INDUCTION BY HERBIMYCIN A OF CONTACT INHIBITION IN *v-src*-EXPRESSED CELLS

KAYOKO SUZUKAKE-TSUCHIYA, YUKARI MORIYA and MAKOTO HORI

Showa College of Pharmaceutical Sciences,
5-1-8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan

YOSHIMASA UEHARA

National Institute of Health,
2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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Herbimycin A, an inhibitor of $pp60^{src}$ tyrosin kinase, caused *src* oncogene-expressed cells to become sensitive to contact inhibition, but did not affect *ras* oncogene-expressed cells. The cell lines tested were temperature sensitive *v-src*- and temperature sensitive *v-ras*-integrated nontransformed rat kidney cell line (NRK) (*src*^{ts}NRK and *ras*^{ts}NRK, respectively) and a wild-type *v-src*-integrated NIH3T3 (*src*3T3). *src*^{ts}NRK cells in densely populated cultures (plated at 1.25×10^4 cells/cm^2), grown at 33°C in the presence of 0.45 μg/ml of herbimycin A, ceased the cell cycle at the G_0-G_1 stage within 2 days, and the cells showed normal morphology. Upon removal of herbimycin A, the quiescent cells resumed the cell cycle in concert with morphological alteration from 'normal' to 'transformed', and proceeded through the S and M stages successively in a synchronized manner. Cells in the late S stage, compared with those in other stages of the cell cycle, were more sensitive to the killing effect of 5-fluorodeoxyuridine. Such synchronism of the cell cycle was not observed with sparsely populated cultures (2.5×10^3 cells/cm^2); the cells resumed their asynchronous growth after removal of herbimycin A, although their morphology returned to 'transformed' as in the experiment with the densely populated cultures. The induction by herbimycin A of contact inhibition in densely populated cultures was also observed with *src*3T3 (grown at 37°C) but not with *ras*^{ts}NRK (grown at 33°C).

Herbimycin, a benzoquinoid ansamycin antibiotic originally screened for its herbicidal activity[1], was reisolated as an active substance altering the transformed morphology of *v-src*-expressed cells to the normal morphology[2]. The morphological alteration was accompanied by changes in various phenotypes including organization of cytoskeltons, synthesis of fibronectin[3], requirement for higher concentrations of serum in the culture medium, lowered glucose transport, and loss of colony forming ability in the soft agar medium[4].

Contact inhibition of growth is an important characteristic of normal cells. We found that *v-src*-expressed cells in densely populated cultures became contact-inhibited in the presence of herbimycin and, when the antibiotic was removed, so was the contact inhibition and the cells resumed growth in a synchronized manner. The cells in the late S stage were killed by 5-fluorodeoxyuridine (FUdR) to a greater extent. These results suggest a possible use of herbimycin in cancer chemotherapy in combination with delayed administration of a drug which is selectively toxic to DNA synthesizing cells.

Materials and Methods

Cells and Culture Conditions

A nontransformed rat kidney cell line (NRK) infected with ts25, a T class mutat of Rous sarcoma virus Prague strain[5] (*src*^{ts}NRK), was provided by M. YOSHIDA, Cancer Institute, Tokyo, Japan. ts371 Kirsten murine sarcoma virus-infected NRK[6] (*ras*^{ts}NRK) was provided by T. Y. SHIH, NIH, Bethesda, MD. An NIH3T3 cell line infected with Rous sarcoma virus SR-D (*src*3T3) was provided by S. KAWAI, the Institute of Medical Science, University of Tokyo, Tokyo, Japan. The cell lines were maintained in DULBECCO's modified Eagle medium (DME; Gibco Laboratories, Grand Island, N.Y.) supplemented with 5% heat inactivated calf serum in humidified air with 5% CO_2 at 33°C (*src*^{ts}NRK and *ras*^{ts}NRK) or at 37°C (*src*3T3).

