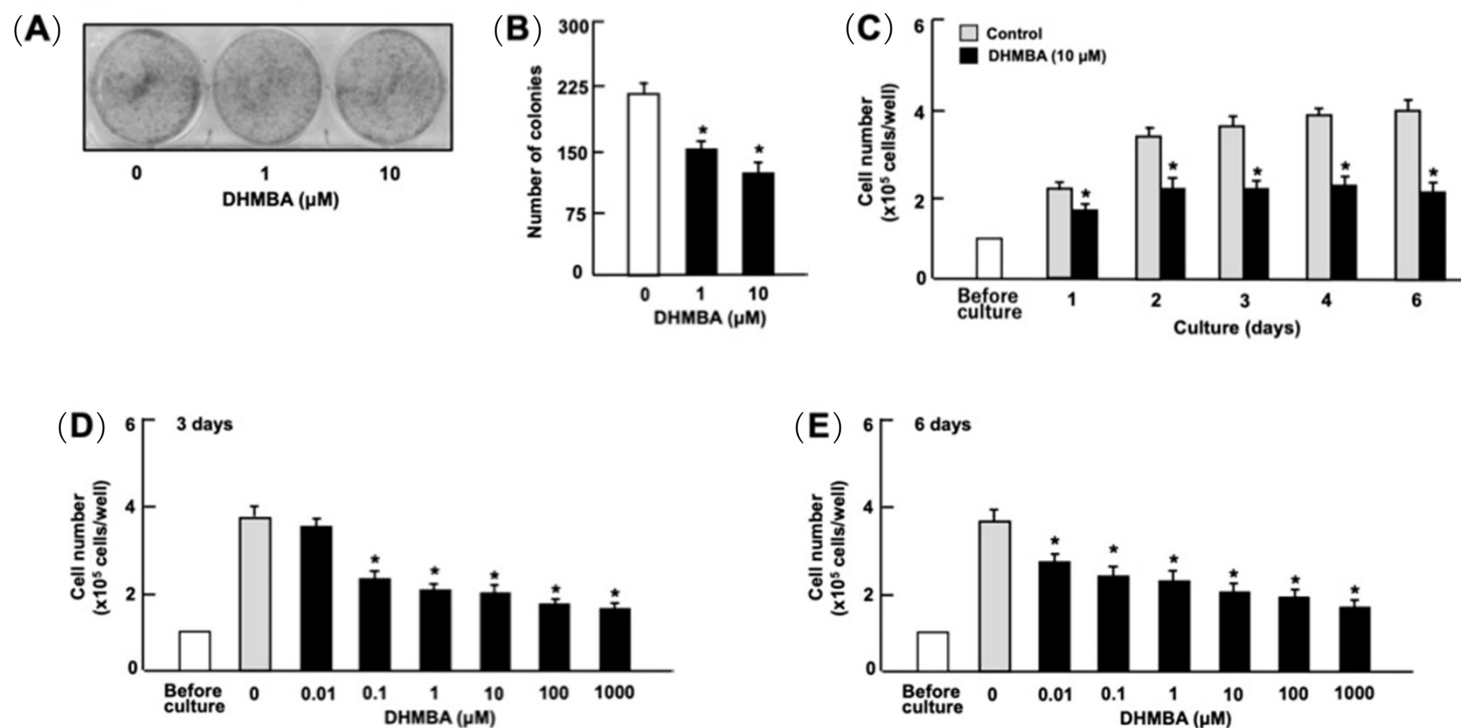


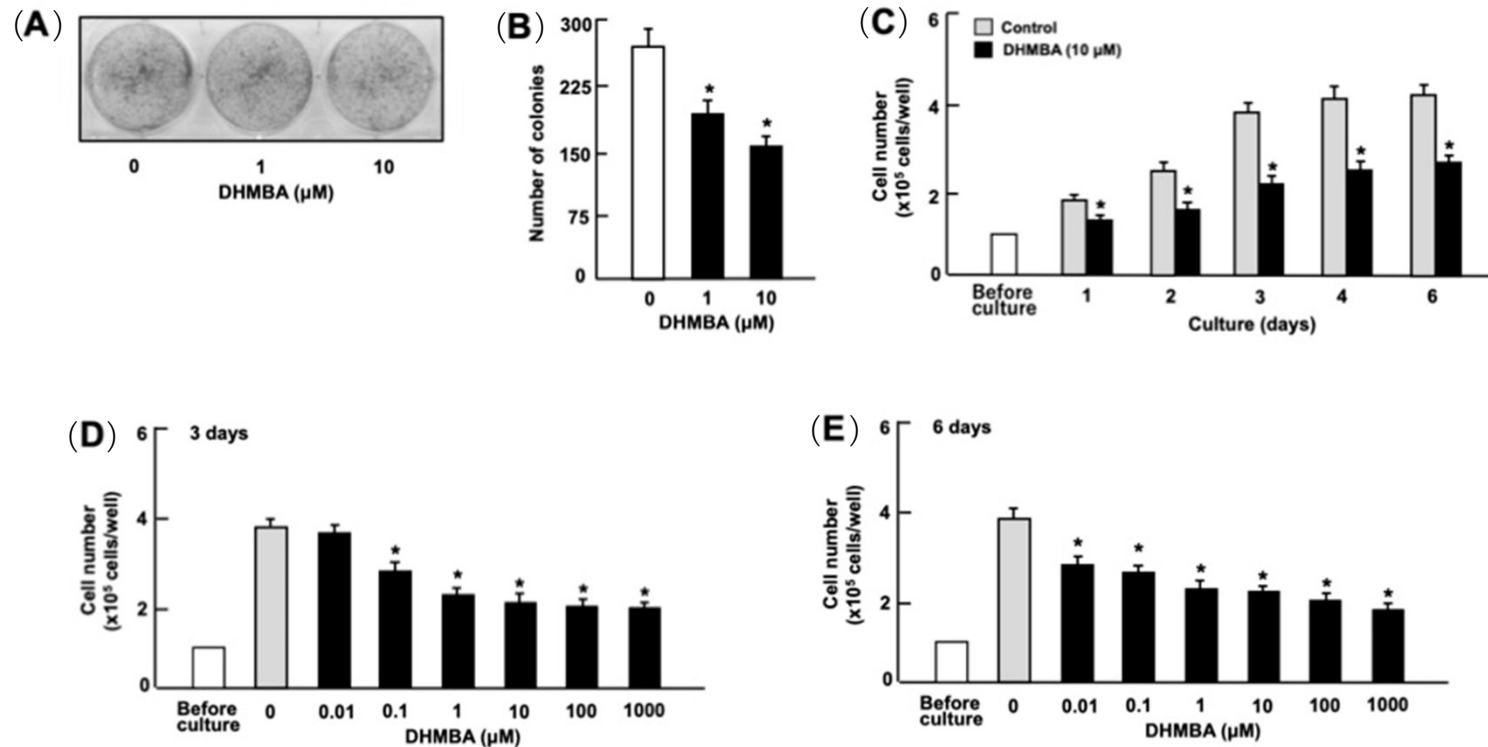
Fig. 1



Explanatory diagram describing an inhibitory effect of 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) on growth of human prostate cancer PC-3 cells.

Fig. 1(A): PC-3 cells (cell number of 1×10^3 /per well of 6-well plate) were cultured under 5% of CO_2 , at 37°C , for 9 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (1 or 10 μM). **Fig. 1(B):** The number of colonies was counted. **Fig. 1(C):** PC-3 cells (1×10^5 cells/per well of 24-well plate) were cultured under 5% of CO_2 , at 37°C , for 1 to 6 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (10 μM). **Figs. 