

As shown in Fig. 1A, treatment of cells with gefitinib decreased the level of not only HSP70 but also HSP90 in a dose-dependent manner; however, the decrease was only clearly observed for HSP70 and not for HSP90, at the concentration of 1 μ M. In contrast, levels of other HSPs (HSP27, HSP47 and HSP60) were not affected by treatment with gefitinib (Fig. 1A). Erlotinib is another inhibitor of EGFR used clinically [42] and here we found that this drug also decreases the levels of HSP70 and HSP90 (Fig. 1B), suggesting that the inhibitory effect of gefitinib on EGFR is involved in the decrease in the level of HSP.

Real-time RT-PCR analysis revealed that treatment of cells with gefitinib at 1 μ M, a concentration determined to be sufficient to decrease the level of HSP70 (Fig. 1A), did not affect the *hsp70* mRNA level; however, this drug decreased the level at concentrations higher than 10 μ M (Fig. 1C).

Suppression of translation of HSP70 by gefitinib

The cellular level of protein is regulated by various factors, such as transcription, mRNA stability, translation and protein stability.

With these possibilities in mind, we then examined which of these factors is affected by gefitinib in relation to HSP70. The results in Fig. 1C suggest that 1 μ M gefitinib does not affect the transcription and mRNA stability of *hsp70*.

To confirm this, a luciferase reporter assay was used to examine the effect of gefitinib on the promoter activity of *hsp70*. Treatment of cells with gefitinib did not affect the luciferase activity in cells carrying a reporter plasmid with an *hsp70* promoter or an HSE inserted upstream of the *luciferase* gene (Fig. 2A), suggesting that gefitinib does not affect the promoter activity of *hsp70*. Further to this, 1 μ M gefitinib did not affect the *hsp70* mRNA level in cells pre-treated with actinomycin D, an inhibitor of transcription (Fig. 2B), suggesting that treatment of cells with 1 μ M gefitinib does not affect *hsp70* mRNA stability. The results in Fig. 1A and B also suggest that treatment of cells with 10 μ M gefitinib affects *hsp70* mRNA stability but not promoter activity.

We then tested whether gefitinib affects the translation and degradation of HSP70. As shown in Fig. 2C, the decrease in the level of HSP70 with 1 μ M gefitinib was not observed in cells pre-

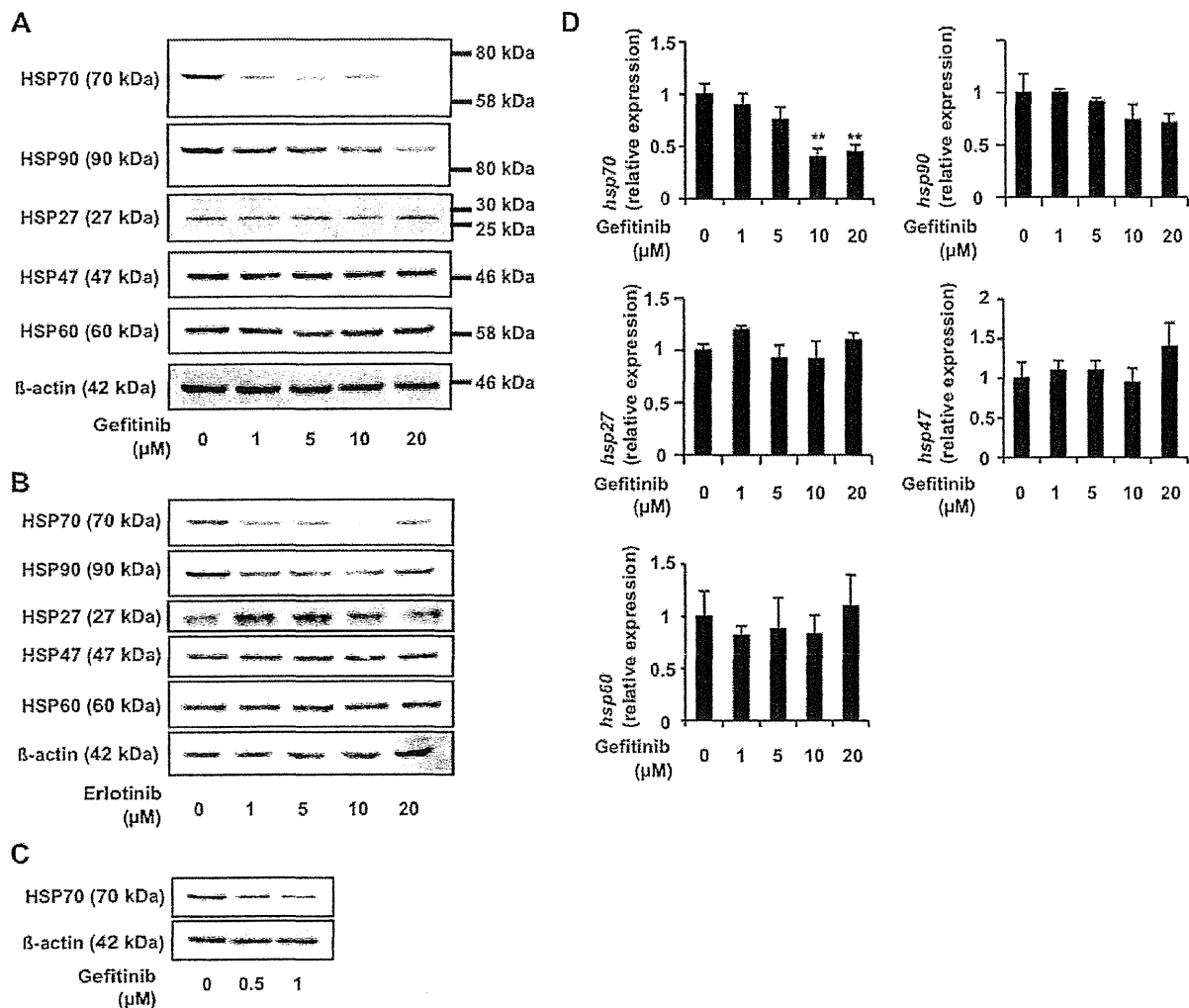


Figure 1. Suppression of expression of HSPs by gefitinib. A549 cells were incubated with the indicated concentration of gefitinib (A, C) or erlotinib (B) for 24 h (A, B) or 12 h (C). Whole-cell extracts were analyzed by immunoblotting with an antibody against HSP70, HSP90, HSP27, HSP47, HSP60 or actin (A, B). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer set for each gene. Values were normalized to the *actin* gene, expressed relative to the control sample (C). Values shown are mean \pm S.D. ($n=3$). ** $P<0.01$. doi:10.1371/journal.pone.0027296.g001

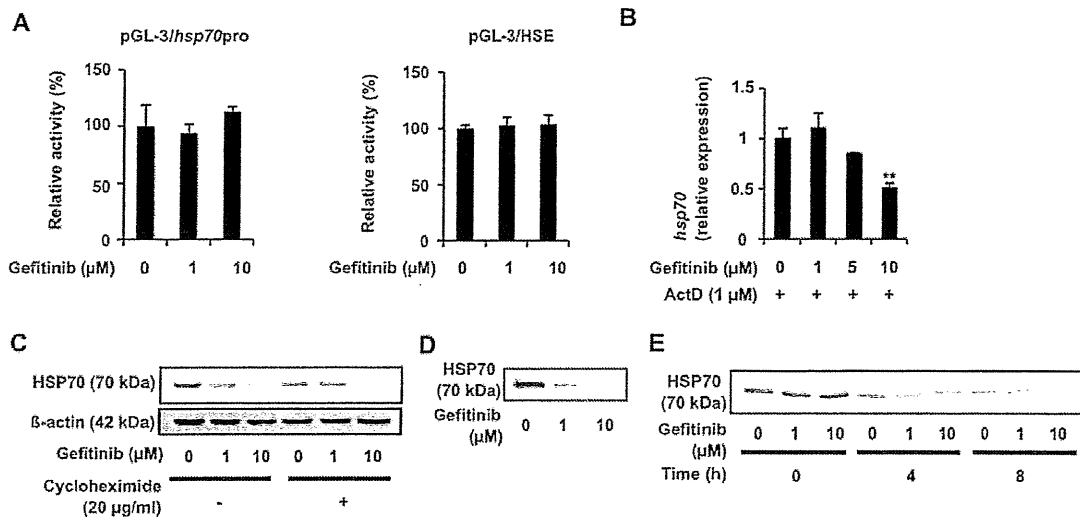


Figure 2. Translational regulation of expression of HSP70 by gefitinib. A549 cells were co-transfected with pRL-SV40 (internal control plasmid carrying the *R. reniformis luciferase* gene) and a pGL-3 derivative (pGL-3/*hsp70*pro or pGL-3/HSE) and cultured for 24 h. Cells were incubated with the indicated concentration of gefitinib for 24 h and *P. pyralis luciferase* activity was measured and normalized for *R. reniformis luciferase* activity. The 100% value of the *P. pyralis luciferase* activity is 6.9×10^4 or 5.8×10^4 units for pGL-3/*hsp70*pro or pGL-3/HSE, respectively (A). A549 cells were pre-incubated with 1 μg/ml actinomycin D (ActD) (B) or 20 μg/ml cycloheximide (C) for 1 h and further incubated for 8 h (B) or 24 h (C) with the indicated concentration of gefitinib (B, C). The mRNA (B) and protein (C) expression was monitored and is expressed as described in the legend of Fig. 1 A549 cells were pulse-labelled for 15 min with [³⁵S]methionine and [³⁵S]cysteine (D, E). Before the pulse-labelling, cells were incubated with the indicated concentration of gefitinib for 8 h (D). Pulse-labelled proteins were chased with excess amounts of Non-radioactively labeled methionine and cysteine for the indicated period in the presence of the indicated concentration of gefitinib (E). Labelled proteins were extracted, immunoprecipitated with antibody against HSP70, subjected to SDS-PAGE and autoradiographed (D, E). Values are mean \pm S.D. ($n=3$). ** $P<0.01$. doi:10.1371/journal.pone.0027296.g002

treated with cycloheximide, an inhibitor of protein synthesis. Furthermore, a protein pulse-labelling experiment showed that the synthesis of HSP70 was inhibited in cells pre-treated with gefitinib (Fig. 2D). These results suggest that 1 μM gefitinib inhibits the translation of HSP70. On the other hand, the results of the pulse-chase experiment suggest that treatment of cells with 1 μM gefitinib does not affect the stability of HSP70; 1 μM gefitinib did not affect the level of labelled HSP70 after chase periods of 4 h or 8 h (Fig. 2E). The results in Fig. 2E also suggest that treatment of cells with 10 μM gefitinib stimulates the degradation of HSP70.

3' UTRs of genes play an important role in translational regulation, including that by miRNAs. Thus, we examined the contribution of this region to gefitinib-induced suppression of expression of HSP70 by a luciferase reporter assay. Treatment of cells with gefitinib significantly decreased the luciferase activity in cells carrying the reporter plasmid in which the 3' UTR of *hsp70* was inserted downstream of the *luciferase* gene (Fig. 3A), suggesting that 3' UTR-mediated modulation of HSP70 translation plays an important role in the gefitinib-induced suppression of HSP70 expression.

The contribution of miRNAs to gefitinib-induced suppression of HSP70 expression was then tested by use of siRNA for Dicer1, a protein essential for maturation of miRNAs. As shown in Fig. 3B, transfection of cells with siRNA for Dicer1 decreased the level of *Dicer1* mRNA, but not that of *hsp70*, suggesting that the miRNA system is not involved in the regulation of *hsp70* mRNA stability. The decrease in the level of HSP70 and inhibition of HSP70 protein synthesis in the presence of 1 μM gefitinib were not observed in cells transfected with siRNA for Dicer1 (Fig. 3C and D), suggesting that the 1 μM gefitinib-dependent inhibition of HSP70 translation is mediated by the miRNA system. However, the 10 μM gefitinib-dependent decrease in the level of HSP70 and

inhibition of protein synthesis of HSP70 were observed even in cells transfected with siRNA for Dicer1 (Fig. 3C and D).

Next, using a database, we searched for miRNAs with sequences complementary to the 3' UTR of *hsp70*. Six candidate miRNAs were found (miR-146a, miR-146b-5b, miR-223*, miR-561, miR-449a and miR-449b). Real-time RT-PCR analysis revealed that among these miRNAs, the levels of miR-146a and miR-146b-5b clearly increased after treatment of cells with 1 μM gefitinib (we used experimental conditions of real-time RT-PCR under which only fully processed miRNA could be detected) (Fig. 4A). Furthermore, transfection of cells with siRNA for Dicer1 suppressed this gefitinib-dependent increase in the levels of these miRNAs (miR-146a, miR-146b-5b) (Fig. 4B) and transfection of cells with synthesized miR-146a and miR-146b-5b mimic RNA fragments decreased the level of HSP70 (Fig. 4C and D) but not that of *hsp70* mRNA (Fig. 4E). These results suggest that a gefitinib-dependent increase in the levels of miR-146a and miR-146b-5b is involved in gefitinib-dependent inhibition of HSP70 translation.

