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DRUG-RESISTANCE GENE TRANSFFR INTO CULTURED-MURINE ERYTHRO-LEUKEMIA CELLS: PREFERENTIAL ACTIVITY IN INDUCING DIFFERENTIATION OF THE TRANSDUCTED CELLS IN VITRO BY DIMETHYL SULFOXIDE AND VITAMIN D_3

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ABSTRACT The gene transfer into various target cells is an important technique in genetic engineering, for such as biomass production or gene therapy because of the critical role it plays in new protein synthesis and intermediary metabolism. We demonstrate here, using a laser microinjector, the introduction of a drug-resistance gene into cultured-murine cells (TSA8). The cells were transformed to Geneticin (G418) resistance by introduction of the neomycin-resistanc gene. The transformed-murine erythroleukemia cells could be induced to differentiate by dimethyl sulfoxide (DMSO). Erythroid differrentiation by vitamin D_3 derivatives was examined in the cells. After the cells were cultured for 5-days with 1.0 % DMSO, as much as 60 % of the cells became benzydine-positive. This differentiation was markedly inhibited by addition of the active form of vitamin D_3, 1α, 25-dihydroxyvitamin D_3 (1α, 25 $(OH)_2D_3$). The active vitamin D_3 was the most potent in inhibition for the DMSO-induced erythroid differentiation. These results are the same results that the vitamin D_3 derivatives and DMSO are involved in erythroid differentiation of normal murine cells, as published elsewhere. These results suggest that the transduction of neomycin-resistance genes is totally ineffective in cell growth and erythroid differentiation for murine cells. These experiments also provide a posibility or model for future various gene replacement therapy in which functional genes can be introduced into various target cells using the laser microinjector.

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INTRODUCTION

Mammalian erythropoiesis is an ideal model system in which to study the regulation of determination and differentiation. Regulation of the rate of red blood cell formation may be achieved by control mechanisms that exert their effect at a number of critical steps. These include (i) proliferation of pluripotent hematopoietic stem cells (CFU-S); (ii) commitment of the hematopoietic stem cells to erythropoiesis; (iii) proliferation of committed erythroid precursor cells (namely, BFU-E (burst-forming unit of erythroid) and CFU-E (colony-forming unit of erythroid)); and (iv) commitment of the erythroid precursor cells to express the program of biosynthetic and morphogenetic activities characteristic of terminal differentiation of this specialized lineage (40, 41).

Established mouse erythroleukemic (MEL) cells have been used to analyse the commitment to terminal differentiation. MEL cells established from mice infected with Friend virus complex appear to be arrested at the proerythroblastic stage of development (2). These cells are not responsive to erythropoietion and, in this respect, differ from normal precursor cells. However, MEL cells can be induced in vitro to undergo a coordinated program resembling the final stage of normal erythroid differentiation by the addition of a variety of chemical reagents (2). Commitment is associated with the entire set of biochemical changes related to the erythroid differentiation.

Erythroid differentiation has been extensively studied in the murine erythroleukemia cells established by Friend et al., as described previously (1). Morphologically, these cells resemble proerythroblasts and show only a small precentage (under 1 %) of spontaneous erythroid differentiation (1). The cells can be induced to differentiate into erythrocytes by such inducers as dimethyl sulfoxide (DMSO) (2), butyric acid (3), and hexamethylene bisacetamide (4). Glucocorticoids (5) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (6) inhibit DMSO-induced differentiation of Friend cells. Erythroid differentiation can be detected by chromatin condensation and other morphological changes resulting in nondividing cells (2), appearance of specific antigens in exythrocyte membranes (7), heme and hemoglobin syntheses (2), and accumulation of globin mRNA (8).

Abe et al. found that the active form of vitamin D_3, 1α, 25-dihydroxyvitamin D_3 (1α, 25 (OH) $_2D_3$), suppresses proliferation and induces differentiation of murine myeloblastic leukemia cells (Ml) into monocyte macrophages (9). Subsequently, several laboratories reported that 1α, 25 $(OH)_2D_3$ is capable of inducing differentiation of not only Ml cells, but also human promyelocytic leukemia cells (HL-60) and human histiocytic monoblast-like lymphoma cells (U937) preferentially along the monocyte-macrophage pathway (10-14).

Recently, Shibuya and Mak established a novel type MEL cell line from the spleen of DBA/2 mice infected with anemiainducing Friend virus complex (FV-A) (15). This line has the capacity to form colonies in semi-solid culture. In the presence of dimethyl sulfoxide

(DMSO) or erythropoietin, TSA8 cells form colonies like those derived from CFU-E. Since a continuous 5-day exposure of cells to DMSO in liquid culture induces hemoglobin synthesis (15), this cell line has the ability to differentiate erythroid cells that are similar to the previously established Friend erythroleukemia cells (2, 8, 16,). However, this cell line is thought to be arrested at an earlier stage than previously described MEL cells for two reasons: its colony-forming ability in semi-solid culture and its responsibility to erythropoietin. Shibuya and Mak described that the level of hemoglobin-positive colonies of this line is maximum after 4-5 days in semi-solid culture (15). However, normal CFU-E from mouse bone marrow or fetal liver exhibit maximum levels within 2-days. TSA8 cells may have weak responsiveness to erythropoietin or may be an earlier stage than the CFU-E stage. In any case, these cells may be suitable for the analysis of commitment and proliferation of the earlier precursor cells.