1(D) and 1(E):** PC-3 cells (1×10^5 cells/per well of 24-well plate) were cultured under 5% of CO_2 , at 37°C , for 3(**Fig. 1D**) and 6(**Fig. 1E**) days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (0.01, 0.1, 1, 10, 100, and 1000 μM). After the culture, cells adhered to the bottom surface of the dish were removed and counted. Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of cultured cells. * $p < 0.001$, comparison with the control (white or gray bar). 1-way ANOVA, Tukey-Kramer post-test.

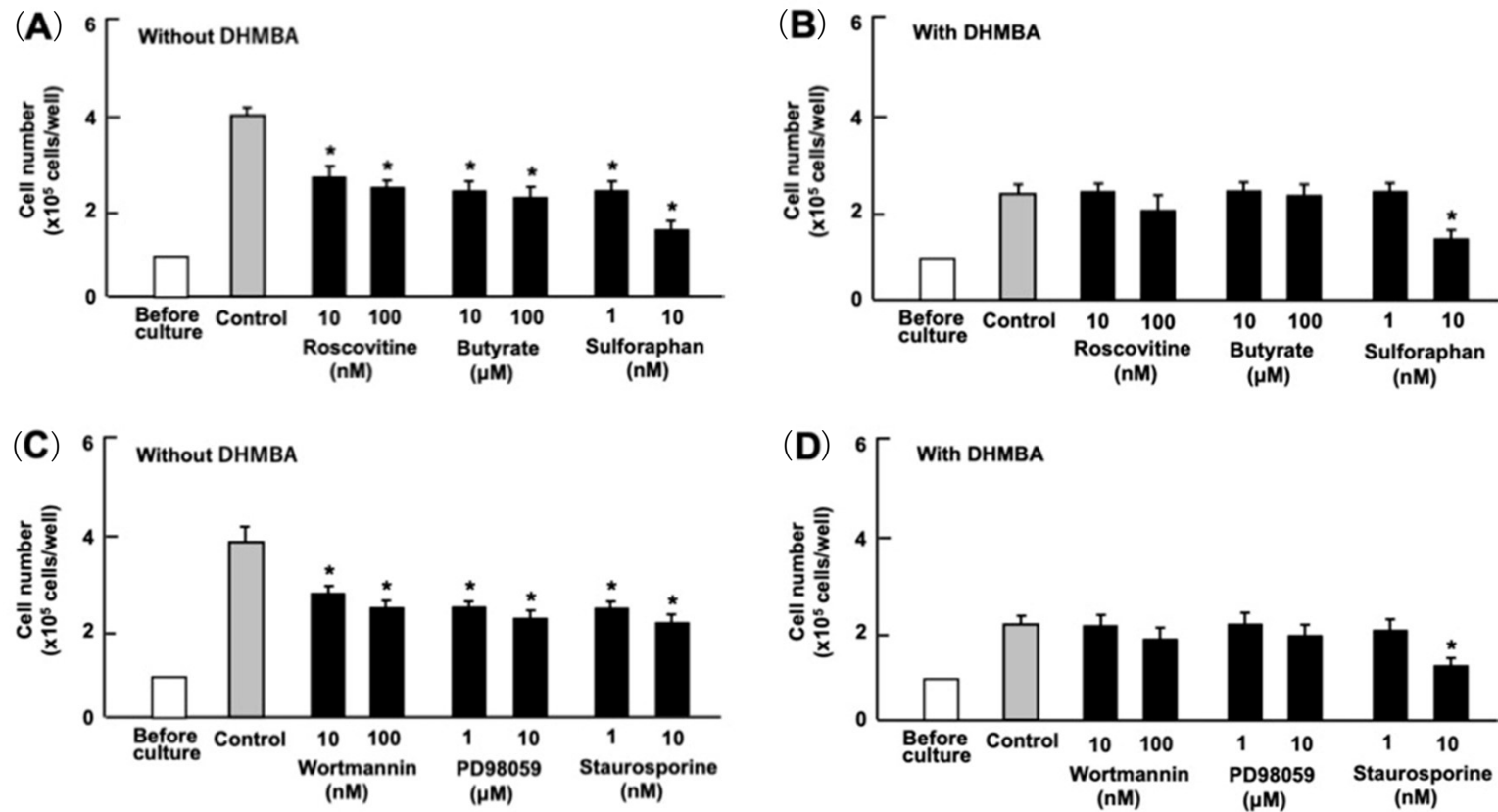
Fig. 2



Inhibitory effect of 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) on growth of human prostate cancer DU-145 cells.

