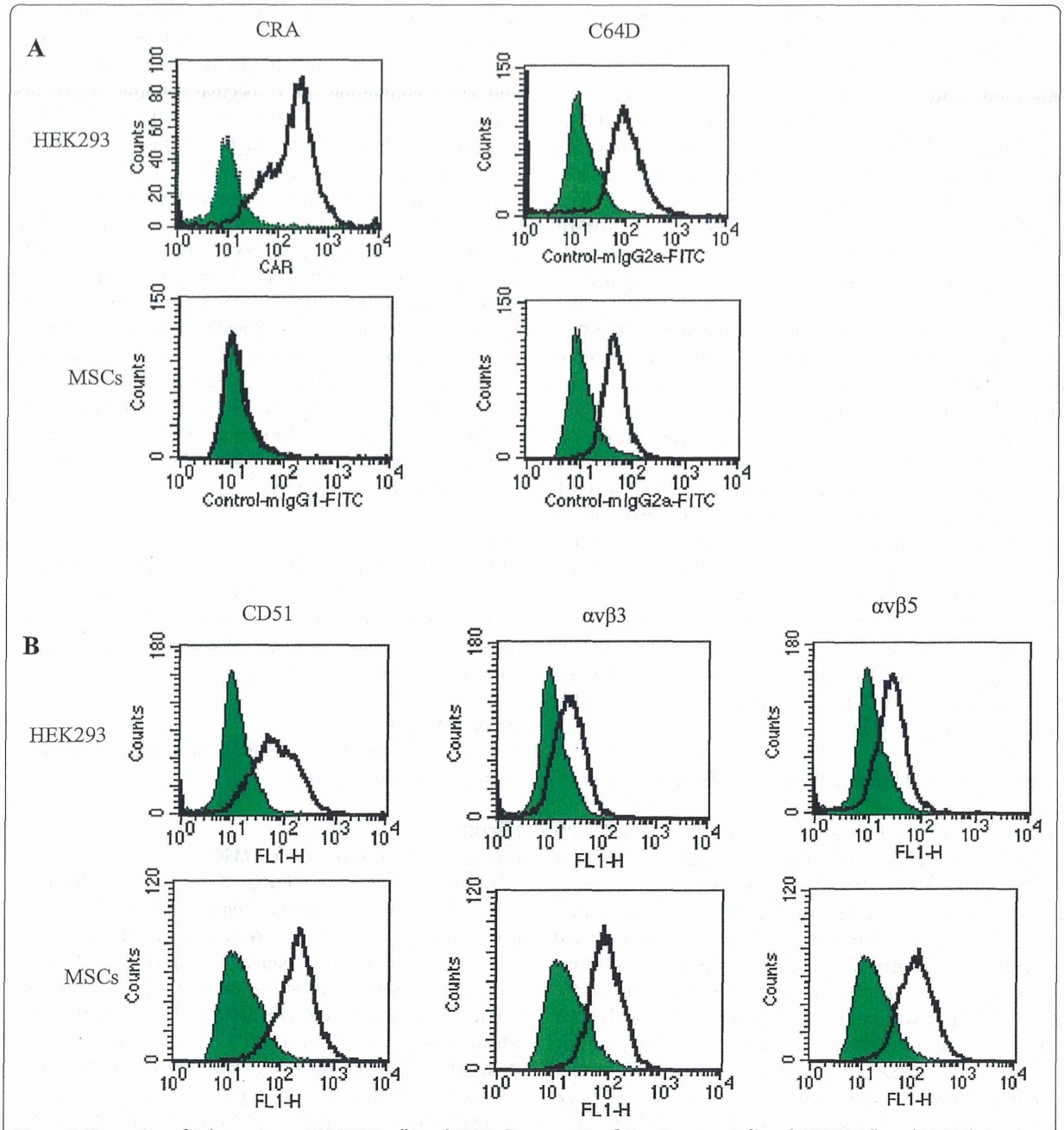

Figure 1 Expression of Ad receptors on HEK293 cells and MSCs. Representative flow cytometry profiles of HEK293 cells and MSCs that were stained with **(A)** anti-CAR, anti-CD46, **(B)** anti-CD51, anti-integrin αvβ3 or anti-integrin αvβ5 Ab. Shaded areas and bold lines show unstained and stained cells, respectively.

cells were not different when they were transduced either with Ad5-GFP or AdF35-GFP (Figure 4). In contrast, GFP positive MSCs cells were undetected with Ad5-mediated transduction and the positive percentages after transduction with AdF35-GFP were lower than those of HEK293 cells. The differential GFP positive rates were attributable to viral infectivity to the cells since both Ad5-GFP and AdF35-GFP used the same CMV promoter.

Anti-tumor effects of MSCs infected with AdF35-IL-28A

IL-28A produced cytotoxic effects on cells expressing the receptor complex, IL-28Rα and IL-10Rβ. We examined

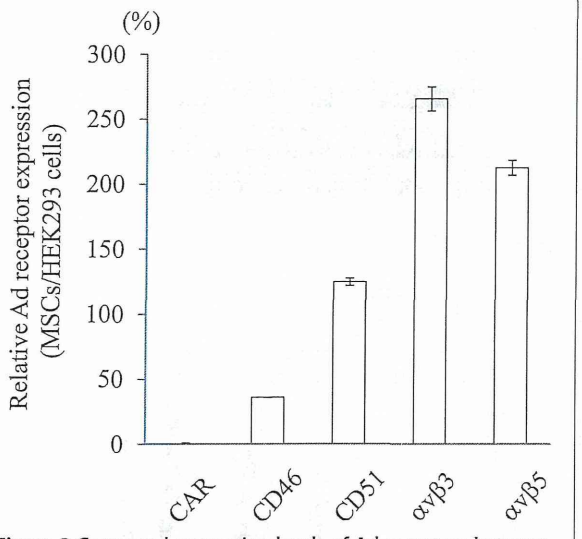

Figure 2 Compared expression levels of Ad receptors between MSCs and HEK293 cells. Mean fluorescence intensity in staining profiles was expressed as an arbitrary FL1 unit and the relative ratios, MSCs/HEK293 cells, are shown as a percentage. SE bars are also shown (n = 3).

the expression on MSCs together with immortalized fibroblasts, OUMS-24 and HFF cells, and esophageal carcinoma TE-11 cells as a reference of normal cells and as a positive control for the IL-28A receptor complex, respectively (Figure 5A) [12]. MSCs and the fibroblasts expressed IL-10Rβ but not IL-28Rα, demonstrating that MSCs were insensitive to IL-28A. In contrast, the esophageal carcinoma cells were positive for both molecules [12].