The introduction of cloned DNA sequences into haematopoietic progenitor cells would provide a novel approach for studying this differentiating in vivo system. One laboratory has reported DNA-mediated transfer of genes into mouse bone marrow cells (17, 18). However, retroviruses offer a number of advantage over DNA-mediated gene transfer procedures, including high efficiency infection of a wide range of cell types in vitro and in vivo, stable and low copy integration into the host chromosome, and a defined integrated provirus structure. For thses reasons recombinant DNA techniques have been utilized to construct high efficiency retrovirus vectors expressing foreing genes (19-23).

We demonstrate here, using a Hitachi-laser microinjector, the introduction of a dominant selectable drug-resistance gene into defined classes of mouse haematopoietic progenitor cells. This microinjector technique would provide a number of advantages over retrovirus-mediated gene transfer procedures, including high efficiency transfection of various cells of animal and plant in vitro and in vivo. We now describe the effects of DMSO and vitamin D_3 on erythroid differentiation of transformed-murine erythroleukemia cells (TSA8).

MATERIALS AND METHODS

Recobinant Retroviruses. The retroviral vector pZIP-NEOSV (X) (24) containg the Tn5 neomycin-resistance gene (25) transcribed from the Moloney murine leukemia virus (MoMu LV) long terminal repeat (LTR) promoter (Fig. 1) was obtained from R. Mulligan and introduced into ψ2 cells (26) to produce "helper-free" viral stocks (27). The MoTN cell line produces a virus containing the Tn5 neomycin-resistance gene transcribed from a herpes simplex thymidine kinase (TK) promoter (Fig. 1) as described (28). The MuLV Neo. 1 cell line produces a virus containing the Tn5 neomycin-resistance gene transcribed in a reverse orientation from a simian virus 40 (SV40) early promoter (Fig. 1) as described (29). MuLV Neo. 1 and MoTN cell lines were kindly provided by A. Bernstein. Expression of the Tn5 neomycin-resistance gene in eukaryotic cells renders cells resistant to the neomycin analogue

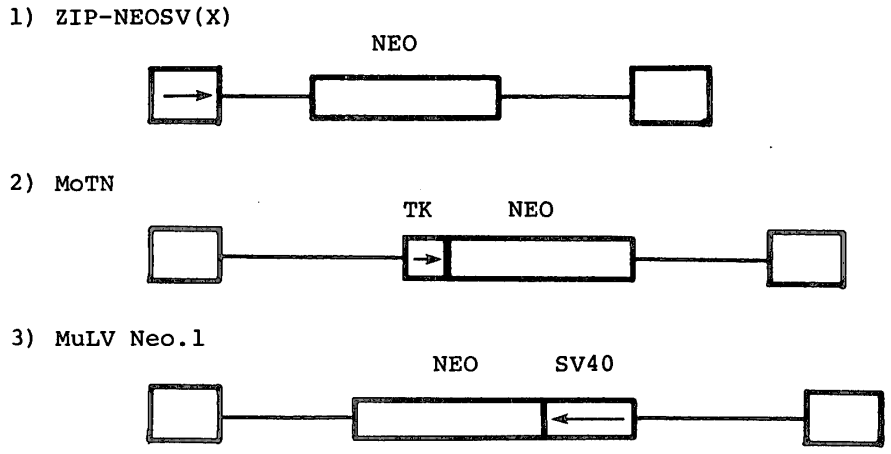

Fig. 1.

Genticin (G418). All three virus genes contain MoMuLV LTRs and are introduced into defined classes of mouse haematopoietic progenitor cells using a Hitachi-laser microinjector.

Cells and Cell Culture. TSA8 cells were established by Shibuya and Mak from anemia-inducing Friend virus complex, FV-A. The cell line was kindly provided by Dr. Mak of the University of Tronto. It was maintained for one year in the laboratory of Professor M. Oishi of the Institute of Applied Microbiology and then for one year in our group. The cells were grown in Iscove's modified Dulbecco medium supplemented with 15 % fetal bovine serum as described (30). For induction, the inducer was added to a suspension of the cells at a density of $2x10^5$ cells/ml. For enhance induction, slightly overgrown cells were passaged and the inducer was added one day after passage, just after the cells had started to grow. The G418 -resistant cells containing transfected-copies of the neomycin-resistance gene were selected for growth in the presence of G418 (0.4mg/ml).

Assay for Transduction of Neo-Retoviral Genes and Diffferentiation. Morphological changes: cytospin slides of the neo-retroviral gene transducted-cells were prepared and stained with Wright-Giemsa solution. Immunofluorescence studies were performed as described (31).

Benzidine staining for differentiation: after cells were washed once in Hanks' balanced salt solution (HBSS) and then suspended in the solution, the suspension was mixed with a equal volume of the freshly prepared staining solution (3 % benzidine in 90 % acetic acid: H_2O: 30 % H_2O_2=1: 5: 0.1, v/v) and the number of benzidine-positive cells was determined using a phase contrasst microscopy (32, 33). At least 200 cells were counted.

