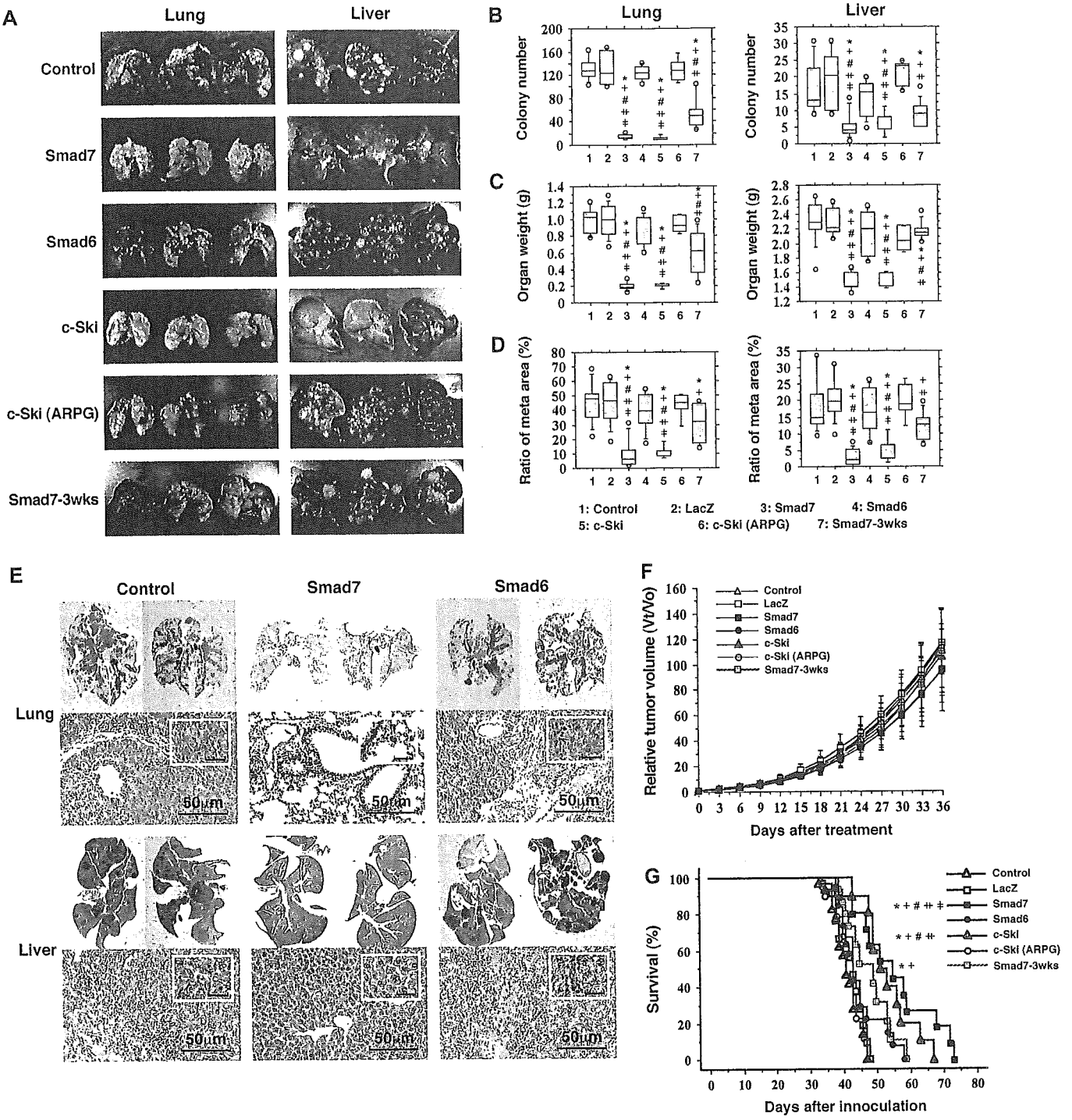

Fig. 1. Smad7 and c-Ski effects on metastasis of JygMC(A) cells. **A)** Mice bearing tumors derived from JygMC(A) cells were treated with virus buffer (Control), AdCMV–Smad7 (Smad7), AdCMV–Smad6 (Smad6), AdCMV–c-Ski (c-Ski), or AdCMV–c-Ski (ARPG) [c-Ski (ARPG)] beginning on day 10 after JygMC(A) cell injection, or treated with AdCMV–Smad7 beginning 3 weeks after JygMC(A) cell injection. Their lungs (**left panels**) and livers (**right panels**) were analyzed on day 46 after inoculation of JygMC(A) cells. Representative examples of the two organs from each group of mice are shown. Lungs of mice in the Control, Smad6, and c-Ski (ARPG) groups show hemorrhage and necrosis because of metastasis. **B–D)** Quantitative evaluation of metastatic lesions by number of metastatic colonies (**B**), organ weight (**C**), and ratio of metastatic area to total area (**D**) in lungs and livers of mice harvested at 5 weeks after inoculation ($n > 10$ mice/group). The data are displayed in box-and-whisker plots; **center horizontal lines** indicate median values, **boxes** delineate interquartile ranges, **whiskers** demarcate values within the 10th–90th percentiles, and **solid circles** indicate values less than the 10th percentile and greater than the 90th percentile. *, statistically significant difference compared with Control (P values ranged from .036 [for ratio of metastatic area in the lung sections, Control versus Smad7–3wks] to .001); +, statistically significant difference compared with LacZ (P values range from .04 [for ratio of metastatic area in lung sections, LacZ versus Smad7–3wks] to <.001); #, statistically significant difference compared with Smad6 (P values range from .013 to <.001); ++, statistically significant difference compared with c-Ski (ARPG) (P values range from .037 [for organ weight in the liver sections, c-Ski (ARPG) versus Smad7–3wks] to .001); ‡, statistically significant difference compared with Smad7–3wks (P values range from .026 [for number of liver colonies, Smad7–3wks versus Smad7] to < .001). **E)** Representative macroscopic and microscopic images of hematoxylin–eosin-stained sections of lung and liver from mice treated with virus buffer (Control) (**left panels**) and from mice treated with AdCMV–Smad7 (**middle panels**) or AdCMV–Smad6 (**right panels**). Original magnification of microscopic findings, ×200 (**scale bar**, 50 μm). Magnification in the boxed areas, ×500 (**scale bar**, 10 μm). **F)** Time course of primary tumor growth in mice treated with intravenous injection of each virus or with virus buffer (Control). Primary tumors were measured every 3 days, and tumor volume was calculated. Each point represents a mean value; **bars** correspond to 95% confidence intervals. **G)** Survival of mice treated with intravenous injection

difference = 14 days [95% CI = 6 days to 22 days], $P<.001$; Smad7 versus Control: median survival = 55 days versus 43 days, difference = 12 days [95% CI = 6 days to 18 days] $P<.001$; c-Ski versus LacZ: median survival = 51 days versus 41 days, difference = 10 days [95% CI = 5 days to 15 days], $P<.001$; c-Ski versus Control: median survival = 51 days versus 43 days, difference = 8 days [95% CI = 3 days to 13 days], $P<.001$); at least 50% of the mice in the Smad7 (7 of 12 mice) and c-Ski (5 of 10 mice) groups survived for 50 days with healthy appearance, and two (17%) of the 12 mice treated with AdCMV–Smad7 ultimately survived for more than 70 days. By contrast, there was no statistically significant difference in survival time between AdCMV-Smad6–treated or AdCMV-c-Ski (ARPG)–treated mice and control mice (Smad6 versus LacZ: median survival = 43 days versus 41 days, difference = 2 days [95% CI = −1 day to 5 days], $P = .11$; Smad6 versus Control: median survival = 43 days versus 43 days, difference = 0 days [95% CI = −3 day to 3 days], $P = .30$; c-Ski(ARPG) versus LacZ: median survival = 44 days versus 41 days, difference = 3 days [95% CI = 1 day to 5 days], $P<.18$; c-Ski(ARPG) versus Control; median survival = 44 days versus 43 days, difference = 1 day [95% CI = −3 day to 5 days], $P = .70$). These results suggest that adenovirus-mediated gene transfer of Smad7 or c-Ski, but not of Smad6 or c-Ski (ARPG), prolonged mouse survival through the inhibition tumor metastasis.

Effect of Delayed Gene Transfer of Smad7 on Metastasis of JygMC(A) Cells

We also examined the effect of Smad7 expression on the growth of tumor cells after the initial development of metastases at the target organs by delaying the initiation of Smad7 adenovirus administration until 3 weeks after inoculation of the cancer cells (Fig. 1). Compared with mice treated with virus buffer only (Control), mice bearing tumors with delayed Smad7 treatment (Smad7–3wks) developed many but statistically significantly fewer metastases in the lung and liver (Smad7–3wks versus Control: median number of lung colonies = 49 versus 128, difference = 79 [95% CI = 57 to 99], $P = .0022$; median lung weight = 0.623 g versus 1.026 g, difference = 0.403 g [95% CI = 0.118 g to 0.635 g], $P = .001$; median ratio of metastatic area in lung = 31.7% versus 48.3%, difference = 16.6% [95% CI = 2.12% to 26.6%], $P = .0357$; Smad7–3wks versus Control: median number of liver colonies = 9.5 versus 13, difference = 3.5 [95% CI = 1.0 to 14.0], $P = .0171$; median liver weight = 1.786 g versus 2.297 g, difference = 0.511 g [95% CI = 0.314 g to 0.744 g], $P = .0021$; ratio of metastatic area in liver = 12.7% versus 15.0%, difference = 2.3% [95% CI = 0.26% to 11.0%], $P = .0498$) (Fig. 1, A–D) and statistically significantly longer survival (Smad7–3wks versus Control: median survival = 43 days versus 49 days, difference = 6 days [95% CI = 3.4 days to 8.6 days], $P = .014$) (Fig. 1, G). These results suggest that Smad7 expression inhibits the development of new metastatic colonies but does not influence the growth or progression of existing metastatic colonies.

Efficacy of Systemic Adenovirus Administration in Tumors and Host Tissues

To determine the efficiency of adenovirus-mediated gene transfer into mice bearing tumors, we evaluated the LacZ expression in lung, liver, and primary tumor tissue after systemic administration of AdCMV–LacZ using X-Gal staining methods. We observed a strong LacZ expression in the liver and weaker expression in the lung and primary tumors of mice on day 4 after adenovirus administration (Fig. 2, A).

We then examined expression of the systemically administered genes encoding Smad6 and Smad7 in mouse lung, liver, and primary tumors by RT–PCR analyses. Mice treated with AdCMV–Smad6 or AdCMV–Smad7 had markedly higher levels of Smad6 and Smad7 mRNAs, respectively, in lung, liver, and primary tumors on day 4 after adenovirus administration than mice treated with AdCMV–LacZ (Fig. 2, B). We further measured mRNA levels of PAI-1, a target gene of TGF-β, and of Id-1, a target gene of BMP, in the primary tumors of mice treated with AdCMV–Smad7, AdCMV–Smad6, or AdCMV–Lac-Z, by using quantitative RT–PCR analysis. Primary tumors from mice treated with AdCMV–Smad7 had statistically significantly less PAI-1 and Id-1 mRNA than those from mice treated with AdCMV–Lac-Z (Fig. 2, C), whereas primary tumors from mice treated with AdCMV-Smad6–infected mice had statistically significantly less Id-1 mRNA. Together, these results demonstrate that systemic administration of AdCMV–Smad6 or AdCMV–Smad7 results in successful gene transfer in the primary tumors as well as in the host tissues and indicate that administration of AdCMV–Smad6 and AdCMV–Smad7 could inhibit endogenous TGF-β superfamily signals.