We then examined possible cytotoxicity of IL-28A released from MSCs in a co-culture experiment. Lung carcinoma OBA-LK1 cells were positive for the IL-28A receptor complex and the growth was suppressed by recombinant IL-28A [11]. We infected MSCs with AdF35-IL-28A or AdF35-LacZ as a control and detected IL-28A released from MSCs with ELISA at 363 ± 4.61 pg/ml/day per 10^3 MSCs. IL-28A-sensitive OBA-LK1 cells were mixed with the MSCs and the viable cell numbers were estimated with the WST assay (Figure 5B). Viability of MSCs that were either uninfected or infected with Ad-IL-28A or Ad-LacZ was not statistically different, showing that expression of IL-28A did not induce growth suppression in MSCs. In contrast, absorbance of a mixed population consisting of OBA-LK1 cells and AdF35-IL-28A-infected MSCs was lower than that of a mixture of OBA-LK1 cells and either uninfected MSCs or AdF35-LacZ-infected MSCs. Absorbance of the cell mixture of

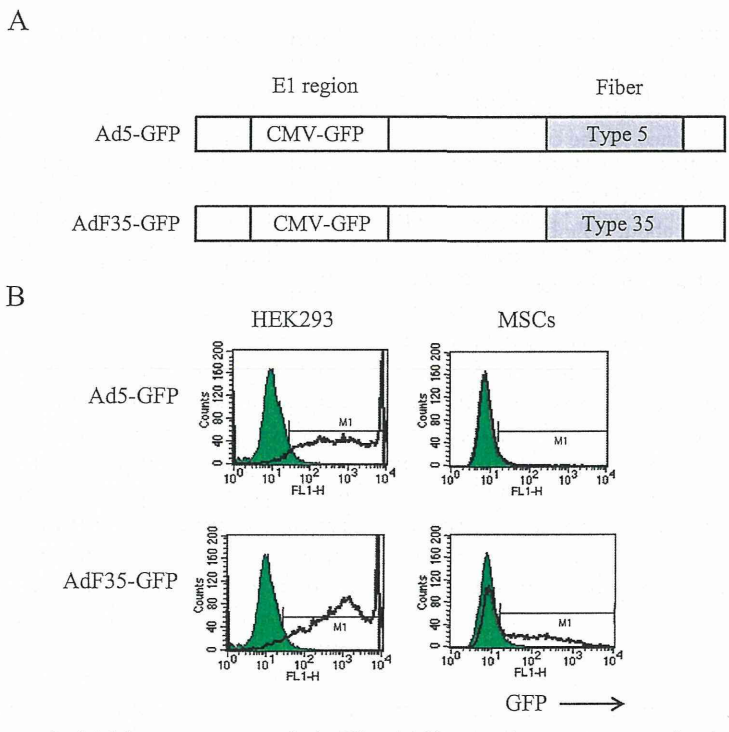

Figure 3 Infectivity of Ad to MSCs. (A) Schematic structures of Ad5-GFP and AdF35-GFP. The E1 region was replaced with the CMV promoter-linked GFP gene. **(B)** Representative flow cytometry profiles of HEK293 cells and MSCs that were infected with Ad5-GFP or AdF35-GFP. M1 indicates positively stained population, and shaded areas and bold lines show uninfected and infected cells, respectively.

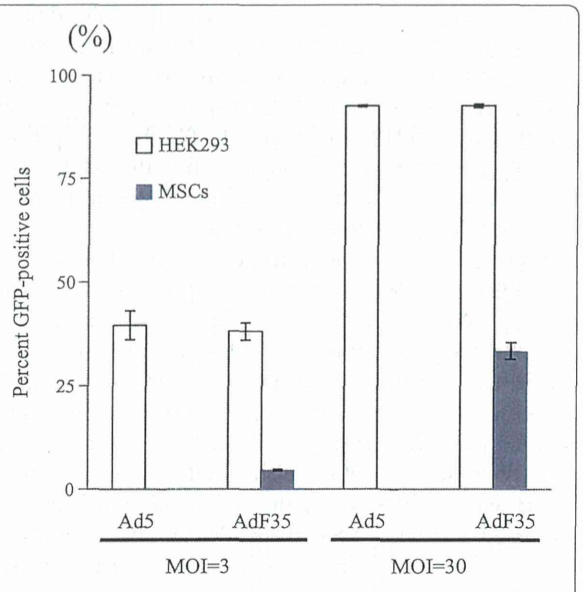

Figure 4 Infectivity of Ad5 and AdF35 to MSCs. MSCs or HEK293 cells were infected with Ad5-GFP or AdF35-GFP (MOI = 3 or 30) and cell populations greater than the brightest 5% of uninfected cells were judged as GFP-positive. SE bars are also shown (n = 3).

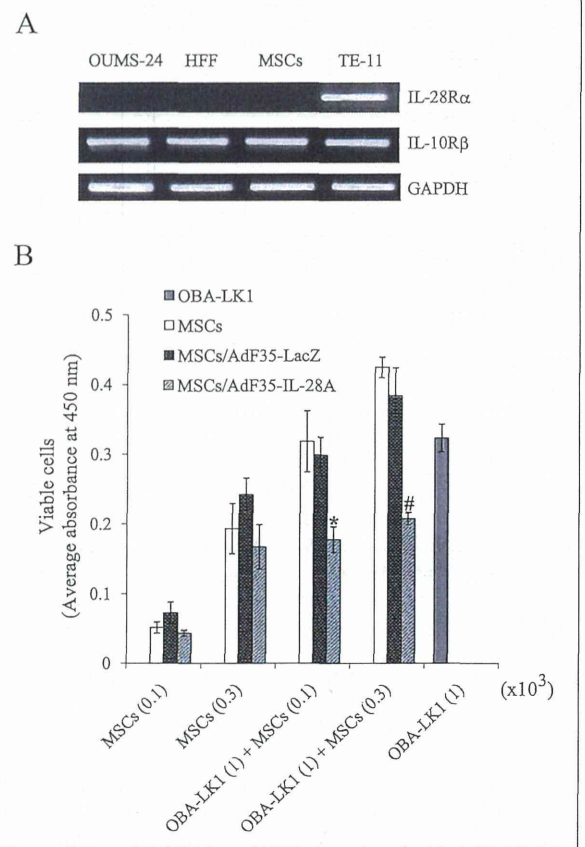

Figure 5 Growth inhibition of MSCs transduced with AdF35-IL-28. **(A)** Expression of IL-28 receptor complexes in human fibroblasts and MSCs. RT-PCR showed IL-28Rα and IL-10β gene products. TE-11 cells were used as a positive control for the gene expression and GAPDH as a loading control. **(B)** MSCs (100 or 300/well), uninfected or infected with AdF35-IL-28A or AdF35-LacZ (MOI = 100), were cultured alone or with OBA-LK1 cells (1,000/well) for 4 days. Viability of each group was detected with the WST-8 reagent and expressed as absorbance at 450 nm. SE bars are also shown (n = 3). *P < 0.05 and #P < 0.01, comparing MSCs infected with AdF35-IL-28A versus MSCs uninfected or infected with AdF35-LacZ.

OBA-LK1 cells and AdF35-IL-28A-infected MSCs was even lower than that of OBA-LK1 cells alone, indicating that IL-28A released from MSCs inhibited growth of OBA-LK1 cells.