RESULTS AND DISCUSSION

Progress in today's biotechnology has given rise to the need for new techniques to incorporate exogenous genes into target cells. Conventionally, gene transfection has been

accomplished by mechanical and chemical processes. The former involves opening tiny holes in cell membranes through which to introduce genes using a very fine needle under a microscope. With the latter process, cell membranes are treated with calcium phosphate to improve their penetration rate in order for genes to slip into the target cell. However, there are drawbacks to both of these processes. The mechanical process requires operational skills, as well as tremendous labor and time to treat cells one by one. The chemical process presents a problem in that its efficiency of cell modification is low. We demonstrated here, using this laser microinjector, the transfection of a drug-resistance gene into cultured-murine cells (TSA8). This new technique has been developed by Hitachi, Ltd. in Japan, which makes high performance gene transfection. This microinjector works in such a way that a fine laser beam is irradiated through the object lens of a microscope upon a particular cell in the culture medium in which the desired genes are suspended. The laser makes a very small self-healing perforation in part of tha cell membrane, enabling the gene to be introduced into the target cell. As a result, the laser microinjector bring about a dramatic improvement in transfection rate and speed, as well as in the efficiency of cell modification. Moreover, and so on, has been put into practice.

The murine TSA8 cells were transformed to Geneticin (G418) resistance by introduction of the neomycin-resistance gene using the laser microinjector (Fig. 2). MoTN-transformed TSA8 cells expressed TK-specific antigens (Fig. 3). This expression was confirmed by the gel electrophoresis detection (Fig. 3). These transformed TSA8 cells also formed teratocarcinomas in syngenic mice at a similar level as did untransfected control cells (Fig. 4). These results sugest that the transduction of neomycin-resistance gene may be ineffective in gene expression and cell growth.

The transformed-murine erythroleukemia TSA8 cells could be induced to differentiate with DMSO. The cells were significantly inhibited with 1.0 % DMSO. The benzidine-positive cells increased on day 2, attained maximum on day 5, and decreased thereafter. Therefore, cells were cultured with 1.0 % DMSO, in which the cells differentiated by about 60 % of them. Therefore, cells were cultured with 1.0 % DMSO and erythroid differentiation was examined on day 5 in following experiment.

Figure 5 shows the effect of 1α, 25 $(OH)_2D_3$ on erythroid differentiation and restricted growth of transfomed-TSA8 cells induced by DMSO. The active vitamin D_3 (1α, 25 $(OH)_2D_3$) inhibited markedly DMSO-induced erythroid differentiatin (Fig. 5B), but it did not affect cell growth (Fig. 5A). On day 5, percentages of the benzydine-positive cells were decreased to 50 % at 0.12nM, 41 % at 1.2nM, 18 % at 12nM, and 15 % at 120nM of vitamin D_3. Figure 6 shows the morphological changes of the TSA8 cells before and after differentiation with 1.0 % DMSO. The nonrounded-cells were observed in DMSO-induced cells. These cells induced with 1.0 % DMSO alone showed morphological changes 2 days after induction. The inhibitory effect of the vitamin D_3 on DMSO-induced erythroid differentiation is similar to