Fig. 2(A): DU-145 cells (1×10^3 cells/per well of 6-well plate) were cultured under 5% of CO_2 , at 37°C , for 9 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (1 or 10 μM). **Fig. 2(B):** The number of colonies was counted. **Fig. 2(C):** DU-145 cells (1×10^5 cells/per well of 24-well plate) were cultured under 5% of CO_2 , at 37°C , for 1 to 6 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (10 μM). **Figs. 2(D) and 2(E):** DU-145 cells (1×10^5 cells/per well of 24-well plate) were cultured under 5% of CO_2 , at 37°C , for 3 (**Fig. 2D**) and 6 (**Fig. 2E**) days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (0.01, 0.1, 1, 10, 100, and 1000 μM). After the culture, cells adhered to the dish were removed and counted. Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of cultured cells. * $p < 0.001$, comparison with the control (white or gray bar). 1-way ANOVA, Tukey-Kramer post-test.

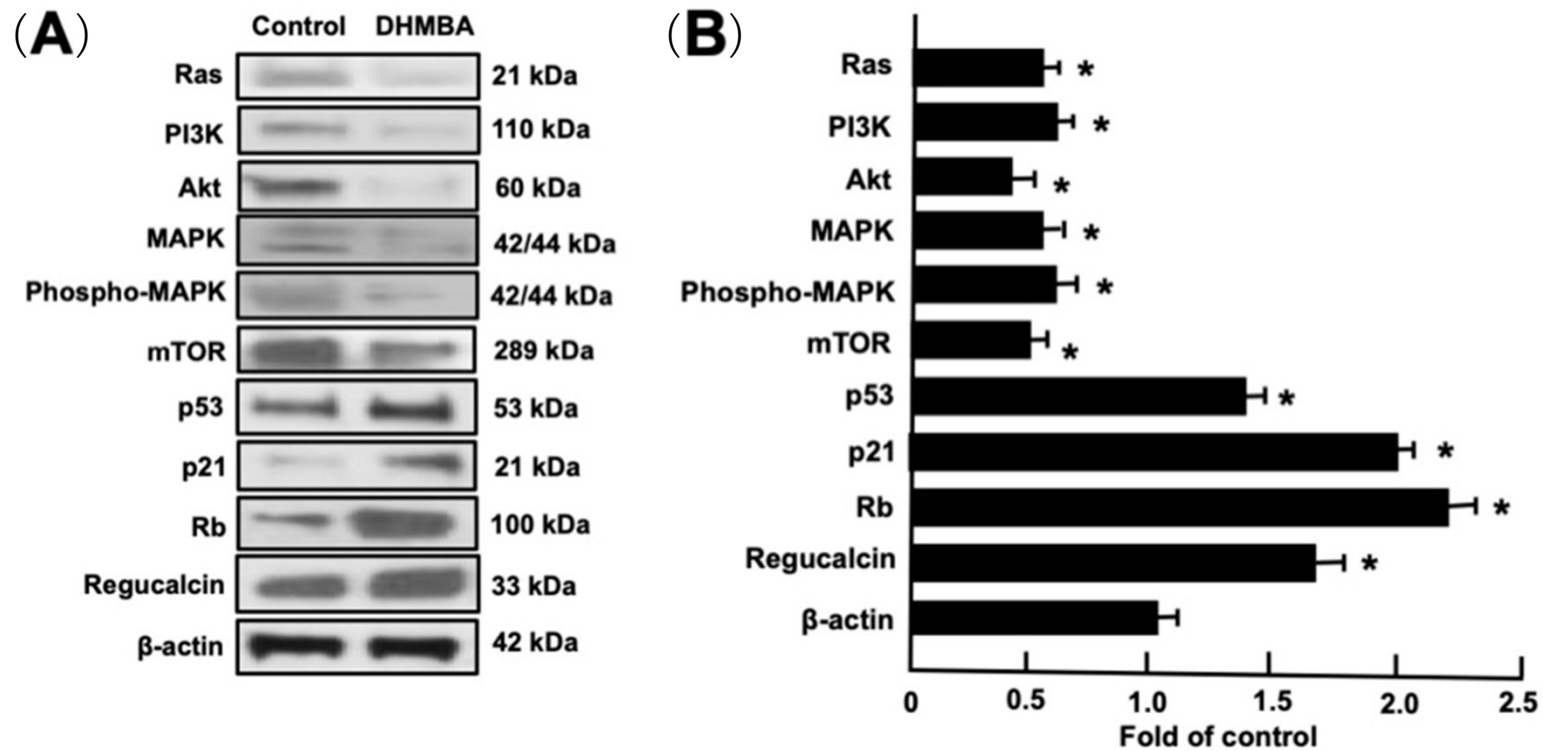
Fig. 3



Inhibitory effect of 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) on growth of human prostate cancer PC-3 cells in the presence of a cell growth inhibitor and an intracellular signaling inhibitor.

Figs. 3(A) and 3(B): PC-3 cells (1×10^5 cells/ml/per well of 24-well plate) were cultured under 5% of CO₂, at 37°C, for 3 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (10 μM) containing roscovitin (10 and 100 nM), butyrate (10 and 100 μM), and sulforaphane (1 and 10 nM) of cell cycle inhibitors. **Figs. 3(C) and 3(D):** PC-3 cells (1×10^5 cells/ml/per well of 24-well plate) were cultured under 5% of CO₂, at 37°C, for 3 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (10 μM) containing wortmannin (10 or 100 nM), PD98059 (1 or 10 μM), and staurosporine (1 or 10 nM). After the culture, cells adhered to the dish were removed and counted. Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of different cultured cells. *p < 0.001, comparison with the control (gray bar). 1-way ANOVA, Tukey-Kramer post-test.

