillary	10	29	
Acinar	2	8	(AIS-MIA versus invasive ADC)
Micropapillary	2	3	
Solid	15	12	0.129
Vascular invasion			
Absent	12	31	
Present	23	33	0.174
Pleural invasion			
Absent	28	54	
Present	7	14	0.944
Adjuvant chemotherapy			
With	15	25	
Without	20	43	0.548

Abbreviations: ADC, adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; IASLC, International Association for the Study of Lung Cancer; ATS, American Thoracic Society; ERS, European Respiratory Society; ALDH1A1, aldehyde dehydrogenase 1A1.

plays a pivotal role as a common language between oncologists/pulmonologists, pathologists, radiologists, molecular biologists, and thoracic surgeons.

In conclusion, although this study is limited because the number of patients was relatively small, the expression of ALDH1A1 is an independent predictor of overall survival. Moreover, the frequency of ALDH1A1-positive ADCs that were papillary predominant was higher than for solid predominant, and AK1C1 expression was found to be significantly lower in papillary predominant ADCs than in noninvasive or solid predominant ADCs, suggesting that the comprehensive histologic subtyping approach in the IASLC/ATS/ERS classification provides new molecular biology insights regarding CSC theory.

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Disclosure

All authors report they have no conflicts of interest associated with this study.

References

1. Jemal A, Siegel R, Xu J, et al. Cancer statistics, 2010. *CA Cancer J Clin.* 2010;60:277–300.
2. Travis WD, Brambilla E, Noguchi M, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol.* 2011;6:244–285.
3. Warth A, Muley T, Meister M, et al. The novel histologic International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification system of lung adenocarcinoma is a stage-independent predictor of survival. *J Clin Oncol.* 2012;30:1438–1446.
4. Xu L, Tavora F, Battafarano R, et al. Adenocarcinomas with prominent lepidic spread: retrospective review applying new classification of the American Thoracic Society. *Am J Surg Pathol.* 2012;36:273–282.
5. Yoshizawa A, Motoi N, Riely GJ, et al. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases. *Mod Pathol.* 2011;24:653–664.
6. Yoshizawa A, Sumiyoshi S, Sonobe M, et al. Validation of the IASLC/ATS/ERS lung adenocarcinoma classification for prognosis and association with EGFR and KRAS gene mutations: analysis of 440 Japanese patients. *J Thorac Oncol.* 2013;8:52–61.
7. Russell PA, Wainer Z, Wright GM, et al. Does lung adenocarcinoma subtype predict patient survival? A clinicopathologic study based on the new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary lung adenocarcinoma classification. *J Thorac Oncol.* 2011;6:1496–1504.
8. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature.* 2001;414:105–111.
9. Cai J, Cheng A, Luo Y, et al. Membrane properties of rat embryonic multipotent neural stem cells. *J Neurochem.* 2004;88:212–226.

10. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell*. 2007;1:555–567.
11. Huang EH, Hynes MJ, Zhang T, et al. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res*. 2009;69:3382–3389.
12. Jiang F, Qiu Q, Khanna A, et al. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res*. 2009;7:330–338.
13. Dozmorov MG, Azzarello JT, Wren JD, et al. Elevated AKR1C3 expression promotes prostate cancer cell survival and prostate cell-mediated endothelial cell tube formation: implications for prostate cancer progression. *BMC Cancer*. 2010;10:672.
14. Seo DC, Sung JM, Cho HJ, et al. Gene expression profiling of cancer stem cell in human lung adenocarcinoma A549 cells. *Mol Cancer*. 2007;6:75.
15. Li X, Wan L, Geng J, et al. Aldehyde dehydrogenase 1A1 possesses stem-like properties and predicts lung cancer patient outcome. *J Thorac Oncol*. 2012;7:1235–1245.
16. Patel M, Lu L, Zander DS, et al. ALDH1A1 and ALDH3A1 expression in lung cancers: correlation with histologic type and potential precursors. *Lung Cancer*. 2008;59:340–349.
17. International Union Against Cancer. *TNM Classification of Malignant Tumours*. 7th ed. Oxford, UK: Wiley-Blackwell; 2009.
18. Hilton J. Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. *Cancer Res*. 1984;44:5156–5160.
19. Yoshida A, Rzhetsky A, Hsu LC, et al. Human aldehyde dehydrogenase gene family. *Eur J Biochem*. 1998;251:549–557.
20. Tanei T, Morimoto K, Shimazu K, et al. Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. *Clin Cancer Res*. 2009;15:4234–4241.
21. Lindahl R. Aldehyde dehydrogenases and their role in carcinogenesis. *Crit Rev Biochem Mol Biol*. 1992;27:283–335.
22. Storms RW, Trujillo AP, Springer JB, et al. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. *Proc Natl Acad Sci U S A*. 1999;96:9118–9123.
23. Kastan MB, Schlaffer E, Russo JE, et al. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood*. 1990;75:1947–1950.
24. Hess DA, Meyerrose TE, Wirthlin L, et al. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. *Blood*. 2004;104:1648–1655.
25. Armstrong L, Stojkovic M, Dimmick I, et al. Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity. *Stem Cells*. 2004;22:1142–1151.
26. Kahlert C, Bergmann F, Beck J, et al. Low expression of aldehyde dehydrogenase 1A1 (ALDH1A1) is a prognostic marker for poor survival in pancreatic cancer. *BMC Cancer*. 2011;11:275.
27. Chang B, Liu G, Xue F, et al. ALDH1 expression correlates with favorable prognosis in ovarian cancers. *Mod Pathol*. 2009;22:817–823.
28. Jez JM, Flynn TG, Penning TM. A new nomenclature for the aldo-keto reductase superfamily. *Biochem Pharmacol*. 1997;54:639–647.
29. Hara A, Matsuura K, Tamada Y, et al. Relationship of human liver dihydrodiol dehydrogenases to hepatic bile-acid-binding protein and an oxidoreductase of human colon cells. *Biochem J*. 1996;313(Pt 2):373–376.
30. Lin HK, Jez JM, Schlegel BP, et al. Expression and characterization of recombinant type 2 3 alpha-hydroxysteroid dehydrogenase (HSD) from human prostate: demonstration of bifunctional 3 alpha/17 beta-HSD activity and cellular distribution. *Mol Endocrinol*. 1997;11:1971–1984.
31. Khanna M, Qin KN, Wang RW, et al. Substrate specificity, gene structure, and tissue-specific distribution of multiple human 3 alpha-hydroxysteroid dehydrogenases. *J Biol Chem*. 1995;270:20162–20168.
32. Kadota K, Nitadori J, Sarkaria IS, et al. Thyroid transcription factor-1 expression is an independent predictor of recurrence and correlates with the IASLC/ATS/ERS histologic classification in patients with stage I lung adenocarcinoma. *Cancer*. 2013;119:931–938.

CHEST

Original Research

LUNG CANCER

Prognostic Factors and the Significance of Treatment After Recurrence in Completely Resected Stage I Non-small Cell Lung Cancer

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Objective: **The objective of this study was to identify the clinicopathologic factors influencing postrecurrence survival (PRS) in and the effect of postrecurrence therapy (PRT) on patients with completely resected stage I non-small cell lung cancer (NSCLC).**

Methods: **We reviewed the data of 919 patients in whom complete resection of stage I NSCLC had been performed.**

Results: **Of the 919 patients, 170 (18.5%) had recurrent disease. Initial PRT was performed in 118 patients (69.1%) (surgery in eight, chemotherapy in 79, radiotherapy in 10, and chemoradiotherapy in 21). On multivariate analyses, PRT (hazard ratio [HR], 0.542; 95% CI, 0.344-0.853; $P = .008$), female sex (HR, 0.487; 95% CI, 0.297-0.801; $P = .005$), and differentiation (HR, 1.810; 95% CI, 1.194-2.743; $P = .005$) demonstrated a statistically significant association with favorable PRS. Bone metastasis (HR, 3.288; 95% CI, 1.783-6.062; $P < .001$), liver metastasis (HR, 4.518; 95% CI, 1.793-11.379; $P = .001$), chemotherapy (HR, 0.478; 95% CI, 0.236-0.975; $P = .040$), epidermal growth factor receptor-tyrosine kinase inhibitors treatment (EGFR-TKIs) (HR, 0.460; 95% CI, 0.245-0.862; $P = .015$), and nonadenocarcinoma (HR, 2.136; 95% CI, 1.273-3.585; $P = .004$) were independently and significantly associated with PRS in the 118 patients who underwent any PRT. Subgroup analysis with a combination of these five PRS factors in the patients who underwent any PRT revealed median PRS times of 42.4 months for 20 patients lacking all five risk factors and 18.8 months for 98 patients with at least one of these risk factors ($P = .001$).**

Conclusions: **PRT, sex, and differentiation were independently associated with PRS. In the patients who underwent any PRT, PRS was related to EGFR-TKIs, chemotherapy, histology, and initial recurrence sites. One challenge for the future will be to create systematic treatment strategies for recurrent NSCLC according to the risk factor status of individual patients.**

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Abbreviations: EGFR-TKI = epidermal growth factor receptor-tyrosine kinase inhibitor; HR = hazard ratio; NSCLC = non-small cell lung cancer; PRS = postrecurrence survival; PRT = postrecurrence therapy; PS = performance status; RFP = recurrence-free proportion

Surgical resection with a curative intent is considered the standard of care for early stage non-small cell lung cancer (NSCLC), but > 20% of patients had recurrence, even in pathologic stage I cases.[1-6] Recurrence after complete resection for stages I to III of NSCLC ranges from 30% to 75%, and has been reported to depend on pathologic staging and follow-up period.[1,6-8] The majority of recurrences occur within the first 2 years,[1,6] although there are several studies showing late recurrences ≥ 5 years after resection.[9-11] Long-term, continuous follow-up is required to establish accurate recurrence rates and patterns.

Although several studies focusing on postrecurrence survival (PRS) of patients in stage I or stage I-III NSCLC have been reported,[2-4,8,12-14] no standard treatment strategy for recurrent disease based on prospective studies has been established. However, a standard treatment strategy is necessary because much longer

follow-up periods and robust protocols are required to evaluate PRS objectively. It is difficult to generalize about multifactorial patient backgrounds, which depend on disease, treatment, and performance status (PS) at recurrence. The prognostic factors predicting PRS or the appropriate treatment are still controversial.

Encouraging new treatments (including epidermal growth factor receptor-tyrosine kinase inhibitors [EGFR-TKIs], anaplastic lymphoma kinase inhibitors, pemetrexed, and bevacizumab) have afforded benefits to certain patients with advanced or recurrent NSCLC.[15-21] Advances in postrecurrence therapy (PRT) may provide improvement in overall survival among the patients who undergo surgery. The objective of the present study was to identify the clinicopathologic factors influencing PRS and their effect of PRT on stage I NSCLC.

Materials and Methods

From January 1990 through December 2007, 1,214 patients underwent complete resection for pathologic stage I NSCLC at our hospital. Complete resection was defined as demonstrating cancer-free surgical margins, both grossly and histologically. All patients underwent radical, anatomic, lobar resection and systematic, mediastinal lymph node dissection. The following exclusion criteria were applied: preoperative chemotherapy, radiation therapy, or both (n = 38); low-grade malignant tumors, including carcinoids, mucoepidermoid carcinomas, or adenoid cystic carcinomas (n = 20); and death within 30 days of operation (n = 9). Of the remaining 1,147 patients, complete follow-up was available for 919 patients, who composed the subjects of this study.