Involvement of EGFR and JNK inhibition in gefitinib-dependent suppression of expression of HSP70

As described above, gefitinib inhibits self-phosphorylation of tyrosine residues in the cytosolic domains of EGFR. Thus, here we tested whether or not gefitinib suppresses expression of HSP70 through inhibition of the EGFR. To do this we used the cell lines H1975 and PC9 which are resistant or sensitive, respectively, to gefitinib-induced inhibition of the self-phosphorylation of EGFR and the resulting modulation of intracellular signal transduction due to mutations in the EGFR [43], [44]. As shown in Fig. 5A, the concentration of gefitinib required for suppression of HSP70 expression was higher and lower in H1975 cells and PC9 cells,

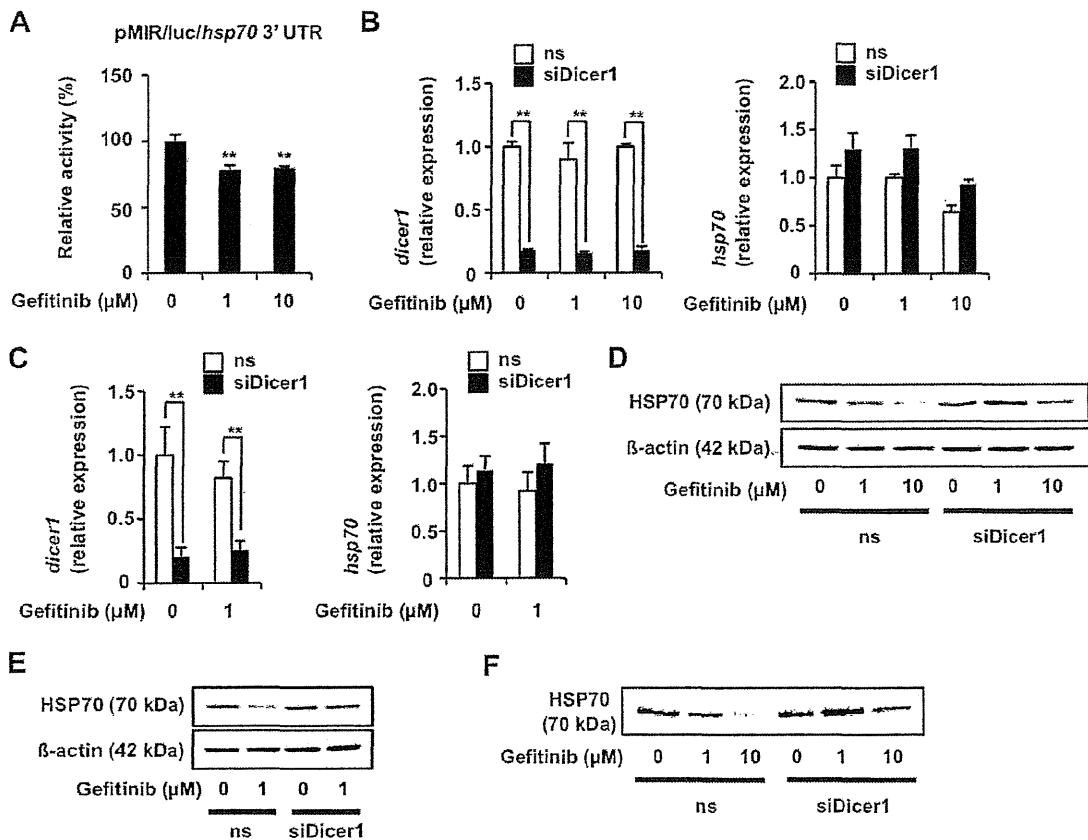


Figure 3. Contribution of miRNA to gefitinib-dependent suppression of expression of HSP70. A549 cells were co-transfected with pRL-SV40 and pMIR/luc/hsp70 3' UTR and cultured for 24 h. Cells were incubated with the indicated concentration of gefitinib for 24 h and luciferase reporter assay was done as described in the legend of Fig. 2. The 100% value of the *P. pyralis* luciferase activity is 1.2×10^5 units (A). A549 cells were transfected with siRNA for Dicer1 (siDicer1) or non-specific siRNA (ns) (B-D). After 24 h, cells were incubated with the indicated concentration of gefitinib for 12 h (B), 24 h (C) or 8 h (D). The mRNA (B) and protein (C) expression was monitored and is expressed as described in the legend of Fig. 1. Pulse-labelling experiments were performed as described in the legend of Fig. 2 (D). Values shown are mean \pm S.D. ($n=3$). ** $P<0.01$; n.s., not significant.

doi:10.1371/journal.pone.0027296.g003

respectively, than for A549 cells, suggesting that gefitinib suppresses expression of HSP70 through an inhibitory effect on the self-phosphorylation of EGFR.

Activation of the EGFR affects various cellular responses through modulation of intracellular signal transduction, such as activation of JNK and ERK [45], [46]. To test whether inhibition of JNK or ERK is involved in gefitinib-dependent suppression of HSP70 expression, we examined the effect of inhibitors of JNK and ERK, SP600125 and U0126, respectively, on the expression of HSP70. Treatment of cells with SP600125 (Fig. 5B) but not U0126 (data not shown) decreased the level of HSP70, suggesting that the inhibition of JNK is involved in the gefitinib-dependent suppression of HSP70 expression.

Effect of gefitinib on bleomycin-induced pulmonary fibrosis

As described in the Introduction, we recently reported that expression of HSP70 protects against bleomycin-induced pulmonary fibrosis [35]. Therefore, the *in vitro* results outlined above imply that gefitinib exacerbates bleomycin-induced pulmonary fibrosis through suppression of HSP70 expression. To test this, we examined the effect of oral daily administration of gefitinib on the

expression of HSP70 in the lungs. As shown in Fig. S2, this administration decreases the level of HSP70. The decrease became apparent at day 3 and was also observed at day 6 (Fig. S2). Therefore, to examine the effect of administration of gefitinib on bleomycin-induced pulmonary fibrosis, mice were orally administered gefitinib once per day for three days before receiving a single intratracheal administration of bleomycin; the administration of gefitinib was then continued, once every two days, for the following 14 days.

Histopathological analysis of pulmonary tissue using hematoxylin and eosin (H & E) staining revealed that the simultaneous oral administration of gefitinib (200 mg/kg) and intratracheal administration of bleomycin (1 mg/kg) produced severe pulmonary damage (thickened and edematous alveolar walls and interstitium, and infiltration of leucocytes) (Fig. 6A). Administration of either gefitinib or bleomycin alone did not cause such clear-cut pulmonary damage (Fig. 6A). Masson's trichrome staining of collagen revealed that administration of bleomycin caused slight collagen deposition, an effect that was greatly exacerbated by simultaneous administration of gefitinib (Fig. 6B). The pulmonary hydroxyproline level (an indicator of collagen levels) was increased by administration of bleomycin alone, and this effect was further

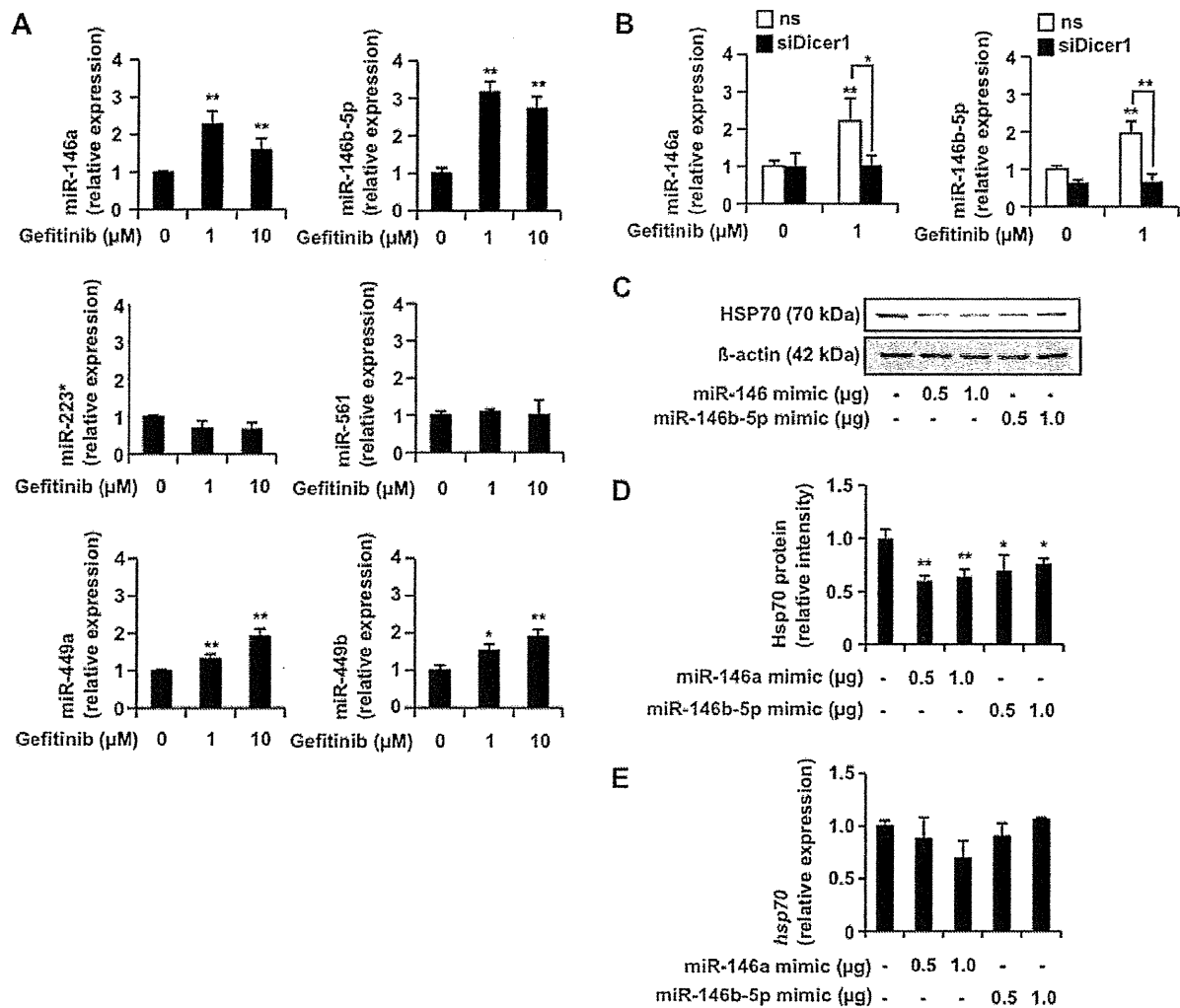


Figure 4. Identification of miRNAs involved in gefitinib-dependent suppression of expression of HSP70. A549 cells were incubated with the indicated concentration of gefitinib for 3 h (A). A549 cells were transfected with siRNA for Dicer1 (siDicer1) or non-specific siRNA (ns) and after 24 h were incubated with the indicated concentration of gefitinib for 3 h (B). A549 cells were transfected with the indicated amount (μg/well) of miRNA mimic RNA fragments for 24 h (C, E). The RNA (A, B, E) and protein (C) expression was monitored and expressed as described in the legend of Fig. 1. The RUN44 non-coding RNA was used for normalization (A, B, E). The intensities of the HSP70 bands were determined and are expressed relative to the control (one of the gels is shown in C) (D). Values shown are mean ± S.D. (n=3). **P<0.01; *P<0.05; n.s., not significant. doi:10.1371/journal.pone.0027296.g004

enhanced by the simultaneous administration of gefitinib (Fig. 6E). Under our experimental conditions, administration of gefitinib alone did not cause collagen deposition nor increase the level of pulmonary hydroxyproline (Fig. 6B and E).

We also performed similar experiments in transgenic mice expressing HSP70. Since these mice are resistant to bleomycin, we used a higher dose (2 mg/kg) of bleomycin than in the previously described experiments. As shown in Fig. 6C, D and F, the gefitinib-dependent enhancement of bleomycin-induced pulmonary damage, collagen deposition and increase in pulmonary hydroxyproline level were not clearly observed in the transgenic mice, suggesting that gefitinib exacerbates bleomycin-induced pulmonary fibrosis through the suppression of HSP70 expression.

To test this idea, we examined the effect of the administration of gefitinib and/or bleomycin on pulmonary expression of HSP70 in wild-type mice and transgenic mice expressing HSP70. To begin

with, we confirmed the overexpression of HSP70 in the lungs of transgenic mice in both the presence and absence of gefitinib administration (Fig. 6G). As shown in Fig. 6H and I, in wild-type mice, administration of gefitinib decreased the pulmonary level of HSP70 in both the presence and absence of simultaneous bleomycin administration. Administration of bleomycin alone also decreased the pulmonary level of HSP70 (Fig. 6H and I). In contrast, such a gefitinib-dependent decrease in the pulmonary level of HSP70 was not observed clearly in transgenic mice expressing HSP70 in both the presence or absence of simultaneous administration of bleomycin (Fig. 6J). We also found that using a lower dose of gefitinib (100 mg/kg) neither exacerbated bleomycin-induced pulmonary fibrosis nor suppressed pulmonary expression of HSP70 (Fig. S3). These results suggest that administration of gefitinib exacerbates bleomycin-induced pulmonary fibrosis through suppression of HSP70 expression.

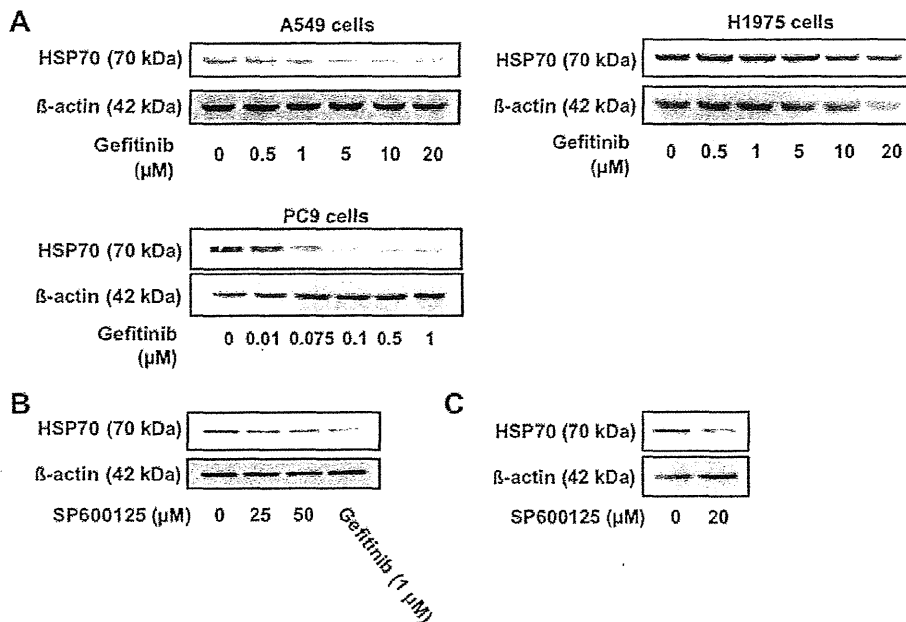


Figure 5. Involvement of inhibition of EGFR and JNK in gefitinib-dependent suppression of expression of HSP70. A549 (A-C) and H1975 and PC9 (A) cells were incubated with the indicated concentration of gefitinib (A, B) or SP600125 (B) for 24 h. Protein expression was monitored and is expressed as described in the legend of Fig. 1.
doi:10.1371/journal.pone.0027296.g005

For further confirmation of this idea, we examined the effect of co-administration of GGA. As shown in Fig. 7A-C, co-administration of GGA clearly suppressed the gefitinib-dependent enhancement of bleomycin-induced pulmonary tissue damage, collagen deposition and increases in the pulmonary hydroxyproline level in wild-type mice. We also examined the effect of GGA on the expression of HSP70 in the lung. As shown in Fig. 7D and E, co-administration of GGA recovered the gefitinib-suppressed expression of HSP70 in the lung, suggesting that GGA suppresses the gefitinib-dependent exacerbation of bleomycin-induced pulmonary fibrosis by inducing the recovery of HSP70 expression.

