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The Study of Epstein–Barr Virus Induction by Cocultivation of EBV-Transformed Cells with a Mammary Carcinoma Cell Line MCF-7*

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Abstract—*EBV was found to be activated by cocultivation of EBV transformed lymphoblastoid cell lines and a mammary carcinoma cell line, MCF-7. Both non-EBV producer and producer cell lines are susceptible to this activation. However not every EBV-transformed cell line can be activated. Although the mechanism of activation was investigated in a number of ways, it is still unknown whether the process requires cell to cell contact, if the inducing agent is secreted in the medium and where in the host cell or the virus cycle it acts.*

INTRODUCTION

THE MECHANISM which regulates the functions of Epstein–Barr virus (EBV) such as the nucleus associated antigen EBNA), early antigen (EA), DNA, viral capsid antigen (VCA) and membrane antigen (MA) expression is not yet known. Nevertheless, this mechanism appears to act at several levels. First, it maintains EBV indefinitely in a latent state in non-producer cell lines or in a latent state with occasional spontaneous activation of the virus productive cycle in a minority of the cells in producer cell lines [1–3]. However, this situation has been found to be affected by a number of experimental manipulations. For instance, some non-producer and producer cell lines are known to be induced by treatment with a number of chemical agents such as 5-bromodeoxyuridine (BrdUrd) and 5-iodo-2-deoxyuridine (IdUrd) [4, 5], mitomycin C [6] or 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) [7]. Physical agents such as temperature, or X-ray treatments have also been used [7]. The state of the virus in the host cell has also been found to be affected by superinfection with the P3HR-1 strain of virus [8]. Furthermore, the state of the virus can be induced to become productive by cell fusion [9, 10]. This paper reports on yet another way by which the relationship between EBV and its host cell can be altered. Cocultivation of certain EBV transformed cell lines with the mammary carcinoma cell line, MCF-7 [11] has been found to activate the virus cycle.

MATERIALS AND METHODS

Cell cultures

Lymphoblastoid cell lines used in this investigation were obtained from Dr. George Klein, Pfizer, Inc. and from this laboratory (see Table 1 for details). Cells were routinely maintained in RPMI-1640 medium supplemented with 100 μg/ml of streptomycin, 100 units of penicillin, 300 μg/ml of glutamine and 10% fetal calf serum and fed every 3–4 days. Medium RPMI-1640 was also used in all the cocultivation experiments. All cultures were kept at 37°C in a humidified 5% CO_2 incubation.

Reagents

The $EA(D)^+$ VCA^+ antiserum (Choma

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Table 1. List of cell lines, their origin and characteristics

Cell lines	Origin	EBV	EBV Production	Notes
B95-8	Marmoset	+	+	[3]
627-76C5	Marmoset	+	+	[13]
627-76D2	Marmoset	+	+	
P3HR-1	BL*	+	+	[14]
Daudi	BL	+	+	[15]
Raji	BL	+	−	[16]
RO-bl	BL	+	−	Adherent derivative of Raji [17]
Ramos	BL	−	−	[18]
EHR-B-Ramos (EBR)	BL	+	−	EBV converted Ramos for G. Klein
JY	PBL†	+	−	Obtained from Pfizer, Inc.
CEM	ALL‡	−	−	[19]
MCF-7	Mammary carcinoma	−	−	Provided by G. Klein [11]

*BL, Burkitt's Lymphoma; †PBL, Peripheral blood lymphocytes; ‡ALL, Acute lymphocytic leukemia.

Himo) with titers of 1:640 (EA) and 1:5120 (VCA) from a nasopharyngeal carcinoma patient was a gift of Dr. George Klein. The EA^- VCA^+ antiserum (L22) with a titer of 1:640 was from a normal person in the laboratory. Fluoreocein-isothiocyanate-conjugated goat antihuman IgG was obtained from Microbiological Associates, Bethesda, Maryland.

Virus for superinfection was prepared by growing 1 l of P3HR-1 cells to a stationary phase. The cells and cellular debris were pelleted out by centrifugation at 1033 ***g*** for 10 min. The supernatant was spun at 13,700 ***g*** for 2 hr after which the pellet is resuspended in 10 ml of medium, clarified at 2324 ***g*** for 10 min. If not used right away, the preparation was stored in 0.5 ml volumes at −70°C.