Synchronization of *src*^{ts}NRK Cells

src^{ts}NRK cells were seeded at 10^5 cells/dish (2 ml, 8 cm^2) (high density cell cultures) or at 2×10^4 cells/dish (2 ml, 8 cm^2) (low density cell cultures). Twelve hours after the cell seeding, 0.45 μg/ml and 0.125 μg/ml herbimycin were added to the high density and the low density cell cultures, respectively. The cells were cultured for 48 hours with herbimycin, with renewal of half volumes of the herbimycin-containing media at 24 hours taking in consideration of the instability of the antibiotic and the consumption of growth factors. About 70% inhibition of cell growth accompanied by alteration of cell morphology to 'normal' was observed with the cultures at both cell densities. The cells were then washed free of herbimycin with DULBECCO's phosphate buffered saline (PBS) and incubated further in the ordinary medium. At the time indicated, the incubation of 2 sets of 3 dishes each was terminated, one set was labeled with [6-^{3}H]TdR (25 Ci/mmol; 0.25 μCi/dish and 1.0 μCi/dish to a high density cell culture and a low density cell culture, respectively) to monitor the rate of DNA synthesis while the other set was subjected to cell counting. Rates of DNA synthesis and cell counts obtained for the three dishes in each set were averaged.

Determination of Rates of DNA Synthesis

src^{ts}NRK cells/dish (2 ml, 8 cm^2) were labeled with [^{3}H]TdR at 33°C for 1 hour. The cells were washed with 1 ml of cold PBS and received 2 ml of cold 5% TCA. The acid-insoluble residues of the cell layers were washed with 1 ml of cold 5% TCA, dissolved in 0.5 ml of 0.5 N KOH, mixed with a liquid scintillation solution and subjected to radioactivity measurement.

Determination of the Killing Effect of FUdR on Synchronized Cells

src^{ts}NRK cells in densely populated cultures were exposed to herbimycin for 2 days, washed free of the antibiotic and allowed to grow in a herbimycin-free medium as in the synchronization experiment. The cells at G_0-G_1, early S, middle S, late S or G_2-M stage were treated with a mixture of FUdR and uridine, both at 5 μg/ml, for 4 hours, and applied to solid support colony formation according to MURAKAMI *et al.*[4] with minor modifications. One ml aliquot of the cell suspension (200 cells/ml) was added to culture dishes containing 4 ml of prewarmed DME medium with 10% serum and 0.05 μg/ml amphotericin B. Colonies were stained with 0.5% crystal violet in 20% MeOH.

Results and Discussion

Synchronization by Herbimycin of *src*^{ts}NRK Cells in Densely Populated Cultures at 33°C

To test if herbimycin causes *v-src* expressed cells to become sensitive to contact inhibition, *src*^{ts}NRK cells in densely or sparsely populated cultures at 33°C (a permissive temperature) were temporarily exposed to herbimycin, washed free of the antibiotic and re-incubated. The logic underlying this experiment was that the cells in densely populated cultures, but not sparsely populated ones, would become contact-inhibited and cease the cell cycle at the G_0-G_1 stage, if herbimycin induced contact-inhibition. Therefore, upon removal of herbimycin, the cells in densely populated cultures should resume growth in a synchronized

Fig. 1. Growth of *src*^{ts}NRK cells after removal of herbimycin.

● High density cell cultures, ▲ low density cell cultures. The time of removal of herbimycin is expressed as 0 hour.

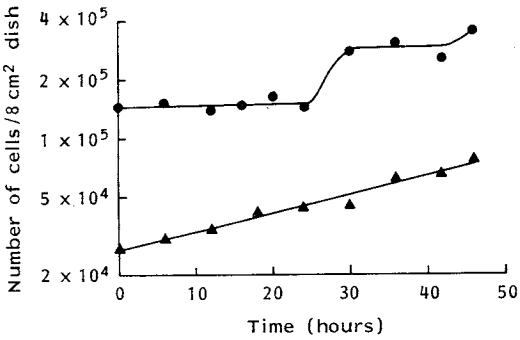


Fig. 2. DNA synthesis by *src*^{ts}NRK cells after removal of herbimycin.

○ High density cell cultures, △ low density cells cultures.