We confirmed growth inhibitory activities of MSCs transduced with AdF35-IL-28A in a different assay (Table 1). OBA-LK1 cells were labeled with PKH 26 and cultured with MSCs for 4 days. We then stained all the cells with Hoechst 33342 and calculated numbers of PKH 28 positive cells among Hoechst 33342 positive cells. Percentages of PKH 28 positive OBA-LK1 cells were about 80% because the PKH 28 labeling was not complete under the experimental condition. OBA-LK1 cells cultured with untransduced MSCs further decreased the PKH 28 positive ratio since PKH 26 negative MSCs were also counted. Percentages of PKH 26 positive cells in cell mixtures were not different between co-culture with uninfected MSCs and that with AdF35-LacZ-infected MSCs irrespective of a ratio of the mixtures. The percentages however significantly lower in co-culture with AdF35-IL-28A-infected MSCs compared with cell mixture with uninfected MSCs or AdF35-LacZ infected MSCs. These data demonstrated that MSCs-derived IL-28A inhibited growth of OBA-LK1 cells.

Transcriptional regulation and growth assistance in MSCs

We investigated whether a putative tumor promoter could activate the *luciferase* gene in non-tumorous MSCs. We therefore examined transcriptional regions of the *midkine*, the *survivin* and the *COX-2* genes for the promoter activity in MSCs with the SV40 T antigen promoter region as a reference (Table 2). These regions are often used for activation of a transgene in a tumor-specific manner. A regulatory region of the *COX-2* gene activated the *luciferase* gene greater than that of the *midkine* or the *survivin* gene. A transcriptional activity of the COX region was greater than that of the SV40 T antigen promoter, but much less than that of the CMV promoter which is commonly used for transgene activations in many cells.

We also examined a possible tumor growth-promoting activity of MSCs with animal experiments (Figure 6). We inoculated YES-2 esophageal carcinoma cells into nude mice without or with MSCs or fibroblasts OUMS-24. Tumor growth of mixed populations, irrespective of

Table 1 Growth suppression of OBA-LK1 cells cultured with transduced MSCs

OBA-LK1	MSCs infected with	Mixed cell ratio (OBA-LK1 : MSCs)	PKH 26 positive cells (Percentage ± SE)[1]
(+)	(–)		80.2 ± 2.0
(+)	None	10 : 1	57.4 ± 4.2*
(+)	AdF35-LacZ	10 : 1	65.6 ± 4.1*
(+)	AdF35-IL28A	10 : 1	47.6 ± 2.7*
(+)	None	10 : 3	67.0 ± 4.4#
(+)	AdF35-LacZ	10 : 3	56.1 ± 2.0#
(+)	AdF35-IL-28A	10 : 3	26.4 ± 1.4#

OBA-LK1 cells (3.3×10^4) stained with PKH 26 were cultured with MSCs at a ratio indicated, and all the cells were then stained with Hoechst 33342 on day 4. [1]Percentages of PKH 26 positive cells among Hoechst 33342 positive cells and SEs are also shown (n = 3).
*P < 0.05 and #P < 0.01, comparing between cells mixed with AdF35-IL-28A-infected MSCs and cells mixed with uninfected MSCs or AdF35-LacZ-infected MSCs.

the ratio or mixed cells, was not different from that of YES-2 cells alone, demonstrating that MSCs, like fibroblasts, did not support tumor growth of YES-2 esophageal carcinoma cells co-injected.

Discussion

We showed that MSCs were resistant to Ad5-mediated gene transfer but were transduced with fiber-modified AdF35 vectors. The transduction preference was linked with the Ad receptor expression on MSCs, which were negative for CAR but positive for CD46 molecules. Comparison of receptor expressions between HEK293 cells and MSCs further showed that CD46 expression levels on MSCs were not as great as those on HEK293 cells, which resulted in lower transduction efficacy of AdF35 to MSCs than to HEK293 cells. Interestingly, expression levels of αvβ3 and αvβ5 integrin molecules were greater on MSCs than on HEK293 cells. Nevertheless, transduction of MSCs with Ad5 was not detected, indicating that the integrin molecules did not play a role as an auxiliary receptor in MSCs although the integrin molecules were demonstrated to be the major receptor of Ad5 in CAR-deficient cells [23]. In addition, the integrin molecules were shown to enhance AdF35-mediated gene transduction [24], but the present study suggested that elevated expression levels of integrin molecules on MSCs cells could not restore transduction efficacy of AdF35 to MSCs to the same level as to HEK293 cells. Many factors seem to be involved in mechanisms underlying Ad infectivity and the mechanisms can be different among cell types tested. They may include a possible threshold level of the receptor expression necessary for Ad infection and a presumable reciprocal interaction among the receptor molecules. CD46 is not a sole receptor for AdF35 and the expression levels were relatively low in freshly isolated MSCs [25]. Nevertheless, the MSCs from adult donors were infected with AdF35 [25], and we presume that Ad vectors bearing the type 35 fiber is currently one of the efficient vectors for gene transfer into MSCs.

Table 2 Promoter activity of transcriptional regulatory regions in MSCs

Transcriptional regulatory region	Luciferase activity (average ± SE)[1]
(–)	16.8 ± 8.9
SV40 T antigen	100.0 ± 11.5*
Midkine	66.8 ± 27.6*
Survivin	47.5 ± 14.7*
COX-2	368.2 ± 182.8*
CMV	6323.4 ± 2067.3*

[1]Relative luciferase activity was calculated based on the SV40 T antigen promoter-mediated activity as 100%. SEs are shown (n = 3).
*P < 0.05, comparing between the COX-2 region and either the SV40 T antigen, the survivin regulatory region, the midkine regulatory region or the CMV promoter.

Usage of gene modified MSCs has several advantages over direct Ad administrations in the anti-tumor activity. Transduced MSCs, injected intratumorally, tend to localize at the tumor sites in contrast to Ad which are subjected to a rapid washout from the injection sites [26]. Propensity of MSCs to migrate into tumors is thus favorable for MSCs-mediated anti-tumor effects [27] although the property was dependent on an experimental system [28]. Administration of MSCs into a tumor site also needs careful consideration since MSCs can promote the tumor growth due to the ability to constitute and maintain the microenvironments around tumors. We thereby examined the possible growth-enhancing activity *in vivo* and showed that MSCs did not support tumor growth of esophageal carcinoma cells co-injected. MSCs were not able to enhance tumor growth in nude mice but the possibility of tumor promoting actions needs to be studied in different experimental models. On the other hand, the present study showed that untransduced MSCs did not achieve any anti-tumor effects by themselves. The growth suppressing activity of transduced MSCs was thus attributable to IL-28A since the suppression was dependent on cell numbers of *IL-28*-transduced but not on *β-galactosidase*-transduced MSCs. A property of expressed transgene products is also crucial for therapeutic efficacy of MSCs-mediated gene delivery. For example, IL-28A not only induces tumor cell death through apoptosis but activates inmate and acquired immunity through augmented natural killer activities and facilitated antigen presentation [12,14-17]. Moreover, IL-28A in vivo influences and modulates tumor microenvironments such as inhibition of angiogenesis, which can be mediated by other cytokines [13].