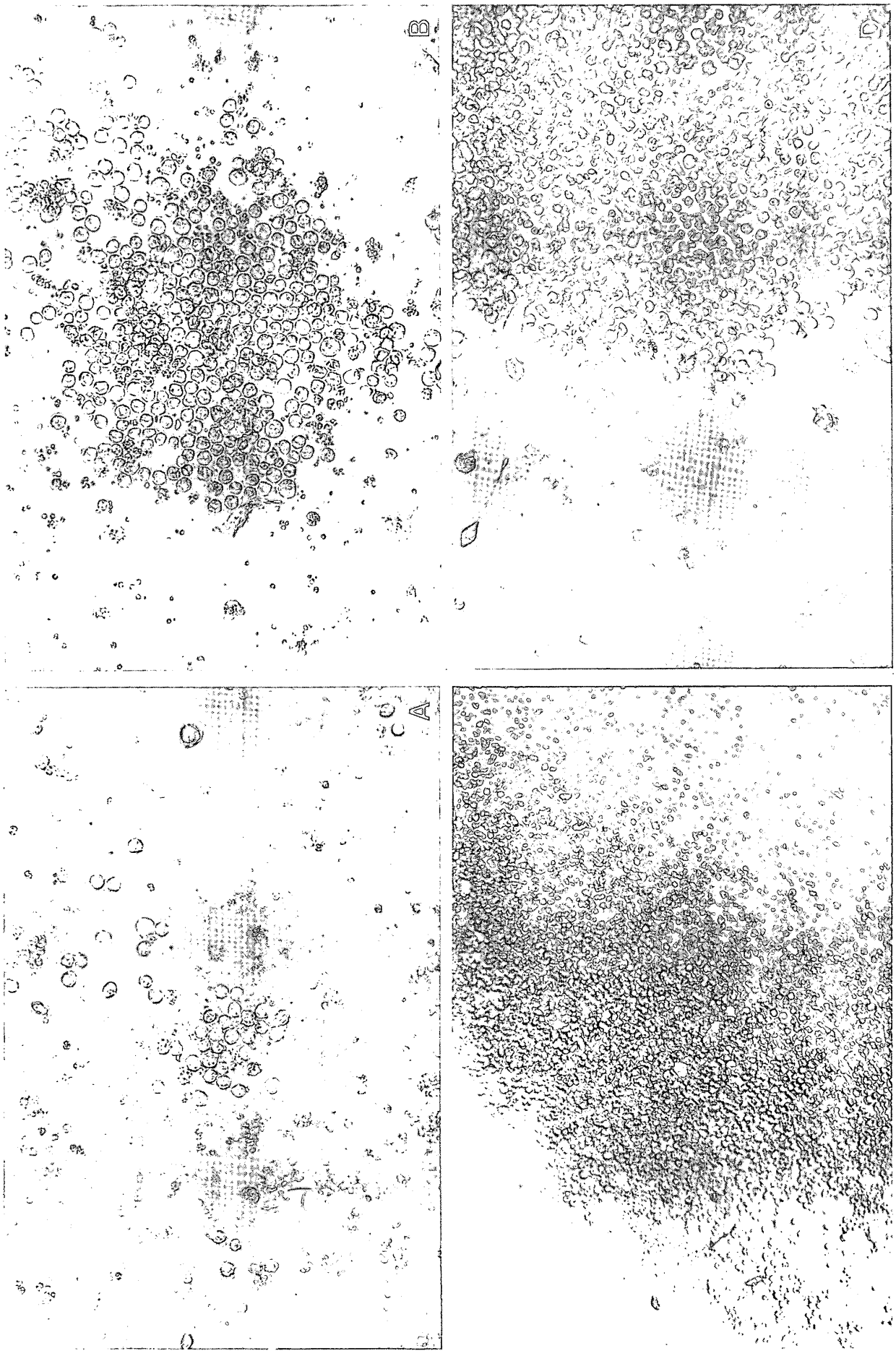

Fig. 2.

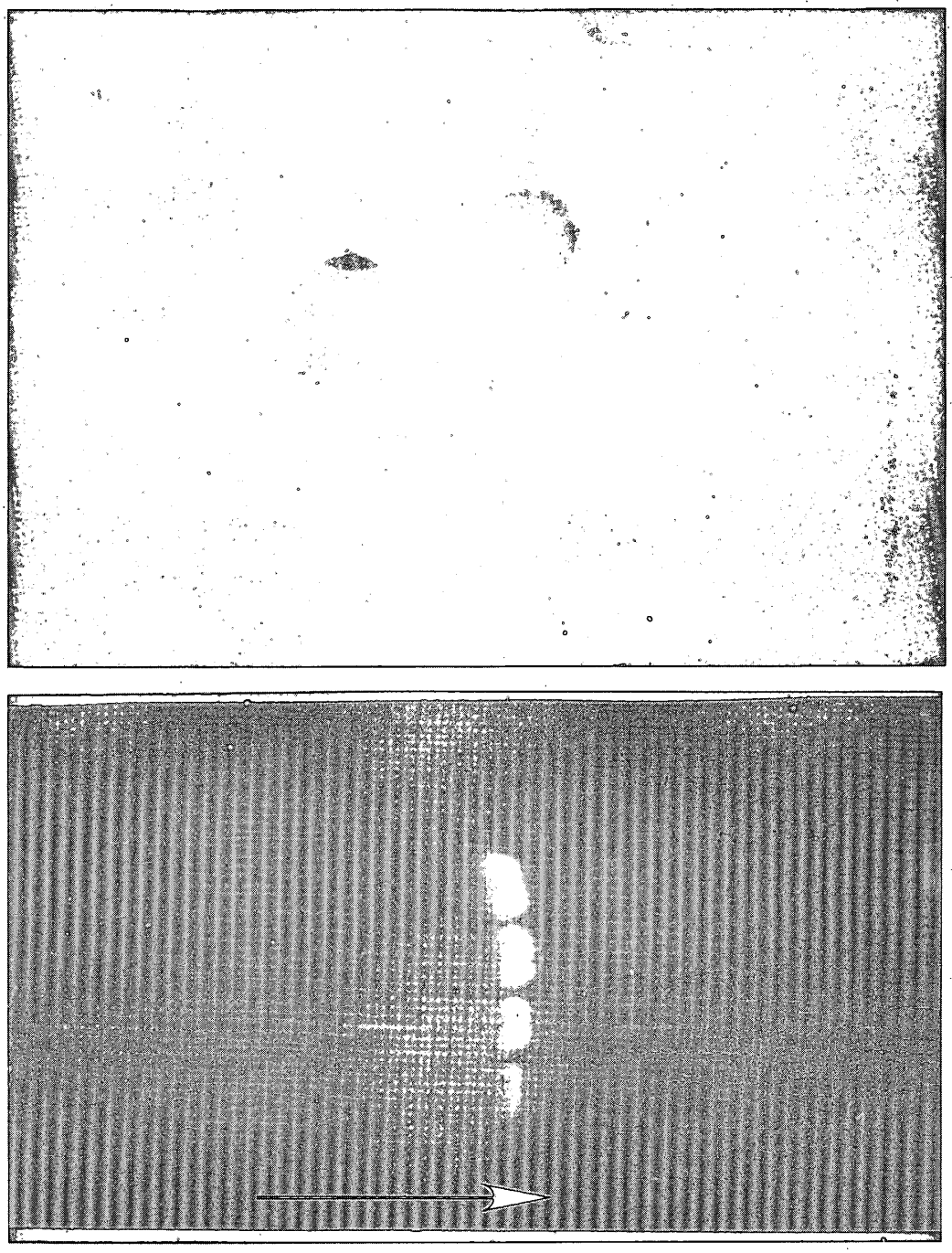

Fig. 3.