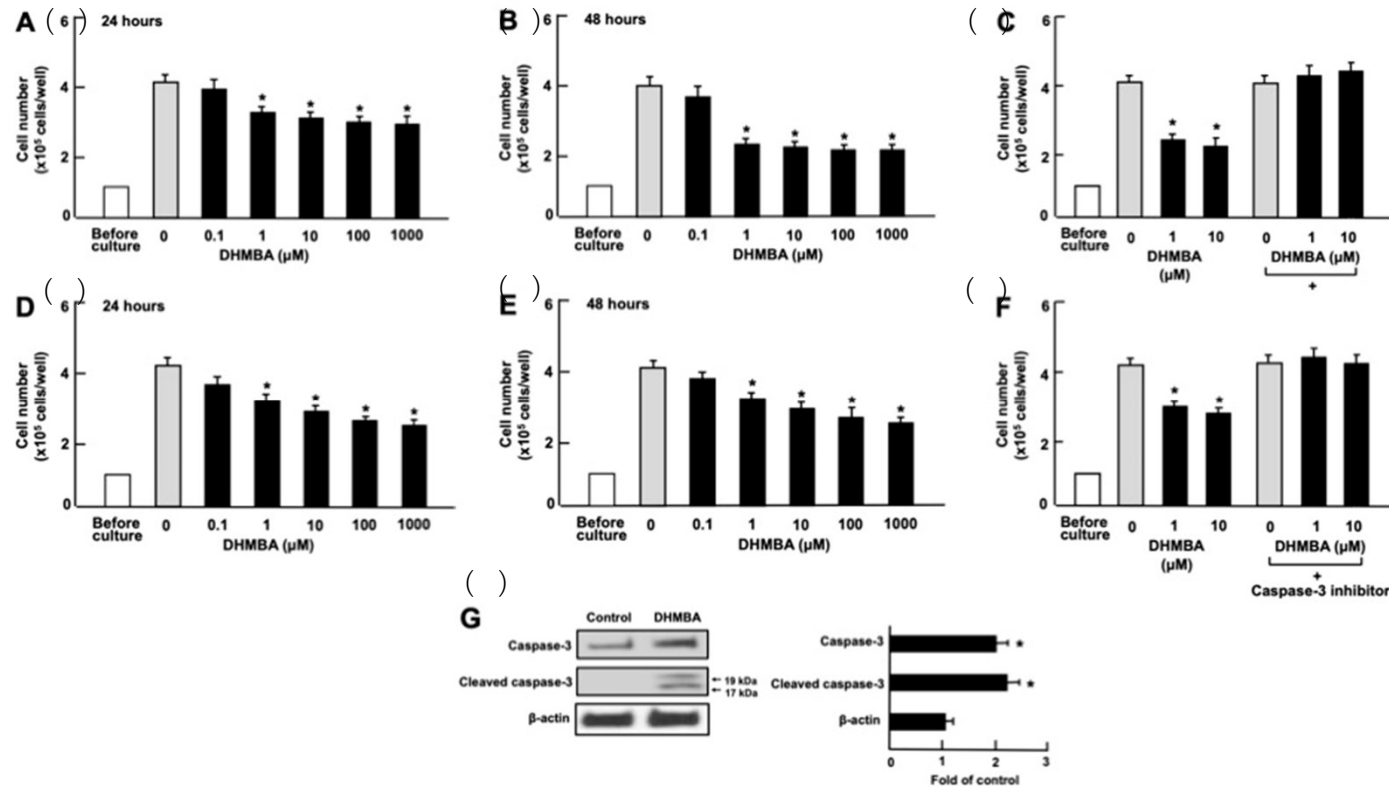
Fig. 4



Effect of DHMBA on an expression level of protein molecules involved in enhancement of cell growth of prostate cancer cells.

PC-3 cells (1×10^6 cells/10 ml, 100-mm dish) were cultured under 5% of CO_2 , at 37°C , for 3 days in DMEM culture fluid containing 10% of FBS, 1% of P/S, and 1% of fungizone. In addition, the culture fluid contained DHMBA ($10 \mu\text{M}$). After the culture, the cells were adjusted by adding a cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing proteolytic enzyme and a protein phosphatase activity inhibitor. Using $40 \mu\text{g}$ of a protein sample solution, the product was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE, 12%). After that, protein molecules were transferred to a nylon membrane. Immunoblot was performed on the membrane using an antibody that specifically bound to the protein molecules. **Fig. 4(A)**: As band of each of protein molecules, representative one obtained from 4 dishes of different cultured cells is shown. **Fig. 4(B)**: Mean values and standard deviations of % values compared with a control group of a value obtained by measuring a concentration of each band are shown. * $p < 0.01$, comparison with the control. 1-way ANOVA, Tukey-Kramer post-test.

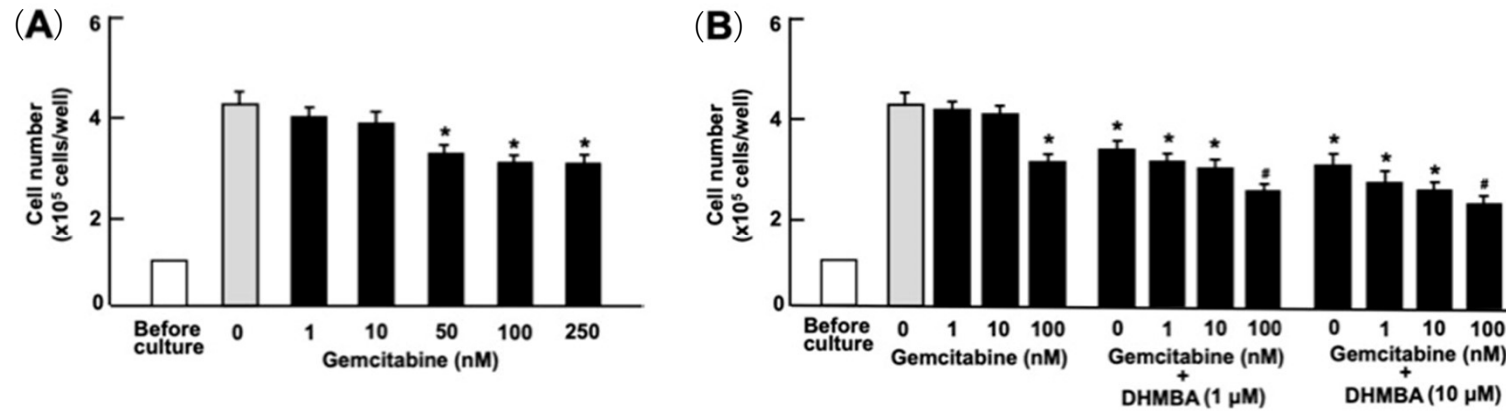
Fig. 5



Effect of 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) on cell death of human prostate cancer PC-3 and DU-145.