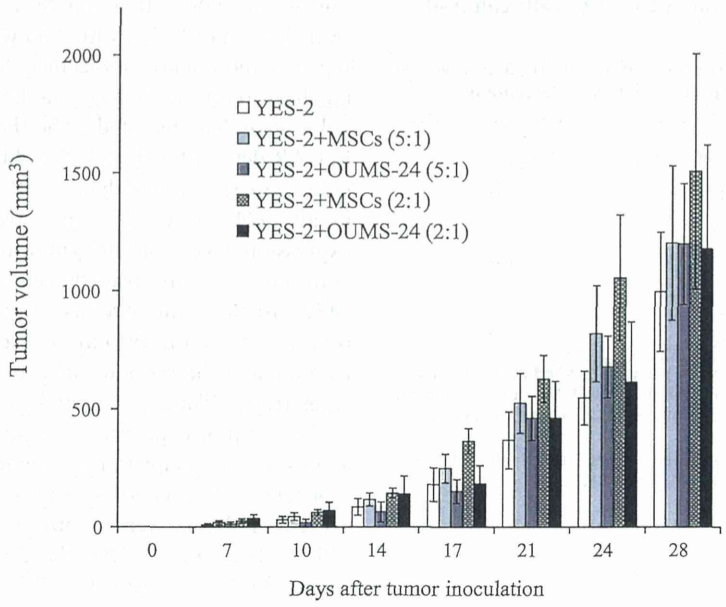

Figure 6 MSCs did not influence growth of co-injected tumors. YES-2 cells and MSCs or OUMS-24 cells were inoculated subcutaneously into BALB/c *nu/nu* mice at the ratios indicated. Average tumor volumes and SE bars are shown (n = 6).

Recently, several reports demonstrated anti-tumor effects produced by MSCs-mediated delivery of replication-competent Ad into tumors [28,29] and a clinical research revealed benefits of such autologous MSCs in neuroblastoma patients [30]. Tumor cells were initially used for cell-mediated delivery of replication-competent Ad [31] since tumor cells well supported Ad replication compared with non-transformed cells. MSCs may not be effective in the light of production of progenitor Ad because of the low proliferation rate. We examined the promoter activity of transcriptional regulatory regions which activated the *Ad E1A* gene and subsequently enabled Ad replication-competent within tumors [32-34]. The *COX-2* region gave greater activities than the *midkine* or the *survivin* region. Expression levels of midkine and survivin in adult tissues are often associated with proliferation rates of cells [35] and relatively low promoter activities of these regions could reflect the low growth rates of MSCs. Instead, COX-2 expression can be liked with inflammatory responses [36,37]. Elevated promoter activity of the *COX-2* region in MSCs may be related to MSCs' propensity to migrate toward inflammatory sites. Nevertheless, a promoter activity of the *COX-2* region was much lower than that of the CMV promoter which is commonly used to activate a transgene in replication-incompetent Ad. These data suggest that MSCs are a suitable cell-mediate vehicle for CMV promoter-driven replication-incompetent Ad rather than for replication-competent Ad in which the *E1A* is activated by an exogenous transcriptional regulatory region.

Conclusions

We demonstrated anti-tumor effects of MSCs-mediated delivery of IL-28A to lung carcinoma cells in the vicinity. A local administration of gene-modified MSCs can deliver the gene product to targets and is one of the cell therapies for cancer. AdF35 is a better vector than conventional type 5 Ad in transducing MSCs because of its enhanced infectivity. Immune responses against gene-modified MSCs are less significant as long as MSCs are autologously isolated. Nevertheless, when MSCs are infected with replication-competent Ad, cell-mediated immunity against viral gene-loaded MSCs will be generated. Further investigations are required for the cell-mediated immunity in the light of Ad-loaded MSCs and for the strategy to evade host immunity such as use of immunosuppressive agents.

Abbreviations

MSCs: Mesenchymal stem cells; Ad: Adenoviruses; CAR: Coxsackie adenovirus receptor; IFN: Interferon; IL: Interleukin; FITC: Fluorescein isothiocyanate; Ab: Antibody; GFP: Green fluorescent protein; LacZ: β-galactosidase; MOI: Multiplicity of infection; RT-PCR: Reverse transcription-polymerase chain reaction; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ELISA: Enzyme-linked immunosorbent assay; CMV: Cytomegalovirus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TS, KK, QL and SO conducted experiments, YT, HS and KH analyzed the data, KT and NY organized the experiments, NY and MT prepared the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grant-in-Aid for Research on seeds for Publicly Essential Drugs and Medical Devices from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-aid from the Nichias Corporation which does not have any patents, products in development or marketed products to declare.

Sources of support
Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Grant-in-Aid for Research on seeds for Publicly Essential Drugs and Medical Devices from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-aid from the Nichias Corporation.

Author details
[1]Department of Molecular Cell Biology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan. [2]Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, Chiba, Japan. [3]Department of Molecular Biology and Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan. [4]Department of Respirology, Graduate School of Medicine, Chiba University, Chiba, Japan. [5]Department of Surgery, School of Medicine, Toho University, Tokyo, Japan. [6]Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center, Yachiyo, Japan.

Received: 10 September 2014 Accepted: 23 September 2014
Published: 25 September 2014