Growth ratio

Since RO-bl and MCF-7 cells grow as monolayers, they could not be separated when examining the former for EA production. Hence, it was necessary to determine the growth ratio of each cell line in order to compute the percentage of cells induced to make EA. A total of 2×10^6 cells were seeded in 100 × 20 mm plastic dish. Each sample was prepared in triplicate. After 48 hr, cultures were harvested by either trypsinization or scraping with a rubber blade and counted in a hemocytometer. This was not necessary for non-adherent EBV positive cells since they could easily be separated from the EBV negative adherent MCF-7 cells.

Immunofluorescence

Cells were harvested, smeared, air dried, acetone-fixed for 10 min at room temperature and stained for EA and VCA by the indirect method [19]. Smears were stained for VCA with a human EA^-VCA^+ serum (titre 1:640) used at a 1:20 dilution. An EA^+VCA^+ human antiserum with an EA and VCA titres of 1:640 and 1:5120, respectively was used to stain for EA(D) at 1:40 dilution. The FITC-conjugate was used at a 1:20 dilution. All stained smears were counterstained with Evans Blue (100 μg/ml) for 20 min at room temperature, briefly rinsed with phosphate buffered saline (PBS), mounted in 50% glycerol and examined in a Zeiss fluorescence microscope. All reagents were diluted with PBS.

Mixing ratio for RO-bl and MCF-7 cocultivation

One of several factors which was believed

to effect the MCF-7 induction of EBV was the ratio in which the cells are mixed. To ascertain this, cells were mixed in the ratio of RO-bl to MCF-7 ranging from 1:100 to 100:1. for a total of 2×10^6 cells per 100×20 mm plastic dish, incubated for 48 hr and tested for EA induction.

Optimum incubation time for EBV induction

In order to determine the optimum incubation time for EBV induction, RO-bl and MCF-7 cells were mixed in a 1:1 ratio and grown for 4 days while samples were prepared daily and tested for EBV production. Since RO-bl was found to express only EA, it was also necessary to do a similar time course experiment with B95-8 to see how the expression of VCA is affected by MCF-7.

The effect of MCF-7 conditioned medium

The possibility that induction of EBV by MCF-7 is effected through a substance secreted in the medium was checked by using MCF-7 conditioned medium. The medium was prepared from 48 and 72 hr MCF-7 cultures. RO-bl cells were incubated in conditioned medium, undiluted or diluted 5- and 10-fold with fresh medium, harvested 48 hr later and examined for EA.

5-Iodo-2-deoxyuridine induction

RO-bl and MCF-7 cells were mixed as described before and cocultivated in the presence or absence of 50 μg/ml of IdUrd for 48 hr, harvested and tested for EA and VCA, respectively.

Superinfection

Raji and its RO-bl derivative cells were super-infected by incubating 5×10^6 cells with P3HR-1 EBV concentrate ($100\times$) diluted 1:10 at 37°C for 1 hr with occasional shaking. Unadsorbed virus was removed by centrifugation and the cell pellet was resuspended in fresh medium, washed once and then seeded at 2×10^6 per 100×20 mm plastic Falcon dish in which was also seeded 10^6 MCF-7 cells. After 48 hr, the non-attached cells were collected and prepared for immunofluorescence as described before.

Autoradiography

To determine which cells in mixed cultures express EBV antigens, cells were labelled for 48 hr with 0.5 μCi/ml of tritiated thymidine (S.A. 8.5 mCi/mM), cocultivated with the corresponding unlabelled cells for another 48 hr, harvested and stained for immunofluorescence. The slides were then processed basically according to Baserga and Malamud [20]. They were dip-coated with NTB-2 Nuclear Track emulsion (Kodak, Rochester, New York) diluted 1:1 with distilled water, air dried 20–30 min, incubated desiccated for 7 days at 4°C and the autoradiograph developed, after which they were examined for both silver grains and immunofluorescence. Representative samples were photographed.