Preoperative evaluation included physical examination, chest radiography, CT scan of the chest and abdomen, bone scintigraphy, blood examination, and, since the early 2000s, PET scan (recently integrated PET-CT scan). Histologic subtypes of lung cancer were determined according to the World Health Organization classification,[22] and disease stage was determined in accordance with the *TNM Classification for Lung and Pleural Tumours*, 7th ed.[23]

The follow-up schedule consisted of a clinic visit every 3 months in the first year after resection, every 6 months from the second to the fifth year, and annually thereafter on an outpatient basis, and aimed at continuing follow-up for 10 years after resection. Follow-up procedures included physical examination, chest radiography, and blood examination (including serum tumor markers). CT scans of the chest and abdomen was performed every 6 months in the first 2 years, and annually from the third to the fifth year. Whenever any symptoms or signs of recurrence were detected, MRI of the brain and bone scintigraphy were performed.

Recurrences were diagnosed by physical examination and diagnostic imaging. Histologic or cytologic confirmation of the recurrence was made when clinically feasible. Local recurrence was defined as disease recurrence at the surgical margin, ipsilateral hemithorax, or mediastinum. Radiographic lymph node recurrence was defined as enlarged lymph nodes measuring > 1 cm on the short axis by CT scan and/or hypermetabolic lymph nodes on PET-CT scans. Pathologic confirmation of recurrence was made by endobronchial ultrasound-guided transbronchial needle aspiration of enlarged lymph nodes during follow-up. Distant metastasis was defined as disease recurrence in the contralateral lung or outside the hemithorax and mediastinum. A second primary tumor was recorded when a patient presented with a new histologic type, and with clinical features consistent with a new primary tumor. Data collected from our department database of patients, telephone interviews, and correspondence from outside sources during the follow-up periods were included.

Clinical characteristics were retrieved from available clinical records. The following clinicopathologic factors were assessed in the PRS analysis: age, sex, smoking status, primary tumor status (T1 vs T2), tumor size (0-30 mm vs > 30 mm), tumor differentiation (well/moderate vs poor), pathologic vascular invasion, pleural invasion, histology (adenocarcinoma vs others), and extent of resection (single lobe lobectomy vs more extensive resection, namely bilobectomy/pneumonectomy).

Length of the recurrence-free period was calculated in months from date of resection to date of initial recurrence or last follow-up showing no recurrence. To calculate the recurrence-free proportion (RFP), patients who died without recognized recurrence or who were known to have no recurrence at the date of last contact were censored. Length of PRS was measured from date of initial recurrence to date of death from any cause or date on which the patient was last known to be alive. PRS and RFP curves were plotted using the Kaplan-Meier method, and differences in variables were determined using the log-rank test or the Breslow tests. Categorical comparison was performed using the χ^2 test for discrete data and Student *t* test for continuous data. Multivariate analyses were performed using the Cox proportional hazards regression model. A backward stepwise selection procedure was implemented. All tests were two-sided, and *P* values < 0.05 were considered to indicate a statistically significant difference. Statview 5.0 software (SAS Institute Inc) was used for statistical analyses.

Data collection and analyses were approved, and the need to obtain written informed consent from each patient was waived, by the institutional review board at Tokyo Medical University (No. 2133).

Results

Median follow-up time for survivors was 62.0 months (range: 1.4-247.6 months). The RFP was 82.2% at 5 years after operation. Of the 919 patients, 170 (18.5%) had recurrent disease, with a median age of 66 years at the time of initial recurrence. Median PRS time for these patients was 17.6 months (range: 0.4-103.0 months). The 1- and 2-year PRS proportions were 73.5% and 51.4%, respectively (Fig 1).

Table 1 shows 5-year RFPs and univariate/multivariate analyses of recurrence according to clinicopathologic characteristics of patients with stage I NSCLC. Univariate analysis identified five significant risk factors:

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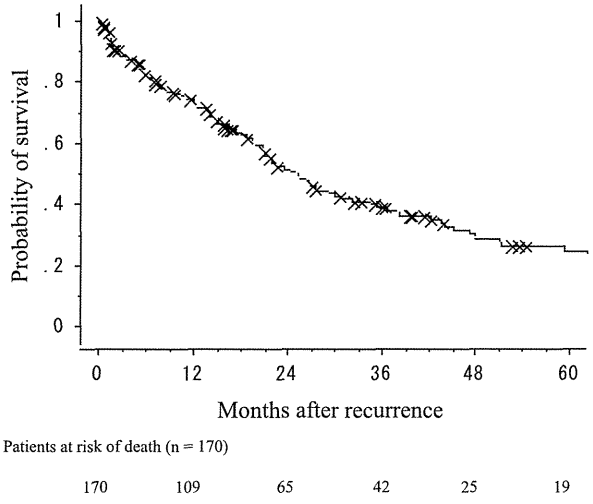

FIGURE 1. Postrecurrence survival curve of patients with non-small cell lung cancer recurrence.

male sex, pathologic vascular invasion, pleural invasion, poorly differentiated carcinoma, and nonadenocarcinoma. Multivariate analysis demonstrated that pathologic vascular invasion (hazard ratio [HR], 2.306; 95% CI, 1.621-3.280; $P < .001$), pleural invasion (HR, 1.489; 95% CI, 1.048-2.115; $P = .026$), and poorly differentiated carcinoma (HR, 1.842; 95% CI, 1.328-2.555; $P < .001$) were statistically significant predictors of recurrence.

Initial recurrence sites and PRT are shown in Table 2. Type of recurrence included only local recurrence in 43 patients (25.3%), distant in 113 (66.5%), and both in 14 (8.2%). Most commonly involved organs were the lung (the site of recurrence in 66 patients: ipsilateral in 23, contralateral/bilateral in 43), followed by regional lymph nodes in 37, brain in 30, bone in 21, and liver in 16. Initial PRT was performed in 118 patients (69.4%), and included surgery for 8, chemotherapy for 79, radiotherapy for 10, and chemoradiotherapy for 21. Surgical resections (n = 8) were performed in three patients with solitary pulmonary metastasis, three with solitary brain metastasis, one with adrenal gland metastasis, and one with chest wall and axillary lymph node involvement. Forty-one patients (24.1%) had no treatment for recurrent disease. Of the 118 patients who underwent any PRT, 66 (55.9%) underwent second-line or subsequent therapy, including

Table 1—*Patient Characteristics and Univariate and Multivariate Analyses of Recurrence*

	Univariate Analysis			Multivariate Analysis		
Factors	Patients, No.	5-y RFP, %	*P* Value	HR	95% CI	*P* Value
Age,[a] y						
<65	439	84.1	...	...	...	...
≥65	480	80.4	.129	...	...	...
Sex						
Male	542	78.0	...	...	...	...
Female	377	87.8	<.001	...	...	...
Smoking status						
Never smoker	347	85.2	.134	...	...	...
Ever smoker	572	80.2	...	...	...	...
T category						
T1	512	84.7	...	...	...	...
T2	407	78.9	.100	...	...	...
Tumor size						
0-30 mm	663	84.0	...	...	...	...
>30 mm	256	81.5	.112	...	...	...
Pathologic vascular invasion						
Absent	481	91.0	...	1	...	...
Present	421	72.1	<.001	2.306	1.621-3.280	<.001
Pleural invasion						
Absent	719	84.9	...	1	...	...
Present	191	71.8	<.001	1.489	1.048-2.115	.026
Histology						
Adenocarcinoma	706	83.8	...	...	...	...
Nonadenocarcinoma	213	76.3	.039	...	...	...
Differentiation						
Well or moderate	656	86.7	...	1	...	...
Poor	216	67.7	<.001	1.842	1.328-2.555	<.001
Type of surgery						
Single lobectomy	873	81.9	...	...	...	...
Bilobectomy or pneumonectomy	46	87.2	.942	...	...	...

HR = hazard ratio; RFP = recurrence-free proportion.
[a]Median age = 65 y.

Table 2—*Initial Recurrence Site and Postrecurrence Therapy*

Data of Recurrence Sites and Postrecurrence Therapies	Patients, No.
Overall	170
Type of recurrence	
Distant	113
Local	43
Both	14
Initial recurrence site	
Ipsilateral lung	23
Contralateral/bilateral lung	43
Regional lymph nodes	37
Malignant effusion/dissemination	13
Stump	9
Brain	30
Bone	21
Liver	16
Adrenal gland	10
Others	14
Postrecurrence therapy	
Initial therapy	
Surgery	8 (lung, 3; brain, 3; adrenal gland, 1; lymph nodes, 1)
Surgery alone	6
Surgery + chemotherapy	3
Chemotherapy	79
Radiation therapy	10
Chemoradiotherapy	21
None	41
Unknown	11
Second-line or the subsequent therapy	66
Chemotherapy	58
EGFR-TKIs	27 (gefitinib, 22; erlotinib, 3; both, 2)
EGFR mutation status/histology	Positive 12 (Ad, 11; Sq, 1) Wild 4 (Ad, 3; LCC, 1) Unknown 11 (Ad, 10; LCC, 1)
Others	7

Ad = adenocarcinoma; EGFR-TKI = epidermal growth factor receptor-tyrosine kinase inhibitor; LCC = large cell carcinoma; Sq = squamous cell carcinoma.

chemotherapy for 58, and EGFR-TKIs for 27 (gefitinib, 22 patients; erlotinib, three patients; and both, two patients). Among the latter 27 patients, *EGFR* mutations were detected in 12; four had wild-type *EGFR*.

Table 3 shows univariate/multivariate analyses of PRS. Univariate analysis identified six significant risk factors for PRS: male sex, smoking, poorly differentiated carcinoma, nonadenocarcinoma, no PRT, and shorter recurrence-free interval (≤24 months; median recurrence-free period was 24 months). Multivariate analysis demonstrated that PRT (HR, 0.542; 95% CI, 0.344-0.853; $P = .008$), female sex (HR, 0.487; 95% CI, 0.297-0.801; $P = .005$), and differentiation (HR, 1.810; 95% CI 1.194-2.743; $P = .005$) had a statistically significant association with favorable PRS.

The results of multivariate analysis of PRS determined that PRT had a strong impact on PRS. Therefore, we further examined PRS in the 118 patients who underwent any PRT (Table 4). Univariate analysis identified nine significant risk factors for PRS: male sex, smoking, poorly differentiated carcinoma, bone metastasis, liver metastasis, no chemotherapy or EGFR-TKI, no second-line therapy, and multiple organ metastases. Multivariate analysis demonstrated that bone metastasis (HR, 3.288; 95% CI, 1.783-6.062; $P < .001$), liver metastasis (HR, 4.518; 95% CI, 1.793-11.379; $P = .001$), chemotherapy (HR, 0.478; 95% CI, 0.236-0.975; $P = .040$), EGFR-TKI therapy (HR, 0.460; 95% CI, 0.245-0.862; $P = .015$), and nonadenocarcinoma (HR, 2.136; 95% CI, 1.273-3.585; $P = .004$) had a statistically significant association with PRS.

Subgroup analysis with a combination of these five PRS factors (no EGFR-TKI and chemotherapy, presence of liver or bone metastasis, nonadenocarcinoma) in patients with recurrence who underwent any PRT revealed median PRS times of 42.4 months for 20 patients lacking all five unfavorable factors and 18.8 months for 98 patients with one of these risk factors, respectively (Fig 2). The difference in PRS was statistically significant between the two groups ($P = .001$).