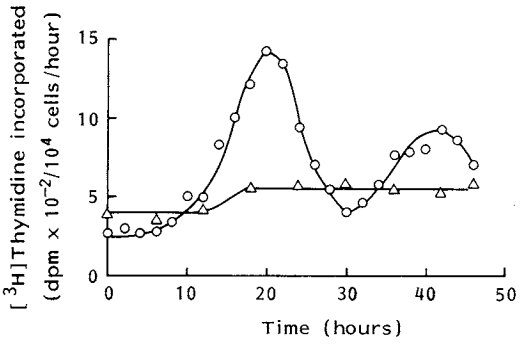
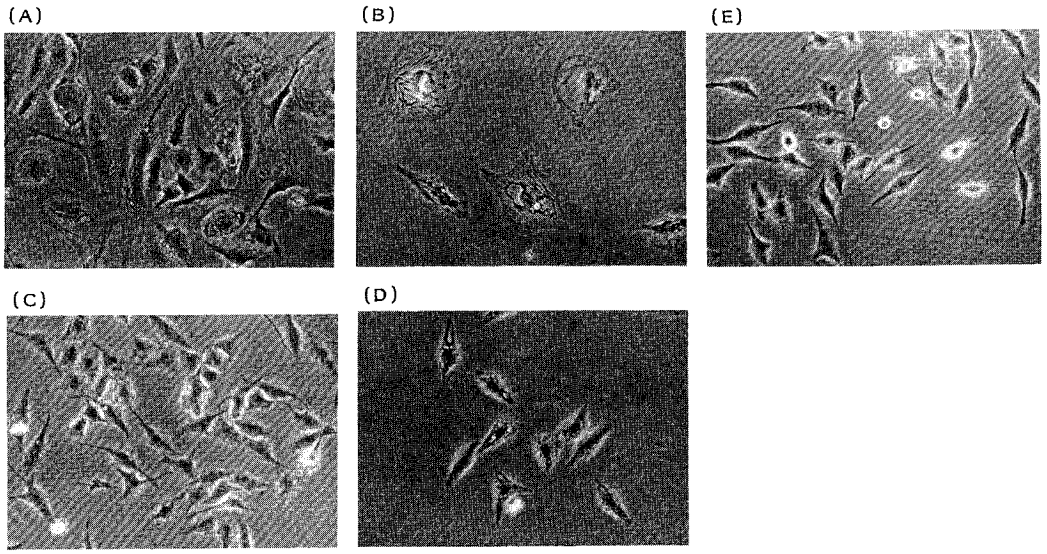


Fig. 3. Phase-contrast micrographs of *src*^{ts}NRK cells.



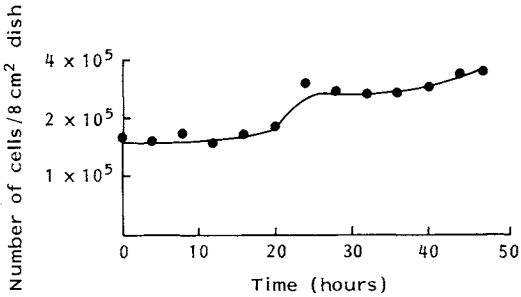
Cells were treated with herbimycin for 48 hours in a densely populated culture (A) or a sparsely populated culture (B), washed free of herbimycin and incubated further in the absence of herbimycin for 18 hours (C and D for the densely and the sparsely populated cultures, respectively). Cells without the herbimycin treatment (E).

manner while those in sparsely populated cultures should grow asynchronously. Cell growth after removal of herbimycin under either condition would be accompanied by morphological alteration from 'normal' to 'transformed'.

The results were as we had expected. It should be noted in Fig. 1 that the number of cells in the densely populated cultures stayed constant at 1.5×10^5 cells/dish for almost 24 hours after removal of herbimycin, then doubled within 6 hours to reach 3.0×10^5 cells/dish, and maintained that density until the end of the observation. DNA synthesis occurred also synchronously (Fig. 2); the first peak appeared 20 hours after the removal of herbimycin, *i.e.*, 10 hours before the cell division. The second peak of DNA

synthesis followed the cell division with a 12 hours' delay. In contrast, the sparsely populated cultures resumed asynchronous growth soon after the removal of herbimycin (Fig. 1), with a nearly constant rate of DNA synthesis (Fig. 2). The alteration of cell morphology from 'normal' to 'transformed' proceeded at similar rates irrespective of cell populations (Fig. 3).

Fig. 4. Growth of *src*3T3 cells after removal of herbimycin.