Discussion

Because the molecular mechanism governing drug-induced ILD (interstitial pneumonia associated with pulmonary fibrosis) is unknown, a suitable animal model is not available at present. Consequently, neither a clinical protocol for the treatment of drug-induced ILD nor an assay system to eliminate candidate drugs with the potential to cause this type of side effect has been established. We recently reported that A771726, an active metabolite of leflunomide, induced EMT of lung epithelial cells both *in vitro* and *in vivo*, and that administration of leflunomide exacerbated bleomycin-induced pulmonary fibrosis, suggesting that the leflunomide-dependent exacerbation of bleomycin-induced pulmonary fibrosis is mediated by the stimulation of EMT of lung epithelial cells. We proposed that examination of the EMT-inducing ability of candidate drugs is useful for screening to eliminate those with the potential side effect of inducing pulmonary fibrosis. We also found that leflunomide-dependent exacerbation of bleomycin-induced pulmonary fibrosis is ameliorated by the simultaneous intratracheal administration of uridine, which suppresses the A771726-dependent induction of EMT *in vitro*, and propose that this administration is beneficial for the

treatment of leflunomide-induced pulmonary fibrosis in humans. In the present study, we followed a similar strategy, focusing on the expression of HSP70, because we had recently reported that expression of HSP70 protects against bleomycin-induced pulmonary fibrosis through cytoprotective and anti-inflammatory effects and, by inhibiting the production of TGF- β 1 and TGF- β 1-dependent EMT of lung epithelial cells [35].

We have found that gefitinib suppresses the expression of HSP70 *in vitro*. The concentration of gefitinib used (1 μ M) is similar to that obtained in plasma when administered at therapeutic levels (about 1-2 μ M) [47]. Thus, we considered that the suppression of HSP70 expression by gefitinib is clinically relevant and investigated the molecular mechanism underlying this effect. We concluded that treatment of cells with gefitinib (1 μ M) decreases the level of HSP70 through inhibition of translation based on observations that (i) the gefitinib-dependent decrease in the level of HSP70 was suppressed by an inhibitor of translation, (ii) the translation of HSP70, measured by pulse-labelling experiments, was inhibited by treatment of cells with gefitinib, and (iii) *hsp70* promoter activity, the level and stability of *hsp70* mRNA and the stability of HSP70 were all unaffected by treatment of cells with gefitinib. Furthermore, we suggested that two miRNAs (miR-146a and miR-146b-5p) are involved in this gefitinib-dependent suppression of HSP70 translation; this was based on our observations that (i) the introduction of the 3' UTR of *hsp70* into a luciferase reporter plasmid caused a gefitinib-dependent decrease in luciferase activity, (ii) the gefitinib-dependent suppression of HSP70 translation was not observed in cells transfected with siRNA for Dicer1, and (iii) gefitinib increased the levels of miR-146a and miR-146b-5p, both of which have the ability to decrease the level of HSP70. Since for various cancer cells and tissues, down-regulation of expression of these miRNAs and up-regulation of HSP70 and the contribution of these to cancer progression have been reported [48], [49], [50], the results

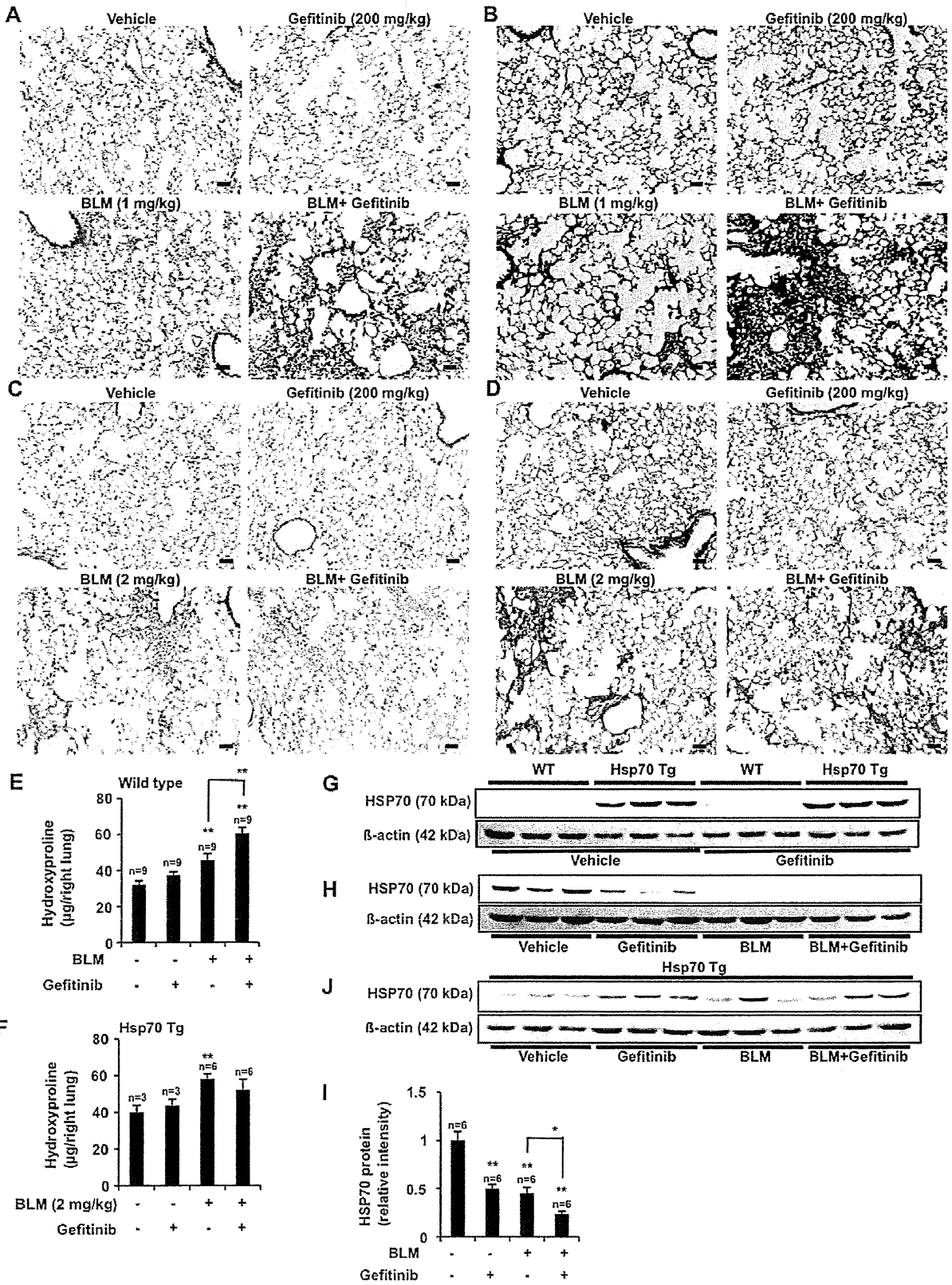


Figure 6. Effect of gefitinib on bleomycin-induced pulmonary fibrosis and pulmonary expression of HSP70. Wild-type mice (A, B, E, G, H) and transgenic mice expressing HSP70 (C, D, F, G, J) were orally administered gefitinib (200 mg/kg) or vehicle once per day for 3 days (from day 0 to day 2), then treated once only with or without 1 mg/kg (for wild-type mice) or 2 mg/kg (for the transgenic mice) bleomycin (BLM) (day 3). Mice were then orally administered gefitinib (200 mg/kg) or vehicle once per 2 days for 14 days (from day 3 to day 17). Sections of pulmonary tissue were prepared at day 17 and subjected to histological examination (H & E staining (A, C) or Masson's trichrome staining (B, D)) (scale bar, 50 μ m) (A-D). Pulmonary hydroxyproline levels at day 17 were determined (E, F). Total protein was extracted from pulmonary tissues at day 17 and analyzed by immunoblotting with an antibody against HSP70 or actin (G, H, J). The intensities of the HSP70 bands were determined (one of the gels is shown in H) and are expressed relative to the control (I). Values are mean \pm S.E.M. * P <0.05; ** P <0.01; n.s., not significant. doi:10.1371/journal.pone.0027296.g006

of this study suggest the latter up-regulation is caused by the former down-regulation.

A decrease in the level of HSP70 was also observed in cells treated with 10 μ M gefitinib; however, the mechanism governing

this decrease seems to be different from that seen with 1 μ M gefitinib, because treatment of cells with 10 μ M gefitinib caused a decrease in the level of *hsp70* mRNA and in the stability of both *hsp70* mRNA and HSP70. We consider that this high con-

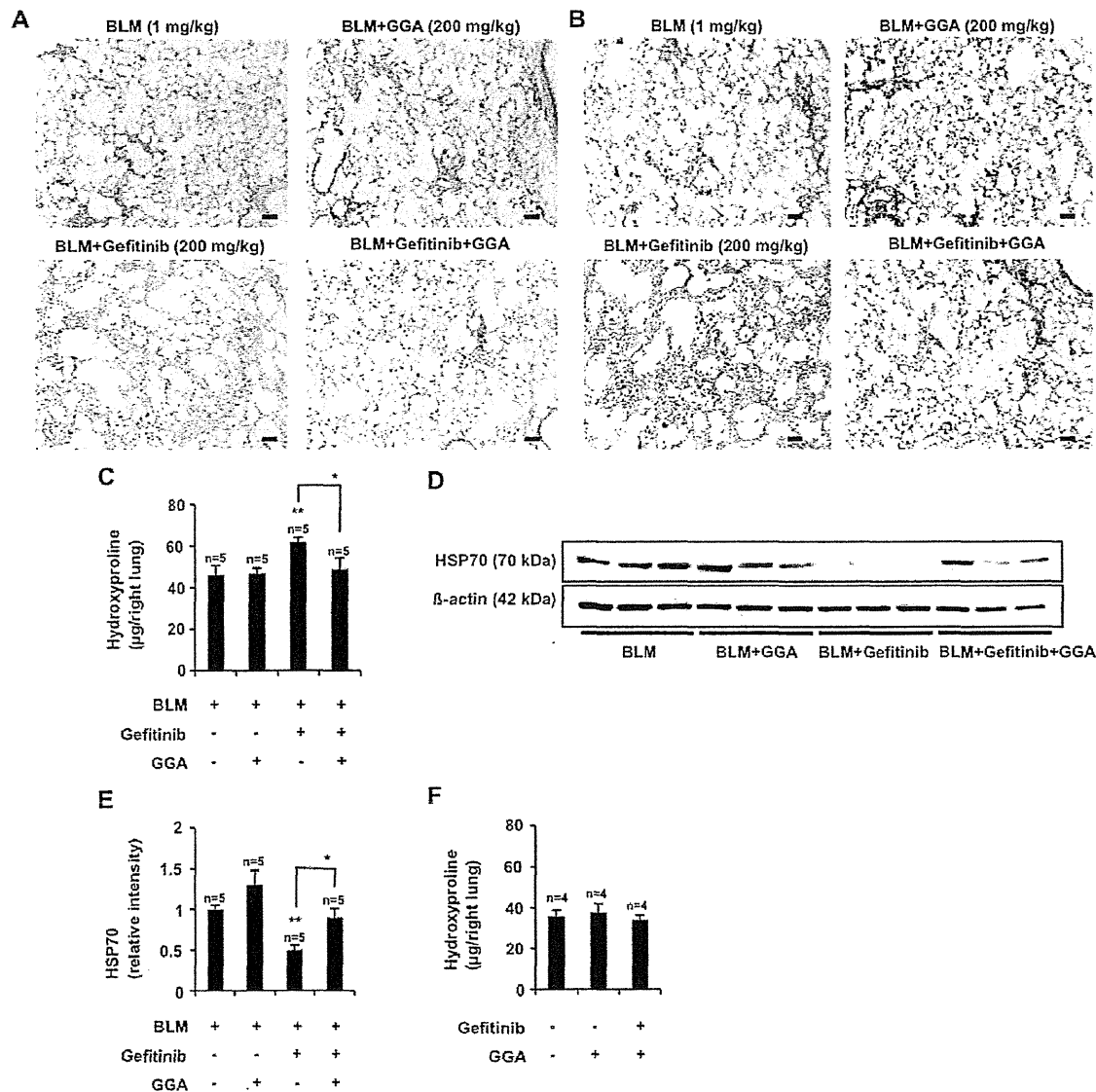


Figure 7. Effect of GGA on gefitinib-dependent exacerbation of pulmonary fibrosis and pulmonary expression of HSP70. Wild-type mice were orally administered gefitinib (200 mg/kg) and/or GGA (200 mg/kg) once per day for 3 days (from day 0 to day 2), then treated once only with 1 mg/kg bleomycin (BLM) (day 3). Mice were then orally administered gefitinib (200 mg/kg) and/or GGA (200 mg/kg) once per 2 days for 14 days (from day 3 to day 17). Sections of pulmonary tissue were prepared at day 17 and subjected to histological examination (H & E staining (A) or Masson's trichrome staining (B)) (scale bar, 50 μ m). The pulmonary hydroxyproline levels were determined at day 17 (C). Total protein was extracted from pulmonary tissues at day 17 and analyzed and expressed as described in the legend of Fig. 6 (D, E). Values are mean \pm S.E.M. ** P <0.01; * P <0.05. doi:10.1371/journal.pone.0027296.g007

centration of gefitinib pleiotropically affects various cellular reactions.