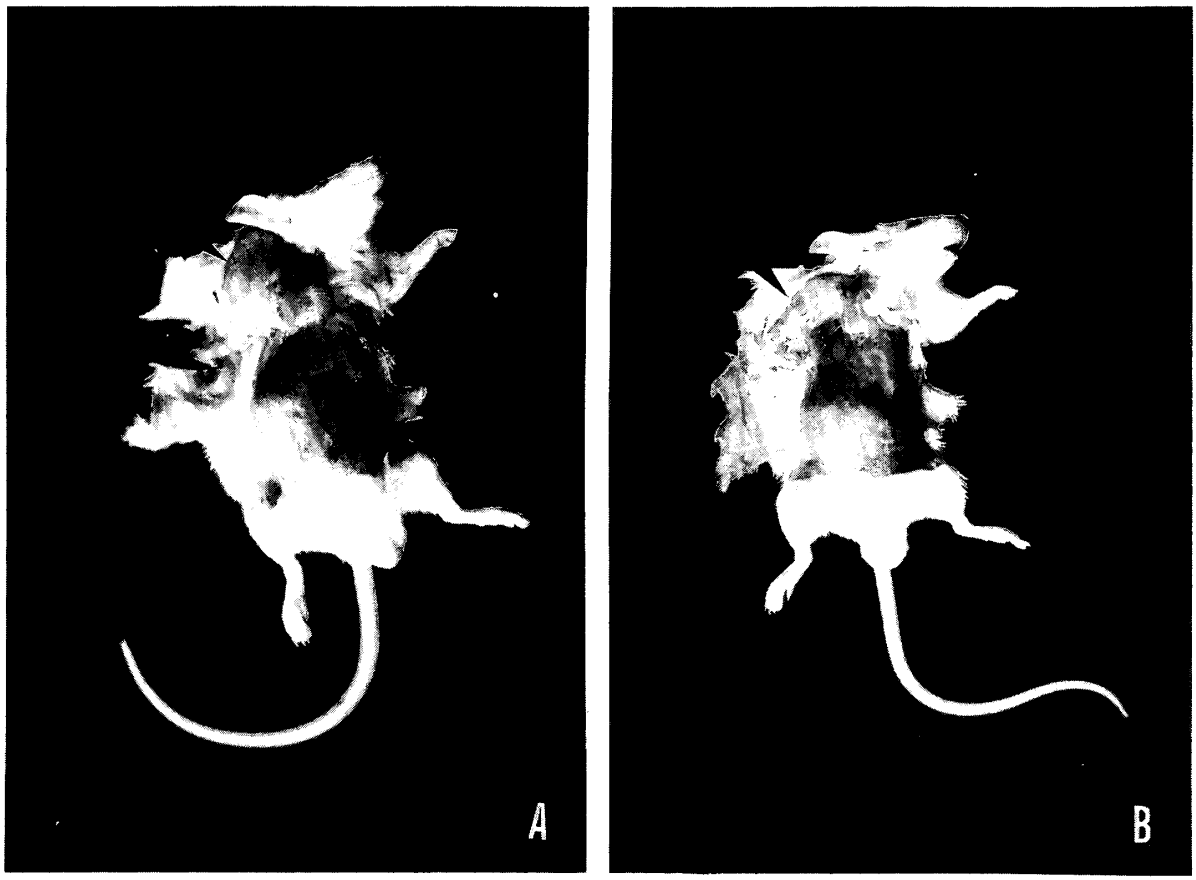

Fig. 4.

Fig. 1. Schematic structure of recombinant retroviruses used to transfect erythroleukemia cells (TSA8). All constructs contain LTR sequences derived from MoMuLV and the Tn5 neomycin-resistance gene. In ZIP-NEOSV (X) the neomycin-resistance gene is transcribed from the retroviral LTR promoter. In MoTN the neomycin-resistance gene is transcribed from a herpes thymidine kinase (TK) promoter. In MuLV Neo. 1 the neomycin-resistance gene is transcribed in a reverse orientation from an SV40 early promoter.

Fig. 2. Micrographs of erythroleukemia cells transfected with recombinant retrovirus genes, and selection in G418. Cells were infected using a laser microinjector in medium and selected in G418 (250 μg/ml). (A) micrograph after 10 days; (B) after 14 days; (C and D) after 21 days from transfection of MoTN genes.

Fig. 3. Immunofluorescence of anti-TK antisera to surface antigen of cells (TSA8) (upper), and TK-crude protein extracts in electrophoresed-gel (lower), as described (37). MoTN-transformed TSA8 cells and gel plate were treated with anti-TK antisera and subsequently to fluorescein isothiocyanate-conjugated anti-mouse Ig.

Fig. 4. Photograph for tumor genesis test of murine erythroleukemia cells (TSA8) before (A) and after (B) transformation with MoTN-transforming genes. Murine erythroleukemia cells can be transplanted into newborn mice treated with anti-erythrocyte serum (Adams et al., 1973 and Miyoshi et al., 1977). The cells were transplanted at a dose of 5 to 10×10^6 cells into each newborn mice. Immediately after the plantation, 0.1 ml of anti-erythrocyte serum which was prepared by sensitizing rabbits with mouse thymocytes was injected intraperitoneally and the inoculation was repeated twice a week thereafter.