Discussion

We set out to identify clinicopathologic factors influencing PRS of patients with stage I NSCLC. Although curative surgical resection is the most effective therapy for stage I NSCLC, a considerable number of patients will develop recurrence. In the current study, overall incidence of recurrence was 18.5%, and median PRS time was 17.6 months. Initial location of recurrence was at a distant site in 74.7%, and the proportions of recurrences within 2 or 3 years after surgery were 48.2% and 66.5%, respectively (unpublished data). Previous studies have reported that the incidence of recurrence in patients with stage I NSCLC was 14% to 36%, with the 1-year survival rate ranging from 30% to 68% (Table 5).[1-6,8,24]

We examined risk factors for recurrence in stage I NSCLC, and identified three: pathologic vascular invasion, pleural invasion, and poorly differentiated carcinoma. These standard pathologic factors have also been reported to be good predictors of overall survival for patients with stage I NSCLC.[25-36] In our study, univariate analysis for PRS identified six significant risk factors (male sex, smoking, poorly differentiated carcinoma, nonadenocarcinoma, no PRT, and shorter recurrence-free interval [≤24 months]), while multivariate analysis revealed that sex, PRT, and differentiation were independent prognostic factors. Only differentiation was a significant predictor of recurrence and poor PRS, and pathologic vascular invasion and pleural invasion had no significant impact on PRS. PRS may be associated

Table 3—*PRS Analyses*

Factors	Univariate Analysis			Multivariate Analysis		
	Patients, No.	Median PRS, mo	*P* Value	HR	95% CI	*P* Value
Age at recurrence,[a] y						
<66	76	18.9	...	...	...	...
≥66	94	15.8	.242	...	...	...
Sex						
Male	118	15.5	...	1	...	...
Female	52	25.6	<.001	0.487	0.297-0.801	.005
Smoking status						
Never smoker	59	25.0	...	...	...	...
Ever smoker	111	14.1	.006	...	...	...
T category						
T1	87	15.8	...	...	...	...
T2	83	19.6	.476	...	...	...
Tumor size						
0-30 mm	132	16.9	...	...	...	...
>30 mm	38	20.9	.632	...	...	...
Pathologic vascular invasion						
Absent	53	15.8	...	...	...	...
Present	113	17.0	.088	...	...	...
Pleural invasion						
Absent	115	15.8	...	...	...	...
Present	53	18.8	.393	...	...	...
Histology						
Adenocarcinoma	124	20.9	...	...	...	...
Nonadenocarcinoma	46	12.4	<.001	...	...	...
Differentiation						
Well or moderate	97	20.8	...	1	...	...
Poor	65	14.1	.002	1.810	1.194-2.743	.005
Type of surgery						
Single lobectomy	162	17.3	.152	...	...	...
Bilobectomy or pneumonectomy	8	19.5	...	...	...	...
Adjuvant therapy						
Without	134	15.9	.547	...	...	...
With	36	21.0	...	...	...	...
Postrecurrence therapy						
Without	41	7.2	...	1	...	...
With	118	21.4	.021	0.542	0.344-0.853	.008
Recurrence free interval						
≤24 mo	82	16.2	...	...	...	...
>24 mo	88	18.4	.021	...	...	...
Type of recurrence						
Distant	127	15.8	...	...	...	...
Local only	43	18.8	.087	...	...	...
Number of recurrent sites						
Single	132	16.8	...	...	...	...
Multiple	38	18.6	.305	...	...	...

PRS = postrecurrence survival. See Table 1 legend for expansion of other abbreviations.
[a]Median age at recurrence = 66 y.

with recurrent disease characteristics, including the recurrence site, PRT, recurrence-free interval, or PS at time of recurrence, rather than with the biologically aggressive characteristics of lung cancer.

Previous studies have demonstrated the survival benefit of PRT in patients with stage I NSCLC. Nakagawa et al[4] and Hung et al[2,3] demonstrated that patients with stage I NSCLC treated either surgically or nonsurgically had a significantly better PRS than those with supportive care alone. In our study, PRT provided a more favorable PRS than that of no treatment, similarly to previous reports. However, the results of PRS in the patients who underwent any PRT showed that surgical resection was not related to a favorable outcome. This may have been because the number of patients who received surgery for recurrent disease was too small to provide any supportive data in terms of survival benefit. However, in cases of surgical resection for recurrent lung metastasis, objective evidence supporting the role of surgery is limited because it

Table 4—*PRS Analyses in 118 Patients Who Underwent Postrecurrence Therapy*

Factors	Univariate Analysis			Multivariate Analysis		
	Patients, No.	Median PRS, mo	*P* Value	HR	95% CI	*P* Value
Age at recurrence, y						
<66	63	22.4	...	...	...	...
≥66	55	19.5	.151	...	...	...
Sex						
Male	79	20.0	...	...	...	...
Female	39	27.2	.002	...	...	...
Smoking status						
Never smoker	43	27.6	...	...	...	...
Ever smoker	75	17.6	.035	...	...	...
Histology						
Adenocarcinoma	84	24.4	...	1	...	...
Nonadenocarcinoma	34	13.9	<.001	2.136	1.273-3.585	.004
Differentiation						
Well or moderate	66	23.1	...	...	...	...
Poor	46	18.8	.019	...	...	...
Lung metastasis						
Absent	68	19.8	...	...	...	...
Present	49	21.4	.053	...	...	...
Brain metastasis						
Absent	96	19.6	...	...	...	...
Present	21	22.6	.584	...	...	...
Bone metastasis						
Absent	100	21.9	...	1	...	...
Present	17	15.8	.001	3.288	1.783-6.062	<.001
Liver metastasis						
Absent	110	21.9	...	1	...	...
Present	7	10.5	.001	4.518	1.793-11.379	.001
Chemotherapy						
Without	15	9.6	...	1	...	...
With	103	22.7	.009	0.478	0.236-0.975	.040
Surgical resection						
Without	110	20.8	...	...	...	...
With	8	33.7	.209	...	...	...
EGFR-TKI therapy						
Without	91	17.0	...	1	...	...
With	27	41.4	.002	0.460	0.245-0.862	.015
Second line therapy						
Without	52	14.0	...	...	...	...
With	66	27.2	.004	...	...	...
Recurrence free interval						
≤24 mo	59	17.0	...	...	...	...
>24 mo	59	22.4	.394	...	...	...
Type of recurrence						
Distant	85	20.8	...	...	...	...
Local only	33	21.8	.086	...	...	...
Number of recurrent sites						
Single	89	21.0	...	...	...	...
Multiple	29	20.8	.049	...	...	...

See Table 1 and 3 legends for expansion of abbreviations.

may be difficult to distinguish second primary tumors from recurrent pulmonary metastasis. Advances in genomic analysis, molecular biologic tools, or diagnostic imaging may enable more accurate diagnosis of a solitary pulmonary lesion.

Among the cohort of 118 patients with any PRT, we identified five independent favorable prognostic factors of PRS by multivariate analysis: the absence of bone or liver metastasis, chemotherapy, EGFR-TKI therapy, and nonadenocarcinoma. Moreover, the result of the study showed an important aspect of a prognostic-factor based risk stratification. Median PRS times were 42.4 months for the patients lacking all five factors and 18.8 months for the patients with one of these risk factors ($P = .001$).

Some authors have found that the site of initial recurrence was a prognostic factor for PRS, which agrees with the current study. Yoshino et al[8] demonstrated

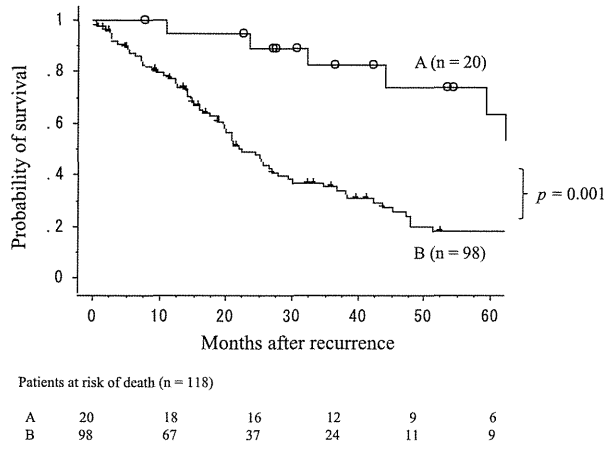

FIGURE 2. Postrecurrence survival curves of the patients lacking (A) all five unfavorable factors (not receiving epidermal growth factor receptor-tyrosine kinase inhibitor therapy and chemotherapy, liver or bone metastasis positive, nonadenocarcinoma), and (B) the patients with one of the five risk factors.

that bone metastasis was a marginally prognostic factor for PRS in patients with stage I-III NSCLC at the first resection. Assessment of bone metastatic type, osteoblastic or osteolytic, may be important as a part of postrecurrence therapeutic strategy because it has been noted that osteoblastic tumors lead to both a better prognosis and activating *EGFR* mutation presence.[37]

Major advances in NSCLC management have resulted from the understanding of molecular biology, development of molecule-targeting agents, and identification of biomarkers for targeted treatment. Since 2002, gefitinib has been used in Japan for the treatment of inoperable or recurrent NSCLC, and we started to administer it around the same period. It is now felt that EGFR-TKIs can improve the survival of some previously treated and untreated patients with advanced NSCLC, with the overall benefit being driven primarily by the subgroup with *EGFR* mutations.[15-17,38,39] EGFR-TKIs have also improved endurance and health-related quality of life compared with platinum-based doublet chemotherapy.[15-17] EGFR-TKIs are, therefore, good candidates for first-line PRT in patients who have had resected adenocarcinoma with distant metastases, but only in those with *EGFR* mutations.

There are several limitations in the present study. This study is retrospective, and bias may exist. First, patient-selection bias regarding PRT was unavoidable. Curative intent therapy or systematic treatment is difficult to perform in patients with poor PS. In the current study, PS or comorbidities at the time of recurrence were not accurately evaluated. Second, distinguishing second primary tumors from recurrent pulmonary metastasis was difficult. Even if a pathologic specimen was obtained, definitive diagnosis could be difficult under the current morphology-based diagnostic criteria. Third, complete follow-up was not available for all eligible patients.

There are presently no clinical guidelines for PRT regarding resected NSCLC based on large-scale prospective studies. Molecularly targeted therapy, chemotherapeutic regimens, and surgical strategies have evolved substantially over the decades. A challenge for the future will be to create systematic treatment strategies for recurrent NSCLC according to the individual patient's recurrent-disease characteristics, including the initial recurrence site, age, sex, PS, or recurrence-free interval, and original tumor characteristics.

CONCLUSION

This study showed that male sex, the absence of PRT, and poorly differentiated carcinoma were independent unfavorable prognostic factors of PRS in patients

Table 5—*PRS of Patients With Stage I Non-small Cell Lung Cancer in Previous Series*

Series/Year	Patients, No.	Incidence of Recurrence, No. (%)	PRS, % (y)	Type of Recurrence	Independent Favorable Factors of PRS
Martini et al[6]/1995	598	159 (26.6)	NR	L/D	NR
al-Kattan et al[1]/1997	123	36 (29.3)	NR	L/D	NR
Nakagawa et al[4]/2008	397	87 (21.9)	67.7 (1)	L/D	Symptom at recurrence, negative
			34.4 (3)		Cervicomediastinum metastases, negative
					Liver metastases, negative
					PRT (surgery/nonsurgery)
Hung et al[2]/2009	933	74 (7.9)	48.7 (1)	L	PRT (surgery, chemotherapy, and/or radiotherapy)
			17.6 (2)		
Hung et al[3]/2010	933	166 (17.8)	30.2 (1)	D	Disease-free interval > 16 mo
			15.1 (2)		PRT
Current series/2013	919	170 (18.5)	73.5 (1)	L/D	PRT
			51.4 (2)		Female sex

D = distant recurrence; L = local recurrence, NR = not reported; PRT = postrecurrence therapy. See Table 3 legend for expansion of other abbreviation.

with resected stage I NSCLC. Moreover, in patients who underwent any PRT, who were receiving EGFR-TKIs and chemotherapy, and with absence of liver or bone metastasis, and with nonadenocarcinoma had a statistically significant association with favorable PRS. Further clinical studies may give more accurate information about the benefits of PRT for survival and lead to the improvement of clinical assessment and therapeutic strategies in recurrent NSCLC.