PC-3 (Figs. 5(A) and 5(B)) and DU-145 (Figs. 5(D) and 5(E)) cells (1×10^5 /in 1 ml of culture fluid per hole using 24-hole plate) were cultured under 5% of CO₂, at 37°C, for 3 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone), and when the cells reached sub-confluence, the culture fluid was exchanged for one containing DHMBA (0.01, 0.1, 1, 10, 100, or 1000 μM), and the cells were cultured for 24 and 48 hours. After the culture, cells adhering to the dish were counted. In another experiment, after culture for 3 days, when the cells reached sub-confluence, for PC-3 (Fig. 5(C)) or DU-145 (Fig. 5(F)) cells, the culture fluid was exchanged for a culture fluid containing DHMBA (1 or 10 μM) and a caspase-3 inhibitor (10 μM), and the cells were counted after the culture for 48 hours. Further, after culture for 3 days, when the cells reached sub-confluence, the culture fluid was exchanged for a culture fluid obtained by adding gemcitabine (0.1, 1, 10, 50 or 100 nM) or CH223191 (1, 10, and 25 μM) to DMEM culture fluid containing DHMBA (1 or 10 μM) or not containing DHMBA, and the cells were counted after culture for 48 hours. After the culture, cells adhered to the dish were removed and counted. Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of different cultured cells. *p < 0.001, comparison with the control (gray bar). 1-way ANOVA, Tukey-Kramer post-test. **Fig. 5(G):** PC-3 cells (1×10^6 cells/10 ml, 100-mm dish) were cultured under 5% of CO₂, at 37°C, for 3 days in DMEM culture fluid containing 10% of FBS, 1% of P/S, and 1% of fungizone. In addition, the culture fluid contained DHMBA (10 μM). After the culture, the cells were adjusted by adding a cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing proteolytic enzyme and a protein phosphatase activity inhibitor. Using 40 μg of a protein sample solution, the product was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE, 12%). After that, the protein molecules were transferred to a nylon membrane. Immunoblot was performed on the membrane using an antibody that specifically bound to the protein molecules. **Fig. 5(A):** As band of each of protein molecules, representative one obtained from 4 dishes of different cultured cells is shown. **Fig. 5(B):** Mean values and standard deviations of % values compared with a control group of a value obtained by measuring a concentration of each band are shown. *p < 0.01, comparison with the control. 1-way ANOVA, Tukey-Kramer post-test.

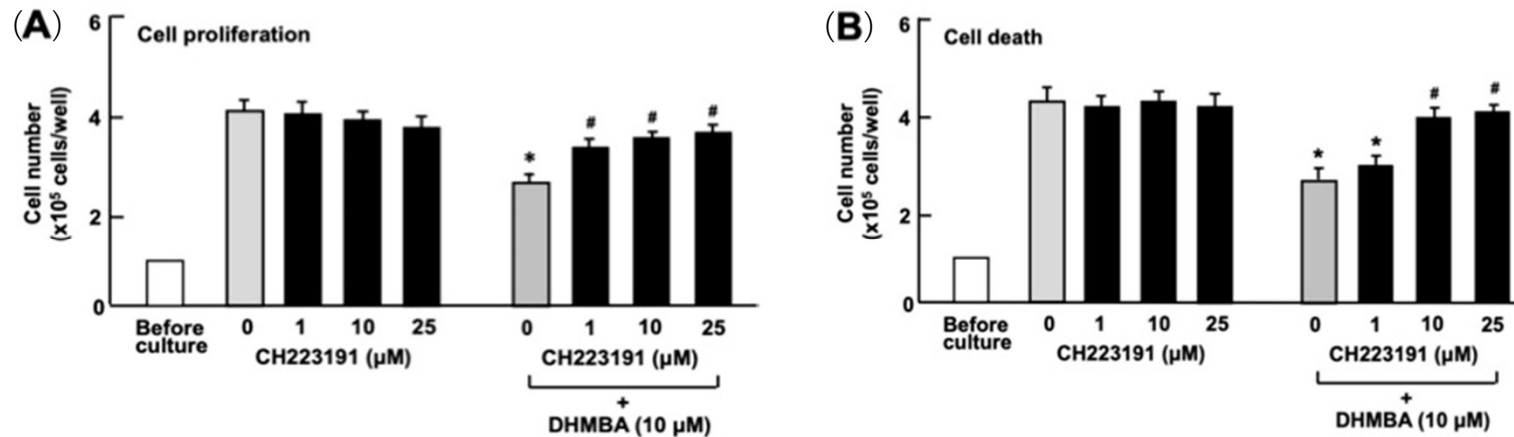
Fig. 6



Effects of 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) on human prostate cancer PC-3 cell death and comparison with an anticancer drug, gemcitabine.

Fig. 6(A): PC-3 cells (1×10^5 /in 1 ml of culture fluid per hole using 24-hole plate) were cultured under 5% of CO₂, at 37°C, for 3 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone), and when the cells reached sub-confluence, the culture fluid was exchanged for one containing gemcitabine (1, 10, 50, 100, or 250 nM), and the cells were cultured for 48 hours. After the culture, cells adhering to the dish were counted. **Fig. 6(B):** After culture for 3 days, when the cells reached sub-confluence, the culture fluid was exchanged for a culture fluid containing DHMBA (1 or 10 μM) and gemcitabine (1, 10, or 100 nM), and the cells were counted after culture for 48 hours. After the culture, cells adhered to the dish were removed and counted. Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of cultured cells. *p < 0.001, comparison with the control (gray bar). 1-way ANOVA, Tukey-Kramer post-test.