Response of JygMC(A) Cells to TGF-β and BMP

We next examined whether TGF-β and BMP signals are transduced in JygMC(A) cells by treating cells in culture with human recombinant TGF-β3 (3 ng/mL) or BMP-4 (50 ng/mL) for 1 hour and examining their effects on phosphorylation of Smad proteins using anti–phospho Smad antibodies. TGF-β3, but not BMP-4, induced phosphorylation of Smad2 (Fig. 3, A), and BMP-4, but not TGF-β3, induced phosphorylation of Smad1 and Smad5 (Fig. 3, B) in JygMC(A) cells. Moreover, TGF-β3, but not BMP-4, increased PAI-1 mRNA levels (Fig. 3, C, upper panel), and BMP-4, but not TGF-β3, increased Id-1 mRNA levels compared with the untreated controls (Fig. 3, C, lower panel), in JygMC(A) cells. These findings suggest that JygMC(A) cells can respond to both TGF-β and BMP.

Metastasis in Mice Bearing Tumors Derived from JygMC(A) Cells that Stably Express Smad7

We next examined whether Smad7 inhibits metastasis through a direct effect on the JygMC(A) cells from which the primary tumors were derived or through an indirect effect on the microenvironment for metastasis in the target organs. We first generated

of each virus or virus buffer (Control), as evaluated by Kaplan–Meier analysis and the log rank test. *, statistically significant difference compared with Control (P values range from .014 to <.001); +, statistically significant difference compared with LacZ (P values range from .006 to <.001); #, statistically significant difference compared with Smad6 (Smad7: $P = .002$; c-Ski: $P = .005$). ++, statistically significant difference compared with c-Ski (ARPG) (Smad7: $P = .001$; c-Ski: $P<.001$). ‡, statistically significant difference between Smad7–3wks and Smad7 ($P = .0483$).

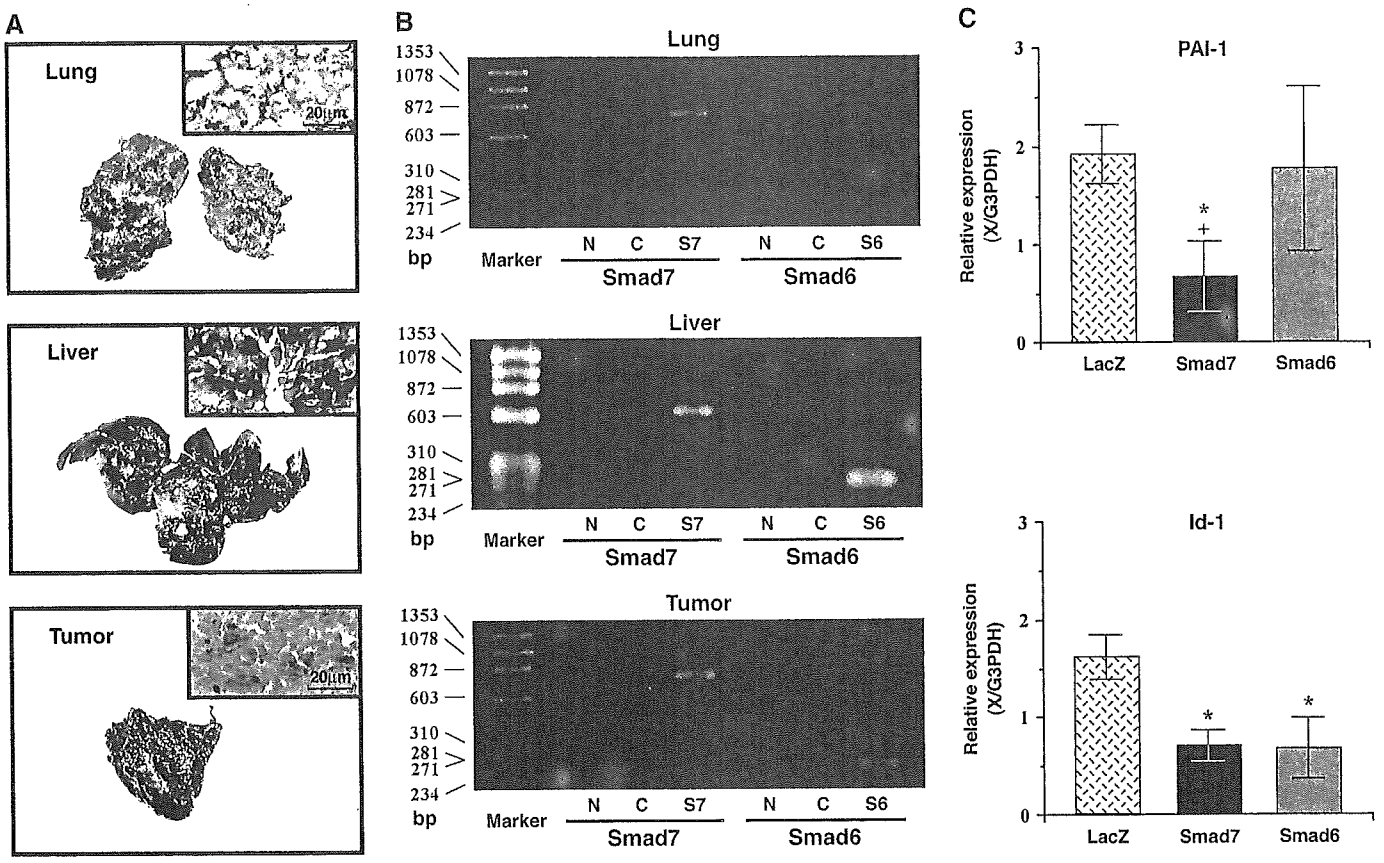

Fig. 2. Expression of exogenous LacZ, Smad7, and Smad6 genes in mice treated with adenovirus-mediated gene transfer. **A)** Lung (**top panel**), liver (**middle panel**), and tumor (**bottom panel**) were obtained from mice treated with systemic administration of AdCMV–LacZ on day 4 after virus administration and subjected to X-Gal staining to detect expression of β-galactosidase encoded by the LacZ gene (**boxed area** in each panel shows the corresponding histologic section; **scale bar** shows 20 μm). **B)** Reverse transcription–polymerase chain reaction (RT-PCR) analysis of mRNA expression of exogenous Smad7 or Smad6 in lung, liver, and tumor of mice treated with systemic administration of AdCMV–Smad7 (S7), AdCMV–Smad6 (S6), or AdCMV–LacZ (C). A PCR for each set of primers was run using water instead of mRNA as a control (N). **C)** Quantitative RT–PCR analysis of mRNA levels for PAI-1 (**top panel**) and Id-1 (**bottom panel**) in the tumors of mice treated with AdCMV–Smad7 (Smad7), AdCMV–Smad6 (Smad6), or AdCMV–LacZ (LacZ). Each value is normalized to the expression of G3PDH and represents a mean of triplicate determinants; **bars** correspond to 95% confidence intervals. Top panel: *, $P<.001$, Smad7 versus LacZ; +, $P<.001$, Smad7 versus Smad6; bottom panel: *, $P<.001$, LacZ versus Smad7 and LacZ versus Smad6.

JygMC(A) cells that stably express Smad6 or Smad7 (Jyg–Smad6 cells and Jyg–Smad7 cells, respectively) or that contained the empty expression vector (Jyg–Empty) as a control. Western blot analyses confirmed that equivalent levels of Smad6 and Smad7 genes were expressed by the respective stable cell lines (data not shown). We injected the flanks of nude mice with each newly constructed stable cell line, Jyg–Smad6, Jyg–Smad7 or Jyg–Empty (n = 40 mice/group) or with parental JygMC(A) cells (n = 30 mice). Mice bearing tumors derived from cells stably transfected with Jyg–Empty and from parental cells (Control) served as the control groups. Similar to the results in the adenovirus-mediated gene transfer experiment (Fig. 1), there were no statistically significant differences in tumor growth among mice injected with any of the three stable cell lines or with parental cell line (Supplemental Fig. 1; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue23). However, mice bearing tumors derived from Jyg–Smad7 cells displayed statistically significantly less metastasis than control mice, as determined by a decrease in the number of metastatic colonies (Fig. 4, B), in organ weight (Fig. 4, C), and in the ratio of metastatic area to total area (Fig. 4, D) in both lung and liver. Also, the Jyg–Smad7 mice survived statistically significantly longer than mice from any of the other groups (Jyg–Smad7 versus Jyg–Empty: median survival = 95 days versus 45 days, difference = 50 days [95% CI = 39 days to 61 days], $P<.001$; Jyg–Smad7 versus Control: median survival = 95 days versus 42 days, difference = 53 days [95% CI = 42 days to 64 days], $P<.001$; Jyg–Smad7 versus Jyg–Smad6: median survival = 95 days versus 43 days, difference = 52 days [95% CI = 41 days to 63 days], $P<.001$); no mice in the Jyg–Smad7 group died before 40 days after tumor cell injection, and 12 (57%) of the 21 mice in this group survived for more than 90 days with a healthy appearance. Two mice ultimately survived for more than 130 days (Fig. 4, E). All of the mice injected with Jyg–Smad7 cells died because of enlarged primary tumors but had little evidence of metastasis. By contrast, mice bearing tumors derived from Jyg–Smad6 cells showed neither a reduction in cancer metastasis (Fig. 4, A–D) nor a statistically significant difference in survival compared with the control mice (Fig. 4, E; $P = .34$ for Jyg–Smad6 versus Jyg–Empty; $P = .16$ for Jyg–Smad6 versus Control).