*src*3T3 cells were seeded at 10^5 cells/dish (2 ml, 8 cm^2) and cultured with 0.3 μg/ml herbimycin for 48 hours.

Effects of Herbimycin on Other Cell Lines

Densely populated cultures of *src*3T3, another cell line expressing *v-src* (not temperature sensitive), were also synchronized by a temporary exposure to herbimycin, accompanied by morphological alterations (Figs. 4 and 5).

It has been reported that herbimycin alters cell morphology of various cell lines expressing tyrosine kinase family oncogenes, with the exception of a

Fig. 5. Phase-contrast micrographs of *src*3T3 cells.

Densely populated cells were incubated for 48 hours in the presence (A) or absence (B) of herbimycin.

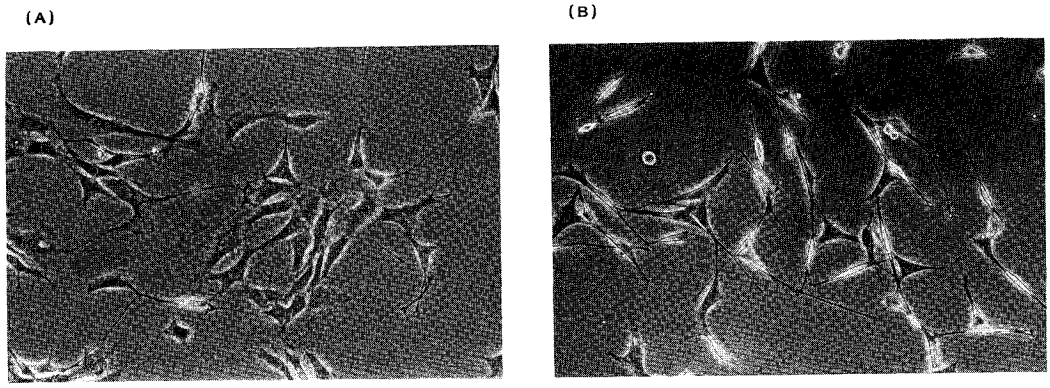
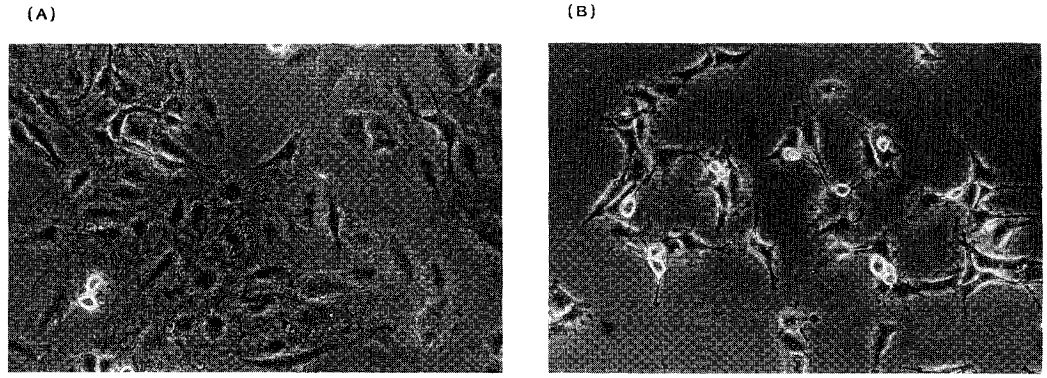


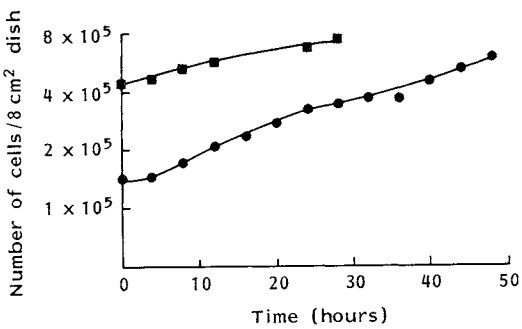
Fig. 6. Phase-contrast micrographs of *ras*^{ts}NRK cells.