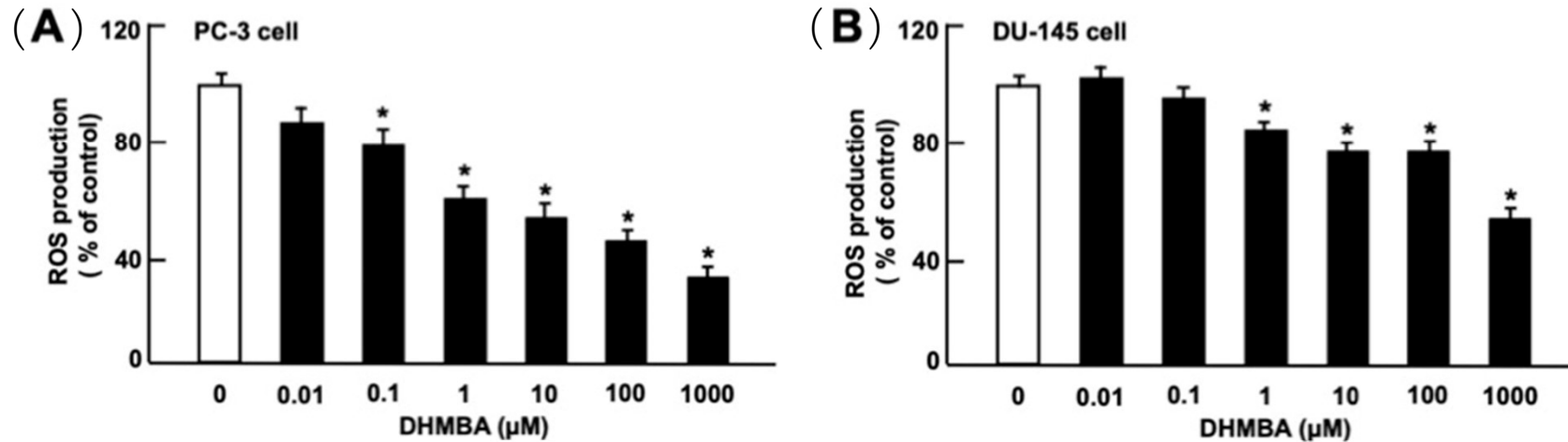
Fig. 7



Effect of an aryl hydrocarbon receptor inhibitor on growth inhibitory and cell death inducing actions of DHMBA on prostate cancer cells.

Fig. 7(A): PC-3 cells (1×10^5 /in 1 ml of culture fluid per hole using 24-hole plate) were cultured under 5% of CO_2 , at 37°C , for 3 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) in the presence of DHMBA (10 μM) and aryl hydrocarbon receptor inhibitor CH223191 (1, 10, or 25 μM) to examine the action on cell growth. **Fig. 7(B):** PC-3 cells (1×10^5 /in 1 ml of culture fluid per hole using 24-hole plate) were cultured under 5% of CO_2 , at 37°C , for 3 days in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone), and when the cells reached sub-confluence, the culture fluid was exchanged for one containing DHMBA (10 μM) and CH223191 (1, 10, or 25 μM), and the cells were cultured for 48 hours. After the culture, cells adhered to the bottom surface of the dish were removed and counted. Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of cultured cells. * $p < 0.001$, comparison with the control (gray bar not containing CH223191 or DHMBA). 1-way ANOVA, Tukey-Kramer post-test.