Effect of Smad7 Expression on Metastasis of Adjacent Tumor Cells

To examine whether JygMC(A) cells that stably express Smad7 influence the microenvironment of surrounding tissues (possibly through the production of soluble factors), we evaluated tumor growth, metastasis, and survival of 5-week-old male

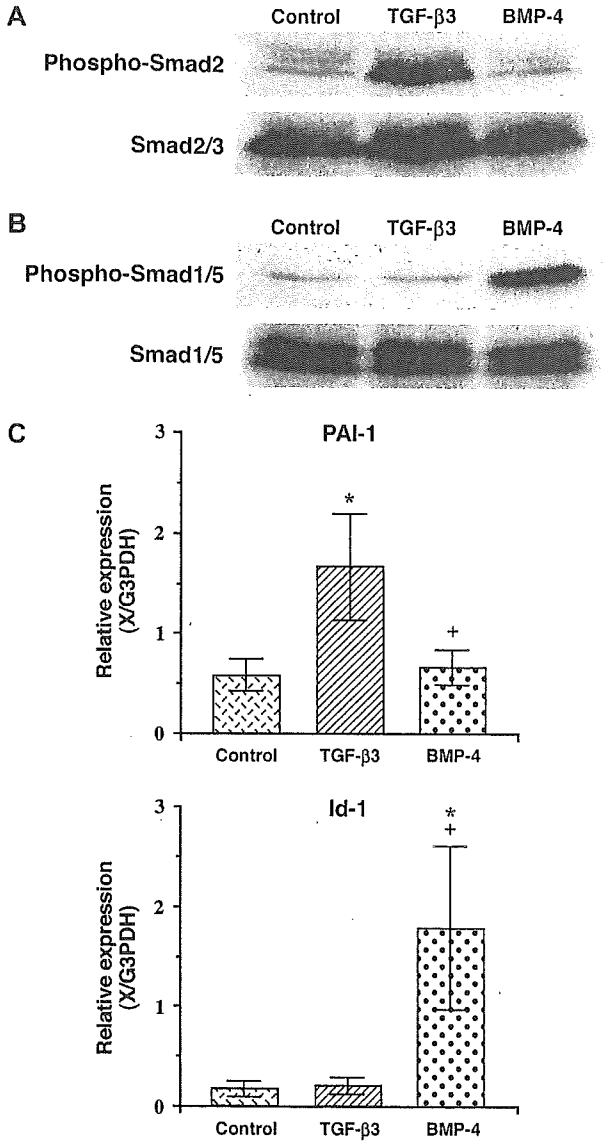

Fig. 3. Response of JygMC(A) cells to TGF-β and BMP. JygMC(A) cells were treated with TGF-β3 (3 ng/mL), BMP-4 (50 ng/mL), or buffer (Control) for 1 hour. Protein lysates were prepared, and equal amounts of each lysate were subjected to western blot analysis using (**A**) an anti–phospho-Smad2 antibody (**top panel**) or an anti–Smad2/3 antibody (**bottom panel**) or (**B**) an anti–phospho-Smad1/5 antibody (**top panel**) or an anti–Smad1/5 antibody (**bottom panel**). **C**) Quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of mRNA levels for PAI-1 (**top panel**) and Id-1 (**bottom panel**) in JygMC(A) cells after 24 hours of treatment with 3 ng/mL TGF-β3, 50 ng/mL BMP-4, or control. Each value is normalized to the expression of G3PDH and represents a mean of triplicate determinants; **bars** correspond to 95% confidence intervals. Top panel: *, $P<.001$, Control versus TGF-β3; +, $P<.001$, TGF-β3 versus BMP-4; Bottom panel *, $P< .001$, Control versus BMP-4; +, $P<.001$, TGF-β3 versus BMP-4.

nude mice that were subcutaneously injected with a mixture of 5×10^6 untransfected parental JygMC(A) cells and 5×10^6 Jyg–Smad7 cells (Cont-Smad7-combined group, n = 30). Mice injected with 5×10^6 parental JygMC(A) cells (Cont-1/2 group, n = 30) or with 5×10^6 Jyg-Smad7 cells (Jyg–Smad7–1/2 group, n = 30) were used as controls. We observed no statistically significant differences between mice bearing tumors derived from the combined cell lines (the Cont-Smad7-combined group) and mice bearing tumors derived from JygMC(A) cells alone (the Cont-1/2 group) with respect to the growth of the primary tumors (Supplemental Fig. 1; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue23), metastasis to lung and liver (Fig. 4, A–D), or animal survival (Fig. 4, E). These findings strongly suggest that the Smad7 protein directly modulates the cancer cells in which it is expressed to reduce their metastatic potential rather than acting through intercellular mediators.

Effect of Smad7 Expression on the Molecular Signature of JygMC(A) Cells

We next attempted to identify proteins whose expression is differentially modulated by Smad7 expression. For this purpose, we used a high-throughput western blotting method that uses mixtures of monoclonal antibodies to evaluate differences in levels of cellular signaling proteins in total cell extracts among different cells or tissues. We were particularly interested in changes in the expression of proteins that are associated with cell adhesion because in the first steps of metastasis, tumor cells lose cell–cell adhesiveness and gain motility *(27)*.

We prepared extracts of JygMC(A) cells, JygMC(A) cells that had been infected with AdCMV–LacZ or AdCMV–Smad7, Jyg–Empty cells, and Jyg–Smad7 cells, and subjected the extracts to a PowerBlot analysis to examine the relative expression levels of 47 signal transducing molecules involved in cell–cell adhesion. Analyses of the resulting antibody array data indicated that expression of nine proteins was increased whereas expression of one protein was decreased in JygMC(A) cells treated with AdCMV–Smad7 relative to parental JygMC(A) as well as in Jyg–Smad7 cells relative to Jyg–Empty cells. Proteins whose expression increased included components of adherens junctions (i.e., E-cadherin, α-catenin, β-catenin, nexillin, profilin, gelsolin, and phosphorylated FAK) and tight junctions (i.e., occludin and ZO-2). However, expression of the mesenchymal marker N-cadherin was statistically significantly lower in cells that expressed exogenous Smad7 than in cells that did not (Smad7 versus Control: mean relative RNA level = 1.15 versus 16.2, difference = 15.05 [95% CI = 12.4 to 17.9], $P = .003$; Jyg–Smad7 versus Jyg–Empty: mean relative RNA level = 1.23 versus 7.54, difference = 6.31 [95% CI = 4.54 to 8.94], $P<.001$). The PowerBlot results were confirmed by western blot analyses of cell extracts (data not shown) as well as by quantitative RT–PCR of RNA isolated from JygMC(A) cells infected with AdCMV–LacZ or AdCMV–Smad7, Jyg–Empty cells, and Jyg–Smad7 cells (Fig. 5). These findings indicate that Smad7 may increase the expression of proteins associated with cell adhesion properties and that Smad7 increases E-cadherin expression but inhibits N-cadherin expression.

Morphology of JygMC(A) Cells that Overexpress Smad6 or Smad7 Expression

Molecular alterations of JygMC(A) cells by Smad7 prompted us to examine the effects of Smad7 expression on cell morphology. JygMC(A) cells exhibited spindlelike, fibroblastic morphology with stress fibers oriented longitudinally (Supplemental Fig. 2; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue23). However, expression of Smad7, but not Smad6, changed the morphology of JygMC(A) cells to a flat, epithelial shape, and markedly increased the number of tight junctions compared with parental cells (Supplemental

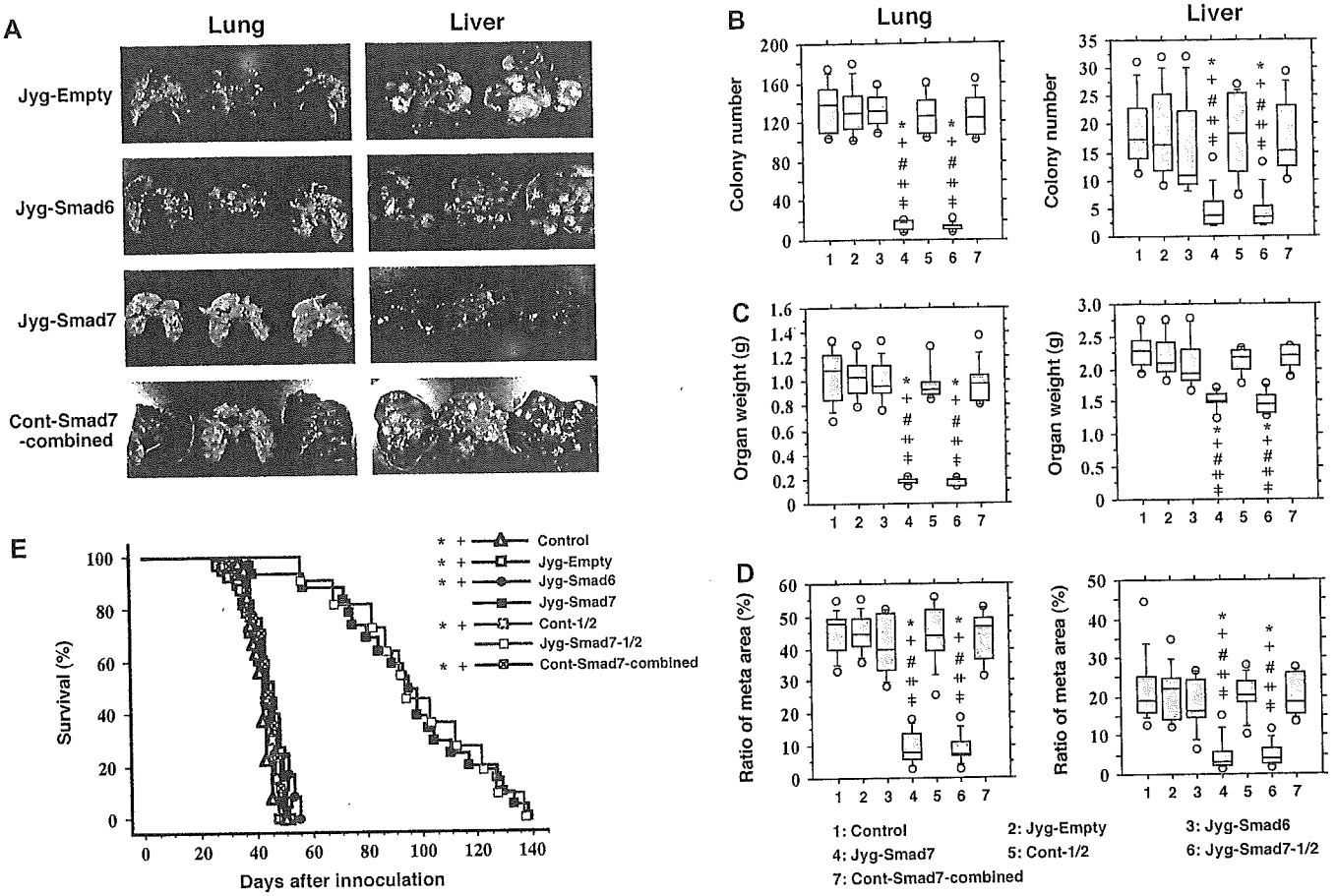

Fig. 4. Effect of stable expression of Smad6 and Smad7 in JygMC(A) cells on in vivo cancer growth and metastasis. **A)** Mice were inoculated with 10^7 JygMC(A) cells stably transfected with empty expression vector (Jyg–Empty) or expression vector containing the gene for Smad6 (Jyg–Smad6) or Smad7 (Jyg–Smad7), or with a mixture of untransfected JygMC(A) cells and Jyg–Smad7 cells (Cont-Smad7–combined) and were analyzed for metastasis in lungs and liver on day 46 after tumor cell injection. Mice inoculated with 5×10^6 parental JygMC(A) cells (Cont-1/2 group) and with 5×10^6 Jyg–Smad7 cells (Jyg-Smad7–1/2 group) were used as controls for Cont-Smad7–combined. Representative pictures of lungs (**left panels**) and livers (**right panels**) of mice from the Jyg–Empty, Jyg–Smad6, and Cont-Smad7–combined groups harvested on the day 46 after the injection of cancer cells are shown. **B–D)** Quantitative evaluation of metastatic lesions by number of metastatic colonies (**B**), organ weight (**C**), and ratio of metastatic area to total area (**D**) in lungs and livers of mice harvested at 5 weeks after inoculation ($n > 10$ mice/group). Data are displayed in box-and-whisker plots; **center horizontal lines** indicate median values, **boxes** delineate interquartile ranges, **whiskers** demarcate values within the 10th–90th percentiles, and **solid circles** indicate values less than the 10th percentile and greater than the 90th percentile. *, $P<.001$, Control versus Jyg–Smad7 or Jyg–Smad7–1/2 in colony number, organ weight, and the ratio of metastatic area in lung as well as liver sections; +, $P<.001$, Jyg–Empty versus Jyg–Smad7 or Jyg–Smad7–1/2 in colony number, organ weight, and the ratio of metastatic area in lung as well as liver sections; #, $P<.001$, Jyg–Smad6 versus Jyg–Smad7 or Jyg–Smad7–1/2 in colony number, organ weight, and the ratio of metastatic area in lung as well as liver sections; ++, $P<.001$, Cont-1/2 versus Jyg–Smad7 or Jyg–Smad7–1/2 in colony number, organ weight, and the ratio of metastatic area in lung as well as liver sections; ‡, $P<.001$, Cont–Smad7–combined versus Jyg–Smad7 or Jyg-Smad7–1/2 in colony number, organ weight, and the ratio of metastatic area in lung as well as liver sections. **E)** Survival of mice bearing tumors derived from each cell line, as evaluated by Kaplan–Meier analysis and the log rank test. *, $P<.001$, Jyg–Smad7 versus Control, Jyg–Empty, Jyg–Smad6, Cont–1/2, or Cont–Smad7–combined; +, $P<.001$, Jyg–Smad7–1/2 versus Control, Jyg–Empty, Jyg–Smad6, Cont–1/2, or Cont–Smad7–combined.