After binding EGF, the EGFR is self-phosphorylated and transduces the signal via various mechanisms, including the activation of JNK. Here, we have found that mutations in the EGFR that alter the concentration of gefitinib required for inhibition of EGFR self-phosphorylation affect the gefitinib-dependent decrease in the level of HSP70. Furthermore, an inhibitor of JNK caused a decrease in the level of HSP70. These results suggest that inhibition of EGFR self-phosphorylation and the resulting inhibition of JNK are involved in the gefitinib-dependent decrease in the level of HSP70. However, the manner by which inhibition of JNK suppresses translation of HSP70 remains unclear.

Administration of gefitinib alone did not produce pulmonary fibrosis under our experimental conditions. As one of the risk factors for gefitinib-induced ILD is pre-existing pulmonary fibrosis, we hypothesized that gefitinib stimulates pulmonary fibrosis in the presence of other fibrosis-inducing stimuli. In fact, we found that administration of gefitinib stimulates bleomycin-induced pulmonary fibrosis. Interestingly, this gefitinib-dependent stimulation was not observed so clearly in transgenic mice expressing HSP70, suggesting that expression of HSP70 is involved in the gefitinib-induced exacerbation of bleomycin-induced pulmonary fibrosis. We found that administration of gefitinib suppressed the pulmonary expression of HSP70 and that this suppression was not observed in the transgenic mice expressing HSP70. Taken together, our findings suggest that gefitinib exacerbates bleomycin-induced pulmonary fibrosis through the suppression of HSP70 expression. This finding is an important step towards elucidating the molecular mechanism of drug-induced ILD, as well as the mechanism governing the ethnic differences in susceptibility to ILD induced by the drug. It is possible that the susceptible phenotype of Japanese patients is due to a specific polymorphism in the *hsp70* and *hsp1* genes and in other genes related to HSP70. Also, examination of the effect of candidate drugs on the expression of HSP70 *in vitro* could be used for screening to eliminate those drugs with the potential to induce ILD. Furthermore, exacerbation of bleomycin-induced pulmonary fibrosis was observed with leflunomide [7] and gefitinib (this study), suggesting this model can be used as an animal model of

drug-induced pulmonary fibrosis and for screening to eliminate candidate drugs with the potential to induce ILD.

As described above, it was recently reported that administration of GGA ameliorates bleomycin-induced pulmonary fibrosis [51], a result that we confirmed in a previous paper [35]. In this study, we found that administration of GGA suppresses the gefitinib-dependent exacerbation of bleomycin-induced pulmonary fibrosis. We consider that this suppression is due to the recovery of HSP70 expression in the lung. As described in the Introduction, a treatment for drug-induced ILD has not been established. We believe that GGA could be beneficial for the treatment of gefitinib-induced ILD, given that the safety of GGA has already been shown clinically.

Supporting Information

Figure S1 Effects of drugs known to induce ILD clinically on expression of HSP70. A549 cells were incubated with the indicated concentration of A771726, amiodarone (AMD), gefitinib or imatinib for 24 h. Whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or β -actin. (TIFF)

Figure S2 Time course profile for gefitinib-dependent suppression of expression of HSP70 *in vivo*. Mice were orally administered gefitinib (200 mg/kg) or vehicle once per day for the indicated periods. Total protein was extracted from pulmonary tissues and protein expression was monitored by immunoblotting with an antibody against HSP70 or β -actin. (TIFF)

Figure S3 Effect of low dose of gefitinib on bleomycin-induced pulmonary fibrosis and pulmonary expression of HSP70. The effect of a low dose of gefitinib (100 mg/kg) on the expression of HSP70 in the lung (A) and pulmonary hydroxyproline levels (B) were monitored as described in the legend of Fig. 6. Values are mean \pm S.E.M. (TIFF)

Author Contributions

Performed the experiments: TN TH TM. Analyzed the data: TN KT TM. Contributed reagents/materials/analysis tools: TN AA TM. Wrote the paper: TN TM.

References

- Olson AL, Swigris JJ, Lezotte DC, Norris JM, Wilson CG, et al. (2007) Mortality from pulmonary fibrosis increased in the United States from 1992 to 2003. *Am J Respir Crit Care Med* 176: 277–284.
- Fumeaux T, Kothmeier C, Joliet P (2001) Outcome of mechanical ventilation for acute respiratory failure in patients with pulmonary fibrosis. *Intensive Care Med* 27: 1868–1874.
- Camus P, Fanton A, Bonniaud P, Camus C, Foucher P (2004) Interstitial lung disease induced by drugs and radiation. *Respiration* 71: 301–326.
- Inoue A, Saijo Y, Maemondo M, Gomi K, Tokue Y, et al. (2003) Severe acute interstitial pneumonia and gefitinib. *Lancet* 361: 137–139.
- Flieder DB, Travis WD (2004) Pathologic characteristics of drug-induced lung disease. *Clin Chest Med* 25: 37–45.
- Moore BB, Hogaboam CM (2008) Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 294: L152–160.
- Namba T, Tanaka KI, Ito Y, Hoshino T, Matoyama M, et al. (2010) Induction of EMT-like phenotypes by an active metabolite of leflunomide and its contribution to pulmonary fibrosis. *Cell Death Differ* 17: 1882–1895.
- Sheppard D (2006) Transforming growth factor beta: a central modulator of pulmonary and airway inflammation and fibrosis. *Proc Am Thorac Soc* 3: 413–417.
- Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, et al. (2007) The myofibroblast: one function, multiple origins. *Am J Pathol* 170: 1807–1816.
- Kisseleva T, Brenner DA (2008) Fibrogenesis of parenchymal organs. *Proc Am Thorac Soc* 5: 338–342.
- Willis BC, Borok Z (2007) TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 293: L525–534.
- Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z (2005) TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res* 6: 56.
- Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, et al. (2006) Alveolar epithelial cell mesenchymal transition develops *in vivo* during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci U S A* 103: 13180–13185.
- Kim KK, Wei Y, Szekeres C, Kugler MC, Wolters PJ, et al. (2009) Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *J Clin Invest* 119: 213–224.
- Blackledge G, Averbuch S (2004) Gefitinib (Iressa, ZD1839) and new epidermal growth factor receptor inhibitors. *Br J Cancer* 90: 566–572.
- Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 350: 2129–2139.
- Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, et al. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304: 1497–1500.
- Kataoka K, Taniguchi H, Hasegawa Y, Kondoh Y, Kimura T, et al. (2006) Interstitial lung disease associated with gefitinib. *Respir Med* 100: 698–704.
- Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, et al. (2010) Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 362: 2380–2388.
- Ando M, Okamoto I, Yamamoto N, Takeda K, Tamura K, et al. (2006) Predictive factors for interstitial lung disease, antitumor response, and survival in

- non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 24: 2549–2556.
21. Kudoh S, Kato H, Nishiwaki Y, Fukuoka M, Nakata K, et al. (2008) Interstitial lung disease in Japanese patients with lung cancer: a cohort and nested case-control study. *Am J Respir Crit Care Med* 177: 1348–1357.
 22. Suzuki H, Aoshiba K, Yokohori N, Nagai A (2003) Epidermal growth factor receptor tyrosine kinase inhibition augments a murine model of pulmonary fibrosis. *Cancer Res* 63: 5054–5059.
 23. Ishii Y, Fujimoto S, Fukuda T (2006) Gefitinib prevents bleomycin-induced lung fibrosis in mice. *Am J Respir Crit Care Med* 174: 550–556.
 24. Richter K, Haslbeck M, Buchner J (2010) The heat shock response: life on the verge of death. *Mol Cell* 40: 253–266.
 25. Mathew A, Morimoto RI (1998) Role of the heat-shock response in the life and death of proteins. *Ann N Y Acad Sci* 851: 99–111.
 26. Hirakawa T, Rokutan K, Nikawa T, Kishi K (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. *Gastroenterology* 111: 345–357.
 27. Tomisato W, Takahashi N, Komoto C, Rokutan K, Tsuchiya T, et al. (2000) Geranylgeranylacetone protects cultured guinea pig gastric mucosal cells from indomethacin. *Dig Dis Sci* 45: 1674–1679.
 28. Tang D, Kang K, Xiao W, Wang H, Calderwood SK, et al. (2007) The anti-inflammatory effects of heat shock protein 72 involve inhibition of high-mobility-group box 1 release and proinflammatory function in macrophages. *J Immunol* 179: 1236–1244.
 29. Suemasu S, Tanaka K, Namba T, Ishihara T, Katsu T, et al. (2009) A role for HSP70 in protecting against indomethacin-induced gastric lesions. *J Biol Chem* 284: 19705–19715.
 30. Matsuda M, Hoshino T, Yamashita Y, Tanaka K, Maji D, et al. (2010) Prevention of UVB radiation-induced epidermal damage by expression of heat shock protein 70. *J Biol Chem* 285: 5848–5858.
 31. Tanaka K, Tsutsumi S, Arai Y, Hoshino T, Suzuki K, et al. (2007) Genetic evidence for a protective role of heat shock factor 1 against irritant-induced gastric lesions. *Mol Pharmacol* 71: 985–993.
 32. Tanaka K, Namba T, Arai Y, Fujimoto M, Adachi H, et al. (2007) Genetic Evidence for a Protective Role for Heat Shock Factor 1 and Heat Shock Protein 70 against Colitis. *J Biol Chem* 282: 23240–23252.
 33. Asano T, Tanaka K, Yamakawa N, Adachi H, Sobue G, et al. (2009) HSP70 confers protection against indomethacin-induced lesions of the small intestine. *J Pharmacol Exp Ther* 330: 458–467.
 34. Hoshino T, Murao N, Namba T, Takehara M, Adachi H, et al. (2011) Suppression of Alzheimer's disease-related phenotypes by expression of heat shock protein 70 in mice. *J Neurosci* 31: 5225–5234.
 35. Tanaka K, Tanaka Y, Namba T, Azuma A, Mizushima T (2010) Heat shock protein 70 protects against bleomycin-induced pulmonary fibrosis in mice. *Biochem Pharmacol* 80: 920–931.
 36. Kim VN (2005) MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 6: 376–385.
 37. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, et al. (2010) miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J Exp Med* 207: 1589–1597.
 38. Namba T, Homan T, Nishimura T, Mima S, Hoshino T, et al. (2009) Up-regulation of S100P expression by non-steroidal anti-inflammatory drugs and its role in anti-tumorigenic effects. *J Biol Chem* 284: 4158–4167.
 39. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
 40. Chu CY, Rana TM (2006) Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS Biol* 4: e210.
 41. Tanaka K, Ishihara T, Azuma A, Kudoh S, Ebina M, et al. (2010) Therapeutic effect of lecithinized superoxide dismutase on bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 298: L348–360.
 42. Gridelli C, Bareschino MA, Schettino C, Rossi A, Maione P, et al. (2007) Erlotinib in non-small cell lung cancer treatment: current status and future development. *Oncologist* 12: 840–849.
 43. Ono M, Hirata A, Kometani T, Miyagawa M, Ueda S, et al. (2004) Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol Cancer Ther* 3: 465–472.
 44. Van Schaeybroeck S, Kyula J, Kelly DM, Karaiskou-McCaul A, Stokesberry SA, et al. (2006) Chemotherapy-induced epidermal growth factor receptor activation determines response to combined gefitinib/chemotherapy treatment in non-small cell lung cancer cells. *Mol Cancer Ther* 5: 1154–1165.
 45. Miyata H, Sasaki T, Kuwahara K, Serikawa M, Chayama K (2006) The effects of ZD1839 (Iressa), a highly selective EGFR tyrosine kinase inhibitor, as a radiosensitizer in bile duct carcinoma cell lines. *Int J Oncol* 28: 915–921.
 46. Moon DO, Kim MO, Lee JD, Choi YH, Lee MK, et al. (2007) Molecular mechanisms of ZD1839 (Iressa)-induced apoptosis in human leukemic U937 cells. *Acta Pharmacol Sin* 28: 1205–1214.
 47. Li J, Karlsson MO, Brahmeh J, Spitz A, Zhao M, et al. (2006) GYP3A phenotyping approach to predict systemic exposure to EGFR tyrosine kinase inhibitors. *J Natl Cancer Inst* 98: 1714–1723.
 48. Li Y, Vandenboom TG, 2nd, Wang Z, Kong D, Ali S, et al. (2010) miR-146a suppresses invasion of pancreatic cancer cells. *Cancer Res* 70: 1486–1495.
 49. Hurst DR, Edmonds MD, Scott GK, Benz CG, Vaidya KS, et al. (2009) Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. *Cancer Res* 69: 1279–1283.
 50. Leu JL, Pirmkina J, Frank A, Murphy MB, George DL (2009) A small molecule inhibitor of inducible heat shock protein 70. *Mol Cell* 36: 15–27.
 51. Fujibayashi T, Hashimoto N, Jijima M, Hasegawa Y, Kojima T, et al. (2009) Protective effect of geranylgeranylacetone, an inducer of heat shock protein 70, against drug-induced lung injury/fibrosis in an animal model. *BMJ Pulm Med* 9: 45.