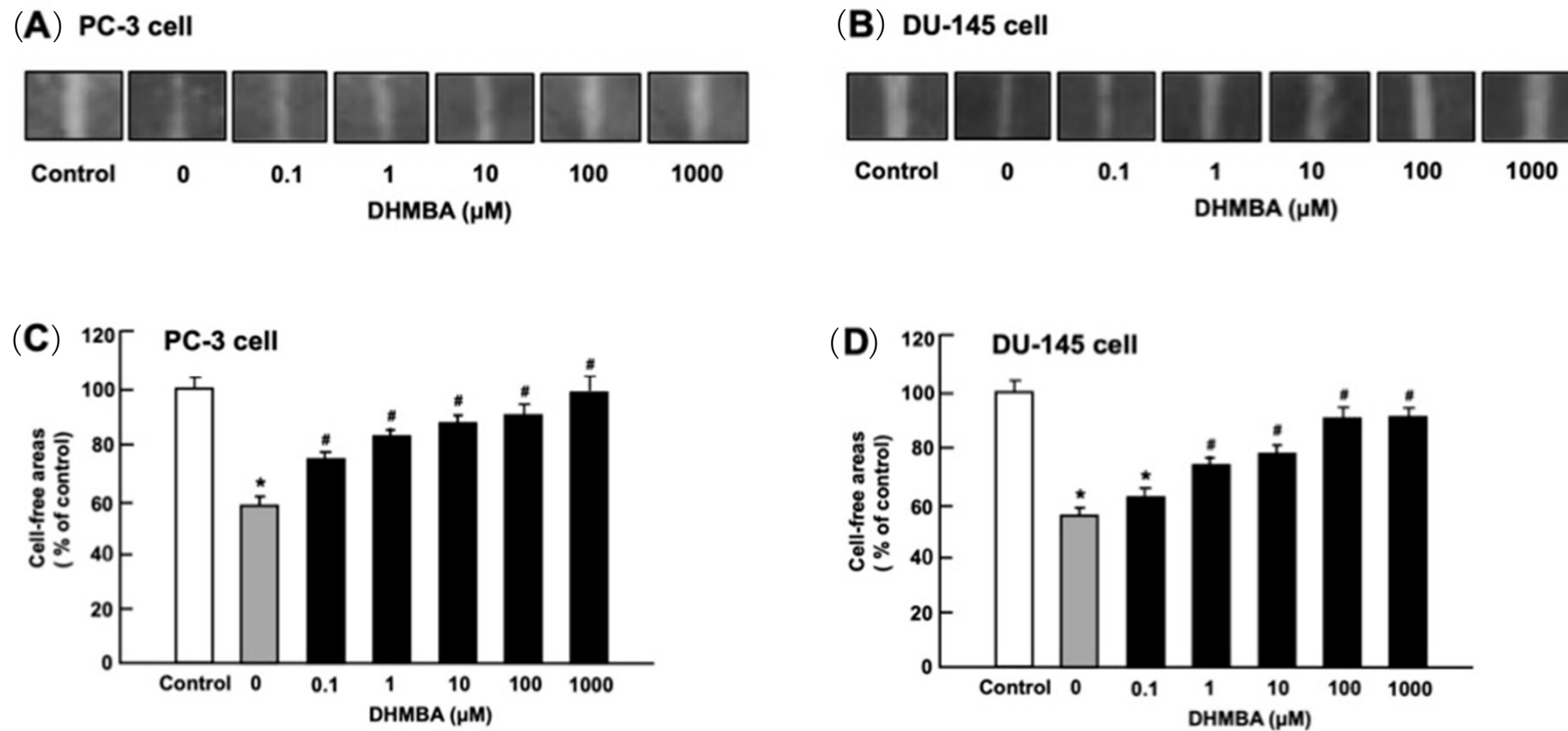
Fig. 8



Inhibitory effect of DHMBA on active oxygen production of prostate cancer cells.

PC-3 and DU-145 cells ($2.5 \times 10^4/0.1$ ml of culture fluid per hole using 96-hole plate) were cultured under 5% of CO_2 , at 37°C , for 24 hours in DMEM culture fluid (containing 10% of FBS, 1% of P/S, and 1% of fungizone) containing DHMBA (0.1, 1, 10, 100, or 1000 μM). After the culture, the cells were washed with PBS, 0.1 ml and 25 μM of an H_2DCFDA solution was added, and incubation was performed for 45 minutes. After the cells were washed with PBS, fluorescence intensity of cells was measured using a fluorescence measurement plate reader at wavelength Ex/Em = 485/535 nm. The result was displayed as % of fluorescence intensity relative to the control group to which DHMBA was not added. Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of cultured cells. * $p < 0.001$, comparison with the control (white bar). 1-way ANOVA, Tukey-Kramer post-test.

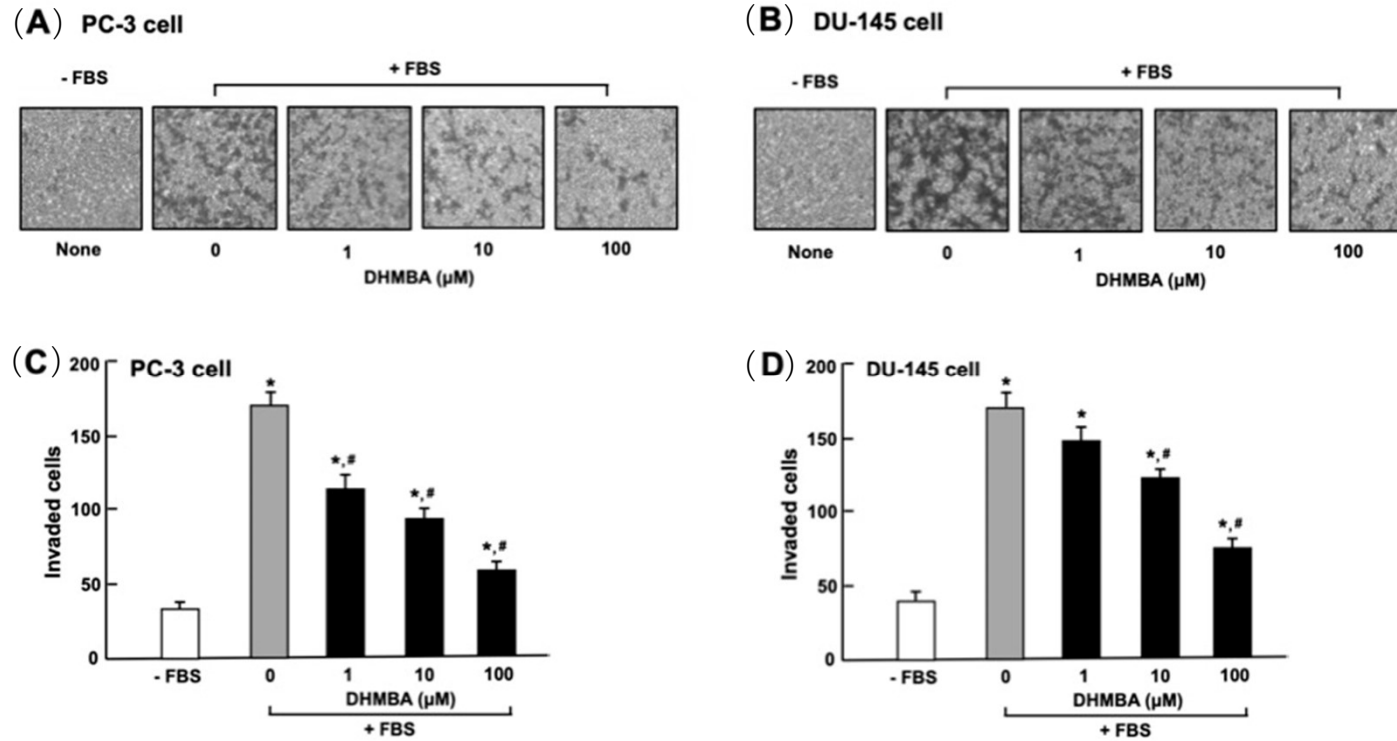
Fig. 9



Inhibitory effect of DHMBA on migration of prostate cancer cells.