Fig. 2; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue23).

Effect of Smad7 Expression on JygMC(A) Cell Motility and Invasion In Vitro

We examined the effects of Smad6 or Smad7 expression on the behavior of JygMC(A) cells in vitro. We used JygMC(A) cells, JygMC(A) cells that had been infected with AdCMV–Smad6 (Smad6), AdCMV–Smad7 (Smad7), or AdCMV–LacZ (LacZ), and Jyg–Empty, Jyg–Smad6, and Jyg–Smad7 cells in these assays. Cell growth assays demonstrated that Smad6 or Smad7 expression by both adenovirus-mediated and stable methods did not affect cancer cell proliferation. There was no statistically significant difference in cancer cell growth ratios among any of the cancer cell lines we used [JygMC(A) cells, JygMC(A) cells that had been infected with AdCMV–Smad6, AdCMV–Smad7, or AdCMV–LacZ, and Jyg–Empty, Jyg–Smad6, and Jyg–Smad7 cells]. (Fig. 6, A). These results are consistent with our in vivo data showing that Smad7 gene transfer did not influence primary tumor growth.

We next used a cell migration scratch wound healing assay of tissue culture cell monolayers to examine the effects of Smad6 and Smad7 expression on the motility of JygMC(A) cells in vitro. We found that statistically significantly fewer JygMC(A) cells that expressed Smad7, but not Smad6, migrated into the midportion of a trisected wound area than either parental JygMC(A) cells or JygMC(A) cells infected with AdCMV–LacZ (Smad7 versus LacZ: median number of migrating cells = 5 versus 49.5, difference = 44.5 [95% CI = 35 to 51], $P<.001$; Smad7 versus Control: median number of migrating cells = 5 versus 52.5, difference = 47.5 [95% CI = 36 to 53], $P<.001$; Smad6 versus

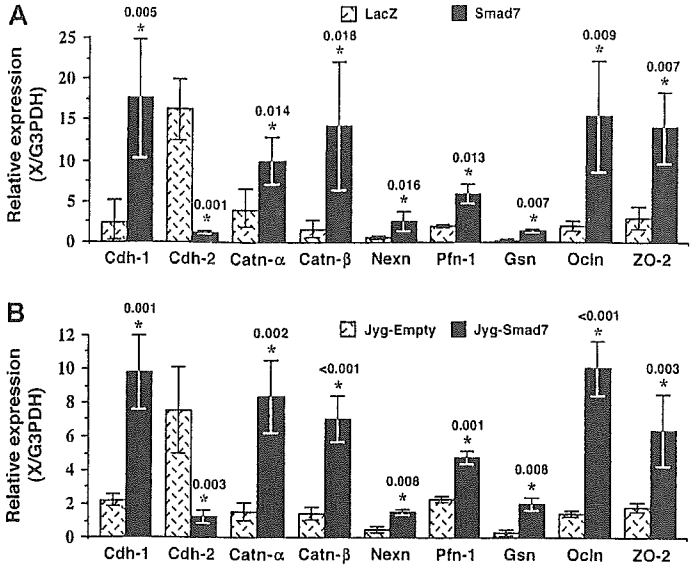

Fig. 5. Effects of Smad7 gene expression on the adhesion properties of JygMC(A) cells. Quantitative reverse transcription–polymerase chain reaction analyses of **A)** JygMC(A) cells infected with AdCMV–LacZ (LacZ) or AdCMV–Smad7 (Smad7) and of **B)** JygMC(A) cells stably transfected with pCAG-IP–Smad7 (Jyg–Smad7) or pCAG-IP–empty (Jyg–Empty). *, statistically significant difference between the Smad7 and control groups for each molecule. Each value is normalized to the expression of G3PDH and represents a mean of triplicate determinants; **bars** correspond to 95% confidence intervals. Cdh1 = E-cadherin; Cdh2 = N-cadherin; Catn-α = α-catenin; Catn-β = β-catenin; Nxn = nexillin; Pfn-1 = profilin 1; Ocln = occludin; Gsn = gelsolin; ZO-2 = zonula occludens 2.

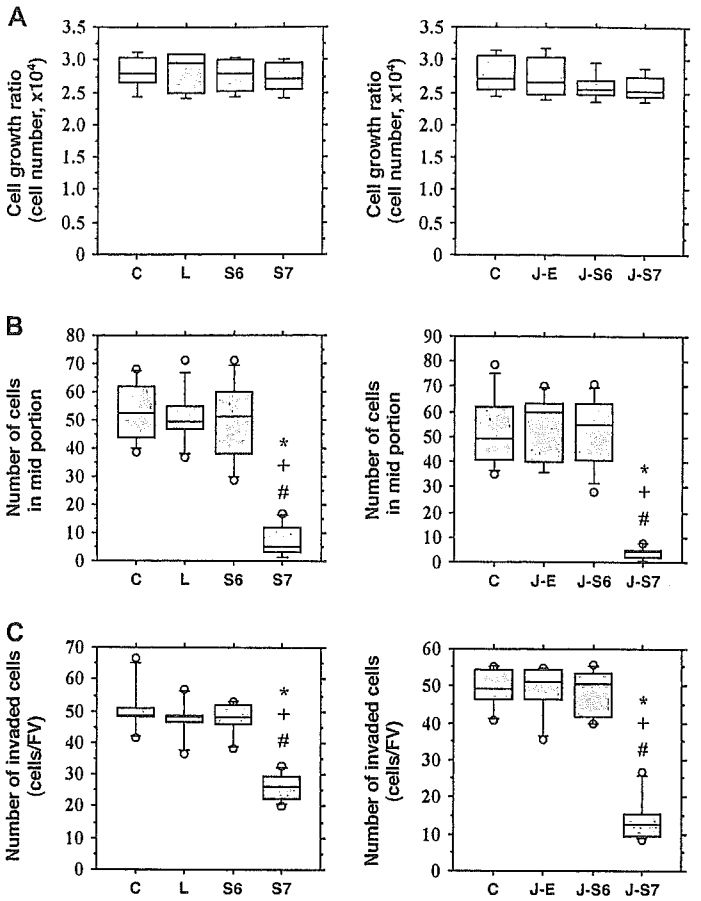

Fig. 6. Effect of Smad7 expression on JygMC(A) cell motility and invasion in vitro. Effects of adenovirus-mediated transfer (**left panels**) and stable transfection (**right panels**) of genes encoding Smad6 and Smad7 on the proliferation (**A**), migration (**B**), and invasion (**C**) of JygMC(A) cells. Parental JygMC(A) cells (i.e., uninfected, untransfected cells) were used a control. The data are displayed in box-and-whisker plots; **center horizontal lines** indicate median values, **boxes** delineate interquartile ranges, **whiskers** demarcate values within the 10th–90th percentiles, and **solid circles** indicate values less than the 10th percentile and greater than the 90th percentile. *, $P<.001$, Smad7 versus Control or Jyg–Smad7 versus Control in migration assay (**middle panels**) and in invasion assay (**bottom panels**); +, $P<.001$, Smad7 versus LacZ or Jyg–Smad7 versus Jyg–Empty in migration assay (**middle panels**) and in invasion assay (**bottom panels**); #, $P<.001$, Smad7 versus Smad6 or Jyg–Smad7 versus Jyg–Smad6 in migration assay (**middle panels**) and in invasion assay (**bottom panels**). C = control; L = AdCMV–LacZ, S6 = AdCMV–Smad6; S7 = AdCMV–Smad7; J-E = Jyg–Empty; J-S6 = Jyg–Smad6; J-S7 = Jyg–Smad7; FV = field of view.

LacZ: median number of migrating cells = 51.5 versus 49.5, difference = 2 [95% CI = −14 to 13], $P>.99$; Smad6 versus Control: median number of migrating cells = 51.5 versus 52.5, difference = 1.0 [95% CI = −14 to 11], $P = .88$); Jyg–Smad7 versus Jyg–Empty: median number of migrating cells = 4 versus 60, difference = 56 [95% CI = 36 to 62], $P<.001$; Jyg–Smad7 versus Control: median number of migrating cells = 4 versus 49, difference = 45 [95% CI = 36 to 60], $P<.001$) (Fig. 6, B; Supplemental Fig. 3, A; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue23). We further investigated the effect of Smad7 expression on the invasive ability of JygMC(A) cells with the use of a Matrigel invasion assay. We found that statistically significantly fewer JygMC(A) cells expressing Smad7, but not Smad6, than control cells invaded through the filter (Smad7 versus LacZ: median number of invading cells = 26 versus 48, difference = 22 [95% CI = 15 to 28], $P = .0022$; Smad7 versus Control: median number of invading cells = 26 versus 49, difference = 23 [95% CI = 17 to 34], $P = .0022$; Smad6 versus LacZ: median number of invading cells = 48.1 versus 48.2, difference = 0.1 [95% CI = −9 to 6.5], $P>.998$); Smad6 versus Control: median number of invading cells = 48.1 versus 48.9, difference = 0.8 [95% CI = −13 to 5.1], $P = .936$); Jyg–Smad7 versus Jyg–Empty: median number of invading cells = 12.8 versus 50.9, difference = 38.1 [95% CI = 23 to 44], $P = .002$; Jyg–Smad7 versus Control: median number of invading cells = 13 versus 49, difference = 36 [95% CI = 28 to 42], $P = .002$; Jyg–Smad6 versus Jyg–Empty: median number of invading cells = 50.6 versus 50.9, difference in location = 0.3 [95% CI = −12 to 7.8], $P = .9372$; Jyg–Smad6 versus Control: median number of invading cells = 50.6 versus 46.8, difference = 3.8 [95% CI = −8.4 to 7.7], $P = .1$) (Fig. 6, C; Supplemental Fig. 2, B, Available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue23). These results suggest that the in vitro migratory and invasive abilities of JygMC(A) cells were inhibited by Smad7 expression.

Discussion

In this study, we have shown that adenovirus-mediated systemic gene transfer of Smad7, but not Smad6, statistically significantly inhibited metastasis of mouse mammary carcinoma JygMC(A) cells and prolonged mouse survival. We also demonstrated that Smad7 gene transfer increased the expression of major components of adherens and tight junctions, modified the profiles of cadherin expression by increasing E-cadherin expression and decreasing N-cadherin expression, and inhibited the migratory and invasive abilities of the JygMC(A) cells.