RESULTS

Growth ratio

For the purpose of computing the percentage of EA^+ cells in mixed cultures, the growth ratio was determined. Table 2 shows the relative growth proportions. The results show that RO-bl grows about 1.5 times as fast as MCF-7 within the tested time period of 48 hr. The percentage of EA^+ cells in RO-bl $\times$ MCF-7 cocultures was adjusted for the unequal growth rate by multiplying the unadjusted percentage by 2/3 for the difference in growth rate and by the dilution factor due to mixing with the non-EBV producing cells of MCF-7. However for cocultures of MCF-7 and non-adherent cells, this computation was not necessary since the two cell types can be easily separated.

Table 2. The relative growth rate of RO-bl and MCF-7

Cell line	Innoculum	Cells after 48 hr ($\times 10^6$)
RO-bl	2×10^5	4.7 ± 0.5
RO-bl + MCF-7	1×10^6 each	3.6 ± 0.5
MCF-7	2×10^6	2.9 ± 0.5

Optimum conditions for EBV induction

Two parameters were examined with respect to their effect on the induction of EBV by MCF-7. The first of these was the effect of the ratio of cells in cocultures. Results presented in Fig. 1 show that there is a direct relationship between the ratio of MCF-7 in coculture with RO-bl and the level of EBV induction. That is, it appears that the higher the ratio of MCF-7 to RO-bl, the greater the induction of the production of EBV antigens. Figure 1 also shows the data without adjustments for unequal growth rates and mixture ratio (see curve). It shows that it is easiest to

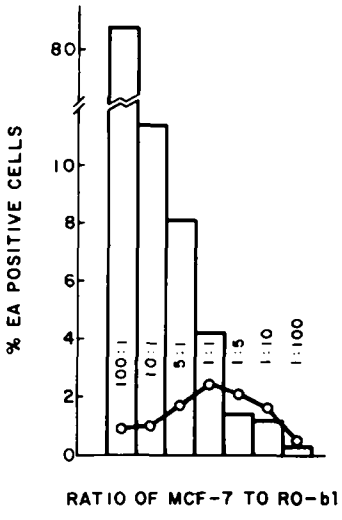

Fig. 1. The effect of cell mixture ratio in cocultures MCF-7 and RO-bl cells were mixed in different ratios as indicated and prepared for EBV induction as described in the Method. The histograms show the % of EA⁺ cells after adjustments for unequal growth rate and dilution due to mixing. The curve shows the data without adjustments for unequal growth rate and dilution. These results are the average of three or more experiments.

detect EBV induction in cocultures when cells were mixed in a 1:1 ratio.

The second parameter examined was the optimum incubation period which seemed to vary from cell line to cell line. Results presented in Fig. 2 show that RO-bl was maximally induced by the second or third day after which the induction level declined. In contrast, Fig. 3 shows that B95-8 was maximally induced as early as 24 hr and the level of EBV induction also remained high throughout the next three days when the experiment was terminated.

Pattern of EBV induction in various cell lines

Whether or not the induction of EBV by MCF-7 cocultivation was a phenomenon unique to RO-bl was tested by cocultivating it

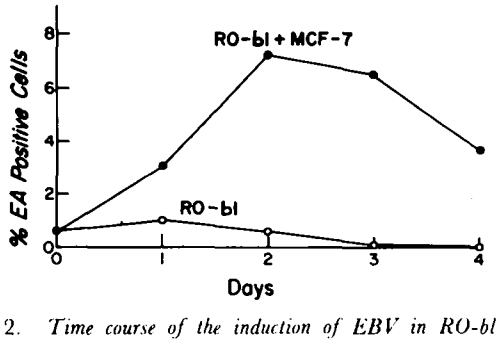

Fig. 2. Time course of the induction of EBV in RO-bl by cocultivation with MCF-7. ○—○ RO-bl; ●—● RO-bl + MCF-7. These are the averages of three experiments.