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Author contributions: Dr Shimada had full access to all of the data in the study. Dr Saji takes responsibility for the accuracy of the data analysis and Dr Ikeda takes responsibility for the integrity of the data.
Dr Shimada: contributed to the design and coordination of the study, prepared the manuscript, read and approved the final manuscript, and served as principal author.
Dr Saji: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Yoshida: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Kakihana: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Honda: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Nomura: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Usuda: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Kajiwara: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Ohira: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Ikeda: contributed to the design and coordination of the study, revised the article for important intellectual content, and read and approved the final manuscript.
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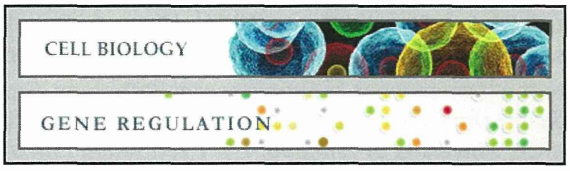
References

1. al-Kattan K, Sepsas E, Fountain SW, Townsend ER. Disease recurrence after resection for stage I lung cancer. *Eur J Cardiothorac Surg*. 1997;12(3):380-384.
2. Hung JJ, Hsu WH, Hsieh CC, et al. Post-recurrence survival in completely resected stage I non-small cell lung cancer with local recurrence. *Thorax*. 2009;64(3):192-196.
3. Hung JJ, Jeng WJ, Hsu WH, et al. Prognostic factors of post-recurrence survival in completely resected stage I non-small cell lung cancer with distant metastasis. *Thorax*. 2010;65(3):241-245.
4. Nakagawa T, Okumura N, Ohata K, Igai H, Matsuoka T, Kameyama K. Postrecurrence survival in patients with stage I non-small cell lung cancer. *Eur J Cardiothorac Surg*. 2008;34(3):499-504.
5. Harpole DH Jr, Herndon JE II, Young WG Jr, Wolfe WG, Sabiston DC Jr. Stage I nonsmall cell lung cancer. A multivariate analysis of treatment methods and patterns of recurrence. *Cancer*. 1995;76(5):787-796.
6. Martini N, Bains MS, Burt ME, et al. Incidence of local recurrence and second primary tumors in resected stage I lung cancer. *J Thorac Cardiovasc Surg*. 1995;109(1):120-129.
7. Martin J, Ginsberg RJ, Venkatraman ES, et al. Long-term results of combined-modality therapy in resectable non-small-cell lung cancer. *J Clin Oncol*. 2002;20(8):1989-1995.
8. Yoshino I, Yohena T, Kitajima M, et al. Survival of non-small cell lung cancer patients with postoperative recurrence at distant organs. *Ann Thorac Cardiovasc Surg*. 2001;7(4):204-209.
9. Maeda R, Yoshida J, Hishida T, et al. Late recurrence of non-small cell lung cancer more than 5 years after complete resection: incidence and clinical implications in patient follow-up. *Chest*. 2010;138(1):145-150.
10. Martini N, Rusch VW, Bains MS, et al. Factors influencing ten-year survival in resected stages I to IIIa non-small cell lung cancer. *J Thorac Cardiovasc Surg*. 1999;117(1):32-36.
11. Okada M, Nishio W, Sakamoto T, Harada H, Uchino K, Tsubota N. Long-term survival and prognostic factors of five-year survivors with complete resection of non-small cell lung carcinoma. *J Thorac Cardiovasc Surg*. 2003;126(2):558-562.
12. Endo C, Sakurada A, Notsuda H, et al. Results of long-term follow-up of patients with completely resected non-small cell lung cancer. *Ann Thorac Surg*. 2012;93(4):1061-1068.
13. Sugimura H, Nichols FC, Yang P, et al. Survival after recurrent nonsmall-cell lung cancer after complete pulmonary resection. *Ann Thorac Surg* 2007;83(2):409-417.
14. Williams BA, Sugimura H, Endo C, et al. Predicting post-recurrence survival among completely resected nonsmall-cell lung cancer patients. *Ann Thorac Surg*. 2006;81(3):1021-1027.
15. Maemondo M, Inoue A, Kobayashi K, et al; North-East Japan Study Group. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med*. 2010;362(25):2380-2388.
16. Mitsudomi T, Morita S, Yatabe Y, et al; West Japan Oncology Group. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol*. 2010;11(2):121-128.
17. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med*. 2009;361(10):947-957.
18. Paz-Ares L, de Marinis F, Dediu M, et al. Maintenance therapy with pemetrexed plus best supportive care versus placebo plus best supportive care after induction therapy with pemetrexed plus cisplatin for advanced non-squamous non-small-cell lung cancer (PARAMOUNT): a double-blind, phase 3, randomised controlled trial. *Lancet Oncol*. 2012;13(3):247-255.
19. Reck M, von Pawel J, Zatloukal P, et al; BO17704 Study Group. Overall survival with cisplatin-gemcitabine and bevacizumab or placebo as first-line therapy for nonsquamous non-small-cell lung cancer: results from a randomised phase III trial (AVAiL). *Ann Oncol*. 2010;21(9):1804-1809.
20. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med*. 2006;355(24):2542-2550.
21. Scagliotti GV, Parikh P, von Pawel J, et al. Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer. *J Clin Oncol*. 2008;26(21):3543-3551.
22. Travis WD, Brambilla E, Muller-Hermelink HK, et al. *World Health Organization Classification of Tumours: Pathology and Genetics of Tumors of the Lung, Pleura, Thymus and Heart*. Lyon, France: IARC Press; 2004.

23. International Union Against Cancer. *TNM Classification of Malignant Tumours*. 7th ed. Oxford, England: Wiley-Blackwell; 2009.
24 Jones DR, Daniel TM, Denlinger CE, et al. Stage IB nonsmall cell lung cancers: are they all the same? *Ann Thorac Surg*. 2006;81(6):1958-1962.
25. Bréchot JM, Chevret S, Charpentier MC, et al. Blood vessel and lymphatic vessel invasion in resected nonsmall cell lung carcinoma. Correlation with TNM stage and disease free and overall survival. *Cancer*. 1996;78(10):2111-2118.
26. Ichinose Y, Yano T, Asoh H, Yokoyama H, Yoshino I, Katsuda Y. Prognostic factors obtained by a pathologic examination in completely resected non-small-cell lung cancer. An analysis in each pathologic stage. *J Thorac Cardiovasc Surg*. 1995; 110(3):601-605.
27. Kobayashi N, Toyooka S, Soh J, et al. Risk factors for recurrence and unfavorable prognosis in patients with stage I non-small cell lung cancer and a tumor diameter of 20 mm or less. *J Thorac Oncol*. 2007;2(9):808-812.
28. Maeda R, Yoshida J, Ishii G, et al. Long-term survival and risk factors for recurrence in stage I non-small cell lung cancer patients with tumors up to 3 cm in maximum dimension. *Chest*. 2010;138(2):357-362.
29. Maeda R, Yoshida J, Ishii G, Hishida T, Nishimura M, Nagai K. Prognostic impact of intratumoral vascular invasion in non-small cell lung cancer patients. *Thorax*. 2010;65(12): 1092-1098.
30. Maeda R, Yoshida J, Ishii G, et al. Poor prognostic factors in patients with stage IB non-small cell lung cancer according to the seventh edition TNM classification. *Chest*. 2011; 139(4):855-861
31. Miyoshi K, Moriyama S, Kunitomo T, Nawa S. Prognostic impact of intratumoral vessel invasion in completely resected pathologic stage I non-small cell lung cancer. *J Thorac Cardiovasc Surg*. 2009;137(2):429-434.
32. Ruffini E, Asioli S, Filosso PL, et al. Significance of the presence of microscopic vascular invasion after complete resection of Stage I-II pT1-T2N0 non-small cell lung cancer and its relation with T-Size categories: did the 2009 7th edition of the TNM staging system miss something? *J Thorac Oncol* 2011;6(2):319-326.
33. Shimada Y, Ishii G, Hishida T, Yoshida J, Nishimura M, Nagai K. Extratumoral vascular invasion is a significant prognostic indicator and a predicting factor of distant metastasis in non-small cell lung cancer. *J Thorac Oncol*. 2010;5(7):970-975.
34. Shimizu K, Yoshida J, Nagai K, et al. Visceral pleural invasion is an invasive and aggressive indicator of non-small cell lung cancer. *J Thorac Cardiovasc Surg*. 2005;130(1):160-165.
35. Shimizu K, Yoshida J, Nagai K, et al. Visceral pleural invasion classification in non-small cell lung cancer: a proposal on the basis of outcome assessment. *J Thorac Cardiovasc Surg*. 2004;127(6):1574-1578.
36. Tsuchiya T, Akamine S, Muraoka M, et al. Stage IA non-small cell lung cancer: vessel invasion is a poor prognostic factor and a new target of adjuvant chemotherapy. *Lung Cancer*. 2007;56(3):341-348.
37. Garfield D, Normanno N, Cadranel J. Prognostic factor for non-small cell lung cancer with bone metastases at the time of diagnosis. *Lung Cancer*. 2012;78(2):168.
38. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med*. 2004;350(21):2129-2139.
39. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*. 2004;304(5676):1497-1500.

Cell Biology:

Transforming Growth Factor-β Induces Transcription Factors MafK and Bach1 to Suppress Expression of the Heme Oxygenase-1 Gene



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Transforming Growth Factor-β Induces Transcription Factors MafK and Bach1 to Suppress Expression of the Heme Oxygenase-1 Gene*[S]

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Background: TGF-β suppresses early carcinogenesis but accelerates malignant progression by activating invasion and metastasis. *HO-1* is induced in response to oxidative stress and protects cells from oxidative injury.
Results: TGF-β suppresses *t*BHQ-inducible expression of *HO-1* through induction of MafK and Bach1.
Conclusion: TGF-β suppresses a protective response to electrophiles.
Significance: This work provides the first evidence that TGF-β affects electrophilic responses.

Transforming growth factor-β (TGF-β) has multiple functions in embryogenesis, adult homeostasis, tissue repair, and development of cancer. Here, we report that TGF-β suppresses the transcriptional activation of the heme oxygenase-1 (*HO-1*) gene, which is implicated in protection against oxidative injury and lung carcinogenesis. *HO-1* is a target of the oxidative stress-responsive transcription factor Nrf2. TGF-β did not affect the stabilization or nuclear accumulation of Nrf2 after stimulation with electrophiles. Instead, TGF-β induced expression of transcription factors MafK and Bach1. Enhanced expression of either MafK or Bach1 was enough to suppress the electrophile-inducible expression of *HO-1* even in the presence of accumulated Nrf2 in the nucleus. Knockdown of MafK and Bach1 by siRNA abolished TGF-β-dependent suppression of *HO-1*. Furthermore, chromatin immunoprecipitation assays revealed that Nrf2 substitutes for Bach1 at the antioxidant response elements (E1 and E2), which are responsible for the induction of *HO-1* in response to oxidative stress. On the other hand, pretreatment with TGF-β suppressed binding of Nrf2 to both E1 and E2 but marginally increased the binding of MafK to E2 together with Smads. As TGF-β is activated after tissue injury and in the process of cancer development, these findings suggest a novel mechanism by which damaged tissue becomes vulnerable to oxidative stress and xenobiotics.

Transforming growth factor-β (TGF-β) regulates multiple biological functions such as cell proliferation, differentiation, apoptosis, and morphogenesis (1, 2). Upon ligand binding, type II serine/threonine kinase receptors activate type I receptors, and activated type I receptors phosphorylate Smad proteins. Phosphorylated receptor-regulated Smads (R-Smads; Smad2 and Smad3) form heteromeric complexes with common partner Smad (Co-Smad; Smad4) and accumulate in the nucleus. Activated Smad complexes regulate expression of target genes by binding to specific DNA sequences together with various cobinding transcription factors and recruiting coactivators or corepressors (3). Differential expression of cobinding transcription factors contributes to the cell type- and context-dependent cellular responses to TGF-β.