The spread of tumor cells from a primary site to distant organs is a critical stage in cancer metastasis that involves both a loss of cell–cell adhesion and a gain of invasive properties *(28)*. Many studies in experimental systems as well as in human patients have revealed that alterations in the adhesive properties of tumor cells are associated with tumor progression and metastasis *(29–33)*. Decreased expression or mutation of major components of adherens junctions is associated with the initiation of cancer metastasis *(29,31,32,34–43)*. Moreover, increases in or reconstitution of such adhesive properties has been shown to suppress metastasis in many different neoplastic tissues *(44–47)*. Here we have shown that Smad7 expression substantially increased the expression of major components of adherens junctions, such as E-cadherin, and tight junctions, such as occludin. Thus, Smad7 expression may enhance the adhesive properties of cancer cells through the formation of adherens junctions and tight junctions in cancer cells, and thereby contribute to the inhibition of metastasis.

We also found that expression of N-cadherin was statistically significantly lower in JygMC(A) cells that expressed Smad7 than in JygMC(A) cells that did not. N-cadherin enhances the motility and invasive ability of various types of tumor cells *(48–52)*. It has been repeatedly reported that de novo expression of N-cadherin in tumor cells is associated with the loss of functional E-cadherin *(53–55)*. Also, TGF-β has been implicated in the switching of cadherin properties from E-cadherin to N-cadherin. This cadherin switch has been found to occur during the epithelial-to-mesenchymal transition (EMT) and may promote cancer metastasis *(56–58)*. Our finding that cancer cells with Smad7 expression have increased expression of N-cadherin and decreased expression of E-cadherin (Fig. 5) raises the intriguing possibility that a Smad7-mediated "cadherin switch" from expression of mesenchymal, promigratory cadherins (e.g., N-cadherin) to expression of epithelial, proadhesive cadherins (e.g., E-cadherin) inhibits tumor invasion and metastasis, and that this cadherin switch may be associated with Smad7-mediated inhibition of TGF-β signaling.

In agreement with the above findings, morphologic analysis of cancer cells with Smad7 expression revealed that Smad7 expression changed the morphology of JygMC(A) cells from a spindlelike, fibroblastic shape to a flat, epithelial shape, and markedly strengthened the cell–cell adhesion with increased numbers of tight junctions. In vitro migration and invasion assays revealed that JygMC(A) cell motility was statistically significantly lower in cells that expressed exogenous Smad7 than in cells that did not. These results, taken together with our data on the expression of adhesion properties (Fig. 5) and delayed gene transfer of Smad7, suggest that Smad7 gene expression inhibits the development of new metastases but does not influence the growth or progression of already-existing metastatic colonies by increasing the cell–cell interactions within primary tumors.

Several studies *(2,6,59)* have shown that the extracellular domain of TβR-II, a soluble TGF-β antagonist, suppresses metastasis in mouse breast cancer models, suggesting that inhibition of TGF-β signaling is essential for the prevention of metastasis. In agreement with these findings, we found that only Smad7 and c-Ski, which effectively inhibit TGF-β–regulated signals *(60–61)*, but not Smad6, which does not, prevented metastasis in our mouse model. Although Smad7, c-Ski, and the extracellular domain of TβR-II all block TGF-β signaling, their modes of action differ. The extracellular domain of TβR-II blocks binding of TGF-β to its specific receptors. Because TGF-β acts in an autocrine fashion in some cancer cells *(8)* and might bind to its receptors on the surface of the cells, TGF-β that is secreted in autocrine fashion may escape from the inhibitory effect of the extracellular domain of TβR-II. By contrast, Smad7 and c-Ski may efficiently block TGF-β superfamily signaling inside the cells. Therefore, Smad7 and c-Ski may exhibit their effects more efficiently than the extracellular domain of TβR-II. However, Smad7 and c-Ski primarily inhibit Smad-mediated signaling, but not other types of signaling *(62)*. Furthermore, Smad7 and c-Ski have been reported to elicit biologic activities that are independent of TGF-β signals, such as the induction of apoptosis in prostate carcinoma cells *(63)*. However, we detected no differences in apoptosis between the control JygMC(A) cells and Smad7-expressing JygMC(A) cells (data not shown). It will be interesting to compare the effects on the inhibition of metastasis of Smad7 and c-Ski with those of the extracellular domain of TβR-II in a future study.

We found that the c-Ski (ARPG) mutant, which was previously shown to inhibit TGF-β signaling *(17)*, did not inhibit cancer metastasis. Although it is not known whether BMP signaling can facilitate the progression of certain tumors in a similar fashion to TGF-β signaling, BMPs regulate the growth of some cancers *(64,65)*. Our results show that Smad7 and c-Ski, which inhibit both TGF-β and BMP signaling, efficiently inhibited the development of metastasis, suggesting that the blocking of both TGF-β and BMP signals is required for the inhibition of cancer metastasis. However, our study is limited because we have not shown directly that inhibition of TGF-β signaling is sufficient to inhibit metastasis of JygMC(A) cells.

In conclusion, our results indicate that TGF-β superfamily signals are important for regulating cell–cell interactions and cancer metastasis, and that blocking these signals by systemic expression of Smad7 may be a novel strategy for the prevention of cancer metastasis, especially among patients with advanced-stage disease.

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NOTES

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Intrinsic Mechanism of Estradiol-Induced Apoptosis in Breast Cancer Cells Resistant to Estrogen Deprivation

Joan S. Lewis, Kathleen Meeke, Clodia Osipo, Eric A. Ross, Noman Kidawi, Tianyu Li, Eric Bell, Navdeep S. Chandel, V. Craig Jordan

Background: **We previously developed an estrogen receptor (ER)–positive breast cancer cell line (MCF-7:5C) that is resistant to long-term estrogen deprivation and undergoes rapid and complete apoptosis in the presence of physiologic concentrations of 17β-estradiol. Here, we investigated the role of the mitochondrial apoptotic pathway in this process.** ***Methods:*** **Apoptosis in MCF-7:5C cells treated with estradiol, fulvestrant, or vehicle (control) was investigated by annexin V–propidium iodide double staining and 4′,6-diamidino-2-phenylindole (DAPI) staining. Apoptosis was also analyzed in MCF-7:5C cells transiently transfected with small interfering RNAs (siRNAs) to apoptotic pathway components. Expression of apoptotic pathway intermediates was measured by western blot analysis. Mitochondrial transmembrane potential (ψ_m) was determined by rhodamine-123 retention assay. Mitochondrial pathway activity was determined by cytochrome *c* release and cleavage of poly(ADP-ribose) polymerase (PARP) protein. Tumorigenesis was studied in ovariectomized athymic mice that were injected with MCF-7:5C cells. Differences between the treatment groups and control group were determined by two-sample *t* test or one-factor analysis of variance. All statistical tests were two-sided.** ***Results:*** **MCF-7:5C cells treated with estradiol underwent apoptosis and showed increased expression of proapoptotic proteins, decreased ψ_m, enhanced cytochrome *c* release, and PARP cleavage compared with cells treated with fulvestrant or vehicle. Blockade of Bax, Bim, and p53 mRNA expression by siRNA reduced estradiol-induced apoptosis relative to control by 76% [95% confidence interval (CI) = 73% to 79%, *P*<.001], 85% [95% CI = 90% to 80%, *P*<.001], and 40% [95% CI = 45% to 35%, *P*<.001], respectively, whereas blockade of FasL by siRNA had no effect. Estradiol caused complete regression of MCF-7:5C tumors in vivo.** ***Conclusion:*** **The mitochondrial pathway of apoptosis plays a critical role in estradiol-induced apoptosis in long-term estrogen-deprived breast cancer cells. Physiologic concentrations of estradiol could potentially be used to induce apoptosis and tumor regression in tumors that have developed resistance to aromatase inhibitors. [J Natl Cancer Inst 2005;97:1746–59]**

It is generally believed that the balance between proliferation and apoptosis influences the response of tumors to treatments such as chemotherapy *(1)*, radiotherapy *(2)*, and hormonal therapy *(3)*. It has been suggested that, when these treatments fail, dysregulation of apoptosis may be the cause. Apoptosis is a form of programmed cell death that is executed by a family of proteases called caspases, which can be activated either by cell-surface death receptors (i.e., the extrinsic pathway) or by perturbation of the mitochondrial membrane (i.e., the intrinsic pathway) *(4)*. Components of the extrinsic pathway include the death receptors FasR/FasL, DR4/DR5, and tumor necrosis

Affiliations of authors: Fox Chase Cancer Center, Philadelphia, PA (JSL, VCJ, EAR, TL); Robert H. Lurie Comprehensive Cancer Center (KM, CO, NK) and Department of Medicine (EB, NSC), Northwestern University, Chicago, IL.

Correspondence to: V. Craig Jordan, OBE, PhD, DSc, Vice President and Research Director for Medical Sciences, Alfred G. Knudson Chair of Cancer Research, Fox Chase Cancer Center, 333 Cottman Ave., Philadelphia, PA 19111 (e-mail: V.Craig.Jordan@fccc.edu).

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REVIEW ARTICLE

Shigeo Horie

ADPKD: molecular characterization and quest for treatment

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Abstract
Autosomal-dominant polycystic kidney disease (ADPKD) is a common hereditary disease that features multiple cystogenesis in various organs and vascular defects. The genes responsible for ADPKD, *PKD1*, and *PKD2* have been identified, and the pathological processes of the disease are becoming clearer. This review focuses on recent findings about the molecular and cellular biology of ADPKD, and especially on *PKD1*. *PKD1* and its product, polycystin-1, play pivotal roles in cellular differentiation because they regulate the cell cycle and because polycystin-1 is a component of adherens junctions. A possible link between polycystin-1 and PPARγ is discussed. The extraordinarily fast research progress in this area in the last decade has now reached a stage where the development of a remedy for ADPKD might become possible in the near future.