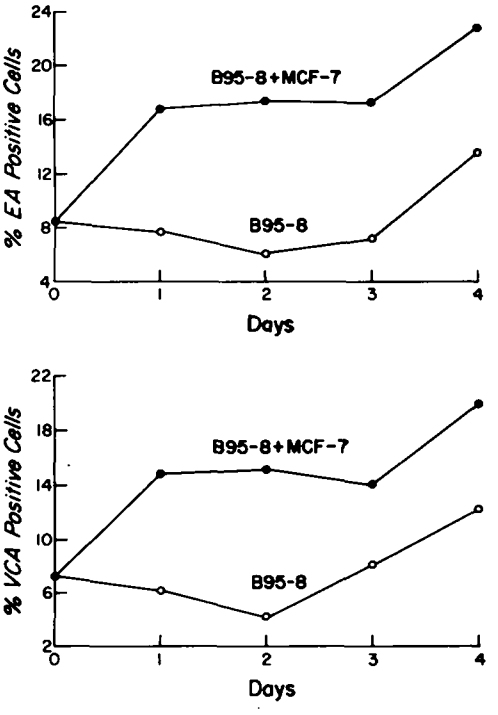

Fig. 3. Time course of the induction of EBV in B95-8. Only non-adherent B95-8 cells were examined for both control and experimental samples. EA and VCA induction are shown in Figs. 3 (A and B), respectively. ○--○ B95-8; ●—● B95-8 + MCF-7. These are the averages of four experiments.

with other cell lines. The results are presented in Fig. 4. First, not all EBV carrying cells were affected in the same way; some such as B95-8, Daudi and Raji were inducible while others such as P3HR-1, EHR-B-Ramos (EBR) and JY were not significantly induced if not totally refractile. Second, MCF-7 induced only EBV positive cells while EBV free cell lines were totally uninducible. Third, non-EBV producer cell lines such as Raji appeared to produce only EA and not VCA upon cocultivation with MCF-7. It should be pointed out that although Fig. 4 may imply that there is no difference between EA and VCA induction in B95-8, this could not be demonstrated with the reagents used. To determine whether there is no difference, it would be necessary to compare two discordant sera (EA^-VCA^+ and EA^+VCA^-). Unfortunately the latter type of reagent was not yet available and the author is not aware of any such serum at the present time.

The question whether the induction of EBV in EBV-transformed cells by cocultivation is a general phenomenon or if it is peculiar to MCF-7 is itself a major undertaking which was not sufficiently studied here. However, preliminary results suggest that various cell lines differ with respect to the ability to induce EBV in transformed cells.

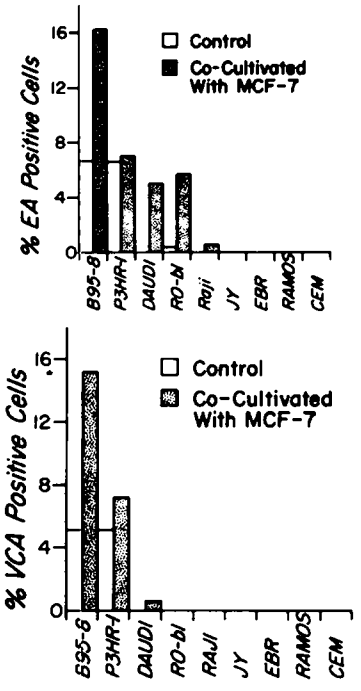

Fig. 4. The pattern of induction of EBV in different lymphoblastoid cell lines by cocultivation with MCF-7. EA and VCA induction are shown in Figs. 4 (A and B), respectively. EBR is an abbreviation of EHR-B-Ramos. These are the averages of three to five experiments.

Combined autoradiography and immunofluorescence

In order to determine which cells in the mixture make EA, an experiment combining autoradiography and immunofluorescence was performed. The theoretical prediction was that if only EBV positive cells were inducible, then in an experiment in which ^{3}H-TdR labelled B95-8 or RO-bl cells were mixed with unlabelled MCF-7 cells, both fluorescence and isotope labels would be found in the same cells. In mixtures in which only MCF-7 cells were labelled with ^{3}H-TdR, isotope and fluorescence would be found in separate cells. As shown in Fig. 5 (A and B), in a coculture of unlabelled B95-8, both isotope label and fluorescence appeared in the same cells. It was also observed that in cultures in which MCF-7 was labelled and B95-8 was unlabelled, no isotope label was found in fluorescent cells [Fig. 5 (C and D)]. A similar result was obtained when MCF-7 was cocultivated with RO-bl.

Superinfection and IdUrd induction

In some non-EBV producer cells, the synthesis of EBV antigens can be induced by superinfection and IdUrd treatment. It was interesting to determine whether EBV induction by either superinfection or IdUrd can be enhanced by the presence of MCF-7 cells. As shown in Table 3, MCF-7 has no significant effect on superinfection. However, induction by IdUrd is amplified almost threefold (Table 4). In both cases, no VCA was detected.