Densely populated cells were incubated for 48 hours in the presence (A) or absence (B) of herbimycin.



quasi-positive effect on NRK integrating K-*ras*[7]. The *quasi*-effect was retested by use of *ras*[ts]NRK in which p21, the oncogene product, is heat-labile. The cell morphology of *ras*[ts]NRK after exposure to herbimycin for 48 hours at 33°C resembled closely the morphology observed at 39°C, *i.e.*, the normal morphology (Fig. 6). However, herbimycin did not make this cell line sensitive to contact inhibition, even though a wide range of drug concentrations and cell densities was tested. In a typical experiment, confluent cells with flattened morphology obtained by exposure to 3 μg/ml herbimycin for 48 hours (Fig. 6) resumed asynchronous growth after removal of herbimycin (Fig. 7). In addition, the normal-like morphology did not return to the original form ('transformed') as readily as did those of *v-src*-integrated cells (Fig. 8 vs. Fig. 3). Anchorage dependence for growth is another characteristic of normal cells. It has also been reported that herbimycin does not cause K-NRK (a cell line expressing *v*-K-*ras*, not temperature sensitive) to become anchorage-dependent for growth[4]. Therefore, it should be concluded that herbimycin has no effect on the function of *ras* oncogene and that the morphological alteration (Fig. 6) is the result of an unknown effect of the antibiotic. Alteration by herbimycin of all phenotypes, including cell morphology, from 'transformed' to 'normal' may be limited to cells expressing tyrosine kinase group oncogenes, as proposed[4].

Fig. 7. Growth of *ras*[ts]NRK cells after removal of herbimycin.

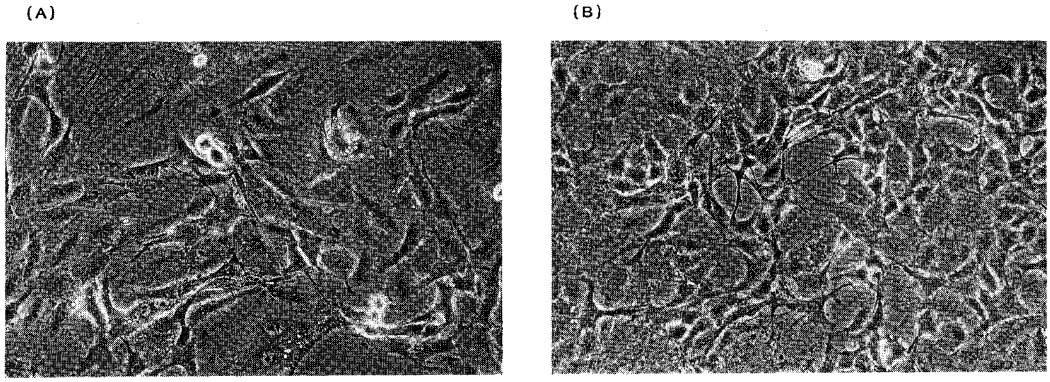


Cells were seeded at 10^5 cells/dish (2 ml, 8 cm^2) (●) or 2×10^5 cells/dish (2 ml, 8 cm^2) (■). After 12 hours of incubation, herbimycin was added at 3 μg/ml to the former or 2 μg/ml to the latter and incubation was continued.

A Possible Application of Inhibitors of Oncogene Products to Cancer Chemotherapy

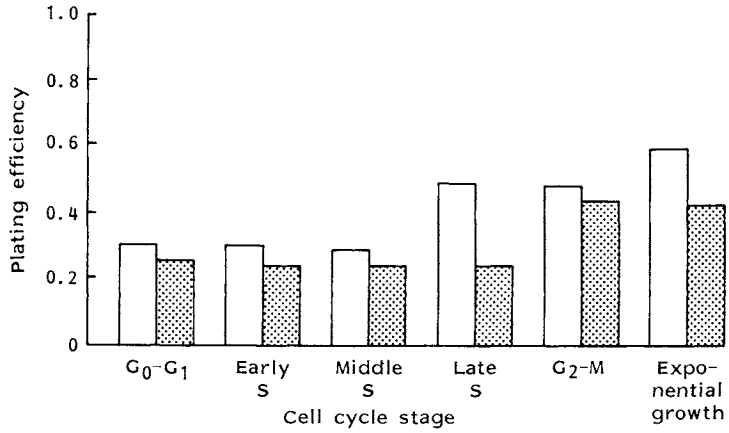
Generally, inhibition and/or inactivation of oncogene products should alter the cell phenotype from 'transformed' to 'normal'. The effect of herbimycin on *v-src*-expressing cells is a good example. We have also observed a similar effect of oxanosine, a guanosine analog, on *v-ras*-expressing cells[6]. This type of effect, however, should be reversible; in other words, it should not cause either permanent alteration of the phenotype or cell death

Fig. 8. Phase-contrast micrographs of *ras*[ts]NRK cells.