Key words ADPKD · *Pkd1* · Polycystin1 · Pioglitazone · Endothelial dysfunction · PPARγ

Autosomal-dominant polycystic kidney disease (ADPKD)

Autosomal-dominant polycystic kidney disease (ADPKD) is the most common genetic renal disease and affects about 1 in 1000 to 1 in 2000 people. Genes responsible for ADPKD, *PKD1* and *PKD2*, have already been cloned. The estimated number of ADPKD patients in Japan is about 30000, and it accounts for 3%–5% of dialysis patients.[1] ADPKD is characterized by the formation of multiple cysts in the cortex and medulla of both kidneys. ADPKD patients develop renal failure because of multiple renal cyst development and a decrease in functional nephrons due to renal parenchymal atrophy and fibrosis. The diagnostic criteria for ADPKD in Japan are given in Table 1. Recently, the Research Committee for Progressive Renal Failure of the Ministry of Health, Labour, and Welfare created clinical practice guidelines for ADPKD. Higashihara et al.[1,2] made the first national survey of ADPKD patients in Japan, which found that half of the ADPKD patients progress to end-stage renal disease by the age of 60. On the other hand, there are also many patients who have no problems in leading a normal life other than a mild decrease in renal function. Therefore, it is not correct to say, as in the old textbooks, that ADPKD patients will eventually need dialysis.[1,2] The pathological features of ADPKD include glomerular fibrosis, renal tubular atrophy, interstitial fibrosis, and infiltration of inflammatory cells. The renal cysts originate from the epithelia of the nephrons and the renal collecting system, and are lined by a single layer of cells that have higher rates of cellular proliferation and are less differentiated than normal tubular cells. Renal cysts feature changes in the extracellular matrix that form the basal lamina and an accumulation of cystic fluid due to abnormal fluid secretion. Cystic epithelial cells have high proliferation rates and lose polarized cell architecture.[3]

Clinical features of ADPKD

The clinical manifestations of ADPKD include abdominal mass, chronic flank or back pain, gross hematuria, urinary tract infection, and urolithiasis. In addition to causing progressive renal failure, renal cysts can be complicated by hemorrhage, rupture, infection, nephrolithiasis, and intractable pain. As well as in the kidneys, cysts frequently develop, in the liver, especially in females.[1] They can also be seen in the pancreas, ovaries, and thyroid glands. It was recently found that even in ADPKD patients with normal renal function, close to 70% suffer from hypertension. Increased blood pressure has been attributed to activation of the renin–angiotensin system,[4] but a primary defect in the

S. Horie (✉)
Department of Urology, Teikyo University School of Medicine,
2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan
Tel. +81-3-3964-2497; Fax +81-3-3964-8934
e-mail: shorie@med.teikyo-u.ac.jp

Table 1. Diagnostic criteria for autosomal-dominant polycystic kidney disease (ADPKD) (Research Committee for Progressive Renal Failure of the Ministry of Health, Labour and Welfare, Japan)

(1) If patients have a family history of ADPKD
Patients in whom more than 3 cysts can be detected in each kidney by ultrasonic tomography (or computed tomography (CT)).
(2) If patients have no family history of ADPKD
1. In patients aged 15 or younger, if more than 3 cysts are detected in each kidney by CT and if the diseases listed below can be excluded as possibilities
2. In patients aged 16 or older, if more than 5 cysts are detected in each kidney by CT and if the diseases listed below can be excluded as possibilities
Diseases that should be excluded
Multiple simple renal cysts
Renal tubular acidosis
Cystic dysplasia of the kidney
Multicystic kidney
Multilocular cysts of the kidney
Medullary cystic kidney
Acquired cystic disease of the kidney

blood vessels has also begun to be recognized as a symptom of ADPKD.[5] Moreover, in many cases, such patients develop left ventricular hypertrophy before developing hypertension.[6] Twenty percent of patients suffer intracranial aneurysm, and 8% suffer intracranial hemorrhage.[7] Marked angiogenesis is seen surrounding the renal cysts. These newly created blood vessels have an abnormal alignment of arterial muscular layers. Such blood vessels can easily rupture, which can cause pain and macroscopic hematuria (M. Sato and O. Hotta, personal communication). Furthermore, ADPKD can be complicated by cardiac valvular abnormalities, colonic diverticuli, and inguinal hernia. These extrarenal manifestations feature the abnormal structural integrity of smooth muscle cells. In summary, ADPKD is a systemic disease since the genes responsible are widely expressed, and mutations in these genes can affect a variety of extrarenal tissues.[8]

Genetics of ADPKD

ADPKD is caused by mutations in two genes, named *PKD1* and *PKD2*.[9,10] Mutations of *PKD1* located on chromosome 16p13.3 are responsible for 85% of the ADPKD cases, whereas mutations of *PKD2* on chromosome 4q21–23 are responsible for the rest of the cases (15%). The clinical manifestations of patients with ADPKD caused by mutations of *PKD1* are identical to those of patients with ADPKD caused by mutations of *PKD2*. However, *PKD2* patients have a later onset of symptoms and a slower rate of progression to renal failure. *PKD2* patients also have a longer renal survival (69.1 years) than *PKD1* patients (53.0 years).[11]

The *PKD1* gene, consisting of 46 exons, encodes a 14.1-kb mRNA transcript that is translated into a protein, polycystin-1, composed of 4302 amino acids.[12] The region of the gene extending from exon 1 to exon 33 is duplicated at six other sites on chromosome 16p, and these duplicated genes express mRNA transcripts.[13] These duplicated genes have made the analysis of mutations of *PKD1* very difficult. Recently, a long-range polymerase chain reaction (PCR), denaturing HPLC (DHPLC), and the protein truncation test have identified mutations in the duplicated region of the *PKD1* gene.[14–16] Various types of mutations have been observed across the entire gene, including splice site, in-frame, and out-of-frame deletions and insertions, nonsense mutations, and missense mutations in *PKD1*.[17] More than 100 mutations of *PKD1* have been identified. Mutations in the 5′ end of the gene appear to be associated with an earlier onset of disease than mutations in the 3′ end.[18] The position of the mutations in *PKD1* is predictive for the development of intracranial aneurysms.[19] *PKD2*, a simpler 15-exon gene, encodes a smaller 5.6-kb transcript. *PKD2* encodes polycystin-2, a 110-kD protein that is part of the transient receptor potential family of cation channels.[10] More than 75 mutations of *PKD2* have been identified, again mainly of the inactivating type.[20] The pathogenic mechanism of ADPKD is thought to be a combination of two mutations. The first mutation is either a point mutation, or a frameshift or nonsense mutation due to the insertions or deletions of bases in the allele of a gene in one of the two chromosomes of the germline cells. The second is a mutation in somatic cells of the kidneys and other target organs while having normal epithelial cell alleles. These mutations result in a loss of *PKD1* or *PKD2* expression in the target organs. This mechanism is very similar to the "2 hit" theory, where cancer is believed to be induced when tumor suppressor genes on both chromosomes are defective.[21–24] However, if each renal cyst arises from a second hit of a somatic mutation, a relatively high rate of somatic mutagenesis would be required to develop the large number of cysts in ADPKD. Indeed, recent studies indicate that the rate of somatic mutations in kidney epithelial cells is more than ten times higher than in cells in other organs.[25] The reason for the high rate of somatic mutagenesis in the kidney is not known. Cyst formation may happen even without "2 hit." If polycystins are essential for the maintenance of epithelial differentiation in target organs such as the kidney, the change in gene expression might trigger the alternation of cellular architecture. Thus, ADPKD could be happening by one or both of the two chance processes, a fluctuation in the gene expression of polycystins and/or a second hit of somatic mutation. This explains, at least in part, why there are different rates of disease progress between patients with polycystic kidney disease.[26] There is also another possibility where mutated parenchymal cells can be functional for cystogenesis. A recent study shows that the conditionally targeted disruption of TGFβ1 receptor in fibroblasts causes epithelial neoplasm such as forestomach cancer and intraepithelial cancer in the prostate.[27] This article shed light on the significance of the epithelial–parenchymal interaction for epithelial tumorigenesis. Lymphocytes obtained from *Pkd2* mutants have decreased cellular level of calcium,[28] which might promote inflammation or regional angiogenesis. Although still highly hypothetical, the role of parenchymal cells in the pathogenesis of ADPKD warrants further study.

Gene products of PKD genes

Polycystin-1 is a large (>460kD) membrane protein with a long extracellular N-terminal which contains various distinct protein motifs, including two leucine-rich repeats flanked by cysteine-rich domains, a C-type lectin domain, a WSC domain, and 16 immunoglobin-like domains called PKD repeats. Polycystin-1 is predicted to have 11 transmembrane domains and a short intracellular C-terminal.[29] These motifs may function as protein–protein or protein–carbohydrate interactions. The intracellular portion of the protein has many sites for phosphorylation, and responds to regulators of signal transduction.[30] Because highly homologous genes have been cloned in nematodes, sea urchins, blowfishes, and mice, this gene is believed to play an important role in life processes that also exist in lower organisms.[17] Polycystin-1 is expressed in many tissues, including the kidney, brain, heart, bone, muscle, and vascular smooth muscle cells.[31]

The gigantic size of *PKD1* made it difficult to carry out gene expression analysis in cultured cells. It was recently discovered that when *PKD1* is expressed in distal tubular cells, polycystin-1 inhibits apoptosis of renal tubular cells and promotes lumen formation.[32] In order to further examine the function of *PKD1* in vivo, several groups have generated Pkd1, the mouse ortholog of *PKD1* in mutant mice (Table 2). Most recently, Muto et al.[33] made mutant mice ($Pkd1^{-/-}$) by targeted disruption of exon 2–6, which has the shortest residual portion of *Pkd1*. The phenotype of a $Pkd1^{-/-}$ embryo in this study was almost identical to those of $Pkd1^{L}$,[34] $Pkd1^{del17\text{-}21geo}$,[35] and $Pkd1^{null}$.[36] Among mutants with a C57BL/6-129 background, $Pkd1^{L/L}$ has the most severe disease, judging by its embryonic lethality, since it does not survive to E16.5. Mutant polycystin-1 of $Pkd1^{L/L}$, L3946*, is much longer than the truncated proteins created by a frame shift in $Pkd1^{null/null}$ and $Pkd1^{-/-}$. Thus, the relationship between disease phenotype and severity and the length of the respective mutant polycystin-1 is not linear.

Analysis of staged embryos showed that $Pkd1^{-/-}$ died in utero, starting on embryonic day (E) 14.5. $Pkd1^{-/-}$ exhibited profound edema and focal hemorrhages, presumably due to vascular fragility. The hearts of $Pkd1^{-/-}$ at E12.5 showed hemorrhagic pericardial effusion, a double-outlet right ventricle (DORV), and a ventricular septal defect. In the $Pkd1^{-/-}$ kidney, renal development is normal until around E15, when cystic structures arise in the center of the organ in the portion that is most developmentally advanced. Thus, polycystin-1 is required for the final maturation of renal epithelia. At E18.5, the renal parenchyma of $Pkd1^{-/-}$ was replaced by numerous cysts (Fig. 1). Cardiac conotruncal defects, such as a double-outlet right ventricle and ventricular septal defects, are considered to be a primary cause of embryonic lethality at this time. Neural crest cells migrate

Table 2. Mutant polycystin-1 and disease phenotype

Mutant	mRNA	Hydrops fetalis	Polyhydramnios	Hemorrage	DORV	Renal cysts	Alive at E16.5[a]	Strain	Ref.
$Pkd1^{-/-}$	Exon 1	Y	Y	Y	Y	E15.5	11%	C57BL/6-129	33
$Pkd1^{null}$	Exon 1–4	Y	Y	ND	ND	E15.5	21%	C57BL/6-129	36
$Pkd1^{del17\text{-}21geo}$	Exon 1–16	Y	ND	Y	Y	ND	0%	129	35
$Pkd1^{L}$	Exon 1–43	Y	ND	Y	ND	E14.5	0%	C57BL/6-129	34

[a] % homozygotes out of total embryos
DORV, double outlet of right ventricle; Y, presence of a phenotype; ND, not described in the reference