Table 3. Superinfection of Raji with P3HR-1 EBV in the presence of MCF-7 cells

Cells	P3HR-1 EBV	%EA$^+$ Cells	%VCA$^+$ Cells
Raji	–	0	0
Raji	+	2.5	0
Raji + MCF-7	–	0.6	0
Raji + MCF-7	+	1.6	0

Table 4. IdUrd induction of EBV in the presence of MCF-7 cells

Cells	50 μg/ml Idurd	%EA$^+$ Cells	%VCA$^+$ Cells
RO-bl	–	0.5	0
RO-bl	+	3.8	0
RO-bl + MCF-7	–	1.3	0
RO-bl + MCF-7	+	8.9	0

DISCUSSION

Current information about EBV indicates that the virus has a very complex regulatory mechanism which controls the relationship between it and its host cells. It also appears that the control of some of the various aspects of the virus-host relationship is exerted by both virus and host cells [9]. In addition to what has been reported by others, this paper describes yet another aspect of the regulation. Data presented here show that MCF-7, a human mammary carcinoma cell line, is capable of inducing EBV in EBV transformed lymphocytes. Although many EBV positive cells are induced by MCF-7, there are also others which are not induced. What determines whether EBV in a particular cell line can be induced by cocultivation with MCF-7 cells is not yet known. Moreover, the induction of EBV by MCF-7 does not seem to follow the pattern of spontaneous EBV production [8]. For example, P3HR-1 which is a producer is not induced by MCF-7. However,

the susceptibility to MCF-7 induction appears to follow that of superinfection and IdUrd induction. For instance, Daudi and Raji are both susceptible to superinfection and chemical induction whereas P3HR-1 is refractory to both.

EBV induction by MCF-7 was found to be complex. The mechanism of the induction was studied in a number of ways. Whether the induction is effected though a soluble diffusible substance was tested by using MCF-7 conditioned medium as the inducing agent. However, this was found to be unable to induce EBV in the tested cell line (RO-bl). Although this may indicate that cell to cell contact is needed for the induction to occur, it may also indicate that the inducing substance is very labile. Furthermore, it may indicate that the agent is secreted in a very minute quantity and that to be effective it has to be continuously secreted. It was thought that the inducing substance might be a hormone or some serum proteins. One of this possibility was α-lactoalbumin which is known to be secreted by MCF-77 [11]. A commercial preparation of bovine α-lactoalbumin (Sigma) was tested, but various concentrations (5–50 μg/ml) were found to have no detectable effect on RO-bl cells.

Another possibility which was tested was that the induction is effected through cell fusion. However, using a combination of fluorescence and ^{3}H-TdR labels, it was found that the results were incompatible with the cell fusion hypothesis [see Fig. 5 (C and D)]. This shows that the induction of EBV by MCF-7 is not due to heterokaryon or hybrid formation as has been reported by others for other cell lines [9, 10].

The last question to be examined was where in the virus cycle or where in the host cell the inducing agent acts. The evidence presented here indicates that the inducing agent acts early in the virus cycle. For instance, in non-producer cell lines such as Raji and its derivative RO-bl the first antigen detected following cocultivation with MCF-7 is EA. This, of course, does not eliminate the possibility of early appearance of other virus antigens which were not examined in these experiments. The data also show that the inducing agent does not affect the transition of the virus cycle from the EA to the VCA phase since in Raji and its derivative only EA is induced. This is a further confirmation of an early observation that although the synthesis of VCA requires the synthesis of EA, its control appears to be independent of that of EA [9]. Because of the lack of a suitable anti-serum (EA^+VCA^-), it could not be determined whether there is a difference between EA and VCA induction. Since no EBV infected persons are known to have EA^+VCA^- anti-serum, such a reagent could only be produced when purified EA becomes readily available. Whether the inducing agent described herein acts on the cell surface, in the cytoplasm or nucleus is still unknown. It also remains to be elucidated whether this induction phenomenon is peculiar only to MCF-7.