References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: **Multilineage potential of adult human mesenchymal stem cells.** *Science* 1999, **284**:143–147.
2. Hamada H, Kobune M, Nakamura K, Kawano Y, Kato K, Honmou O, Houkin K, Matsunaga T, Niitsu Y: **Mesenchymal stem cells (MSC) as therapeutic cytoreagents for gene therapy.** *Cancer Sci* 2005, **96**:149–156.
3. Barry FP, Murphy JM: **Mesenchymal stem cells: clinical applications and biological characterization.** *Int J Biochem Cell Biol* 2004, **36**:568–584.
4. Studeny M, Marini FC, Dembinski JL, Zompetta C, Cabreira-Hansen M, Bekele BN, Champlin RE, Andreeff M: **Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents.** *J Natl Cancer Inst* 2004, **96**:1593–1603.
5. Tuve S, Wang H, Ware C, Liu Y, Gaggar A, Bernt K, Shayakhmetov D, Li Z, Strauss R, Stone D, Lieber A: **A new group B adenovirus receptor is expressed at high levels on human stem and tumor cells.** *J Virol* 2006, **80**:12109–12120.
6. *Shayakhmetov DM, Papayannopoulou T, Stamatoyannopoulos G, Lieber A:* **Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector.** *Virol* 2000, **74**:2567–2583.
7. Yu L, Shimozato O, Li Q, Kawamura K, Ma G, Namba M, Ogawa T, Kaiho I, Tagawa M: **Adenovirus type 5 substituted with type 11 or 35 fiber structure increases its infectivity to human cells enabling dual gene transfer in CD46-dependent and -independent manners.** *Anticancer Res* 2007, **27**:2311–2316.
8. Gaggar A, Shayakhmetov DM, Lieber A: **CD46 is a cellular receptor for group B adenoviruses.** *Nat Med* 2003, **9**:1408–1412.
9. Li Q, Kawamura K, Tada Y, Shimada H, Hiroshima K, Tagawa M: **Novel type III interferons produce anti-tumor effects through multiple functions.** *Front Biosci (Landmark Ed)* 2013, **18**:909–918.
10. Lasfar A, Abushahba W, Balan M, Cohen-Solal KA: **Interferon lambda: a new sword in cancer immunotherapy.** *Clin Dev Immunol* 2011, **2011**:349575.
11. Fujie H, Tanaka T, Tagawa M, Kaijun N, Watanabe M, Suzuki T, Nakayama K, Numasaki M: **Antitumor activity of type III interferon alone or in combination with type I interferon against human non-small cell lung cancer.** *Cancer Sci* 2011, **102**:1977–1990.
12. Li Q, Kawamura K, Ma G, Iwata F, Numasaki M, Suzuki N, Shimada H, Tagawa M: **Interferon-λ induces G1 phase arrest or apoptosis in oesophageal carcinoma cells and produces anti-tumour effects in combination with anti-cancer agents.** *Eur J Cancer* 2010, **46**:180–190.
13. Lasfar A, Lewis-Antes A, Smirnov SV, Anantha S, Abushahba W, Tian B, Reuhl K, Dickensheets H, Sheikh F, Donnelly RP, Raveche E, Kotenko SV: **Characterization of the mouse IFN-λ ligand-receptor system: IFN-λs exhibit antitumor activity against B16 melanoma.** *Cancer Res* 2006, **66**:4468–4477.
14. Sato A, Ohtsuki M, Hata M, Kobayashi E, Murakami T: **Antitumor activity of IFN-λ in murine tumor models.** *J Immunol* 2006, **176**:7686–7694.
15. Numasaki M, Tagawa M, Hidaka M, Suzuki T, Nakamura A, Okada M, Iwakura Y, Aiba S, Yamaya M: **IL-28 elicits antitumor responses against murine fibrosarcoma.** *J Immunol* 2007, **178**:5086–5098.
16. Abushahba W, Balan M, Castaneda I, Yuan Y, Reuhl K, Raveche E, de la Torre A, Lasfar A, Kotenko SV: **Antitumor activity of type I and type III interferons in BNL hepatoma model.** *Cancer Immunol Immunother* 2010, **59**:1059–1071.
17. Lasfar A, Cohen-Solal KA: **Emergence of IFN-lambda as a potential antitumor agent.** In *Targets in Gene Therapy.* 15th edition. Edited by You YP. Croatia: InTech; 2011.
18. Fushimi K, Iijima M, Gao C, Kondo T, Tsuji T, Hashimoto T, Mihara K, Namba M: **Transformation of normal human fibroblasts into immortalized cells with the mutant p53 gene and X-rays.** *Int J Cancer* 1997, **70**:135–140.
19. Compton T: **An immortalized human fibroblast cell line is permissive for human cytomegalovirus infection.** *J Virol* 1993, **67**:3644–3648.
20. Miyauchi M, Yoshida Y, Tada Y, Narita M, Maeda T, Bahar R, Kadomatsu K, Muramatsu T, Matsubara S, Nakagawara A, Sakiyama S, Tagawa M: **Expression of herpes simplex virus-thymidine kinase gene controlled by a promoter region of the midkine gene confers selective cytotoxicity to ganciclovir in human carcinoma cells.** *Int J Cancer* 2001, **91**:723–727.
21. Kawamura K, Yu L, Tomizawa M, Shimozato O, Ma G, Li Q, Sato A, Yang Y, Suzuki T, Abdel-Aziz NM, Tagawa M: **Transcriptional regulatory regions of the survivin gene activate an exogenous suicide gene in human tumors and enhance the sensitivity to a prodrug.** *Anticancer Res* 2007, **27**:89–93.
22. Yamamoto M, Alemany R, Adachi Y, Grizzle WE, Curiel DT: **Characterization of the cyclooxygenase-2 promoter in an adenoviral vector and its application for the mitigation of toxicity in suicide gene therapy of gastrointestinal cancers.** *Mol Ther* 2001, **3**:385–394.
23. Lyle C, McCormick F: **Integrin αvβ5 is a primary receptor for adenovirus in CAR-negative cells.** *Virol J* 2010, **7**:148.
24. Murakami S, Sakurai F, Kawabata K, Okada N, Fujita T, Yamamoto A, Hayakawa T, Mizuguchi H: **Interaction of penton base Arg-Gly-Asp motifs with integrins is crucial for adenovirus serotype 35 vector transduction in human hematopoietic cells.** *Gene Ther* 2007, **14**:1525–1533.
25. Knaän-Shanzer S, van de Watering MJ, van der Velde I, Gonçalves MA, Valerio D, de Vries AA: **Endowing human adenovirus serotype 5 vectors with fiber domains of species B greatly enhances gene transfer into human mesenchymal stem cells.** *Stem Cells* 2005, **23**:1598–1607.
26. Seo SH, Kim KS, Park SH, Suh YS, Kim SJ, Jeun SS, Sung YC: **The effects of mesenchymal stem cells injected via different routes on modified IL-12-mediated antitumor activity.** *Gene Ther* 2011, **18**:488–495.
27. Wang GX, Zhan YA, Hu HL, Wang Y, Fu B: **Mesenchymal stem cells modified to express interferon-β inhibit the growth of prostate cancer in a mouse model.** *J Int Med Res* 2012, **40**:317–327.
28. Hakkarainen T, Särkioja M, Lehenkari P, Miettinen S, Ylikomi T, Suuronen R, Desmond RA, Kanerva A, Hemminki A: **Human mesenchymal stem cells lack tumor tropism but enhance the antitumor activity of oncolytic adenoviruses in orthotopic lung and breast tumors.** *Hum Gene Ther* 2007, **18**:627–641.
29. Yong RL, Shinojima N, Fueyo J, Gumin J, Vecil GG, Marini FC, Bogler O, Andreeff M, Lang FF: **Human bone marrow-derived mesenchymal stem cells for intravascular delivery of oncolytic adenovirus Delta24-RGD to human gliomas.** *Cancer Res* 2009, **69**:8932–8940.
30. García-Castro J, Alemany R, Cascalló M, Martínez-Quintanilla J, Arriero Mdel M, Lassaletta A, Madero L, Ramírez M: **Treatment of metastatic neuroblastoma with systemic oncolytic virotherapy delivered by autologous mesenchymal stem cells: an exploratory study.** *Cancer Gene Ther* 2010, **17**:476–483.
31. Hamada K, Desaki J, Nakagawa K, Zhang T, Shirakawa T, Gotoh A, Tagawa M: **Carrier cell-mediated delivery of a replication-competent adenovirus for cancer gene therapy.** *Mol Ther* 2007, **15**:1121–1128.
32. Yu L, Hamada K, Namba M, Kadomatsu K, Muramatsu T, Matsubara S, Tagawa M: **Midkine promoter-driven suicide gene expression and -mediated adenovirus replication produced cytotoxic effects to immortalised and tumour cells.** *Eur J Cancer* 2004, **40**:1787–1794.
33. Ono HA, Davydova JG, Adachi Y, Takayama K, Barker SD, Reynolds PN, Krasnykh VN, Kunisaki C, Shimada H, Curiel DT, Yamamoto M: **Promoter-controlled infectivity-enhanced conditionally replicative adenoviral vectors for the treatment of gastric cancer.** *J Gastroenterol* 2005, **40**:31–42.