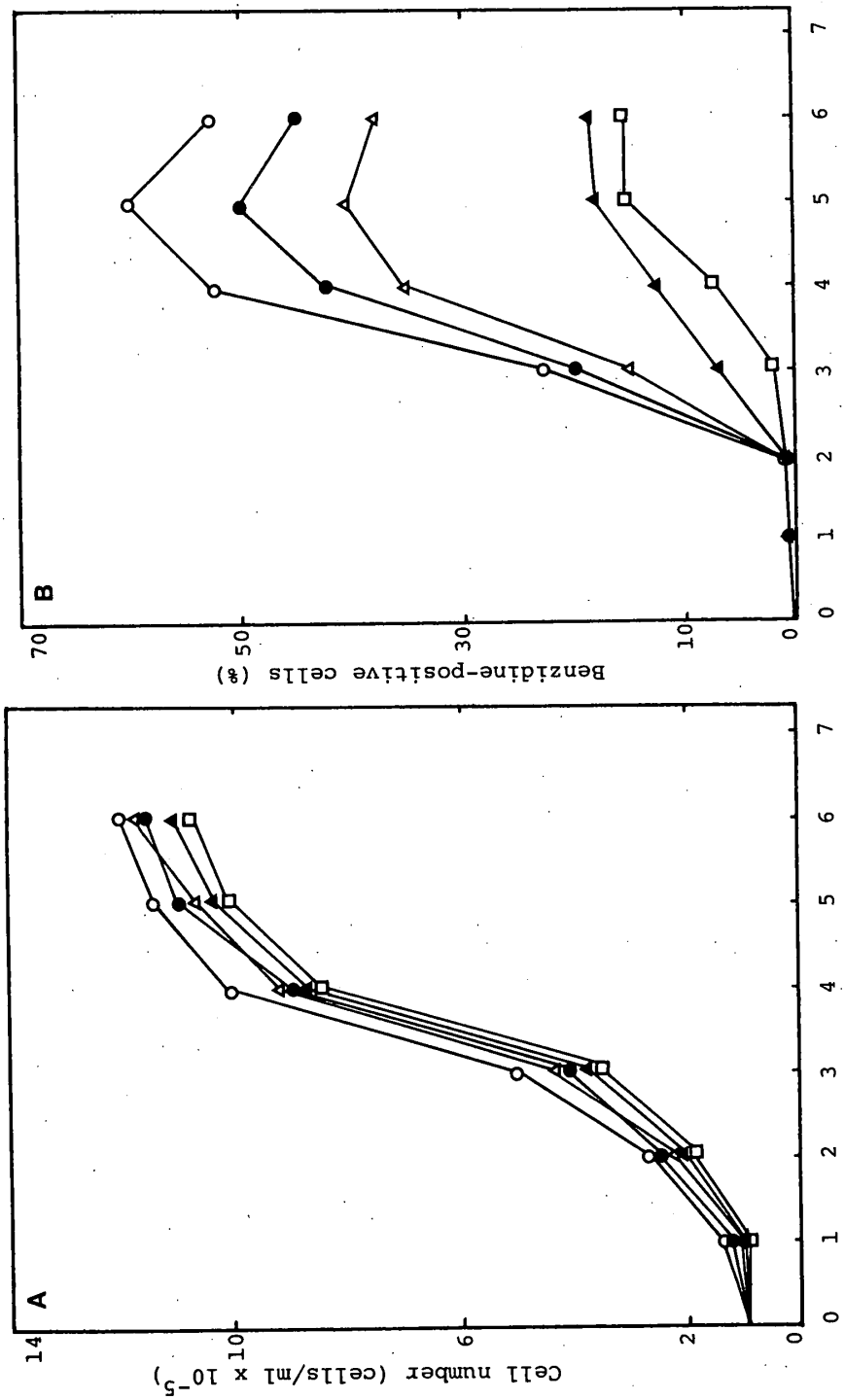

Fig. 5. Effects of 1α, 25 $(OH)_2$ D_3 on the growth (A) and differentiation (B) of transformed-TSA8 cells induced by 1.0% DMSO. Cells were cultured with 1.0% DMSO and graded concentrations of 1α, 25 $(OH)_2D_3$ for 6 days. (A) Typical dose response curves of 1α, 25 $(OH)_2$ D_3 in cell growth in the presence of 1.0% DMSO. (B) Inhibitory effects of graded concentrations of 1α, 25 $(OH)_2$ D_3 on 1.0% DMSO-induced benzidine-positive cells. ○, 1.0% DMSO alone; ●, 1.0% DMSO plus 0.12 nM; △, plus 1.2nM; ▲, plus 12nM; □, plus 120nM of 1α, 25 $(OH)_2$ D_3. Cell counts were performed with a hemocytometer on aliquotes of cell suspensions. Data are means ± S. E. of 3-determinations.

that of dexamethsone (5) and TPA (6).

DMSO inhibited proliferation and enhanced differentiation of transfomed-TSA8 cells. On the other hand, the acvitvated vitamin D_3 inhibited DMSO-induced erythroid differentiation, but the vitamin did not stimulate cell proliferation. Thus, these result suggest that the cell proliferation and differrentiation may be regulated separately. The vitamin D_3 alone