TGF-β is a potent inhibitor of epithelial cell proliferation; therefore, it acts as a tumor suppressor in the early stages of carcinogenesis. On the other hand, cancer cells develop resistance to TGF-β-inducible growth inhibition in the advanced stages of carcinogenesis. At these later stages, TGF-β promotes epithelial-mesenchymal transition, invasion, and metastasis in certain types of cancer cells without TGF-β receptor abnormalities (4). Therefore, TGF-β is thought to act as a double-edged sword in cancer development (5). However, the multifunctional effects of TGF-β on cancer initiation and progression have not been fully elucidated.

Detoxification and export of xenobiotics are crucial for the maintenance of cellular homeostasis and protection against carcinogenic agents (6). Nrf2, a member of the cap 'n' collar family of basic region leucine zipper transcription factors (7), is a key transcriptional regulator of detoxification enzymes, transporters, and antioxidative molecules. Nrf2 forms heterodimers with small Maf proteins (MafF, MafG, and MafK), binds to antioxidant response elements

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⌘ *Author's Choice*—Final version full access.
[S] This article contains supplemental Figs. 1 and 2.
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(AREs),[2] and activates transcription of target genes. Mice lacking Nrf2 fail to induce phase II detoxifying enzymes and antioxidative molecules in response to oxidative stress, indicating that Nrf2 has a critical role in cellular defense against xenobiotics and oxidative stress (8).

ARE-mediated transcriptional activities are regulated by the combinations and relative levels of cap 'n' collar molecules and small Maf proteins. The Nrf2-small Maf heterodimer is essential for the activation of ARE-mediated transcription. On the other hand, small Maf homodimers have been reported to suppress ARE-mediated transcription (7). Other cap 'n' collar molecules, including Bach1, form heterodimers with small Maf proteins and suppress ARE-mediated transcription (9). For example, the MafK-Bach1 heterodimer interacts with AREs in the enhancer region of the heme oxygenase-1 (*HO-1*) gene and suppresses its transcription. Heme, an inducer of *HO-1*, displaces Bach1 from the AREs, which is followed by binding of Nrf2 to the AREs, and increases in *HO-1* expression (10–12). HO-1 catalyzes the rate-limiting step in heme catabolism and generates carbon monoxide, ferric iron, and biliverdin. Carbon monoxide and ferric iron can activate Nrf2, suggesting that HO-1 acts as a cytoprotective factor in both suppression of oxidative stress and activation of Nrf2 (13–15).

In this study, we examined the effect of TGF-β on the expression of *HO-1*. We found that TGF-β induces expression of *MafK* and *Bach1* and that these genes are essential for suppression of *HO-1* by TGF-β signaling.

EXPERIMENTAL PROCEDURES

Cells and Culture—293T and NMuMG cells were obtained from the American Type Culture Collection. These cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Invitrogen). Mouse mammary carcinoma JygMC(A) cells were cultured as described previously (16).

DNA Constructs—Expression constructs encoding ALK5T204D and FLAG-Smads (17, 18) and the luciferase reporters pNQO1-ARE-luc (19) and pHO1-luc (20) were described previously. cDNAs for Nrf2, MafF, MafG, MafK, and Bach1 were cloned into the pcDEF3 vector before use. For establishment of cells stably expressing FLAG-MafK or FLAG-Bach1, corresponding cDNAs were cloned into the pCAGIP vector (21).

DNA Transfection—293T and NMuMG cells were transfected using FuGENE 6 transfection reagent (Roche Diagnostics) or Lipofectamine 2000 (Invitrogen) following the manufacturers' recommendations. For establishment of cell lines stably expressing MafK or Bach1, NMuMG cells were transfected with pCAGIP-FLAG-MafK or pCAGIP-FLAG-Bach1, respectively; cloned; and maintained in the presence of puromycin (1 μg/ml; Sigma).

RNA Interference—NMuMG and JygMC(A) cells were transfected with 40 nM small interfering RNA (siRNA) directed against MafK or Bach1 using Lipofectamine 2000 (Invitrogen). In the case of double knockdown of MafK and Bach1, cells were transfected with a 40 nM concentration of each siRNA. Sequences of siRNA are listed in Table 1. Control siRNA was purchased from Invitrogen (Stealth™ RNAi Negative Universal Control Medium). A pSUPER-puro vector (Oligoengine) expressing a short hairpin RNA against human and mouse Smad4 (pSUPER-sh-Smad4) was described previously (22). NMuMG cells transfected with pSUPER-sh-Smad4 were cloned and maintained in the presence of puromycin (1 μg/ml).

TABLE 1
Sequence information for interference RNA

Name	Sequence
siRNA MafK 1	
Sense	5′-CGAUGAUGAGCUGGUGUCCAUGUCA-3′
Antisense	5′-UGACAUGGACACCAGCUCAUCAUCG-3′
siRNA MafK 2	
Sense	5′-GGGCUAAUGUCUGUGUUCCUGUGUG-3′
Antisense	5′-CACACAGGAACACAGACAUUAGCCC-3′
siRNA Bach1 1	
Sense	5′-GAAGGCUGCUCAAGCAACUUGGAAA-3′
Antisense	5′-UUUCCAAGUUGCUUGAGCAGCCUUC-3′
siRNA Bach1 2	
Sense	5′-ACUGUGAGGUGAAGCUGCCAUUCAA-3′
Antisense	5′-UUGAAUGGCAGCUUCACCUCACAGU-3′

Luciferase Assay—Luciferase activities were measured using Luciferase Assay Systems (Promega) using a luminometer (MicroLumat, Berthold). The obtained luciferase activities were normalized to β-galactosidase activities of cotransfected pcDNA1.2/V5-GW/*lacZ* (Invitrogen).

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)—Total RNA was prepared using ISOGEN (Nippon Gene). RT was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), and PCR was performed using Ex Taq polymerase (Takara Bio). PCR primers are listed in Table 2.

Immunoprecipitation and Immunoblot Analysis—Cells were solubilized in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2000 KIU/ml aprotinin, 1 μg/ml leupeptin). After clearing by centrifugation, total cell lysates or immunoprecipitates obtained using the indicated antibodies were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred to a mixed ester nitrocellulose membrane (Hybond-C Extra, GE Healthcare) and subjected to immunoblot analysis. Anti-Nrf2 (H-300, Santa Cruz Biotechnology), anti-HO-1 (Stressgen), anti-NAD(P)H:quinone oxidoreductase 1 (NQO1) (Abcam), anti-α-tubulin (Cell Signaling Technology), anti-β-actin (Sigma), anti-hemagglutinin (HA) (3F10, Roche Applied Science), anti-FLAG (M2, Sigma), anti-Smad2/3 (clone 18, BD Transduction Laboratories), anti-Smad4 (Santa Cruz Biotechnology), anti-phospho-Smad2 (23), anti-MafK (24), and anti-Bach1 (11) were used as primary antibodies for the immunoblot analysis. Reacted primary antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies (GE Healthcare) and SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). For reblotting, membranes were stripped according to the manufacturer's protocol.

Immunofluorescence Microscopy—To detect Nrf2 and Smad2, NMuMG cells were fixed in 4% formaldehyde. The fixed cells

[2] The abbreviations used are: ARE, antioxidant response element; ALK5, activin receptor-like kinase-5; DEM, diethyl maleate; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1; *t*BHQ, *tert*-butylhydroquinone; KIU, kallikrein-inactivating unit.

TABLE 2
Primers for semiquantitative PCR
GCSH, heavy chain of γ-glutamylcysteine synthetase.

Name	Sequence	Product length	Annealing temperature	Cycle number
		bp	*°C*	
NQO1				
Sense	5′-AGAAGAGAGGATGGGAGGTA-3′	354	55	25
Antisense	5′-TGGTGATAGAAAGCAAGGTC-3′			
HO-1				
Sense	5′-GGGTGACAGAAGAGGCTAAG-3′	319	55	22
Antisense	5′-CATTGGACAGAGTTCACAGC-3′			
GCSH				
Sense	5′-ATTGTTATGGCTTTGAGTGC-3′	353	55	25
Antisense	5′-GCATCATCCAGGTGTATTAA-3′			
β-Actin				
Sense	5′-GCTCATAGCTCTTCTCCAGGG-3′	396	55	22
Antisense	5′-TGAACCCTAAGGCCAACCGTG-3′			
MafF				
Sense	5′-AACACGCCGCACCTGTCGGA-3′	194	55	25
Antisense	5′-GACTTCTGCTTCTGCAGCTC-3′			
MafG				
Sense	5′-AGAAGGAGGAGCTGGAGAAG-3′	257	55	25
Antisense	5′-GCATCCGTCTTGGACTTTAC-3′			
MafK				
Sense	5′-AGCGATGATGAGCTGGTGTC-3′	206	55	25
Antisense	5′-AGCTTCTCCACCTCCTGCTG-3′			
Bach1				
Sense	5′-GTGCAGAGTAAAACCGTGAA-3′	499	55	25
Antisense	5′-AGTCATCTCCCAGGCTAATC-3′			
Smad7				
Sense	5′-GGAAGTCAAGAGGCTGTGTT-3′	296	55	25
Antisense	5′-GTCTTCTCCTCCCAGTATGC-3′			

TABLE 3
Primers for ChIP analysis

Name	Sequence	Product length	Annealing temperature	Cycle number
		bp	*°C*	
HO-1 promoter (E1)				
Sense	5′-TGAAGTTAAAGCCGTTCCGG-3′	183	53	35
Antisense	5′-AGCGGCTGGAATGCTGAGT-3′			
HO-1 promoter (E2)				
Sense	5′-GGGCTAGCATGCGAAGTGAG-3′	201	53	35
Antisense	5′-AGACTCCGCCCTAAGGGTTC-3′			

were permeabilized, and nonspecific protein binding was blocked by 1% bovine serum albumin (BSA) and 0.3% Triton X-100 in phosphate-buffered saline (PBS). Cells were incubated with a mixture of anti-Smad2 (BD Transduction Laboratories) and anti-Nrf2 (25) antibodies and then with Texas Red-conjugated anti-mouse IgG (Molecular Probes/Invitrogen) followed by Alexa Fluor 488-conjugated anti-rat IgG (Molecular Probes/Invitrogen). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

Cell Fractionation—Cells were washed with PBS and treated with ice-cold hypotonic buffer (20% glycerol, 20 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20,000 KIU/ml aprotinin). Nuclei (pellet) and cytosol (supernatant) were separated by centrifugation (16,000 × *g*, 5 min). Nuclei were resuspended in SDS-PAGE sample buffer. Proteins in cytosolic fractions were precipitated with methanol and resuspended in SDS-PAGE sample buffer.

DNA Affinity Precipitation—Cells were lysed in 1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2000 KIU/ml aprotinin, 1 μg/ml leupeptin. Equal amounts of lysate protein were incubated with biotinylated double-stranded DNA oligonucleotides and poly(dI-dC) (Roche Applied Science) at 4 °C for 1 h. DNA-protein complexes were captured with streptavidin-agarose for 1 h and subjected to immunoblotting. The sequences of HO-1-ARE probe were as follows: sense, 5′-Bio-TTCGCTGAGTCATGGTTCCC-3′; antisense, 5′-GGGAAC-CATGACTCAGCGAA-3′ (20).