Cells in densely populated cultures were treated with 3 μg/ml herbimycin for 48 hours, washed free of herbimycin, and incubated further in the absence of herbimycin for 24 hours (A) and 48 hours (B).

Fig. 9. Effect of FUdR viability of herbimycin-induced synchronized *src*^{ts}NRK cells.
Open: Without FUdR, closed: with FUdR.



on its own because the oncogenes remain intact. In fact, herbimycin or oxanosine was only partially cytocidal under conditions which afforded the antibiotics to alter the cell morphology of nearly 100% cell population. Then, what would be the merit of finding inhibitors of oncogene products? The synchronism of the cell cycle induced by herbimycin to the densely populated cultures (Figs. 1 and 4) suggests the possible use of oncogene product inhibitors in cancer chemotherapy; they could be effective in combination with a delayed administration of FUdR, Ara C, and so on, which are selectively toxic to DNA synthesizing cells[8]. This possibility was tested. *src*^{ts}NRK cells were first synchronized using herbimycin and the sensitivity of these cells to the killing effect of FUdR was tested at various stages of the cell cycle. As we expected, there was a specific stage of the cell cycle (late S stage) where the cells were killed more efficiently by FUdR than in the other stages of the cell cycle (Fig. 9). The result will be applicable to cancer chemotherapy because tumor cells in the body appear to be confronted with physical barriers of surrounding tissues and, therefore, to become contact-inhibited and synchronized when they are exposed for a certain period of time to herbimycin or other oncogene product inhibitors. Oncogene-specific chemotherapy may become important in the future.

Acknowledgments

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References

1) ŌMURA, S.; Y. IWAI, Y. TAKAHASHI, N. SADAKANE, A. NAKAGAWA, H. ŌIWA, Y. HASEGAWA & T. IKAI: Herbimycin, a new antibiotic produced by a strain of *Streptomyces*. J. Antibiotics 32: 255~261, 1979
2) UEHARA, Y.; M. HORI, T. TAKEUCHI & H. UMEZAWA: Screening of agents which convert 'transformed morphology' of Rous sarcoma virus-infected rat kidney cells to 'normal morphology': Identification of an active agent as herbimycin and its inhibition of intracellular *src* kinase. Jpn. J. Cancer Res. (Gann) 76: 672~675, 1985
3) UMEZAWA, K.; S. ATSUMI, T. MATSUSHIMA & T. TAKEUCHI: Enhancement of fibronectin expression by herbimycin A. Experientia. 43: 614~616, 1987
4) MURAKAMI, Y.; S. MIZUNO, M. HORI & Y. UEHARA: Reversal of transformed phenotypes by herbimycin A in *src* oncogene expressed rat fibroblasts. Cancer Res. 48: 1587~1590, 1988

5) CHEN, Y. C.; M. J. HAYMAN & P. K. VOGT: Properties of mammalian cells transformed by temperature-sensitive mutants of avian sarcoma virus. Cell 11: 513~521, 1977
6) STEIN, R. B.; J. Y. TAI & E. M. SCOLNICK: Molecular cloning of the temperature-sensitive 371 Kirsten murine sarcoma virus and expression in *Escherichia coli* of the mutant and wild-type viral Kirsten *ras* p21 proteins. J. Virol. 60: 782~786, 1986
7) UEHARA, Y.; Y. MURAKAMI, S. MIZUNO & S. KAWAI: Inhibition of transforming activity of tyrosine kinase oncogenes by herbimycin A. Virology 164: 294~298, 1988
8) PATERSON, H.; B. REEVES, R. BROWN, A. HALL, M. Furth, J. BOS, P. JONES & C. MARSHALL: Activated N-*ras* controls the transformed phenotype of HT1080 human fibrosarcoma cells. Cell 51: 803~812, 1987