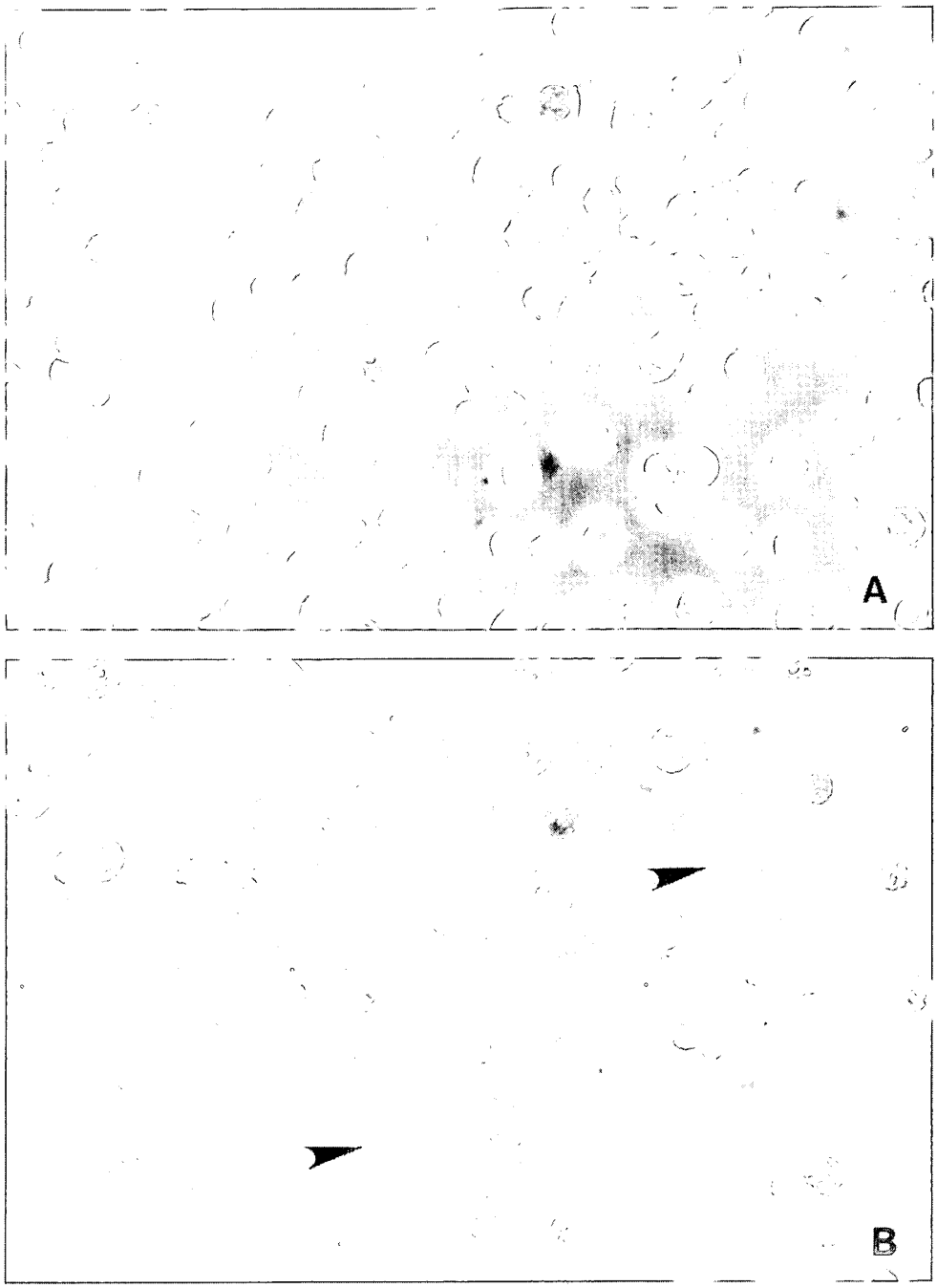

Fig. 6. Morphological changes of TSA8 cells before (A) and after (B) differentiation with 1.0% DMSO. Note the nonrounded-cells (indicated by arrows) in DMSO-induced cells (B). These cells induced with 1.0% DMSO alone showed morphological changes 2 days after induction.

affected nither cell proliferation nor differentiation of transformed-TSA8 cells, although data not shown.

Of the vitamin D_3 derivatives tested, 1α, 25 $(OH)_2D_3$ was the most potent in inhibiting DMSO-induced erythroid differentiation, followed successively by 1α, 24R, 25 $(OH)_3D_3$,1α(OH) D_3,25(OH)D_3, and 24R, 25 $(OH)_2D_3$ (Fig. 7). The order of the potency of vitamin D_3 derivatives in inhibiting erythroid differentiation is closely related to the binding affinity ot the vitamin D_3 derivatives for the cytosol receptor found in chik intestine (34) and human myeloid leukemia cells (HL-60) (35), suggesting that the vitamin inhibits DMSO-induced erythroid differnetiation by a receptor-mediated mechanism.

To further experiment the inhibitory effect of the vitamin D_3 on DMSO-induced erythroid differentiation, we investigated the effect of time of exposure to the vitamin D_3.