Chromatin Immunoprecipitation (ChIP)—ChIP was performed as described previously (17) with modifications. Cells were treated with TGF-β (5 ng/ml) for 1 h before treatment with *tert*-butylhydroquinone (*t*BHQ; 25 μM). After cross-linking with 1% formaldehyde at 37 °C for 15 min, cells were suspended in 500 μl of nuclear lysis buffer (1% SDS, 50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 20,000 KIU/ml aprotinin, 1 μg/ml leupeptin) and sonicated. Soluble chromatin was diluted with 9 volumes of dilution buffer for immunoprecipitation (16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 20,000 KIU/ml aprotinin, 1 μg/ml leupeptin) and incubated with normal rabbit IgG, anti-Nrf2 (H-300, Santa Cruz Biotechnology), anti-MafK (24), anti-Bach1 (11), or anti-Smad2/3 (clone 18, BD Transduction Laboratories) anti-

body with end-over-end rotation at 4 °C overnight followed by incubation with 25 μl of Dynabeads® Protein A (Invitrogen) at 4 °C for 1 h. DNA was extracted from the Dynabeads by means of phenol-chloroform extraction. PCR was performed using Ex Taq polymerase. The PCR primers are described in Table 3. Otherwise, quantitative PCR was performed using qPCR MasterMix for SYBER Green I (Applied Biosystems) and the ABI7500 Fast Sequence Detection system. All samples were run in triplicate in each experiment. Primer sequences are listed in Table 4.

RESULTS

TGF-β Suppresses Electrophile-inducible Expression of HO-1—To examine the effect of TGF-β on expression of *HO-1*, NMuMG cells were treated with *t*BHQ in the absence or presence of TGF-β signaling. mRNA levels of *HO-1* were highly increased 4 h after *t*BHQ stimulation (Fig. 1*A*, *lanes 2* and *3*). However, pretreatment of the cells with TGF-β significantly reduced the induction (Fig. 1*A*, *lanes 4* and *5*). TGF-β alone had no detectable effect on the basal expression levels of *HO-1* (Fig. 1*A*, *lanes 6* and *7*). A representative result of the densitometric quantification of normalized mRNA levels is shown in Fig. 1*A*, *right panel*. TGF-β-mediated suppression of *HO-1* was also examined at the protein level (Fig. 1*B*). These results indicate that TGF-β suppresses *t*BHQ-inducible expression of *HO-1* in NMuMG cells.

TABLE 4
Primers for ChIP analysis and quantitative PCR

Name	Sequence	Product length
		bp
HO-1 promoter (E2)		
Sense	5′-GGGCAGTCTTAAGCAATCCA-3′	146
Antisense	5′-AAGGGTTCAGTCTGGAGCAA-3′	
Smad7		
Sense	5′-TAGAAACCCGATCTGTTGTTTGCG-3′	132
Antisense	5′-CCTCTGCTCGGCTGGTTCCACTGC-3′	

TGF-β Does Not Affect the Stabilization or Nuclear Accumulation of Nrf2—To examine how TGF-β affects *HO-1* expression, we examined the expression and subcellular localization of Nrf2 proteins in NMuMG cells. Because Nrf2 activates the transcription of *HO-1*, we assumed that TGF-β would affect the accumulation or nuclear translocation of Nrf2. However, as shown in Fig. 2, neither the stabilization (Fig. 2*A*) nor the nuclear localization of Nrf2 (Fig. 2, *B* and *C*) was affected by TGF-β.

TGF-β Induces MafK and Bach1—Nrf2 activates transcription of target genes by binding to AREs together with small Maf proteins. However, if small Maf levels rise exceedingly, small Maf homodimers can compete with Nrf2-small Maf heterodimers for the binding to AREs (7). Otherwise, heterodimers of small Mafs and other cap 'n' collar-type transcriptional regulators such as Bach1 can compete for the binding (9). Because TGF-β did not affect the nuclear accumulation of Nrf2, we next examined the effect of TGF-β on the expression of small Mafs and Bach1 and found that TGF-β increased MafK and Bach1 at both mRNA and protein levels (Fig. 3, *A* and *B*). Induction of MafK and Bach1 was suppressed by a TGF-β type I receptor kinase inhibitor, SD-208 (Fig. 3*C*).

MafK and Bach1 Regulate Induction of HO-1—We next examined the effect of MafF, MafG, and MafK on pHO1-luc activities. Transiently expressed MafG and MafK, but not MafF, suppressed the reporter activities (Fig. 4*A*). MafF, MafG, and MafK all formed heterodimers with Nrf2 (Fig. 4*B*), but when small Maf proteins were independently expressed, MafK and MafG, but not MafF bound to *HO-1* ARE DNA fragments (Fig. 4*C*). We then established NMuMG cell lines with stable expression of MafK, MafG, or Bach1. Binding of MafK and MafG to *HO-1* ARE (E2) was confirmed by chromatin immunoprecipitation (Fig. 4*D*). In functional analyses, both *t*BHQ and diethyl maleate (DEM) induced *HO-1* expression in

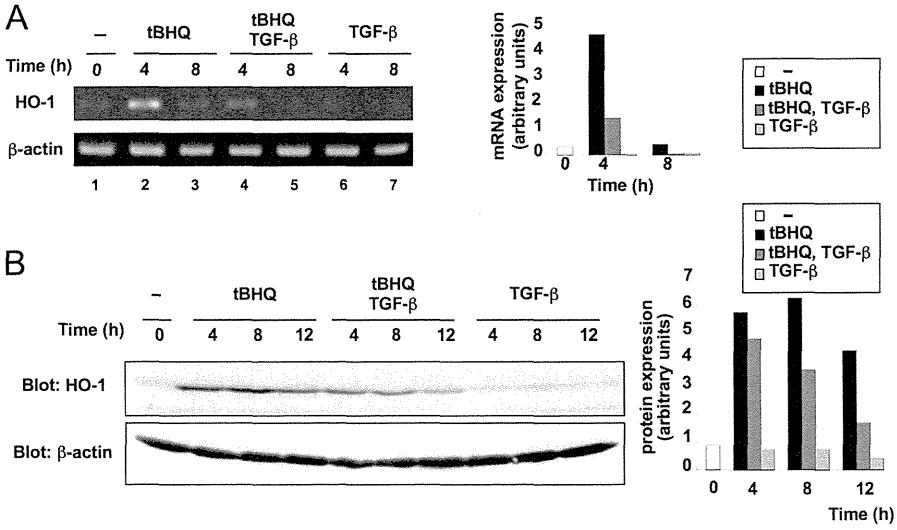

FIGURE 1. **TGF-β suppresses *t*BHQ-inducible expression of the *HO-1*.** *A*, TGF-β suppresses *t*BHQ-inducible mRNA expression of *HO-1*. NMuMG cells were treated with TGF-β (5 ng/ml) for 1 h before stimulation with *t*BHQ (25 μM) and incubated for the indicated times. HO-1 and β-actin mRNAs were detected by semiquantitative RT-PCR (*left panel*). Representative mRNA expression was quantified using NIH ImageJ and normalized to β-actin (*right panel*). *B*, NMuMG cells were treated as in *A*. Immunoblot analysis was performed using anti-HO-1 antibody. β-Actin was examined as a loading control (*left panel*). Quantification of the protein levels was performed using NIH ImageJ and normalized to β-actin (*right panel*). All experiments were repeated more than three times to confirm their reproducibility.

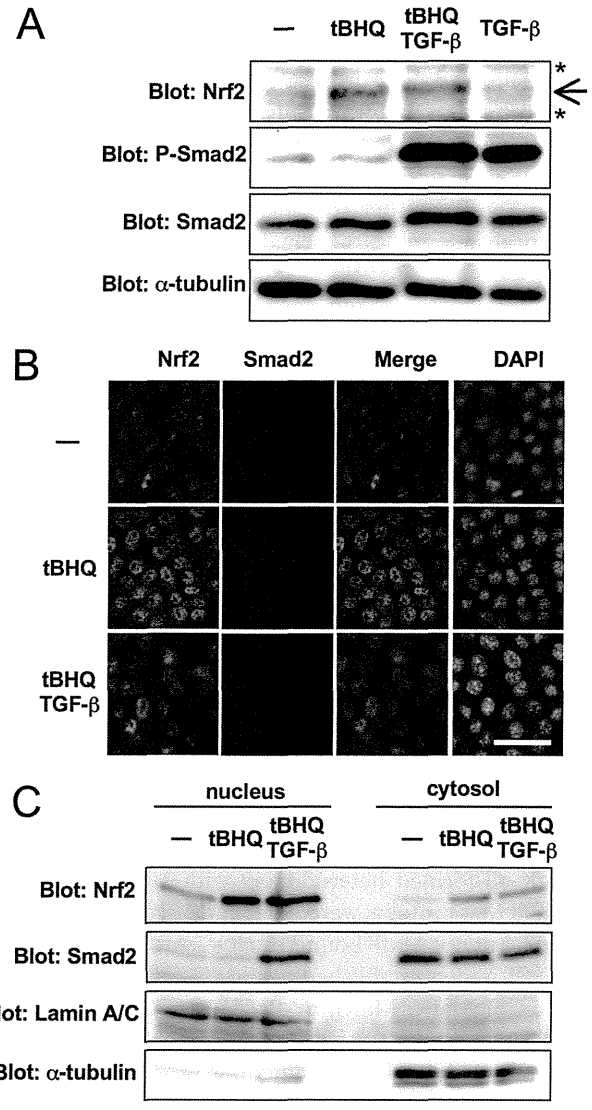

FIGURE 2. **TGF-β does not affect the stabilization and nuclear accumulation of Nrf2.** *A*, NMuMG cells were treated with TGF-β (5 ng/ml) for 1 h before stimulation with *t*BHQ (25 μM) and incubated for an additional 4 h. Immunoblot analysis was performed using anti-Nrf2, -phospho-Smad2 (*P-Smad2*), -Smad2 and -α-tubulin antibodies as indicated. *Arrow*, specific band for Nrf2; *, nonspecific bands. *B*, NMuMG cells were treated as in *A*. After fixation, cells were serially stained with anti-Nrf2 (*green*) and anti-Smad2 (*red*) antibodies. Nuclei were counterstained with DAPI. *Scale bar*, 50 μm. *C*, NMuMG cells were treated as in *A*. Nuclear and cytosolic fractions were isolated and analyzed by immunoblotting using antibodies for Nrf2 and Smad2. Lamin A/C was used as a nuclear protein marker, and α-tubulin was used as a cytosolic protein marker.

NMuMG cells. However, these effects were completely blocked by overexpression of MafK (Fig. 5, *A* and *B*). MafK also decreased pHO1-luc activities in both the absence and the presence of *t*BHQ or DEM (Fig. 5*C*). On the other hand, reduction of MafK by siRNA strikingly enhanced *t*BHQ-inducible expression of *HO-1* mRNA (Fig. 5*D*). However, stable overexpression of MafG did not suppress *t*BHQ- and DEM-inducible expression of *HO-1* (supplemental Fig. 2). We also established NMuMG cells stably expressing FLAG-Bach1 (Fig. 5*E*). Bach1 had a nearly identical effect as that of MafK on *HO-1* expression (Fig. 5, *E–H*) except for a stronger induction of *HO-1* in the absence of both electrophiles and TGF-β (Fig. 5*H*). Furthermore, double knockdown of MafK and Bach1 highly increased both the basal and the *t*BHQ-inducible expression of *HO-1* and abolished the suppressive effect of TGF-β on *t*BHQ-inducible *HO-1* expression (Fig. 5*I*). Endogenous MafK and Bach1 also suppressed *HO-1* in breast cancer cells. Knockdown of MafK or Bach1 in JygMC(A) cells clearly increased expression of *HO-1* (Fig. 5*J*).