ORIGINAL
ARTICLEImprovement of cognitive function in Alzheimer's disease model mice by genetic and pharmacological inhibition of the EP₄ receptor

Tatsuya Hoshino,*† Takushi Namba,† Masaya Takehara,† Naoya Murao,† Takahide Matsushima,‡ Yukihiko Sugimoto,† Shuh Narumiya,§ Toshiharu Suzuki‡ and Tohru Mizushima*†

*Faculty of Pharmacy, Keio University, Tokyo, Japan

†Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

‡Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

§Faculty of Medicine, Kyoto University, Kyoto, Japan

Abstract

Amyloid- β peptide (A β), which is generated by the β - and γ -secretase-mediated proteolysis of β -amyloid precursor protein (APP), plays an important role in the pathogenesis of Alzheimer's disease (AD). We recently reported that prostaglandin E₂ (PGE₂) stimulates the production of A β through both EP₂ and EP₄ receptors and that activation of the EP₄ receptor stimulates A β production through endocytosis and activation of γ -secretase. We here found that transgenic mice expressing mutant APP (APP23) mice showed a greater or lesser apparent cognitive deficit when they were crossed with mice lacking EP₂ or EP₄ receptors, respectively. Mice lacking the EP₄ receptor also displayed lower levels of A β plaque deposition and less neuronal and synaptic loss than control

mice. Oral administration of a specific EP₄ receptor antagonist, AE3-208 to APP23 mice, improved their cognitive performance, as well as decreasing brain levels of A β and suppressing endocytosis and activation of γ -secretase. Taken together, these results suggest that inhibition of the EP₄ receptor improves the cognitive function of APP23 mice by suppressing A β production and reducing neuronal and synaptic loss. We therefore propose that EP₄ receptor antagonists, such as AE3-208, could be therapeutically beneficial for the prevention and treatment of AD.

Keywords: Alzheimer disease, aging, inflammation, memory, neurodegeneration.

J. Neurochem. (2012) **120**, 795–805.

Alzheimer's disease (AD) is the most common neurodegenerative disorder of the central nervous system and the leading cause of adult onset dementia, affecting 5% of the population over the age of 65. Pathological characters of AD are accumulation of neurofibrillary tangles and senile plaques and senile plaques are composed of amyloid- β peptides (A β), such as A β 40 and A β 42 (Mattson 2004). In order to generate A β , β -amyloid precursor protein (APP) is first cleaved by β -secretase and then by γ -secretase (Sisodia and St George-Hyslop 2002). Monomeric A β easily self-assembles to form oligomers and protofibrils, which play an important role in the induction of the neuronal and synaptic loss that results in cognitive decline (Haass and Selkoe 2007). γ -Secretase is composed of four core components, including presenilin (PS)-1 and PS-2 (Haass 2004). Early onset familial AD is linked to three genes, *app*, *ps1* and *ps2* (Haass 2004),

strongly suggesting that A β is a key factor in the pathogenesis of AD. Consequently, cellular factors that affect the production of A β represent good targets for drugs to prevent or treat AD.

It has been suggested that inflammation is important in the pathogenesis of AD; chronic inflammation has been observed

Received July 28, 2011; revised manuscript received October 28, 2011; accepted October 28, 2011.

Address correspondence and reprint requests to Tohru Mizushima, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. E-mail: mizushima-th@pha.keio.ac.jp

Abbreviations used: A β , amyloid- β peptide; AD, Alzheimer's disease; APP, β -amyloid precursor protein; COX, cyclooxygenase; CTF, C-terminal fragment; LTP, long-term potentiation; NeuN, neuronal nuclei; NSAIDs, non-steroidal anti-inflammatory drugs; PGE₂, prostaglandin E₂; PKA, protein kinase A; PS, presenilin.

in the brains of AD patients, and trauma to the brain and ischemia, both of which can activate inflammation, are major risk factors for the disease (Ikonovic *et al.* 2004; Wyss-Coray 2006). Cyclooxygenase (COX), which exists as two subtypes, COX-1 and COX-2, is essential for the synthesis of prostaglandin E₂ (PGE₂), a potent inducer of inflammation. COX-1 is expressed constitutively, whereas COX-2 expression is induced under inflammatory conditions and is responsible for the progression of inflammation (Srinivasan and Kulkarni 1989; Smith *et al.* 1998). It has been suggested that the COX-2-mediated production of PGE₂ plays an important role in the pathogenesis of AD. For example, elevated levels of PGE₂ and over-expression of COX-2 have been observed in AD patient brains (Kitamura *et al.* 1999; Montine *et al.* 1999); the extent of COX-2 expression correlates with the degree of progression of AD pathogenesis (Ho *et al.* 2001); transgenic mice constitutively over-expressing COX-2 show aging-dependent memory dysfunction (Andreasson *et al.* 2001); PGE₂ stimulates the production of reactive oxygen species in microglia and activates β -secretase (Liang *et al.* 2005); and prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of COX, delays the onset and reduces the risk of AD (in t' Veld *et al.* 2001; Imbimbo *et al.* 2010). Thus, in order to identify molecular targets for the development of AD drugs, it is important to understand the molecular mechanism involved in the PGE₂-mediated progression of the disease.

We recently reported that PGE₂ stimulates the production of A β in cells stably expressing a form of APP with two mutations (K651N/M652L; APPsw) that elevates cellular and secreted levels of A β (Hoshino *et al.* 2007). Using agonists and antagonists specific for each of the four PGE₂ receptors (EP₁, EP₂, EP₃ and EP₄ receptors), we found that both EP₂ and EP₄ receptors are involved in the PGE₂-stimulated production of A β *in vitro* (Hoshino *et al.* 2007). With respect to the mechanism underpinning this stimulation, we also recently demonstrated that activation of the EP₂ receptor stimulates the production of A β through activation of adenylate cyclase, an increase in the cellular level of cAMP and activation of protein kinase A (PKA) (Hoshino *et al.* 2009). In contrast, EP₄ receptor activation causes its co-internalization with PS-1 (γ -secretase) into endosomes, a process that activates γ -secretase (Hoshino *et al.* 2009). Furthermore, we showed that deletion of the EP₂ or EP₄ receptor decreases brain levels of A β in transgenic mice expressing APPsw (APP23, a mouse model for AD), suggesting that EP₂ or EP₄ receptor activation stimulates the production of A β *in vivo* (Hoshino *et al.* 2007). These previous results suggest that EP₂ and/or EP₄ receptors could represent valuable molecular targets for the treatment of AD. However, the effect of deletion of these receptors on other AD-related phenotypes, such as neuronal and synaptic loss and cognitive deficits, has not been tested. The effect on cognitive performance is particularly important, because

functional phenotypes (cognitive dysfunction) and pathological phenotypes (such as an increase in the brain level of A β) are not always directly linked (Roberson *et al.* 2007; Kanninen *et al.* 2009). In this study, we therefore examined the effect of EP₂ and EP₄ receptor inhibition on cognitive function in APP23 mice, revealing that genetic inhibition of the EP₄ receptor but not the EP₂ receptor not only suppresses neuronal and synaptic loss but also improves cognitive performance. Similarly, oral administration of AE3-208, an EP₄ receptor-specific antagonist, improved the cognitive function of the APP23 mice. These results suggest that the EP₄ receptor is a valuable molecular target for the development of drugs to prevent or treat AD.

Materials and methods

Materials and animals

See Appendix S1.

Morris water maze test

The Morris water maze test was conducted in a circular 90- or 150-cm diameter pool filled with water at a temperature of 22.0 \pm 1°C, as described previously (Kobayashi *et al.* 2000; Huang *et al.* 2006), with some minor modifications. Details are described in Appendix S1.

ELISA for A β and β - and γ -secretase-mediated peptide cleavage assay

A β 40 and A β 42 levels and β - and γ -secretase activity in the brain were determined as described previously (Hoshino *et al.* 2007). Details are described in Appendix S1.

Thioflavin-S staining and immunohistochemical and immunofluorescence analyses

Thioflavin-S staining and immunohistochemical and immunofluorescence analyses were performed as detailed in Appendix S1.

Statistical analysis

All values are expressed as the mean \pm standard error of the mean (SEM). One- or two-way ANOVA followed by the Tukey test was used to evaluate differences between more than two groups. The Student's *t*-test for unpaired results was used for the evaluation of differences between two groups. Differences were considered to be significant for values of $p < 0.05$.

Results

Effect of deletion of EP₂ or EP₄ receptor on cognitive function in APP23 mice

We first used a Morris water maze to compare the spatial learning and memory of 6-month-old APPsw/EP₂^{-/-} and APPsw/EP₄^{-/-} mice with that of APPsw/EP₂^{+/+} and APPsw/EP₄^{+/+} mice, respectively. Mice were trained for 7 days to learn the location of a hidden platform, and the time required to reach the platform (escape latency) was

measured. As shown Fig. 1(a), APPsw/EP₂^{-/-} mice required a longer time than APPsw/EP₂^{+/+} animals to reach the platform, suggesting that EP₂ receptor deletion exacerbates the cognitive deficit in the APP23 mice. In contrast, the APPsw/EP₄^{-/-} mice tended to take less time to reach the platform than the corresponding control animals (APPsw/EP₄^{+/+}) (Fig. 1b), suggesting that deletion of the EP₄ receptor ameliorates the cognitive deficit. These differences did not reflect differences in swimming ability, because swimming speed and the ability to locate a visible platform were similar between the groups (data not shown).

Given that the above results suggest that the EP₄ receptor may represent the better potential molecular target for the development of AD drugs, we next compared AD-related phenotypes, such as the formation of plaques and neuronal and synaptic loss, between four strains of mice (WT/EP₄^{+/+}, WT/EP₄^{-/-}, APPsw/EP₄^{+/+} and APPsw/EP₄^{-/-}). We first repeated the Morris water maze test using 6-month-old mice, under slightly different experimental conditions (such as the

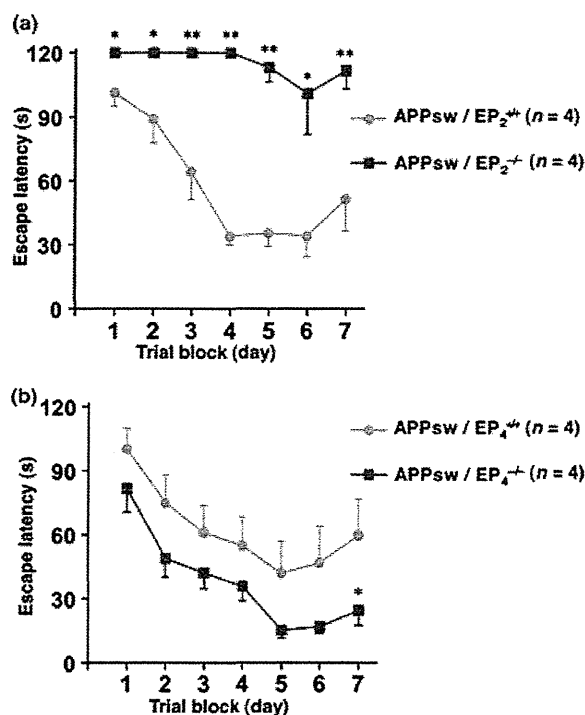


Fig. 1 Effects of deletion of the EP₂ or EP₄ receptor on spatial learning and memory in APP23 mice. Cognitive behavioral tests were carried out, using the Morris water maze, on 6-month-old APPsw/EP₂^{+/+} and APPsw/EP₂^{-/-} (a) or APPsw/EP₄^{+/+} and APPsw/EP₄^{-/-} (b) mice as described in the Materials and methods. Swimming paths in a circular 150-cm diameter pool were tracked for 120 s and the average (four tests) escape latency in each trial block was determined for 7 days. Values are given as mean ± SEM. Student's *t*-test: ***p* < 0.01 and **p* < 0.05.

size of swimming pool and tracking period). As shown Fig. 2(a), APPsw/EP₄^{+/+} mice required a longer time than WT/EP₄^{+/+} mice to reach the platform and this result is consistent with previous reports (Van Dam *et al.* 2003). Again, this difference did not reflect reduced swimming ability, as the swimming speed and the ability to locate a visible platform were similar between the four strains (data not shown). APPsw/EP₄^{-/-} mice required a shorter time to reach the platform than APPsw/EP₄^{+/+} mice (Fig. 2a). Furthermore, there was no significant difference in the escape latency between APPsw/EP₄^{-/-} and WT/EP₄^{+/+}

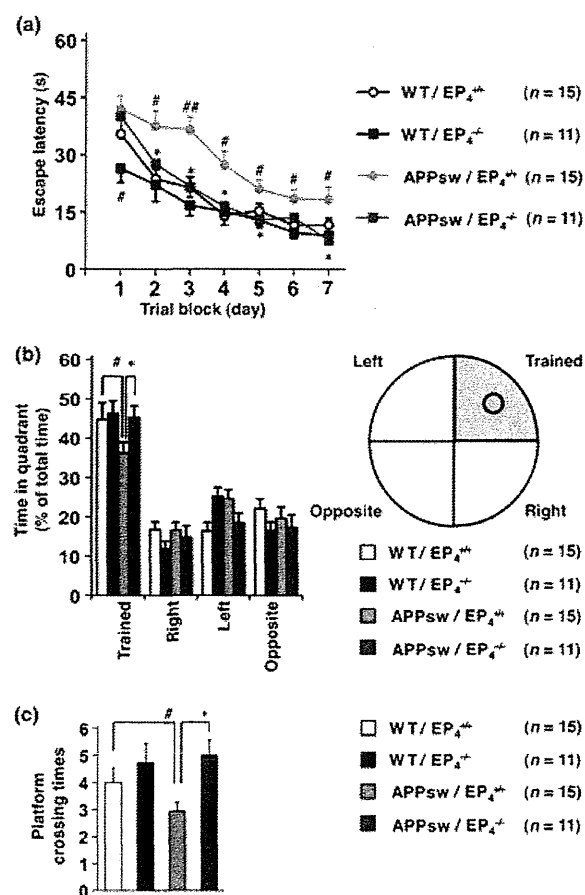


Fig. 2 Effects of deletion of the EP₄ receptor on spatial learning and memory in APP23 mice. Cognitive behavioral tests were carried out on 6-month-old WT/EP₄^{+/+}, WT/EP₄^{-/-}, APPsw/EP₄^{+/+} and APPsw/EP₄^{-/-} mice. The swimming path in a circular 90-cm diameter pool was tracked for 60 s (a). Mice were subjected to a transfer test in which the platform was removed. The spatial memory for a platform location was estimated by per cent search time in each quadrant (the platform had been located in the 'trained' quadrant) (b) or platform crossing times (c). Values are given as mean ± SEM. One-way (b, c) or two-way (a) ANOVA followed by Tukey test: **p* < 0.05, versus APPsw/EP₄^{+/+} mice; ***p* < 0.01 and #*p* < 0.05, versus WT/EP₄^{+/+} mice.