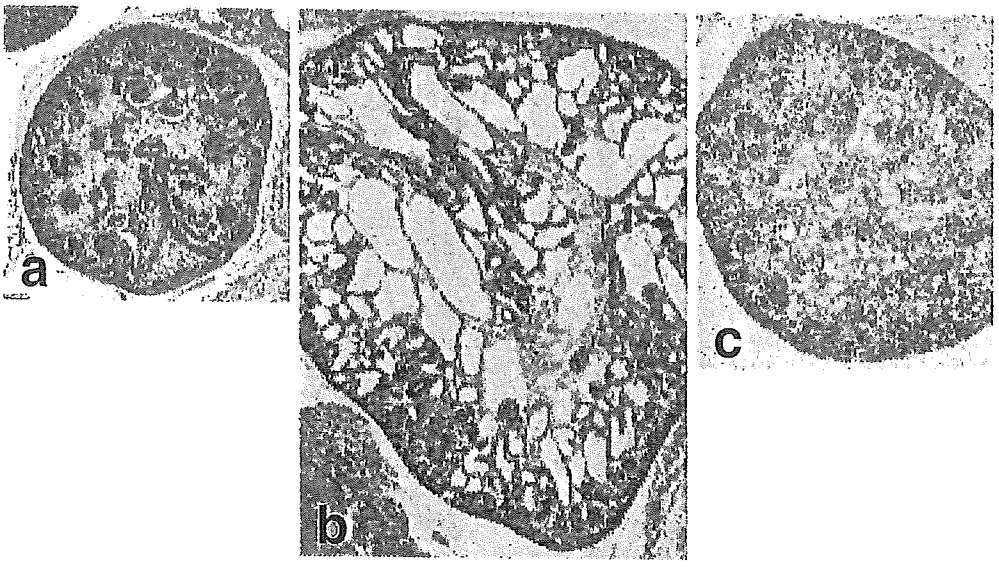

Fig. 1a–c. Pioglitazone treatment inhibited cystogenesis in $Pkd1^{-/-}$ embryonic kidneys E18.5 (H&E ×40).[33] **a** Wild-type; **b** $Pkd1^{-/-}$; **c** pioglitazone-treated $Pkd1^{-/-}$. A kidney of $Pkd1^{-/-}$ (**b**) developed numerous renal cysts, whereas pioglitazone-treated $Pkd1^{-/-}$ (**c**) develops significantly smaller cysts

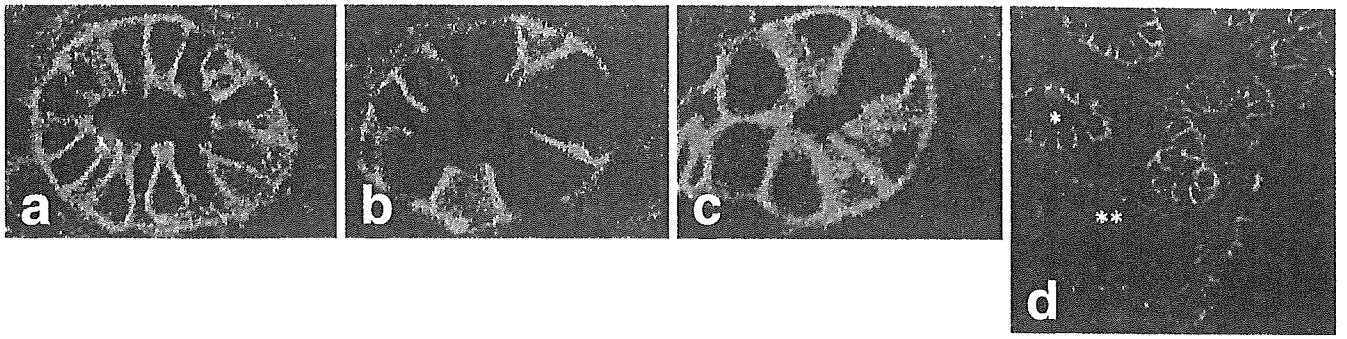

Fig. 2a–d. Decrease in the expression of E-cadherin in *Pkd1*$^{-/-}$ renal tubules and the effect of pioglitazone treatment.[33] **a** wild-type; **b** *Pkd1*$^{-/-}$; **c** pioglitazone-treated *Pkd1*$^{-/-}$ (×600); **d** renal cystogenesis in *Pkd1*$^{-/-}$. *, a nondilated tubule; **, a developing cyst (×200). **a** A normal renal tubule showing strong expression of E-cadherin in basolateral membrane. **b,c** Loss of expression of E-cadherin was noted in a tubule of *Pkd1*$^{-/-}$ which was recovered in pioglitazone-treated *Pkd1*$^{-/-}$. **d** A developing cyst loses expression of E-cadherin. The taller column cells of a nondilated tubule* are losing expression of E-cadherin at basal membranes. Basolateral expression of E-cadherin is extensively lost in shorter cells constituting a cyst**

to form the cardiac conotruncus.[33] Since polycystin-1 is expressed in cells of neural crest origin,[37] the dysfunction of these cells due to the loss of *Pkd1* can be inferred to be the cause of abnormal conotruncal development. Several studies have identified polycystin-1 in the plasma membrane of tubular epithelial cells, especially in the distal nephron and collecting ducts.[38–40] Polycystin-1 has been identified in cell junctional complexes, including adherens junctions and desmosomes. Polycystin-1 binds to adhesive molecules such as β-catenin and E-cadherin, and is localized in the basolateral side of the cell membrane.[41–45] In *Pkd1*$^{-/-}$ mice, the basolateral expression of E-cadherin in developing tubules was diminished in fetal kidneys where cysts had not yet started to form (Fig. 2).[32] These data indicate that the contribution of polycystin-1 in a complex of adherens junction proteins may be essential to maintain the differentiation and polarization of renal tubular cells.

Polycystin-2, the gene product of *PKD2*, is a membrane protein with six transmembrane segments. Several studies have shown that the polycystin-2 channel conducts divalent cations including calcium.[46–48] It was revealed that polycystin-2 exists in the endoplasmic reticulum, and it interacts with polycystin-1 located in the cell membrane to regulate intracellular calcium.[49–51] Thus, if either *Pkd1* or *Pkd2* is defective, abnormalities will occur in intracellular calcium regulation. The observation that animals with homozygous mutations of either *Pkd1* or *Pkd2* die in utero in the later stages of gestation suggests that both gene products are essential for normal development. In ADPKD, the polarity of the epithelial cells of the renal tubules is disrupted, which may result in mislocalization of Na/K-ATPase and EGF receptor (EGFR). It is certainly conceivable that a derangement of calcium regulation by the defective cooperation of polycystin-1 and -2 affects the differentiation and morphogenesis of renal tubules.[52] Polycystins and other proteins associated with renal cystic diseases have recently been identified in primary cilia in renal tubular epithelia.[53,54] These findings have suggested that abnormalities in cilia formation and function may play a role in the pathogenesis of PKD. The role of polycystins in cilia is discussed in detail in a recent review paper.[55]

Molecular signature of ADPKD

Cyst epithelia feature hyperproliferative states as well as loss of cellular polarity.[56] Micropolyps are frequently observed projecting into the lumen. Several studies have shown that the proliferation of tubular epithelial cells in transgenic mice also leads to renal cystic disease.[57–59] Horie et al.[60] first identified that HGF, a growth factor that activates a tyrosine kinase receptor, and cyclic adenosine monophosphate (cAMP) are disparately involved in renal cystic disease. The concentration of HGF was high in proximal cysts, while cAMP was more abundant in distal cysts. Since this research, many studies have shown that cystogenesis is associated with the activation of tyrosine kinase-mediated signaling and cAMP-mediated signaling. HGF is a growth factor and plays a major role in mesenchymal–epithelial interaction. HGF binds to c-Met, a tyrosine kinase receptor. HGF is a potent tubulogenic factor for cultured LLC-pK1, MDCK, and mIMCD-3 cells.[61–63] For proper tubulogenesis, the coordinated expression of growth factors or cytokines, extracellular matrices, and adhesion molecules are necessary. Transgenic mice that over-express HGF do develop renal cystic disease.[64] In *Pkd1*$^{-/-}$ kidney, Gab1, an adapter cytoplasmic protein associated with activated EGFR and c-MET,[65,66] was constitutively activated. EGFR is over-expressed and abnormally localized on the apical (luminal) surface of cyst-lining epithelia.[33] Cyst fluids contain mitogenic concentrations of EGF, and this EGF is secreted into the lumens of cysts in amounts that can induce cellular proliferation.[67] In *bpk* mice, an animal model of renal cystic disease, EGFR is over-expressed. EGFR tyrosine kinase inhibitor, EKI-785, inhibited renal cystogenesis and extended the life-span of bpk mice.[68] In a developing cyst in *Pkd1*$^{-/-}$ kidneys, the cyst epithelial cells

were shorter in height than normal tubular cells, and there was a remarkable decrease in the expression of E-cadherin (see Fig. 2) and constitutive activation of EGFR. A recent report describes the relation between the disruption of cell polarity and the induction of cell proliferation by the activation of a tyrosine kinase. In growth-arrested mammary acini,[69] the activation of ErbB2 affects cell polarity and induces cell proliferation, which emphasizes that uncontrolled activation of tyrosine kinase-mediated signaling leads to a modification of cytoarchitecture. Hence, the decrease in adherens junction (AJ) proteins and the constitutive activation of EGFR and Gab1 in *Pkd1*$^{-/-}$ kidney might be mutually associated with the loss of polycystin-1. Inhibitors of tyrosine kinase may have a role in slowing the expansion of cysts and the decline in renal function. Currently, it still remains a challenge to develop a small-molecule tyrosine kinase inhibitor that allows patients to maintain good compliance without suffering from significant adverse effects.

cAMP is involved in the cell proliferation and fluid secretion of cyst epithelium. First, increasing intracellular cAMP enhances cyst formation and enlargement in in vitro culture systems.[70,71] Furthermore, maneuvers that raise intracellular cAMP, such as the addition of forskolin which activates adenylate cyclase, the addition of the membrane-permeable cAMP analogue 8-bromo-cAMP, and the addition of the receptor-mediated agonists arginine vasopressin, desmopressin, secretin, vasoactive intestinal polypeptide, and prostaglandin E2, stimulate fluid secretion both in monolayers of cultured epithelial cells established from ADPKD patients and in isolated ADPKD cysts.[72–75] Yamaguchi et al.[75] and Hanaoka and Guggino[76] showed that epithelial cells derived from ADPKD have a different response to cAMP from a mixed population of normal renal epithelial cells isolated from the cortex. cAMP stimulated DNA synthesis and cell proliferation in ADPKD epithelial cells, but not in epithelial cells from the renal cortex. The actions of cAMP on ADPKD cells are mediated through PKA activation of the ERK pathway at a locus distal to receptor tyrosine kinase.[75] These authors found that cAMP and EGF had complementary and additive effects on the rate at which ADPKD cells proliferate. Previous reports showed that cAMP inhibited the action of EGF to stimulate the proliferation of MDCK cells[77] and mesangial cells[78] by inhibiting the MAP kinase cascade. However, in cells derived from ADPKD, forskolin and cAMP agonists activated the ERK/MAPK cascade and stimulated cellular proliferation. The loss of polycystin-1 may be the cause of this phenotypic switch from cAMP-induced inhibition to cAMP-induced stimulation of cell proliferation. Cross-talk between intracellular Ca^{2+} and cAMP signaling pathways may, at least, be associated with this phenotype switch.[79] A precise molecular dissection of the action of cAMP-mediated cascade on the ERK/MAPK cascade in ADPKD will make clear how the loss of polycystins affects the cross-talk of protein kinases. Understanding this mechanism will lead to new drug development.