Recruitment of Nrf2, MafK, and Bach1 to ARE Sites in the HO-1 Promoter—ARE sites (E1 and E2) in the promoter region of *HO-1* are essential for its transcriptional regulation. To determine whether changes in *HO-1* expression are associated with altered binding of Nrf2, MafK, and Bach1 to ARE sites *in vivo*, we performed chromatin immunoprecipitation assays. When cells were treated with *t*BHQ, Nrf2 binding to both E1 and E2 (mainly to E1) increased. These interactions were reduced when cells were treated with TGF-β before *t*BHQ stimulation (Fig. 6*A*). Binding of MafK to E1 and E2 increased after *t*BHQ stimulation together with Nrf2, but its binding to E2 was not decreased by TGF-β treatment (Fig. 6*B*). In contrast, Bach1 binding to E2 was highest in the absence of *t*BHQ and was reduced when cells were treated with *t*BHQ (Fig. 6*C*).

Binding of Smad3 and MafK on ARE Sites in the HO-1 Promoter—Binding of MafK or Bach1 and Smad3 was examined by coprecipitation assays. Both MafK and Bach1 bound to Smad3. MafK bound to Smad3 in the presence of TGF-β signaling. On the other hand, Bach1 bound to Smad3 in the absence of TGF-β signaling (Fig. 7, *A* and *B*). Binding of Smad2/3 on ARE (E2) in the promoter region of *HO-1* was detected by chromatin immunoprecipitation assays in the presence of TGF-β signaling (Fig. 7*C*).

DISCUSSION

In this study, we proved that TGF-β suppresses the electrophile-inducible transcriptional activation of *HO-1*. This is the first report on the regulation of the Nrf2-mediated oxidative stress responses by TGF-β signaling. Intriguingly, TGF-β did not reduce the nuclear accumulation of Nrf2 provoked by electrophiles, but the recruitment of Nrf2 to AREs (E1 and E2) in the promoter region of *HO-1* was clearly suppressed by TGF-β. Consistent with the previous report that MafK-Bach1 heterodimers repress *HO-1* expression (12), knockdown of MafK and Bach1 enhanced *HO-1* expression and abolished the suppressive effect by TGF-β (Fig. 5*I*). These results suggest that TGF-β signaling generally antagonizes cytoprotective responses mediated by the Keap1-Nrf2 system. Therefore, we further investigated the effect of TGF-β on Nrf2-mediated activation of other Nrf2 target genes. NQO1 and the heavy and light chains of γ-glutamylcysteine synthetase were examined, and all of these target genes were suppressed by TGF-β, although the extents of the suppression were different in different genes (data not shown). Furthermore, the constitutively active TGF-β type I receptor ALK5T204D significantly suppressed Nrf2-mediated activation of pNQO1-ARE-luc together with Smad3 (data not shown). The reporter activity was further suppressed by the addition of Smad4. These results suggested that TGF-β suppresses the transcriptional activity of Nrf2 through activation of the Smad signaling pathway. However, suppression of these Nrf2-target genes was not canceled by

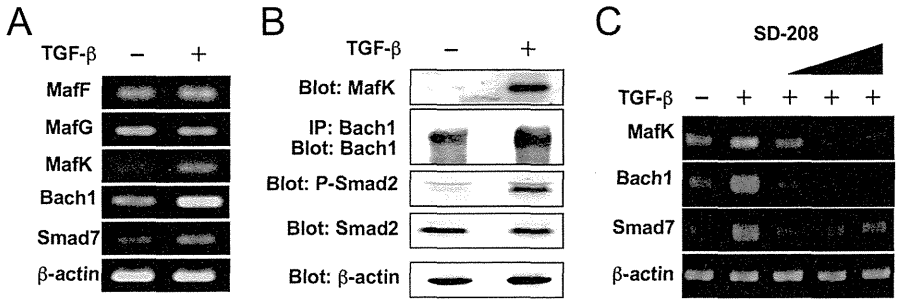

FIGURE 3. **TGF-β induces MafK and Bach1.** *A*, NMuMG cells were treated with TGF-β (5 ng/ml) for 1 h. mRNA for the small Maf family of transcription factors (MafF, MafG, and MafK), Bach1, Smad7, and β-actin was detected by semiquantitative RT-PCR. *B*, NMuMG cells were treated with TGF-β (5 ng/ml) for 4 h. Immunoblot analysis was performed using anti-MafK, -Bach1, -phospho-Smad2 (*P-Smad2*), -Smad2, and -β-actin antibodies as indicated. Bach1 was detected after immunoprecipitation (*IP*) with anti-Bach1 antibody to increase the sensitivity. *C*, NMuMG cells were treated with a TGF-β type I receptor kinase inhibitor, SD-208 (0, 0.03, 0.1, and 0.3 μM) for 30 min before treatment with TGF-β (5 ng/ml) for 1 h. MafK, Bach1, Smad7, and β-actin mRNAs were detected by semiquantitative RT-RCR.

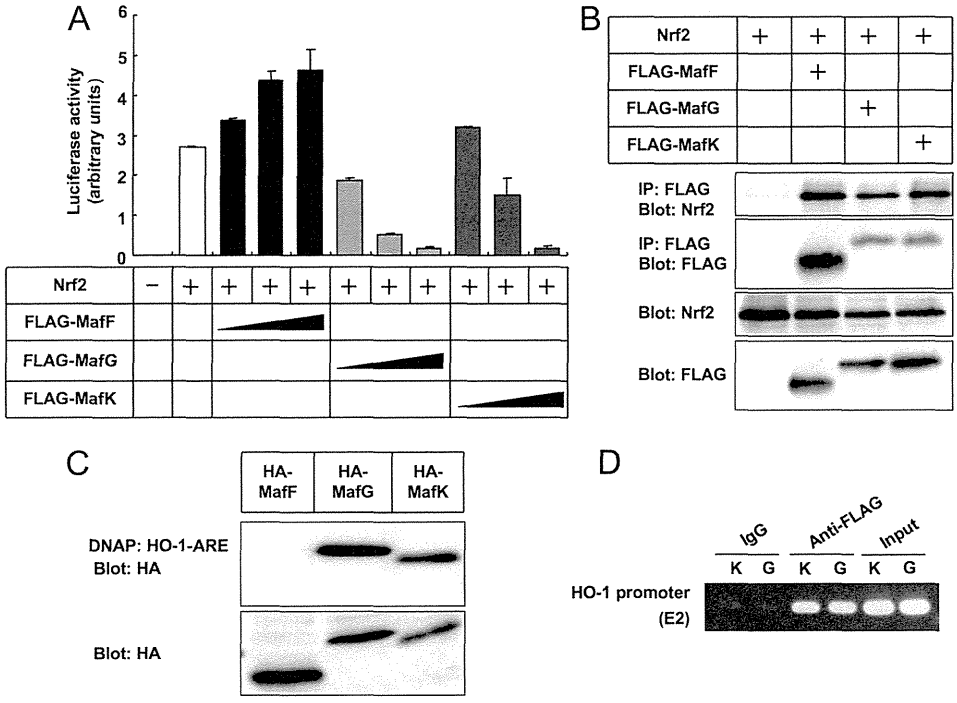

FIGURE 4. **MafK and MafG, but not MafF, suppress transcriptional activity of *HO-1*.** *A*, pHO1-luc reporter activities were activated by overexpression of Nrf2, and the effects of MafF, MafG, and MafK were examined. *Error bars* represent S.D. *B*, interaction between Nrf2 and small Mafs was examined by coprecipitation assays in 293T cells. MafF, MafG, and MafK all coprecipitated Nrf2. *C*, binding of small Mafs to AREs from *HO-1*. HA-tagged MafF, MafG, and MafK were expressed in 293T cells, and the cell lysates were incubated with biotinylated double-stranded DNA fragments (Table 3) and precipitated with avidin beads. Coprecipitated proteins were detected with anti-HA antibody. *D*, chromatin immunoprecipitation analysis using anti-FLAG antibody detected binding of MafK and MafG to the *HO-1* promoter region including ARE (E2) in NMuMG-MafK (*K*) and NMuMG-MafG (*G*) cells, respectively. *DNAP*, DNA affinity precipitation; *IP*, immunoprecipitation.

knockdown of MafK and Bach1 (data not shown). *HO-1* was the only gene in which suppression by TGF-β was canceled by knockdown of MafK and Bach1. Therefore, TGF-β probably suppresses different target genes in different molecular mechanisms. Consistent with this notion, both *HO-1* and *NQO1* are regulated by Nrf2, and these genes contain ARE sites in their promoter regions, but Bach1 interacts specifically with AREs in *HO-1*. Subtle differences in AREs or their flanking sequences affect the binding affinities of the Maf-containing dimers, resulting in different contributions of each dimer to the ARE-dependent gene regulation. Indeed, both Nrf2-MafG heterodimer and MafG homodimer bind to the consensus Maf recognition element with high affinity but bind differentially to the suboptimal binding sequences degenerated from the consensus (26, 27). Different Maf complexes may be differently regulated by TGF-β signaling.

A discrepancy between the nuclear accumulation and transcriptional activity of Nrf2 has been reported. When human aortic endothelial cells were exposed to oscillating flow, Nrf2 accumulated in the nuclei but did not activate stress response genes. In contrast, when the cells were exposed to laminar flow, Nrf2 accumulated in the nuclei and activated its target genes (28). The analogous finding of the current study suggests that levels of small Mafs and Bach1 or other related transcriptional factors might be involved in nuclear regulation of Nrf2 activities.

The functional analyses described above clearly indicated that MafK and Bach1 are essential for the suppression of *HO-1*