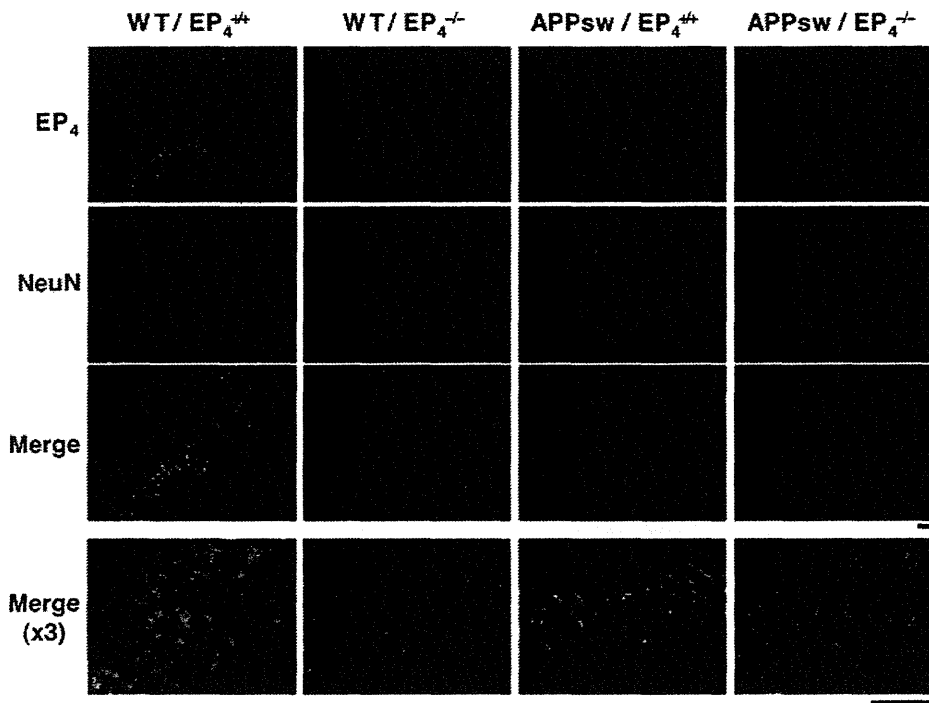


Fig. 3 EP₄ receptor expression in hippocampal neurons. Brain sections from 18-month-old WT/EP₄^{+/+}, WT/EP₄^{-/-}, APPsw/EP₄^{+/+} and APPsw/EP₄^{-/-} mice were immunohistochemically labeled by

immunofluorescence technique with antibodies against the EP₄ receptor and NeuN. The hippocampal CA3 region is shown (scale bar, 100 μm).

mice (Fig. 2a). These results suggest that the expression of APPsw disturbs spatial learning and memory and this effect can be ameliorated by deletion of the EP₄ receptor. WT/EP₄^{-/-} mice took a significantly shorter time to reach the platform than WT/EP₄^{+/+} mice; however, the difference was observed only at day 0 (Fig. 2a).

We then did a transfer test to examine the spatial memory of platform location. After a 7-day training period as described above, each mouse was subjected to a Morris water maze test where the platform was removed and we measured the per cent search time for each quadrant. As shown in Fig. 2(b), the percentage of time spent in the trained quadrant was lower for the APPsw/EP₄^{+/+} group than for either the WT/EP₄^{+/+} or the APPsw/EP₄^{-/-} mice. The crossing time of the area where the platform had been located was lower in the APPsw/EP₄^{+/+} group than in the WT/EP₄^{+/+} and APPsw/EP₄^{-/-} cohorts (Fig. 2c). There was no significant difference between APPsw/EP₄^{-/-} and WT/EP₄^{+/+} mice or between WT/EP₄^{-/-} and WT/EP₄^{+/+} mice for these indices (Fig. 2b and c). These results showed that deletion of the EP₄ receptor ameliorates the spatial memory deficits of APP23 mice.

We then examined EP₄ receptor expression in the brain of 18-month-old mice (hippocampal CA3 region) by immunofluorescence analysis. As shown in Fig. 3, expression of the

receptor was clearly observed in the brains of WT/EP₄^{+/+} and APPsw/EP₄^{+/+} mice. Staining with antibody against neuronal nuclei (NeuN) confirmed expression of the EP₄ receptor in neurons (Fig. 3), consistent with previous results (Choi *et al.* 2006). However, co-staining with antibody against NeuN and that against glial fibrillary acidic protein (a maker for astrocytes) or F4/80 (a maker for microglia) was not so clear (Fig. S1).

Effect of deletion of EP₄ receptor on Aβ plaque deposition and neuronal and synaptic loss in APP23 mice

We have previously reported that the levels of Aβ₄₀ and Aβ₄₂ in soluble and insoluble brain fractions prepared from 6-month-old APPsw/EP₄^{-/-} mice are lower than those from APPsw/EP₄^{+/+} mice (Hoshino *et al.* 2007), a finding that we confirmed here (Fig. 4a). We also examined Aβ plaque deposition by thioflavin-S staining using 18-month-old mice. As shown in Fig. 4(b) and (c), in both the hippocampus and the cerebral cortex, the level of Aβ plaque deposition was much lower in APPsw/EP₄^{-/-} mice than in APPsw/EP₄^{+/+} animals.

We next determined the number of neurons in the hippocampal CA3 region by NeuN staining using 18-month-old mice. As shown in Fig. 4(d) and (e), the number of NeuN-positive cells (neurons) was significantly

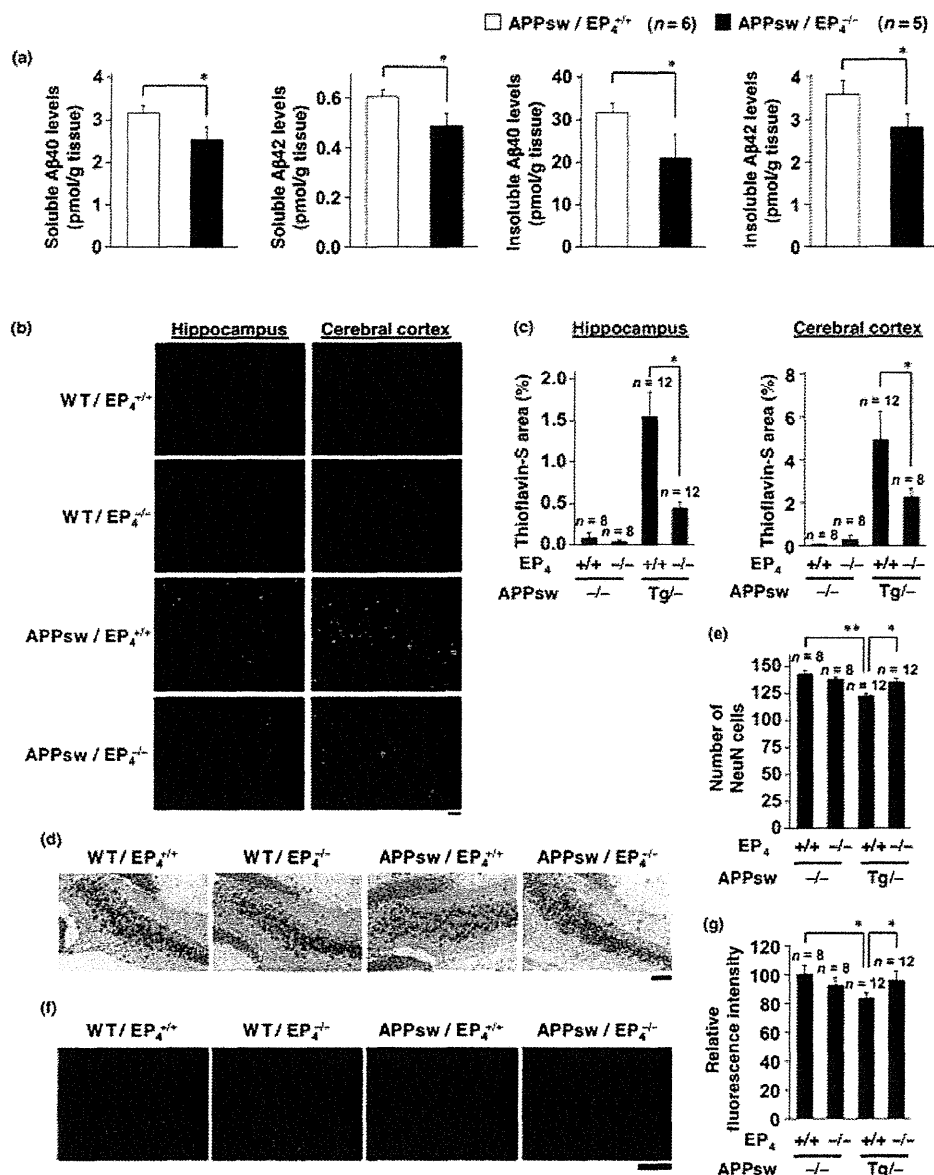


Fig. 4 Effects of EP₄ receptor deletion on Aβ levels, Aβ plaque deposition and neuronal and synaptic loss in APP23 mice. Soluble and insoluble fractions were prepared from the brains of 6-month-old APPsw/EP₄^{+/+} and APPsw/EP₄^{-/-} mice. The amounts of Aβ40 and Aβ42 in each fraction were determined by ELISA as described in the Materials and methods (a). Values are given as mean ± SEM. Student's *t*-test: **p* < 0.05. Brain sections from 18-month-old APPsw/EP₄^{+/+}, APPsw/EP₄^{-/-}, WT/EP₄^{+/+} and WT/EP₄^{-/-} mice were applied to

thioflavin-S staining (scale bar, 200 μm) (b) or immunohistochemical analysis with an antibody against NeuN (scale bar, 100 μm) (d) or immunofluorescence analysis with an antibody against synaptophysin (scale bar, 50 μm) (f). The relative area positive for thioflavin-S staining (c), the number of NeuN-positive cells in the hippocampal CA3 region (e) and the relative fluorescence intensity in the region (g) were determined (three sections per brain). Values are given as mean ± SEM. One-way ANOVA followed by Tukey test: ***p* < 0.01; **p* < 0.05.

higher in the WT/EP₄^{+/+} and APPsw/EP₄^{-/-} brain sections than in the APPsw/EP₄^{+/+} tissue, suggesting that the neuronal loss induced by Aβ was ameliorated by deletion of the EP₄ receptor. Similar results were observed for the

hippocampal CA1 region (Fig. S2). We also estimated the number of synapses by synaptophysin staining using 18-month-old mice. The level of synaptophysin was higher in sections from both WT/EP₄^{+/+} and APPsw/EP₄^{-/-} mice

than in those from APPsw/EP₄^{+/+} mice (Fig. 4f and g), indicating that A β -induced synaptic loss was suppressed by deletion of the EP₄ receptor. Taken together, these results suggest that deletion of the EP₄ receptor decreases the level of A β and A β plaque deposition in the brain and protects against A β -induced neurodegeneration. To confirm this further, stereological quantification of cell number that is more reliable should be performed in future studies.

Effect of oral administration of AE3-208 on AD-related phenotypes in APP23 mice

The results described above suggest that pharmacological inhibition of the EP₄ receptor ameliorates AD-related phenotypes in APP23 mice. In order to test this, we used an EP₄ receptor-specific antagonist, AE3-208. The K_i values of AE3-208 obtained by competition binding assay are 1.3, 30, 790 and 2400 nM for EP₄, EP₃, FP and TP, respectively, and more than 10 μ M for the other prostanoid receptors (Kabashima *et al.* 2002). We have previously reported that AE3-208 suppresses the PGE₂-stimulated production of A β *in vitro* (Hoshino *et al.* 2007). APP23 and wild-type mice were fed either AE3-208-supplemented chow or a control diet between the ages of 3 and 6 months (the average dose of AE3-208 was calculated to be 17.8 mg/kg body weight/day). No significant differences were observed in the amount of chow consumed by the four groups of mice (APP23 or wild-type mice fed AE3-208-supplemented or control chow) during the experimental period. We then examined the spatial learning and memory of the 6-month-old animals in a Morris water maze test. Swimming speed and ability to locate a visible platform were indistinguishable between the four groups (data not shown). However, APP23 mice fed AE3-208-supplemented chow took significantly less time to find the hidden platform than the mice fed control chow (Fig. 5a). No significant difference in the escape latency was recorded between wild-type mice fed AE3-208-supplemented chow and wild-type mice fed control chow (Fig. 5a). These results suggest that the deficit in spatial learning and memory in the APP23 mice can be ameliorated by oral administration of AE3-208.

As shown in Fig. 5(b), the amount of time spent in the trained quadrant showed a tendency to be greater for the APP23 mice fed AE3-208-supplemented chow than for the mice fed control chow. Furthermore, the crossing time of the area where the platform had been located was significantly greater in the former case (Fig. 5c). However, the difference in Fig. 5(b) was not statistically significant and we have no clear explanation for the discrepancy between the 'time in quadrant' and 'platform crossings' outcomes.