PC-3 and DU-145 cells (cell number of 2×10^5 /1 ml of culture fluid per hole using 24-hole plate) were cultured under 5% of CO_2 , at 37°C , for 48 hours in DMEM culture fluid containing 10% of FBS, 1% of P/S, and 1% of fungizone, and a straight line scratch was performed using a pipette tip (200 μl) when the cells reached confluence. Free cells were washed with PBS to be removed, the above-described culture fluid (containing 0.1, 1, 10, 100, or 1000 μM of DHMBA) was added, and the cells were cultured for 48 hours. After the culture, intervals of cell-free parts were measured. **Figs. 9(A) and 9(B)**: Cells on which crystal violet stain was performed. **Figs. 9(C) and 9(D)**: An interval after culture was compared with an interval before the culture when scratch was performed, and the result was indicated as % relative to the control (the value when DHMBA was not contained). Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of cultured cells. * $p < 0.001$, comparison with the control (white bar). # $p < 0.001$, comparison with the control not containing DHMBA (gray bar). 1-way ANOVA, Tukey-Kramer post-test.

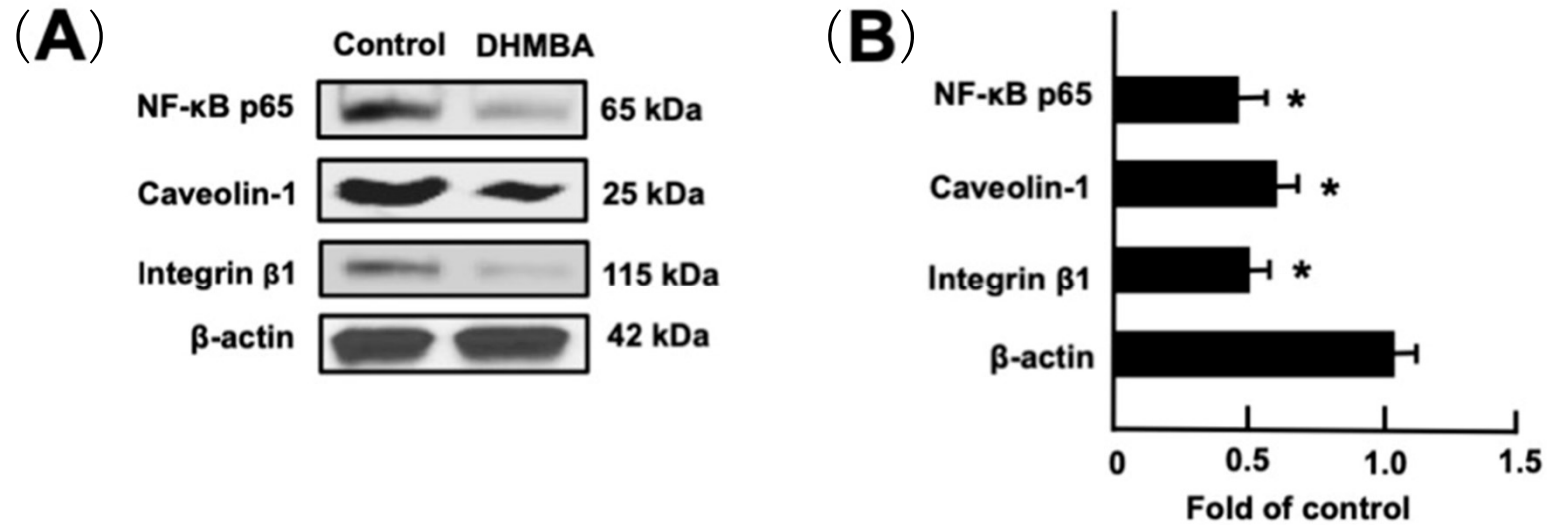
Fig.10



Inhibitory effect of DHMBA on invasion of prostate cancer cells.

Cell invasion activities of prostate cancer PC-3 and DU-145 cells were measured using Transwell chambers. 500 μl of a suspension of PC-3 or DU-145 cells (cell number of 2.5×10^4 /ml, DMEM not containing FBS) was added. This cell suspension contained DHMBA (0, 1, 10, or 100 μM). After addition of the cell suspension to the chambers, it was incubated for 1 hour to cause the cells to adhere to the surface of gel. 0.75 ml of the culture fluid not containing FBS and a culture fluid containing 10% of FBS were added to the lower surface of the chambers. The cells on this plate were cultured under 5% of CO₂, 95% of air, at 37°C, for 24 hours. After the culture, the culture fluid on the upper surface of the chambers was removed, the filter was collected and placed in 70% of cold ethanol (1 ml) for 30 minutes to fix the cells infiltrated with the gel on the lower surface of the filter. After that, the product was immersed in a solution (containing 0.5% of crystal violet in 0.75 ml and 20% of methanol) for 30 minutes for staining. After the staining, the product was washed with PBS and dried. After the drying, the number of the stained cells was counted by the microscope (Olympus MTV-3, Olympus Corporation, Tokyo, Japan). Videos of the stained cells were photographed. **Figs. 10(A) and 10(B)**: Representative images are shown. **Figs. 10(C) and 10(D)**: Each piece of data is shown with mean values and standard deviations of values obtained from 8 wells of cultured cells. *p < 0.001, comparison with the control not containing FBS (white bar). #p < 0.001, comparison with the control not containing DHMBA (gray bar). 1-way ANOVA, Tukey-Kramer post-test.

Fig.11



Effect of DHMBA on an expression level of protein molecules involved in enhancement of migration and invasion of prostate cancer cells.

PC-3 cells (cell number of $1 \times 10^6/10$ ml, 100-mm dish) were cultured in DMEM culture fluid containing 10% of FBS, 1% of P/S, and 1% of fungizone under 5% of CO_2 , at 37°C , for 3 days. In addition, the culture fluid contained DHMBA ($10 \mu\text{M}$). After the culture, the cells were adjusted by adding a cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing proteolytic enzyme and a protein phosphatase activity inhibitor. Using $40 \mu\text{g}$ of a protein sample solution, the product was separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE, 12%). After that, the protein molecules were transferred to a nylon membrane. Immunoblot was performed on the membrane using an antibody that specifically bound to the protein molecules. **Fig. 11(A)**: As band of each of protein molecules, representative one obtained from 4 dishes of different cultured cells is shown. **Fig. 11(B)**: Mean values and standard deviations of % values compared with a control group of a value obtained by measuring a concentration of each band are shown. * $p < 0.01$, comparison with the control. 1-way ANOVA, Tukey-Kramer post-test.