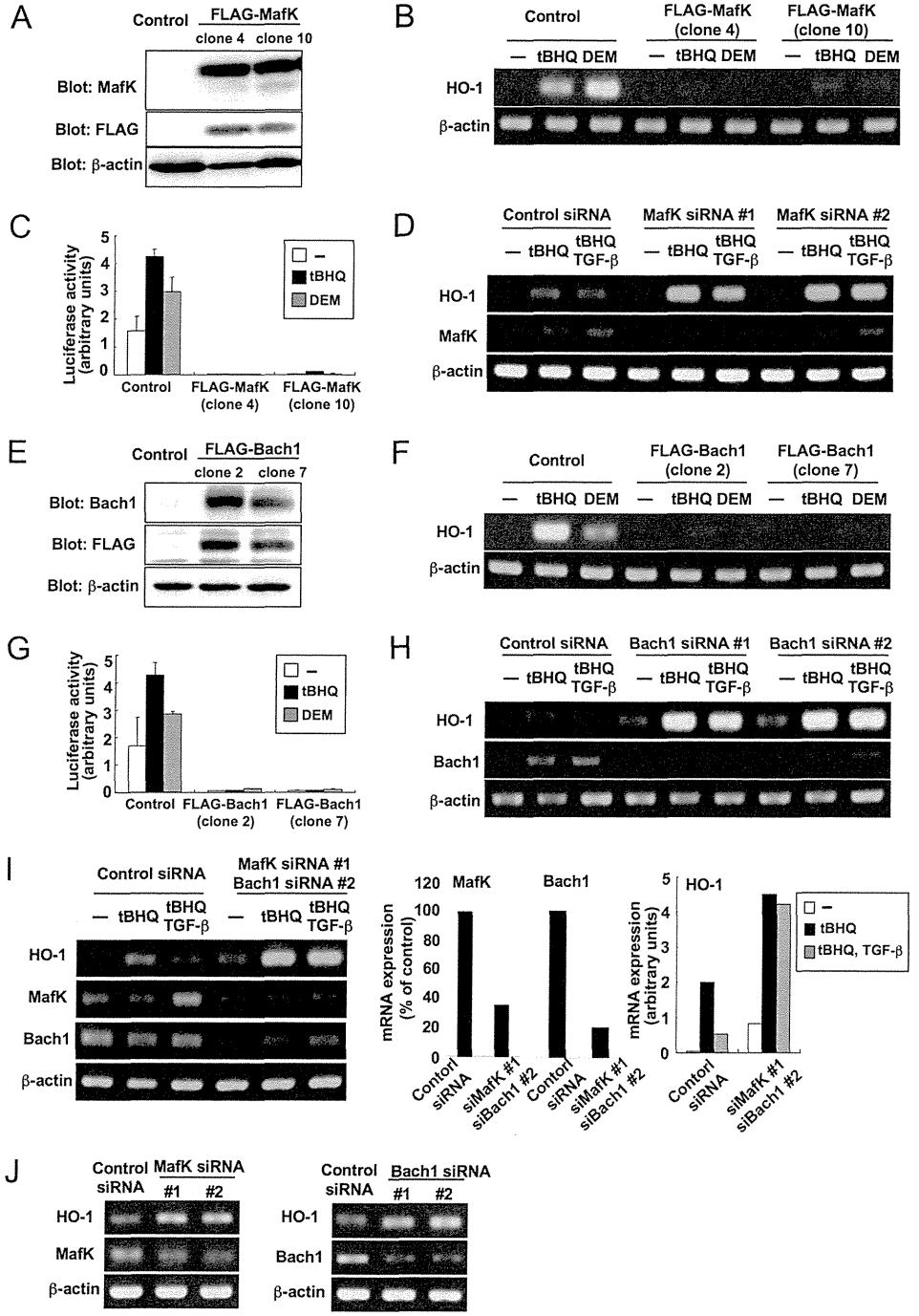

FIGURE 5. **MafK and Bach1 regulate expression of *HO-1*.** *A* and *B*, MafK suppresses *HO-1* induction by *t*BHQ or DEM. *A*, establishment of NMuMG cells stably expressing FLAG-MafK (clones 4 and 10). *Control* represents NMuMG cells transfected with empty vector. *B*, impaired induction of *HO-1* in NMuMG-MafK cells. NMuMG-MafK cells were treated with *t*BHQ (25 μM) or DEM (100 μM) for 4 h. HO-1 and β-actin mRNAs were detected by semiquantitative RT-PCR. *C*, pHO-1-luc activities are inactivated in NMuMG-MafK cells. Six hours after transfection with pHO-1-luc, cells were treated with *t*BHQ (25 μM) or DEM (100 μM) for 12 h. *Error bars* represent mean ± S.D. *D*, knockdown of MafK enhances induction of *HO-1*. NMuMG cells were transfected with MafK siRNA 1 or 2 as described under "Experimental Procedures." Cells were then treated with TGF-β (5 ng/ml) for 1 h and stimulated with *t*BHQ (25 μM) for 4 h. HO-1, MafK, and β-actin mRNAs were detected by semiquantitative RT-PCR. *E* and *F*, Bach1 suppresses induction of *HO-1* by *t*BHQ and DEM. *E*, establishment of NMuMG cells stably expressing FLAG-Bach1 (clones 2 and 7). *F*, impaired induction of *HO-1* in NMuMG-Bach1 cells. NMuMG-Bach1 cells were treated with *t*BHQ (25 μM) or DEM (100 μM) for 4 h. HO-1 and β-actin mRNAs were examined by semiquantitative RT-PCR. *G*, pHO-1-luc activities are suppressed in NMuMG-Bach1 cells. Six hours after transfection with pHO-1-luc, cells were treated with *t*BHQ (25 μM) or DEM (100 μM) for 12 h. *Error bars* represent mean ± S.D. *H*, knockdown of Bach1 enhances induction of *HO-1* and impairs the suppressive effects of TGF-β. NMuMG cells were transfected with Bach1 siRNA 1 or 2 as described under "Experimental Procedures." Cells were treated with TGF-β (5 ng/ml) for 1 h before treatment with *t*BHQ (25 μM) for 4 h. HO-1, Bach1, and β-actin mRNAs were detected by semiquantitative RT-PCR. *I*, double knockdown of MafK and Bach1 enhances induction of *HO-1* and almost completely impairs the suppressive effects of TGF-β. NMuMG cells were transfected with MafK siRNA 1 and Bach1 siRNA 2 as described under "Experimental Procedures." Cells were then treated with TGF-β (5 ng/ml) for 1 h before treatment with *t*BHQ (25 μM) for 4 h. HO-1, MafK, Bach1, and β-actin mRNAs were detected by semiquantitative RT-PCR. Representative mRNA expression was quantified using NIH ImageJ and normalized to β-actin (*right panel*). *J*, knockdown of MafK or Bach1 enhances expression of *HO-1* in breast cancer cells. JygMC(A) cells were transfected with MafK siRNA or Bach1 siRNA as indicated. HO-1, MafK, Bach1, and β-actin mRNAs were detected by semiquantitative RT-RCR.

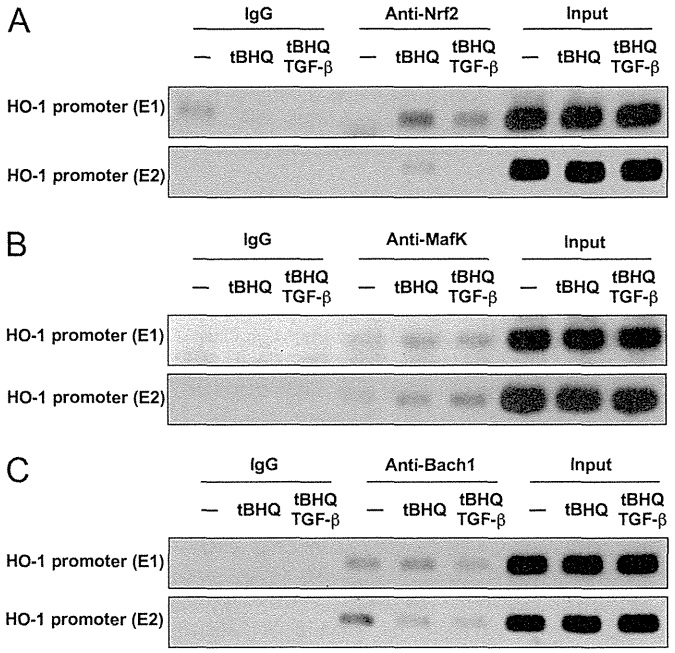

FIGURE 6. **Effects of TGF-β on the recruitment of Nrf2, MafK, and Bach1 to AREs (E1 and E2) in the *HO-1* promoter.** NMuMG cells were treated with TGF-β (5 ng/ml) for 1 h before stimulation with *t*BHQ (25 μM) for 4 h. After fixation, soluble chromatin was immunoprecipitated using anti-Nrf2 (*A*), anti-MafK (*B*), or anti-Bach1 (*C*) antibody as indicated. *HO-1* promoter fragments containing AREs (E1 and E2) were amplified by PCR. *Input*, total chromatin solution analyzed as a control.

by TGF-β signaling (Fig. 5). ChIP analyses (Fig. 6) also revealed that *t*BHQ treatment substitutes Nrf2 for Bach1 in the E1 and E2 elements of *HO-1* and that TGF-β reduces Nrf2 in both elements. However, TGF-β signaling increased MafK binding only marginally, and Bach1 binding to both E1 and E2 was reduced. These results suggest that the displacement of Nrf2 from E1 and E2 was not simply the result of the direct competitive binding between Nrf2 and MafK/Bach1. Consistent with this, we detected Smad2/3 on ARE (E2) in the presence of TGF-β signaling (Fig. 7). The exact molecular mechanism of MafK, Bach1, Smads, and possibly MafG in the TGF-β-dependent suppression of *HO-1* remains to be elucidated.

TGF-β markedly elevated expression of MafK and Bach1 in NMuMG cells. Transcriptional regulation of tissue-specific expression of the *MafK* gene was analyzed previously in transgenic mice harboring the *lacZ* gene as a reporter. Two alternative promoters were identified in the *MafK* gene, and the upstream and downstream promoters mediate the mesodermal and neuronal expressions, respectively (29, 30). A hematopoietic enhancer was also identified in the 3′-region of the *MafK* gene (31). However, the regulatory regions responsible for the induction by TGF-β have not been identified. We found that Smad4 was indispensable for the expression of MafK and suppression of *HO-1* by TGF-β (supplemental Fig. 1, C and E). Conversely, Bach1 expression was constitutively activated by knockdown of Smad4 (supplemental Fig. 1E).

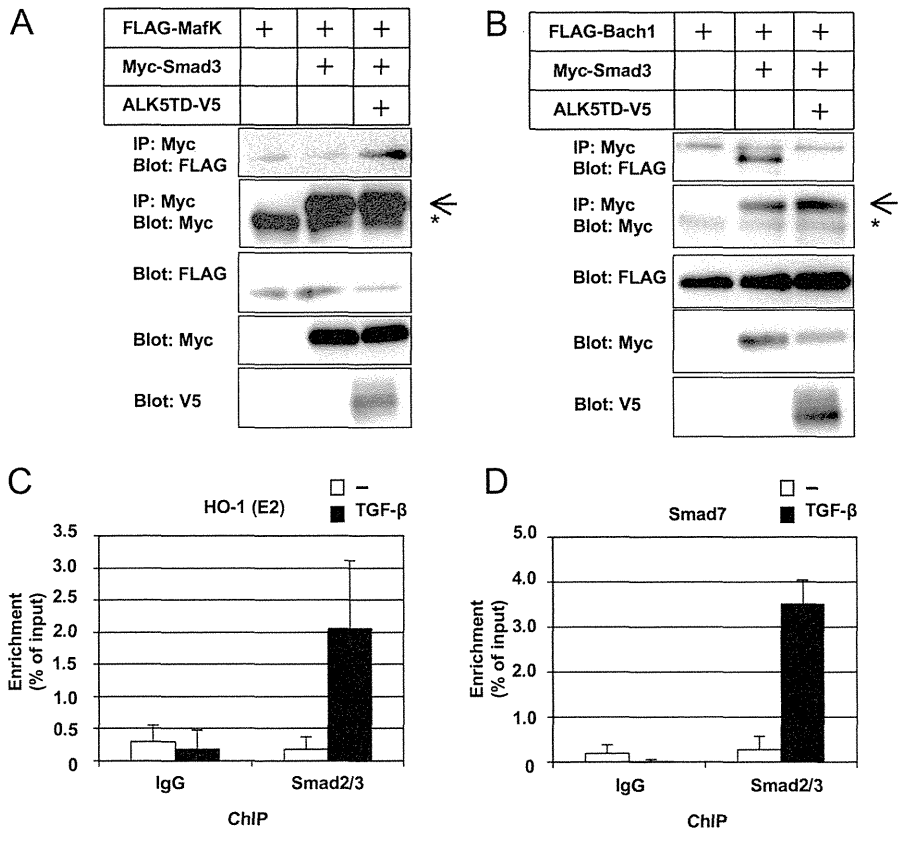

FIGURE 7. **Cooperative functions of TGF-β/Smad signaling with MafK and Bach1.** *A* and *B*, binding of Smad3 to MafK (*A*) and Bach1 (*B*). FLAG-MafK, FLAG-Bach1, Myc-Smad3, and a constitutively active TGF-β type I receptor (ALK5TD-V5) were transfected to 293T cells as indicated, and binding of MafK or Bach1 and Smad3 was examined by immunoprecipitation (*IP*) with anti-Myc antibody followed by immunoblotting with anti-FLAG antibody. *Arrow*, Myc-Smad3; *, IgG. *C* and *D*, chromatin immunoprecipitation analyses using anti-Smad2/3 antibody detected binding of Smad2/3 to the *HO-1* promoter region including ARE (E2) in NMuMG cells (*C*). Binding of Smad2/3 to the Smad7 promoter region was used as a positive control (*D*). *Error bars* represent S.D.