34. Zhu ZB, Chen Y, Makhija SK, Lu B, Wang M, Rivera AA, Yamamoto M, Wang S, Siegal GP, Curiel DT, McDonald JM: **Survivin promoter-based conditionally replicative adenoviruses target cholangiocarcinoma.** *Int J Oncol* 2006, 29:1319–1329.
35. Yu L, Ugai S, O-Wang J, Namba M, Kadomatsu K, Muramatsu T, Matsubara S, Sakiyama S, Tagawa M: **Cell growth- and p53-dependent transcriptional activity of the midkine promoter confers suicide gene expression in tumor cells.** *Oncol Rep* 2003, 10:1301–1305.
36. Burke SJ, Collier JJ: **The gene encoding cyclooxygenase-2 is regulated by IL-1β and prostaglandins in 832/13 rat insulinoma cells.** *Cell Immunol* 2011, 271:379–384.
37. Ulivi V, Giannoni P, Gentili C, Cancedda R, Descalzi F: **p38/NF-kB-dependent expression of COX-2 during differentiation and inflammatory response of chondrocytes.** *J Cell Biochem* 2008, 104:1393–1406.

doi:10.1186/1471-2407-14-713
Cite this article as: Suzuki *et al.*: Mesenchymal stem cells are efficiently transduced with adenoviruses bearing type 35-derived fibers and the transduced cells with the IL-28A gene produces cytotoxicity to lung carcinoma cells co-cultured. *BMC Cancer* 2014 14:713.

OPEN

Citation: Cell Death and Disease (2014) 5, e1517; doi:10.1038/cddis.2014.475

www.nature.com/cddis

Zoledronic acid induces apoptosis and S-phase arrest in mesothelioma through inhibiting Rab family proteins and topoisomerase II actions

S Okamoto[1,2], Y Jiang[1,3], K Kawamura[1], M Shingyoji[4], Y Tada[2], I Sekine[5], Y Takiguchi[5], K Tatsumi[2], H Kobayashi[6], H Shimada[7], K Hiroshima[8] and M Tagawa*[,1,3]

Zoledronic acid (ZOL), a nitrogen-containing bisphosphonate, produced anti-tumor effects through apoptosis induction or S-phase arrest depending on human mesothelioma cells tested. An addition of isoprenoid, geranylgeraniol but not farnesol, negated these ZOL-induced effects, indicating that the ZOL-mediated effects were attributable to depletion of geranylgeranyl pyrophosphates which were substrates for prenylation processes of small guanine-nucleotide-binding regulatory proteins (small G proteins). ZOL-treated cells decreased a ratio of membrane to cytoplasmic fractions in RhoA, Cdc42 and Rab6 but less significantly Rac1 proteins, indicating that these proteins were possible targets for ZOL-induced actions. We further analyzed which small G proteins were responsible for the three ZOL-induced effects, caspase-mediated apoptosis, S-phase arrest and morphological changes, using inhibitors for respective small G proteins and siRNA for Cdc42. ZOL-induced apoptosis is due to insufficient prenylation of Rab proteins because an inhibitor of geranlygeranyl transferase II that was specific for Rab family proteins prenylation, but not others inhibitors, activated the same apoptotic pathways that ZOL did. ZOL suppressed an endogenous topoisomerase II activity, which was associated with apoptosis and S-phase arrest in respective cells because we detected the same cell cycle changes in etoposide-treated cells. Inhibitors for geranlygeranyl transferase I and for RhoA produced morphological changes and disrupted actin fiber structures, both of which were similar to those by ZOL treatments. These data demonstrated that anti-tumor effects by ZOL were attributable to inhibited functions of respective small G proteins and topoisomerase II activity, and suggested that cellular factors were involved in the differential cell cycle changes.
Cell Death and Disease (2014) **5,** e1517; doi:10.1038/cddis.2014.475; published online 13 November 2014

Bisphosphonates (BPs), synthetic analogues of pyrophosphates, are clinically in use for diseases with excessive bone absorption such as osteoporosis and malignancy-associated hypercalcemia. BPs administered *in vivo* are accumulated in the bone matrix and inhibit activities of osteoclasts.[1] The first generation of BPs, without nitrogen in the structure, is converted into cytotoxic non-hydrolyzable ATP analogues and achieves cytotoxic effects thorough decreased mitochondrial membrane potentials.[2,3] The second and the third generations, containing nitrogen, inhibit farnesyl pyrophosphate synthetase, a key enzyme in the mevalonate pathways, and deplete isoprenoid pools, which subsequently results in decreased prenylation of small guanine-nucleotide-binding regulatory proteins (small G proteins) (Supplementary Figure S1).[4]

Isoprenoid lipids, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are substrates for prenylation processes that mediate farnesylation and geranylgeranylation of small G proteins, respectively.[5,6] Ras family proteins are either farnesylated by farnsyl transferase or geranylgeranylated by geranylgeranyl transferase I. In contrast, the majority of Rho family proteins and Rab family proteins are geranylgeranylated by geranylgeranyl transferase I and II, respectively. These lipid modifications are essential for most of small G proteins to bind to cytoplasmic and organelle membranes where prenylated small G proteins become functional, whereas unprenylated small G proteins remain in the cytoplasm and non-functional.[5]

The nitrogen-containing BPs (N-BPs) also induce cytotoxicity to osteoclasts, which is favorable for enhanced bone mineralization, and recent studies also showed that N-BPs had cytotoxic activities on tumors such as breast and prostate cancer.[7,8] These cytotoxic actions are attributable to a number of mechanisms including apoptosis induction and anti-angiogenesis,[9,10] but it is not well investigated as to which small G proteins produce the cytotoxic effects.