Hormones such as AVP, VIP, and secretin, and the PGE2 cycle regulating salt and water balance, may increase the severity of the disease by stimulating the proliferation of the abnormal cyst epithelial cells and promoting the secretion of fluid into the cysts. cAMP has been found to accumulate in animal models of renal cystic diseases, the PCK rat, and the *pcy* mouse.[80] The PCK rat, a model of human ARPKD, has a splicing mutation (IVS35-2AT) that skips exon 36 and leads to a frameshift in Pkhd1, the rat ortholog of PKHD1.[81] The *pcy* mouse is a model of the human autosomal recessive cystic kidney disease that is caused by a missense mutation in NPHP3, the gene mutated in adolescent NPH.[82] Both models have a defect in urine concentration and eventually progress to renal failure. Renal cAMP expression was significantly ($P < 0.001$) higher in PCK rats and CD1/*pcy* mice than in wild-type animals. The expression of aquaporin-2, a gene positively regulated by cAMP,[83] and VPV2R was also increased. A vasopressin V2 receptor (VPV2R) antagonist and OPC31260 administration lowered renal cAMP, inhibited disease development, and either halted the progression or caused the regression of established disease in both the PCK rat and the pcy mouse. This effect of OPC31260 was further confirmed in the partially targeted disruption of Pkd2 mice.[84] These results indicate that OPC31260 may be an effective treatment for ADPKD. Clinical trials of VPV2R antagonist for ADPKD is now underway in Japan, the United States, and Europe.

In summary, signaling cascades mediated by tyrosine kinases and cAMP are associated with the proliferative nature of cystic epithelial cells. Intervention to the aberrant signaling cascades in ADPKD may be promising, and clinical trials into this possibility will soon be launched.

Cell cycle and polycystin-1

Direct evidence that polycystin-1 regulates cell growth and differentiation comes from a study by Boletta et al.[85] Madin Darby canine kidney cell lines (MDCK) with stable overexpression of the full-length cDNA for human *PKD1* had a reduced rate of growth, were more resistant to apoptosis, and spontaneously formed branching tubules when cultured in three-dimensional collagen gels. A further study[86] showed that polycystin-1 sustains the cell cycle at the G0/G1 phase by inhibiting Cdk (cyclin-dependent kinases)-2 activity through upregulation of $p21^{waf1}$. Murine embryos that lack *Pkd1* have greatly reduced levels of STAT1 activation and $p21^{waf1}$ induction. Over-expression of polycystin-1 activates STAT1 by physically interacting with JAK2, and STAT1 is necessary for polycystin-1 to induce cell growth arrest. Furthermore, polycystin-1 induces STAT3 activation on which HGF-mediated tubulogenesis is dependent.[87] Both polycystin-1 and polycystin-2 are necessary for the activation of JAK2. The channel properties that result from the co-assembly of polycystin-1 and polycystin-2 at the plasma membrane could activate a signaling cascade, which in turn is responsible for the phosphorylation and activation of JAK2. These data suggest that PKD1 may regulate cell differentiation in normal development by the expression of $p21^{waf1}$. Several studies have implicated a role for polycystin-

1 in Wnt signaling. Wnt signaling modulates tubulogenesis at the adherens junction.[88] The C-terminal portion of polycystin-1 stabilizes β-catenin and activates the Wnt pathway.[89] However, a recent study shows that polycystin-1 modulates AP-1 but not Wnt signaling.[90] The roles of polycystin-1 and Wnt signaling are currently elusive.

PPARγ and polycystin-1

Most PKD1 knockout mice die in the midstages of embryonic development. One of the main causes for this mortality rate is cardiac abnormality. A comprehensive gene analysis by a microarray of wild-type and knockout fetal mice revealed that the expression of the peroxisome proliferator-activated receptors (PPAR) γ gene in knockout mice decreased to 1/6th of that in wild-type mice.[34] PPARγ is a nuclear receptor that after binding to a ligand translocates into the nucleus. Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors. PPARs play an important role in the general transcriptional control of numerous cellular processes, including lipid metabolism, glucose homeostasis, cell-cycle progression, cell differentiation, inflammation, and extracellular matrix remodeling. Three PPAR isoforms, designated PPARα, PPARβ, and PPARγ, have been cloned and are differentially expressed in several tissues, including the kidney. PPARγ primarily regulates lipid metabolism and modulates inflammation. PPARγ is the molecular target of the hypolipidemic fibrates, including bezafibrate and clofibrate. PPARγ participates in embryonic development, implantation, and bone formation. PPARγ is a key factor in adipogenesis, and also plays an important role in insulin sensitivity, cell-cycle regulation, and cell differentiation. PPARs regulate gene expression by binding as heterodimers with retinoid X receptors (RXRs) to specific response elements (PPREs) in the promoter regions of target genes. RXRs are also members of the nuclear hormone receptor superfamily that are activated following the binding of 9-*cis* retinoic acid.[91,92] PPARγ is highly expressed in adipose tissue and expressed at lower levels in the renal medulla, urinary bladder, skeletal muscle, liver, heart, vascular smooth muscle cells, endothelial cells, hepatic Stellate cells, bone marrow stromal cells, monocytes/macrophages, and malignant epithelial cells, including breast, colon, prostate, and bladder cancer cells. 15-deoxy-12,14 prostaglandin J2 is the endogenous PPARγ ligand. Unsaturated fatty acids are also weak PPARγ ligands. Furthermore, antidiabetic agents belonging to the thiazolidine family, such as pioglitazone, were discovered to be PPARγ agonists. PPARγ. agonists not only improve the insulin resistance of peripheral tissues, but also have many other functions, such as suppressing vascular smooth muscle cell proliferation, angiogenesis, and inflammation, and inducing the differentiation of cancer cells. Muto et al.[33] explored the possibility that a decrease in PPARγ. may be associated with the phenotype of *Pkd1* knockout mice. Pioglitazone was administered to the dam of *Pkd1* heterozygous knockout mice to conduct transplacental treatment. The homozygous fetuses' survival rate improved significantly and their cardiac abnormalities disappeared. Renal cyst formation was dramatically decreased[33] (see Fig. 1). Pioglitazone simultaneously improved the abnormal targeting of AJ proteins (see Fig. 2) and the constitutive activation of EGFR and Gab1 in the $Pkd1^{-/-}$ kidney. Recent studies have shown that PPARγ has potent effects on cell proliferation and cell cycling. PPARγ ligands can trigger cell-cycle arrest in NIH3T3 cells and HIB-1B cells.[93] PPARγ ligands can also induce the differentiation of breast cancer cells and liposarcoma.[94,95] PPARγ ligands (including troglitazone, ciglitazone, and 15dPGJ2) significantly inhibit growth in cultured human bladder cancer cells, and this action is associated with the induction of cyclin-dependent kinase inhibitors (p21 and p16) and a reduction of cyclin D expression.[96] Another study showed that activation of PPARγ results in colon cancer cell-cycle arrest (G1 phase) and differentiation.[97] These findings may suggest that PPARγ agonists have a compensatory effect on the affected pathway by the loss of polycystin-1.

Endothelial dysfunction in ADPKD

Both *PKD1* and *PKD2* are expressed in the endothelium and vascular smooth muscle lining human blood vessels.[34,98] Wild-type mice express *Pkd1* in the vessels, whereas knockout *Pkd1* mice show edema, localized hemorrhages,[33,34,98] and increased microvascular permeability.[34] Aged $Pkd1^{+/-}$ mice are characterized by an impaired acetylcholine (Ach)-induced endothelium-dependent relaxation of the aorta.[33] A similar impairment of endothelium-dependent vasorelaxation, contrasting with an intact response to exogenous nitric oxide (NO), has also been documented in normotensive ADPKD patients.[99] The endothelial defects in patients with ADPKD were associated with a major reduction in NO_x excretion and in constitutive isoforms of nitric oxide synthase (cNOS) activity in microdissected resistance vessels. Endothelial dysfunction and impaired cNOS activity in ADPKD may predispose a patient to hypertension, which is accompanied by a further sharp deterioration in endothelium-dependent relaxation. These data suggest that endothelial dysfunction, secondary to an impaired release of NO, appears to be a primary defect in ADPKD. NO is the molecular counterpart of the endothelium-derived relaxing factor.[100] In endothelial cells, NO is synthesized from L-arginine by the endothelial NO synthase (eNOS), a constitutively expressed enzyme that is encoded by the eNOS (NOS3) gene located on 7q36.[101] Once released, NO diffuses rapidly through cell membranes and relaxes smooth muscle cells through the production of guanosine 3′,5′-cyclic monophosphate (cGMP). Furthermore, NO inhibits platelet activation, regulates angiogenesis, and controls microvascular permeability.[100] The influence of eNOS on hypertension, coronary vasospasm, atherosclerosis and, most importantly, the progression of diabetic nephropathy,[102,103] led to the hypothesis that it could be a modifier

gene in ADPKD. eNOS has a polymorphism at Glu298. The Glu298Asp is conserved among species,[104] whereas the Asp298 eNOS may yield a cleaved protein and decreased enzymatic activity.[105] Persu et al.[106] assessed the effect of eNOS on renal disease progression in ADPKD in a large series of unrelated Caucasian and Asian patients. The Glu298Asp (E/D298) polymorphism of exon 7 of eNOS was associated with a significantly faster (by 5 years) onset of ESRD in the group of ADPKD males, as well as a lower renal survival in males from *PKD1*-linked families. Thus, lower enzymatic activity and/or partial cleavage of eNOS could be responsible for increased endothelial dysfunction, and possibly for increased systemic blood pressure and/or an alteration of intrarenal microcirculation in ADPKD patients harboring the Asp298 allele. Long-term pioglitazone treatment improved the endothelial dysfunction of *Pkd1*$^{+/-}$.[33] These finding may suggest that PPAR agonists can be effective against the hypertension and endothelial dysfunction of ADPKD. Currently in Japan, pioglitazone is the only available thiazolidine. It has few side effects, and long-term administration of over a year is also possible. A clinical trial is expected to be conducted in the near future.

In ADPKD, an activation of the systemic renin–angiotensin system is also associated with the early onset of hypertension.[107–109] In addition to the hypertensive action, angiotensin II has also been shown to have independent proinflammatory and profibrotic effects in many forms of inflammatory renal diseases, actions that typically involve the activation of TGF-β,[110] monocyte chemotactic protein-1 (MCP-1)-releasing action,[111] and collagen synthesis stimulating action.[112] This interstitial fibrosis accelerates the progression of renal failure. Recently, a study was carried out by a national consortium to compare the renoprotective effects of angiotensin receptor blockade (ARB) and calcium channel blocker in hypertensive ADPKD patients. The results showed that ARB has a renoprotective effect in curbing the progress of renal failure in ADPKD, whereas CCB does not.[113] Among the several forms of ARB, telmisartan, a unique angiotensin II receptor antagonist with selective PPARγ-modulating activity, may be promising for the treatment of hypertensive ADPKD patients owing to its dual action in inhibiting the AII cascade and improving endothelial dysfunction.[114]

Conclusions

Genes responsible for ADPKD have been identified, and pathological processes have also been elucidated at the molecular level. cAMP and tyrosine kinases have been proposed as the key molecules that mediate cystogenesis in ADPKD. Intervening in these signaling pathways will be the targets for the pharmacological treatment of ADPKD. PPARγ agonists in vivo improved the phenotypes and molecular defects of *Pkd1* homozygous mutant mice. Signaling cascades mediated by PPARγ may complement the loss of polycystin-1 in the target organ. PPARγ agonists can also be potential drug candidates for the treatment of ADPKD. The extensive amount of research work since the cloning of PKD1 has now reached a stage where clinical trials will be launched. Further progress in the science and research for a cure for ADPKD will only be made possible by the participation of young diligent researchers.

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