In order to test whether pharmacological inhibition of the EP₄ receptor ameliorates AD-related pathological phenotypes in APP23 mice, we compared the amount of A β 40 and A β 42 in soluble and insoluble fractions prepared from the brains of APP23 mice fed either AE3-208-supplemented or

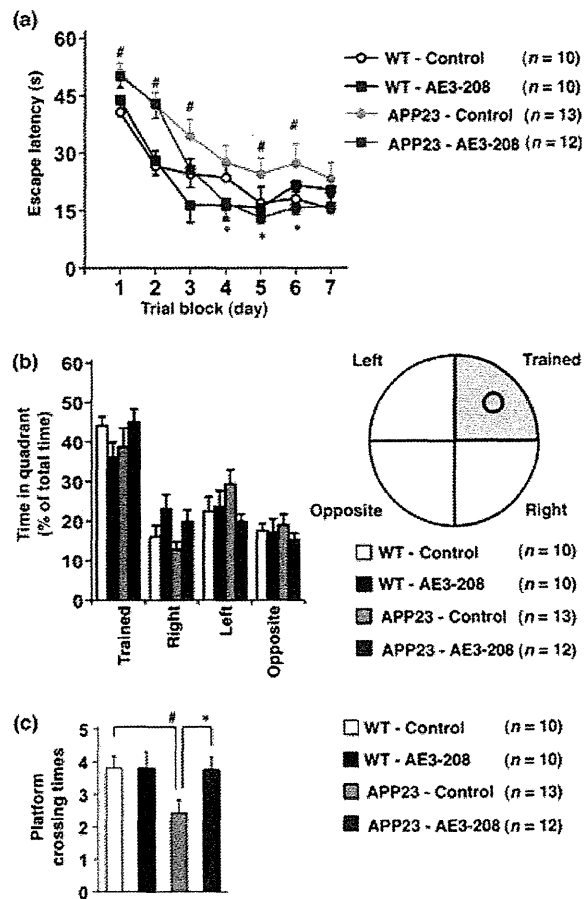


Fig. 5 Effects of oral administration of AE3-208 on spatial learning and memory in APP23 mice. Cognitive behavioral tests were carried out, using the Morris water maze, on 6-month-old wild-type mice (WT) and APP23 mice fed either AE3-208-supplemented chow (160 mg AE3-208/kg chow) or control chow between the ages of 3 and 6 months. Spatial learning and memory were tested as described in the legend of Fig. 2 (a–c). Values are given as mean \pm SEM. One-way (b, c) or two-way (a) ANOVA followed by Tukey test: * p < 0.05, versus APP23-control mice; # p < 0.05, versus WT-control mice.

control chow using 6-month-old mice. As shown in Fig. 6(a), the levels of A β 40 and A β 42 in the insoluble brain fractions from the former group were significantly lower. However, no significant difference was observed in the case of the soluble fractions (Fig. 6a).

We have previously reported that EP₄ receptor activation increases A β levels through its co-internalization into endosomes with PS-1 (γ -secretase), with resulting activation of γ -secretase *in vitro* (Hoshino *et al.* 2009). This finding was supported by our previous *in vivo* demonstration that brain γ -secretase activity is lower in APPsw/EP₄^{-/-} mice than in APPsw/EP₄^{+/+} animals, and that the co-localization of PS-1 with Rab7 (a marker of late endosomes and

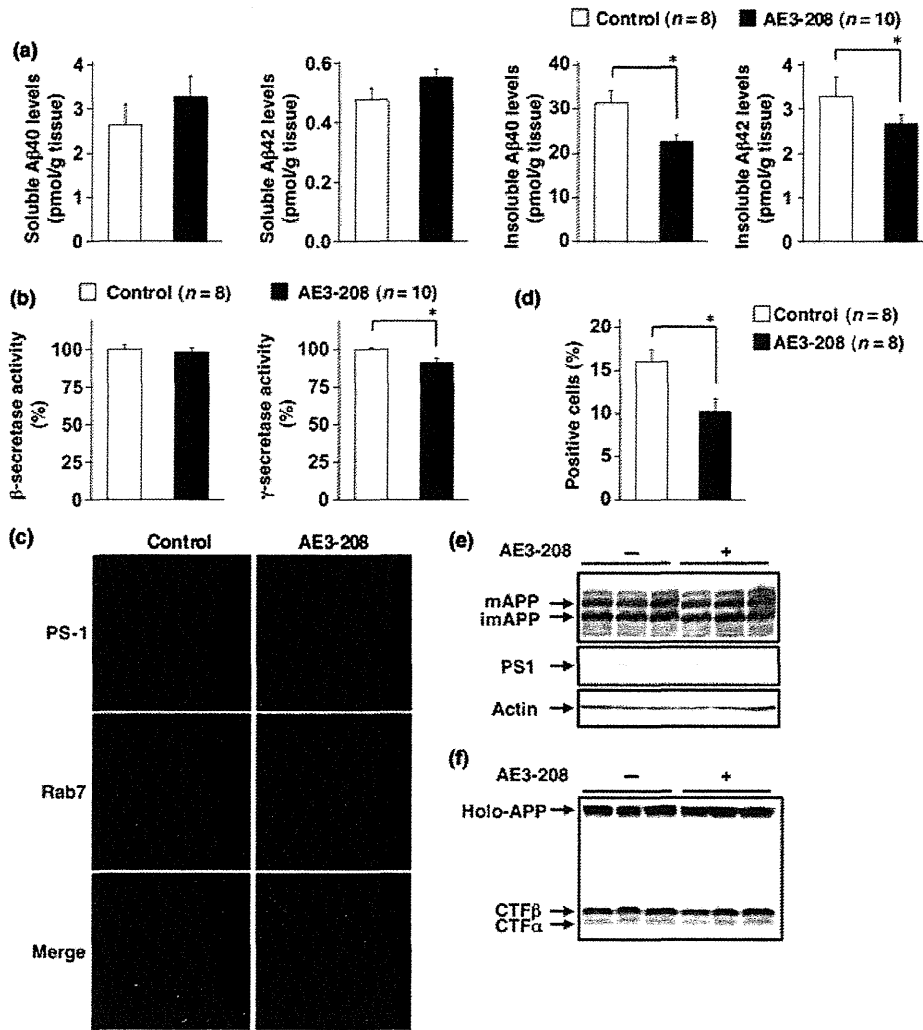


Fig. 6 Effects of oral administration of AE3-208 on A β levels, secretase activity, localization of γ -secretase and APP modulation in the APP23 mouse brain. APP23 mice were treated with AE3-208, as described in the legend of Fig. 5 (a–c). (6-month-old) The amounts of A β 40 and A β 42 were determined as described in the legend of Fig. 4 (a). Membrane fractions were prepared and subjected to a β - or γ -secretase-mediated

peptide cleavage assay as described in the Materials and methods (b). Brain sections were immunostained with antibodies against PS-1 and Rab7 (scale bar, 200 μ m) (c). Cells positive for both PS-1 and Rab7 staining were counted (d). Whole-cell extracts were subjected to immunoblotting with an antibody to APP (e, f), PS-1 (e) or actin (e). Values are given as mean \pm SEM. Student's *t*-test: **p* < 0.05.

lysosomes) is not as apparent in the former group (Hoshino *et al.* 2009). In the present study, we examined the effect of oral administration of AE3-208 on the activity and localization of γ -secretase using 6-month-old mice. As shown in Fig. 6(b), the activity of γ -secretase, but not that of β -secretase, was lower in the brains of APP23 mice fed AE3-208-supplemented chow than in those of mice fed control chow. Furthermore, we found that the co-localization of PS-1 with Rab7 was not as apparent in the former group (Fig. 6c and d). We quantitatively examined the effect of

AE3-208 on the expression of PS-1 staining and found that the effect was not statistically significant (data not shown).

We have previously reported that deletion of the EP₄ receptor in APP23 mice does not affect the modification of APP or α - and β -secretase activity (Hoshino *et al.* 2009), both of which are important for A β production. Here, we examined the effect of oral administration of AE3-208 on these processes using 6-month-old mice. We could separate by sodium dodecyl sulfate–polyacrylamide gel electrophoresis between the mature (*N*- and *O*-glycosylated) and

immature (*N*-glycosylated alone) forms of APP (mAPP and imAPP, respectively) (Tomita *et al.* 1998). As shown in Fig. 6(e), the total amount of APP and the ratio of mAPP and imAPP were similar between APP23 mice fed AE3-208-supplemented chow and those fed control chow, suggesting that the administration of AE3-208 does not affect APP modulation. We also found that the administration of AE3-208 did not affect the level of PS-1 (Fig. 6e). We then examined α - and β -secretase activity by comparing the level of secreted C-terminal fragment (CTF), representing an indirect index of secretase activity. We could not detect a CTF γ band under our experimental conditions. However, as shown in Fig. 6(f), CTF α and CTF β were detected in the APP23 mice and the amounts of CTF α and CTF β were indistinguishable between APP23 mice fed AE3-208-supplemented chow and those fed control chow, thereby suggesting that the administration of AE3-208 does not affect α - or β -secretase activity.

Taken together, these results suggest that the improvement in the cognitive function of the APP23 mice orally administered AE3-208 is mediated by a decrease in the brain levels of A β through suppression of co-internalization of the EP₄ receptor with γ -secretase into endosomes, thereby inhibiting the activation of γ -secretase.

Discussion

We have previously suggested that EP₂ and EP₄ receptors represent valuable molecular targets for the development of drugs to prevent or treat AD by showing that the amount of A β in the brains of APPsw/EP₂^{-/-} and APPsw/EP₄^{-/-} mice is lower than that in the respective control mice (Hoshino *et al.* 2007). However, among the antagonists specific for either the EP₂ or EP₄ receptor, or both, which type offers the most therapeutic potential? In order to address this issue, we herein compared the cognitive performance of APPsw/EP₂^{-/-} or APPsw/EP₄^{-/-} mice with that of their respective wild-type counterparts. This approach was adopted because, although AD is characterized by cognitive impairment, the functional (cognitive) phenotypes and pathological phenotypes (such as an increase in A β levels and A β plaque deposition) of the disease are not always directly linked. For example, some conditions ameliorate cognitive dysfunction in AD model mice without affecting the pathological phenotypes (Roberson *et al.* 2007; Kanninen *et al.* 2009). Our results suggested that APPsw/EP₄^{-/-} mice but not APPsw/EP₂^{-/-} mice display a higher level of cognitive function (spatial learning and memory) than their respective wild-type controls, suggesting that inhibition of the EP₄ receptor might prove the better therapeutic option.

We have previously reported that PGE₂-stimulated production of A β *in vitro* is partially mediated by EP₂ receptor-dependent activation of the cAMP-PKA pathway (Hoshino *et al.* 2009), and that the amount of A β in the brains of

APPsw/EP₂^{-/-} mice is lower than that in control mice (Hoshino *et al.* 2007). Another group has also shown that deletion of the EP₂ receptor in AD model mice reduces A β plaque deposition (Liang *et al.* 2005). Thus, it is surprising that deletion of this receptor exacerbates cognitive dysfunction in APP23 mice, suggesting that deletion of the EP₂ receptor impaired cognitive performance through an A β -independent mechanism. It has previously been reported that A β inhibits long-term potentiation (LTP) through inhibition of the cAMP-PKA pathway (Vitolo *et al.* 2002), and that inhibition of the EP₂ receptor also suppresses LTP via a similar mechanism (Akaneya and Tsumoto 2006). Thus, deletion of the EP₂ receptor may exacerbate cognitive dysfunction in APP23 mice through inhibition of LTP, a process known to be important for memory formation. It was recently reported that deletion of the gene encoding EP₂ receptor in mice without the expression of APPsw have behavioral deficits (Savonenko *et al.* 2009), thus it is unclear whether the observed effects of EP₂ receptor deletion in this study are specific to the AD model. However, it was previously reported that siRNA for EP₄ did not affect LTP (Akaneya and Tsumoto 2006).

We have previously reported that EP₄ receptor activation stimulates the production of A β through its co-internalization with γ -secretase into endosomes, leading to the activation of γ -secretase (Hoshino *et al.* 2009). We also showed that there are lower levels of A β and less endosomal localization of γ -secretase in the brains of APPsw/EP₄^{-/-} mice than in those of APPsw/EP₄^{+/+} animals (Hoshino *et al.* 2007, 2009). Furthermore, in the present study, we have demonstrated that APPsw/EP₄^{-/-} mice display lower levels of A β plaque formation and neuronal and synaptic loss than APPsw/EP₄^{+/+} mice. These results suggest that deletion of the EP₄ receptor ameliorates cognitive dysfunction in APP23 mice by decreasing brain levels of A β and suppressing neurodegeneration.

The findings of the present study also demonstrate that oral administration of the EP₄ receptor-specific antagonist, AE3-208, ameliorates the spatial learning and memory deficits of APP23 mice. AE3-208 has been shown to have some therapeutically beneficial effects, including suppression of tumor growth (Terada *et al.* 2010) and suppression of autoimmune encephalomyelitis (Yao *et al.* 2009). However, it has been reported that AE3-208 exacerbates dextran sodium sulfate-induced colitis, an animal model for ulcerative colitis (Kabashima *et al.* 2002), and that a specific agonist for the EP₄ receptor stimulates bone formation and prevents bone loss (Yoshida *et al.* 2002), suggesting that EP₄ receptor antagonists, including AE3-208, have adverse effects on colitis and osteoporosis, possibilities that must be considered if these agents are to be developed for the clinical treatment of AD. Although the transitional character of orally administered AE3-208 to the brain has not yet been examined, the results of the present study suggest that it can

pass the blood–brain barrier. AD is a chronic disease that requires long-term drug treatment in order to produce therapeutic effects. Thus, this property of AE3-208 would be of great advantage for its clinical use. As for the mechanism underpinning the amelioration of cognitive dysfunction in the APP23 mice following the administration of AE3-208, we believe that this is mediated by a similar mechanism to EP₄ receptor deletion, given that oral administration of AE3-208 decreases levels of A β and γ -secretase activity and inhibits the localization of γ -secretase in endosomes. The soluble A β level was reduced in APPsw/EP₄^{-/-} mice but not in mice administered with AE3-208. This difference would be because of the difference in extent of the inhibition; deletion of the gene encoding EP₄ receptor completely inhibits the function of this protein, whereas administration of the drug may cause partial inhibition. As for the difference between soluble and insoluble A β for the modulation by administration of AE3-208, we have no clear explanation at present. One possible explanation is that the temporal alteration in synthesis of A β may affect more drastically soluble A β level than insoluble one.

Although this study focused on how inflammation affects the pathogenesis of AD through PGE₂ but not on how inflammation is induced in association with AD progression, we examined the effect of inhibition of EP₄ receptor on the activation of astrocytes. As shown in Fig. S3, the expression of glial fibrillary acidic protein (a marker for the activity of astrocytes) was higher in 18-month-old APPsw/EP₄^{+/+} mice than in WT/EP₄^{+/+} and APPsw/EP₄^{-/-} mice. As for AE3-208, because we used 6-month-old mice, the activation of astrocytes by the expression of APPsw was not so clear; however, the activity was a little lower in drug-treated mice than in control mice (Fig. S3). These results suggest that the inhibition of EP₄ receptor suppresses APPsw-mediated activation of astrocytes (inflammation). Based on previously reported results, the activation of EP₄ receptor seems to affect immune systems both positively and negatively. For example, EP₄ receptor-stimulated differentiation of T_H1 cells and production of IL-23 in dendritic cells and resulting inflammation in experimental autoimmune encephalomyelitis were reported (Yao *et al.* 2009). However, in microglia, the activation of EP₄ receptor was reported to suppress the LPS-stimulated production of pro-inflammatory cytokines (Shi *et al.* 2010).