[1]Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, Chiba, Japan; [2]Department of Respirology, Graduate School of Medicine, Chiba University, Chiba, Japan; [3]Department of Molecular Biology and Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan; [4]Department of Thoracic Disease, Chiba Cancer Center, Chiba, Japan; [5]Department of Medical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan; [6]Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan; [7]Department of Surgery, School of Medicine, Toho University, Tokyo, Japan and [8]Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center, Chiba, Japan

*Corresponding author: M Tagawa, Division of Pathology and Cell Therapy, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba, 260-8717, Japan. Tel: +81 43 264 5431; Fax: +81 43 265 4459; E-mail: mtagawa@chiba-cc.jp

Abbreviations: BPs, bisphosphonates; ETO, etoposide; FOH, farnesol; GGOH, geranylgeraniol; N-BPs, nitrogen-containing bisphosphonates; PARP, poly (ADP-ribose) polymerase; siRNA, small interfering RNA; small G proteins, small guanine-nucleotide-binding regulatory proteins; Topo, topoisomerase; ZOL, zoledronic acid

Received 21.7.14; revised 6.10.14; accepted 6.10.14; Edited by A Stephanou

We recently showed that zoledronic acid (ZOL), which is one of the N-BPs to inhibit farnesyl pyrophosphate synthetase, produced cytotoxic activities to human mesothelioma.[11] ZOL treatments induced apoptotic cell death or S-phase arrest in cell cycle, and moreover caused morphological changes from fibroblast-like to spherical shapes. In the present study, we examined what kinds of small G proteins are responsible to these ZOL-mediated effects using inhibitors or small interfering RNA (siRNA) for the respective small G proteins and for prenylating enzymes.

Results

ZOL induced apoptosis and S-phase arrest. We examined ZOL-mediated anti-tumor effects in human mesothelioma cells (Figure 1). Proliferation of four kinds of human mesothelioma cells was suppressed with ZOL treatments (Figure 1a). Cell cycle analyses demonstrated that ZOL increased sub-G1 fractions in MSTO-211H cells, S-phase populations in EHMES-10 cells, and both sub-G1 and S-phase populations in EHMES-1 and JMN-1B cells (Figure 1b). We therefore used MSTO-211H and EHMES-10 cells in further experiments as representative cells that showed increased sub-G1 and S-phase populations, respectively. We then examined signal pathways leading to cell death in MSTO-211H cells (Figure 1c). ZOL treatments decreased expression levels of Mcl-1 and phosphorylated Akt, but increased cleavages of caspase-9, -3 and poly (ADP-ribose) polymerase (PARP). In contrast, ZOL treatments minimally influenced these expression levels in EHMES-10 cells. We also showed that ZOL activated caspase-3, -7, -8 and -9 in MSTO-211H cells (Figure 1d). These data collectively indicated that ZOL induced apoptosis through caspase activations in MSTO-211H, whereas EHMES-10 cells were resistant to the apoptotic signals. ZOL-treated MSTO-211H cells showed dephosphorylation of pRb greater than untreated cells, but phosphorylated levels of pRb were maintained in ZOL-treated EHMES-10 cells compared with those of untreated cells.

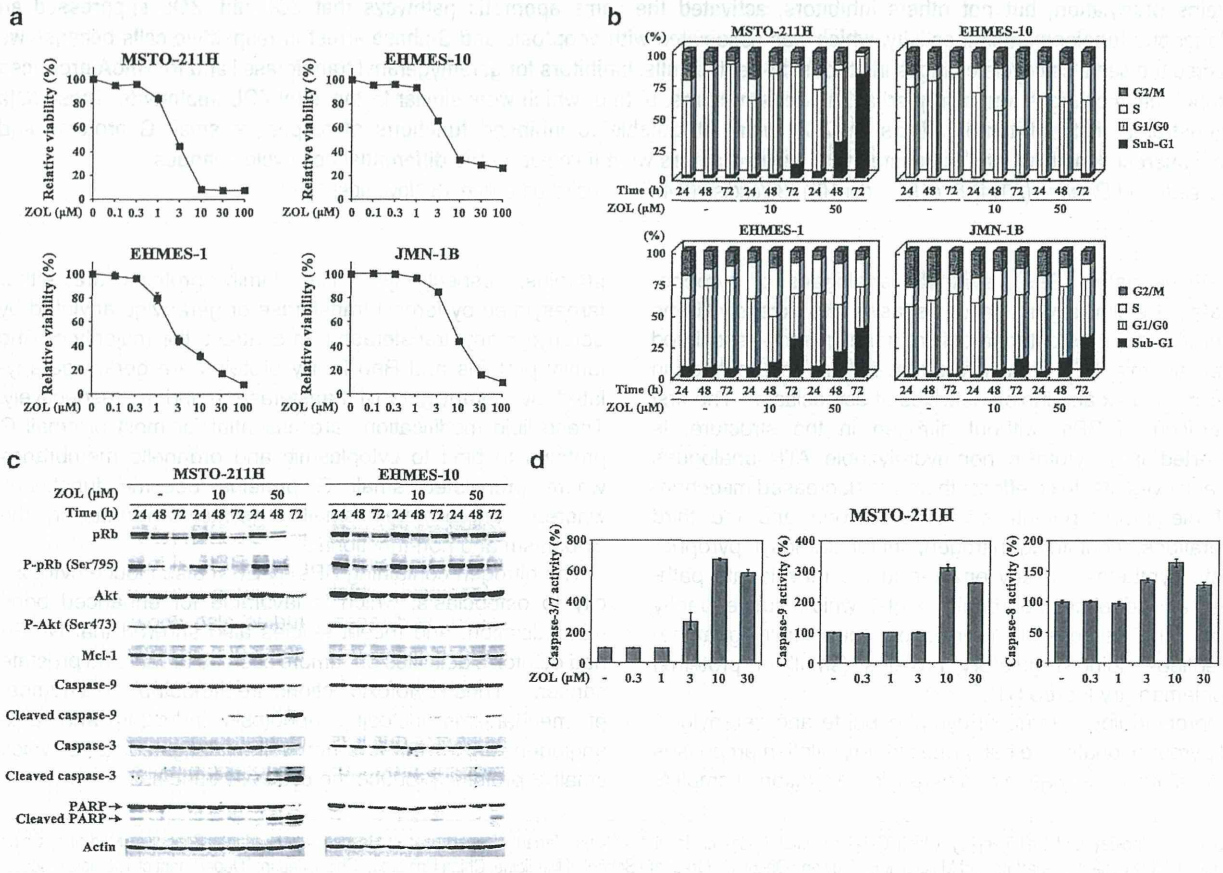

Figure 1 ZOL-mediated apoptosis and S-phase arrest through isoprenoid depletion. (**a**) Viability of cells treated with ZOL was measured with WST assay. The relative viability was calculated based on values of untreated cells as 100%. S.E. bars are shown ($n=3$). (**b** and **c**) Cells treated with different concentrations of ZOL were subjected to cell cycle (**b**) or western blot analyses with actin as the loading control (**c**). (**d** and **f**) Caspase activities in MSTO-211H cells treated with ZOL for 72 h (**d**) and with ZOL and/or GGOH for 48 h (**f**). The relative activity was expressed as a percentage of the untreated case. S.E. bars are shown ($n=3$). (**e** and **g**) Cells treated with agents as indicated for 48 h (MSTO-211H cells) or 72 h (EHMES-10 cells) and were subjected to western blot (**e**) or cell cycle analyses (**g**). Ras with high (arrow) and low (dotted arrow) molecular weights corresponds to unprenylated and prenylated forms, respectively. Actin is used as the control (**e**). (**h**) Lysates of cells treated with 50 μM ZOL were separated into cytoplasm (c) or membrane (m) fraction and then probed with respective antibodies as indicated. (**i**) Differential ratios between cytoplasm and membrane fractions detected in western blot analyses (**h**). The intensity was determined with an imaging analyzer