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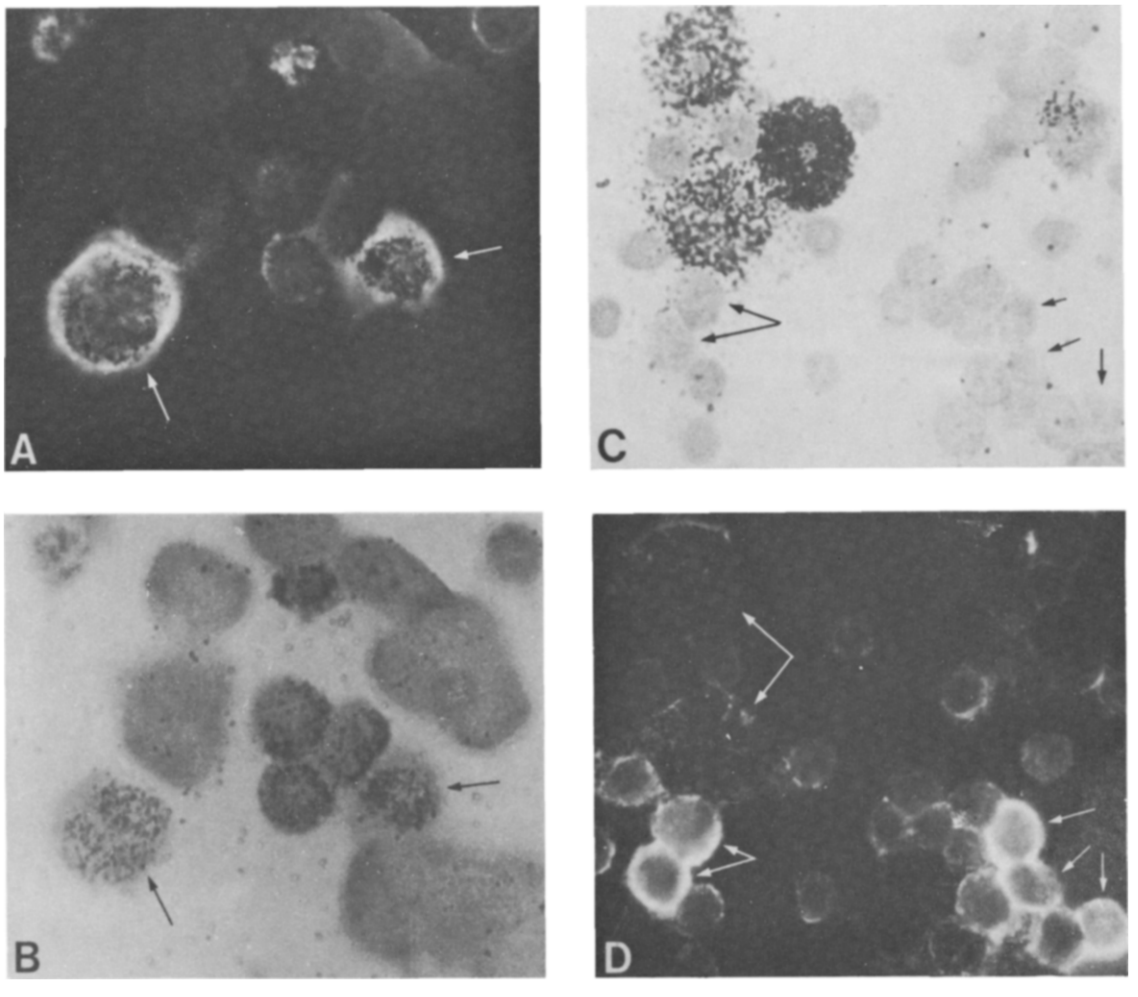

Fig. 5. *Autoradiograph and immunofluorescence of B95-8 cocultivated with MCF-7. A and B are pictures of EA immunofluorescence and autoradiograph of cells prepared from a mixed culture of B95-8 and MCF-7 in which B95-8 was labelled with tritium (see Method). Here, immunofluorescence and isotope label appear in the same cells (arrow). C and D are another set of similar pictures in which MCF-7 were labelled with tritium. In contrast to A and B, the isotope label and immunofluorescence appear in different cells. The observation was also found to be true of RO-b1 cocultivated with MCF-7.*

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