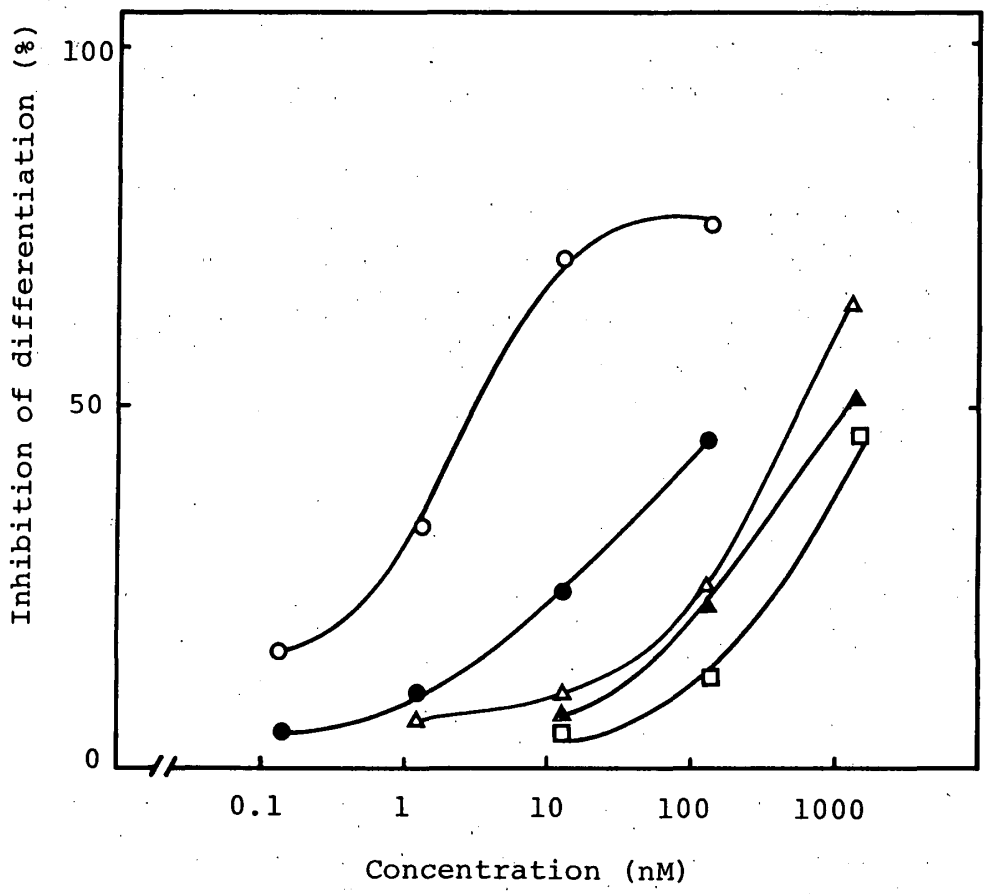

Fig. 7. Inhibition effects of various vitamin D_3-derivatives on DMSO-induced differentiation of MoTN-transformed erythroleukemia cells (TSA8). Cells were cultured for 5 days with 1.0% DMSO and 1α, 25 $(OH)_2$ D_3 (○), 1α, 24R, 25 $(OH)_3$ D_3 (●), 1α (OH) D_3 (△), 25 (OH) D_3 (▲), or 24R, 25 $(OH)_2$ D_3 (□). Cell differentiation was expressed as percentages of benzidine-positive cells in total cells, and inhibition of differentiation was expressed as percentages of the control value (1.0% DMSO alone). Each point represents means ± S. E. of 5 experiments.

Treatment of the TSA8 cells with the vitamin D_3 for 24-72 hr prior to the addition of DMSO was totally ineffective in inhibiting DMSO-induced erythroid differentiation (Table 1). Addition of the vitamin D_3 72 hr after adding DMSO also failed to inhibit differentiation. Thus, these results suggest that the vitamin acts in an early step of the commitment by DMSO. It has been reported that glucocorticoids inhibit globin gene expression induced by DMSO at a transcriptional stage (36).

In conclusion, the naturally occurring hormone, vitamin D_3, is involved not only in myeloid differentiation as reported previously (9-14), but also in erythroid differentiation. The vitamin D_3 induces differentiation of myeloid leukemia cells such as Ml, HL-60 and U937 preferentially along the monocyte-macrophage pathway (9-14). In contrast, the vitamin inhibits erythroid differentiation induced by DMSO. Thus, it may be conceivable that the vitamin D_3 plays a critical role in determining differentiation of bone marrow progenitor cells. Finally, our experiment suggest that the transduction of neomycin-resistance genes is totally ineffectve in cell proliferation and erythroid differentiation for murine cells. Our experiment also provide a posibility or model for future various gene replacement therapy in

Table 1. Effect of culture time with 1α, 25$(OH)_2D_3$ on DMSO-induced differentiation of MoTN-transformed erythroleukemia cells (TSA8)

Culture time with 1α, 25$(OH)_2D_3$ (hr)	Benzidine-positive cells (%)	Percent inhibition (%)
−	59.7±1.3	0
−72−0	58.1±2.1	−2.6
−48−0	58.4±2.5	−2.1
−24−0	59.7±1.9	0
0−120	15.9±2.6	73.3
24−120	16.1±1.9	73.0
48−120	38.9±2.2	34.8
72−120	55.6±3.0	6.8
96−120	59.1±2.7	1.0

Cells were placed in culture at an initial cell density of 1.0×10^5 cells/ml and cultured with 12nM 1α,25$(OH)_2D_3$ for indicated times before or after adding DMSO (final 1.0%). DMSO was added at time 0. Benzidine-positive cells (BPC) were counted 120hr after adding DMSO, at which time cells were recovered by centrifugation. Percent inhibition was calculated according to the following formula : [(BPC (%) with DMSO alone-BPC (%) with DMSO plus 1α,25$(OH)_2D_3$)/BPC (%) with DMSO alone] $\times$100. Data are means±S. E. of 3-experiments.

which functional genes can be introduced into various target cells using the laser microinjector.

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