Geranylgeranyl pyrophosphate inhibited ZOL-mediated apoptosis and S-phase arrest. We investigated the involvement of farnesylation or geranylgeranylation in the ZOL-induced apoptosis and S-phase arrest. We examined an effect caused by addition of farnesol (FOH) and geranylgeraniol (GGOH), which were converted into farnesyl pyrophosphate and geranylgeranyl pyrophosphate, respectively, to determine ZOL's targets among small G proteins. A supplementary use of GGOH in MSTO-211H cells reduced ZOL-mediated downregulation of Mcl-1 expression, dephosphorylation levels of pRb and Akt, and cleavages of caspase-9, -3 and PARP (Figure 1e). Moreover, GGOH treatments inhibited ZOL-mediated activation of caspases (Figure 1f) and increase of sub-G1 fractions (Figure 1g). These data indicated that ZOL induced apoptosis through caspase activations. In contrast, FOH supplements did not influence these ZOL-mediated effects in MSTO-211H cells (Figures 1e and g). ZOL-induced S-phase arrest and phosphorylation of pRb in EHMES-10 cells were also inhibited with GGOH but not with FOH. These data demonstrated that ZOL-mediated apoptosis and S-phase arrest were attributed to reduced functions of geranylgeranylated but not farnesylated small G proteins. Western blot studies showed that ZOL-mediated unprenylation of Rap1A proteins was blocked with GGOH but not FOH administrations in both MSTO-211H and EHMES-10 cells (Figure 1e).

On the other hand, the prenylation status of Ras proteins was differently modulated with ZOL and additional FOH or GGOH, and was not correlated with ZOL-mediated effects. Prenylation of Ras proteins was suppressed by ZOL and further inhibited by additional GGOH in MSTO-211H cells, whereas FOH rather augmented prenylation. The Ras prenylation in EHMES-10 cells remained unchanged with ZOL and with additional FOH treatments, but slightly inhibited by GGOH. These data collectively showed that Ras proteins were not responsible molecules in the ZOL-mediated effects.

ZOL-induced cellular localization of small G proteins. We examined what kinds of small G proteins were unprenylated with ZOL treatments by testing the cellular distributions because some of antibodies specific to the unprenylated form was unavailable. Fractionated cytoplasmic and membrane portions were examined for the expressions of RhoA, Rac1 and Cdc42, belonging to the Rho family, and Rab6, one of the Rab family proteins (Figures 1h and i). ZOL treatments increased cytoplasmic distribution of RhoA, Cdc42, Rab6 and less significantly Rac1 in both of MSTO-211H and EHMES-10 cells. ZOL inhibited the translocation of Ras proteins from cytoplasm to membrane in MSTO-211H cells but did not influence the Ras distribution in EHMES-10 cells. These data indicated that ZOL suppressed membrane localization of small G proteins because of increased

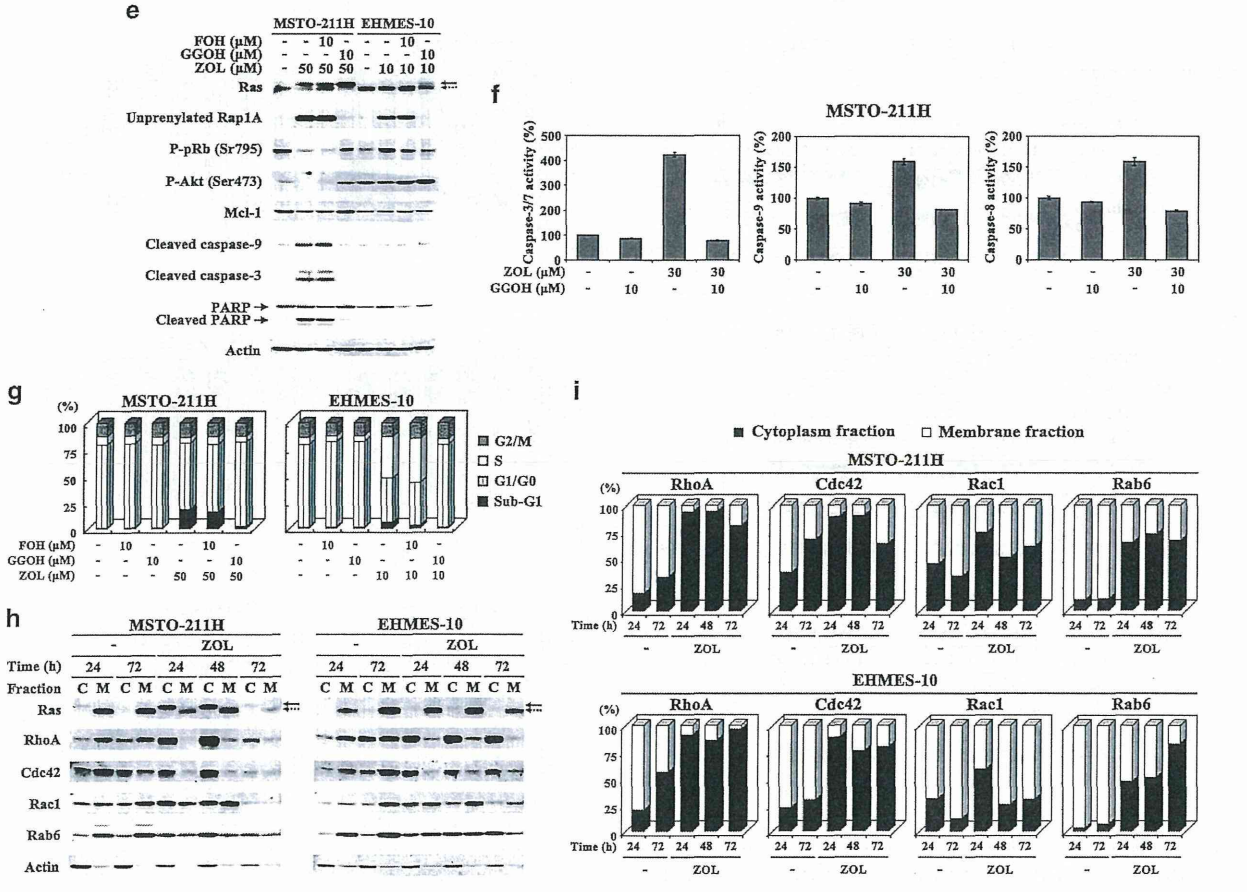

Figure 1 Continued