As described in the introduction, NSAIDs have attracted considerable attention as a new class of drugs for the treatment and prevention of AD, although it should also be noted that some clinical studies have recorded negative results (Imbimbo *et al.* 2010). NSAIDs can be classified into two groups: newly developed COX-2-specific NSAIDs (such as celecoxib) and classical NSAIDs without COX-2 specificity (such as indomethacin). The clinical use of classical NSAIDs is associated with gastrointestinal side effects (Hawkey 2000), as a result of the strong protective effect of prostaglandins on the gastroin-

testinal mucosa (Vane and Botting 1996). Given that it is mainly COX-1, which is expressed in this mucosa, COX-2-specific NSAIDs cause less of an effect on prostaglandin levels in this region, and therefore produce fewer gastrointestinal side effects than classical NSAIDs. However, it has recently been shown that clinical use of COX-2-specific NSAIDs is associated with cardiovascular thrombotic side effects (Ray *et al.* 2004; Singh 2004). These side effects of NSAIDs are likely to prove problematic if the drugs are used long term for the prevention or treatment of AD.

Compared with NSAIDs, we consider that EP₄ receptor-specific antagonists have advantages in relation to both safety and efficacy, based on the following lines of evidence. EP₁ and EP₃ receptors have been reported to be involved in PGE₂-mediated protection of the gastrointestinal mucosa by stimulating the production of bicarbonate and gastric mucosal blood flow, respectively (Takeuchi *et al.* 1997; Araki *et al.* 2000). Therefore, antagonists specific for the EP₄ receptor would be gastrointestinally safer than NSAIDs. However, it is now believed that inflammation has both positive and negative effects in relation to the progression of AD; for example, inflammation activates the phagocytosis of A β by microglia (Shafiq *et al.* 2007; Chakrabarty *et al.* 2010). However, NSAIDs that inhibit overall inflammation inactivate microglial phagocytosis (Yan *et al.* 2003). Therefore, compared with general anti-inflammatory agents, inhibitors that specifically act on the inflammation-mediated progression of AD may be more effective. NSAIDs suppress inflammation through both COX-dependent and COX-independent mechanisms, such as activation of the peroxisome proliferators activated receptor- γ and inhibition of nuclear factor-kB (Tegeger *et al.* 2001), with COX-mediated inhibition and the resulting decrease in PGE₂ levels seen to play a major role in the anti-AD activity of NSAIDs (Qin *et al.* 2003; Heneka *et al.* 2005). Furthermore, it was recently reported that the ability of NSAIDs to decrease PGE₂ levels is important in NSAID-dependent protection of hippocampal LTP against A β toxicity and restoration of A β -mediated suppression of synaptic plasticity and memory function (Kotilinek *et al.* 2008). Based on the findings of the present study, we consider that PGE₂ impairs cognitive performance at least partly through activation of the EP₄ receptor. Thus, we propose that EP₄ receptor-specific antagonists, such as AE3-208, will prove therapeutically more effective than NSAIDs as a result of their greater safety and efficacy. However, although we previously suggested that EP₁ and EP₃ receptors are not involved in PGE₂-stimulated production of A β *in vitro*, it is not clear whether activation of EP₁ and EP₃ receptors affect cognitive performance. Furthermore, modulation of COX-2 expression by activation of EP₄ receptor was also suggested (Shi *et al.* 2010). Therefore, the mechanism by which PGE₂ modulates cognitive performance is unclear at present and understanding of such mechanism is important for the identification of other targets of AD drugs.

Acknowledgements

We thank Dr M. Staufenbiel (Novartis Institutes for BioMedical Research) for providing transgenic mice. We also thank Ono Pharmaceutical Co. (Osaka, Japan) for providing AE3-208. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan, as well as the Japan Science and Technology Agency, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. Authors have no conflicts of interest.

Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Supplementary Materials and methods.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

References

- Akaneya Y. and Tsumoto T. (2006) Bidirectional trafficking of prostaglandin E2 receptors involved in long-term potentiation in visual cortex. *J. Neurosci.* **26**, 10209–10221.
- Andreasson K. I., Savonenko A., Vidensky S. *et al.* (2001) Age-dependent cognitive deficits and neuronal apoptosis in cyclooxygenase-2 transgenic mice. *J. Neurosci.* **21**, 8198–8209.
- Araki H., Ukawa H., Sugawa Y., Yagi K., Suzuki K. and Takeuchi K. (2000) The roles of prostaglandin E receptor subtypes in the cytoprotective action of prostaglandin E2 in rat stomach. *Aliment. Pharmacol. Ther.* **14**(Suppl. 1), 116–124.
- Chakrabarty P., Jansen-West K., Beccard A. *et al.* (2010) Massive gliosis induced by interleukin-6 suppresses Aβ deposition in vivo: evidence against inflammation as a driving force for amyloid deposition. *FASEB J.* **24**, 548–559.
- Choi J. S., Kim H. Y., Chun M. H., Chung J. W. and Lee M. Y. (2006) Expression of prostaglandin E2 receptor subtypes, EP2 and EP4, in the rat hippocampus after cerebral ischemia and ischemic tolerance. *Cell Tissue Res.* **324**, 203–211.
- Haass C. (2004) Take five-BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation. *EMBO J.* **23**, 483–488.
- Haass C. and Selkoe D. J. (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. *Nat. Rev. Mol. Cell Biol.* **8**, 101–112.
- Hawkey C. J. (2000) Nonsteroidal anti-inflammatory drug gastropathy. *Gastroenterology* **119**, 521–535.
- Heneka M. T., Sastre M., Dumitrescu-Ozimek L. *et al.* (2005) Acute treatment with the PPARγ agonist pioglitazone and ibuprofen reduces glial inflammation and Aβ1-42 levels in APPV7171 transgenic mice. *Brain* **128**, 1442–1453.
- Ho L., Purohit D., Haroutunian V. *et al.* (2001) Neuronal cyclooxygenase 2 expression in the hippocampal formation as a function of the clinical progression of Alzheimer disease. *Arch. Neurol.* **58**, 487–492.
- Hoshino T., Nakaya T., Homan T. *et al.* (2007) Involvement of prostaglandin E2 in production of amyloid-beta peptides both in vitro and in vivo. *J. Biol. Chem.* **282**, 32676–32688.
- Hoshino T., Namba T., Takehara M., Nakaya T., Sugimoto Y., Araki W., Narumiya S., Suzuki T. and Mizushima T. (2009) Prostaglandin E2 stimulates the production of amyloid-beta peptides through internalization of the EP4 receptor. *J. Biol. Chem.* **284**, 18493–18502.
- Huang S. M., Mouri A., Kokubo H. *et al.* (2006) Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. *J. Biol. Chem.* **281**, 17941–17951.
- Ikonomic M. D., Uryu K., Abrahamson E. E. *et al.* (2004) Alzheimer's pathology in human temporal cortex surgically excised after severe brain injury. *Exp. Neurol.* **190**, 192–203.
- Imbimbo B. P., Solfrizzi V. and Panza F. (2010) Are NSAIDs useful to treat Alzheimer's disease or mild cognitive impairment?. *Front. Aging Neurosci.* **2**, 1–14.
- Kabashima K., Saji T., Murata T. *et al.* (2002) The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J. Clin. Invest.* **109**, 883–893.
- Kanninen K., Heikkinen R., Mahn T. *et al.* (2009) Intrahippocampal injection of a lentiviral vector expressing Nrf2 improves spatial learning in a mouse model of Alzheimer's disease. *Proc. Natl Acad. Sci. USA* **106**, 16505–16510.
- Kitamura Y., Shimohama S., Koike H., Kakimura J., Matsuoka Y., Nomura Y., Gebicke-Haerter P. J. and Taniguchi T. (1999) Increased expression of cyclooxygenases and peroxisome proliferator-activated receptor-γ in Alzheimer's disease brains. *Biochem. Biophys. Res. Commun.* **254**, 582–586.
- Kobayashi K., Noda Y., Matsushita N. *et al.* (2000) Modest neuropsychological deficits caused by reduced noradrenaline metabolism in mice heterozygous for a mutated tyrosine hydroxylase gene. *J. Neurosci.* **20**, 2418–2426.
- Kotilinek L. A., Westerman M. A., Wang Q. *et al.* (2008) Cyclooxygenase-2 inhibition improves amyloid-beta-mediated suppression of memory and synaptic plasticity. *Brain* **131**, 651–664.
- Liang X., Wang Q., Hand T., Wu L., Breyer R. M., Montine T. J. and Andreasson K. (2005) Deletion of the prostaglandin E2 EP2 receptor reduces oxidative damage and amyloid burden in a model of Alzheimer's disease. *J. Neurosci.* **25**, 10180–10187.
- Mattson M. P. (2004) Pathways towards and away from Alzheimer's disease. *Nature* **430**, 631–639.
- Montine T. J., Sidell K. R., Crews B. C., Markesbery W. R., Marnett L. J., Roberts L. J. 2nd and Morrow J. D. (1999) Elevated CSF prostaglandin E2 levels in patients with probable AD. *Neurology* **53**, 1495–1498.
- Qin W., Ho L., Pompl P. N. *et al.* (2003) Cyclooxygenase (COX)-2 and COX-1 potentiate beta-amyloid peptide generation through mechanisms that involve gamma-secretase activity. *J. Biol. Chem.* **278**, 50970–50977.
- Ray W. A., Griffin M. R. and Stein C. M. (2004) Cardiovascular toxicity of valdecoxib. *N. Engl. J. Med.* **351**, 2767.
- Roberson E. D., Scearce-Lavie K., Palop J. J., Yan F., Cheng I. H., Wu T., Gerstein H., Yu G. Q. and Mucke L. (2007) Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. *Science* **316**, 750–754.
- Savonenko A., Munoz P., Melnikova T., Wang Q., Liang X., Breyer R. M., Montine T. J., Kirkwood A. and Andreasson K. (2009) Impaired cognition, sensorimotor gating, and hippocampal long-term depression in mice lacking the prostaglandin E2 EP2 receptor. *Exp. Neurol.* **217**, 63–73.
- Shafiq S. S., Kyrkanides S., Olschowka J. A., Miller J. N., Johnson R. E. and O'Banion M. K. (2007) Sustained hippocampal IL-1β overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. *J. Clin. Invest.* **117**, 1595–1604.

- Shi J., Johansson J., Woodling N. S., Wang Q., Montine T. J. and Andreasson K. (2010) The prostaglandin E₂ E-prostanoid 4 receptor exerts anti-inflammatory effects in brain innate immunity. *J. Immunol.* **184**, 7207–7218.
- Singh D. (2004) Merck withdraws arthritis drug worldwide. *BMJ* **329**, 816.
- Sisodia S. S. and St George-Hyslop P. H. (2002) Gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? *Nat. Rev. Neurosci.* **3**, 281–290.
- Smith C. J., Zhang Y., Koboldt C. M. *et al.* (1998) Pharmacological analysis of cyclooxygenase-1 in inflammation. *Proc. Natl Acad. Sci. USA* **95**, 13313–13318.
- Srinivasan B. D. and Kulkarni P. S. (1989) Inhibitors of the arachidonic acid cascade in the management of ocular inflammation. *Prog. Clin. Biol. Res.* **312**, 229–249.
- Takeuchi K., Yagi K., Kato S. and Ukawa H. (1997) Roles of prostaglandin E-receptor subtypes in gastric and duodenal bicarbonate secretion in rats. *Gastroenterology* **113**, 1553–1559.
- Tegeder I., Pfeilschifter J. and Geisslinger G. (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* **15**, 2057–2072.
- Terada N., Shimizu Y., Kamba T. *et al.* (2010) Identification of EP₄ as a potential target for the treatment of castration-resistant prostate cancer using a novel xenograft model. *Cancer Res.* **70**, 1606–1615.
- Tomita S., Kirino Y. and Suzuki T. (1998) Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway. Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism. *J. Biol. Chem.* **273**, 6277–6284.
- Van Dam D., D'Hooge R., Staufenbiel M., Van Ginneken C., Van Meir F. and De Deyn P. P. (2003) Age-dependent cognitive decline in the APP23 model precedes amyloid deposition. *Eur. J. Neurosci.* **17**, 388–396.
- Vane J. R. and Botting R. M. (1996) Mechanism of action of anti-inflammatory drugs. *Scand. J. Rheumatol. Suppl.* **102**, 9–21.
- in t' Veld B. A., Ruitenbergh A., Hofman A., Launer L. J., van Duijn C. M., Stijnen T., Breteler M. M. and Stricker B. H. (2001) Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *N. Engl. J. Med.* **345**, 1515–1521.
- Vitolo O. V., Sant'Angelo A., Costanzo V., Battaglia F., Arancio O. and Shelanski M. (2002) Amyloid beta-peptide inhibition of the PKA/CREB pathway and long-term potentiation: reversibility by drugs that enhance cAMP signaling. *Proc. Natl Acad. Sci. USA* **99**, 13217–13221.
- Wyss-Coray T. (2006) Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat. Med.* **12**, 1005–1015.
- Yan Q., Zhang J., Liu H., Babu-Khan S., Vassar R., Biere A. L., Citron M. and Landreth G. (2003) Anti-inflammatory drug therapy alters beta-amyloid processing and deposition in an animal model of Alzheimer's disease. *J. Neurosci.* **23**, 7504–7509.
- Yao C., Sakata D., Esaki Y., Li Y., Matsuoka T., Kuroiwa K., Sugimoto Y. and Narumiya S. (2009) Prostaglandin E₂-EP₄ signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat. Med.* **15**, 633–640.
- Yoshida K., Oida H., Kobayashi T. *et al.* (2002) Stimulation of bone formation and prevention of bone loss by prostaglandin E EP₄ receptor activation. *Proc. Natl Acad. Sci. USA* **99**, 4580–4585.