tors, one must consider the possibility that the structural differences between *erb*B and the EGF receptor may lead to a constitutive, basal level of ligand-independent substrate phosphorylation activity. Alternatively, the specificity of phosphorylation may be altered. The chimeric HER-*erb*B molecule represents a homolog of the v-*fms* oncogene that carries an apparently intact ligand-binding domain. It offers the possibility of characterizing in detail enzymatic parameters of the receptor kinase and represents a model for studying ligand-stimulated transformation in an autocrine or paracrine scenario.

We observed inhibition of EGF-stimulated mitogenesis in Rat1 cells expressing the HER-*erb*B chimera. This effect was dosage-dependent and led to cell death after several days of EGF exposure, which is reminiscent of A431 cells that overexpress normal and altered human EGF receptors (*2, 21*). Whether this phenomenon can also be attributed to the carboxyl-terminal deletion or other criteria, such as receptor overexpression, structural differences between v-*erb*B and c-*erb*B sequences, or incompatibility of avian receptor signaling functions with Rat1 cell signal transduction pathways, is currently unclear.

The hybrid receptor approach used in these experiments may provide a powerful means of identifying growth factor receptor domains that are crucial in the initiation of the cascade of events that leads to normal cell division or oncogenesis.

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Extended Culture of Mouse Embryo Cells Without Senescence: Inhibition by Serum

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Mouse embryo cells cultured in vitro in serum-supplemented media undergo growth crisis, resulting in the loss of genomically normal cells prior to the appearance of established, aneuploid cell lines. Mouse embryo cells established and maintained for multiple passages in the absence of serum did not exhibit growth crisis or gross chromosomal aberration. Cells cultured under these conditions were dependent on epidermal growth factor for survival. Proliferation was reversibly inhibited by serum or platelet-free plasma, suggesting that mouse embryo cultures maintained by conventional procedures are under the influence of inhibitory factors.

MOUSE EMBRYO CELLS CULTURED in conventional serum-supplemented media rapidly lose proliferative potential, leading to growth crisis or senescence followed by the appearance of genomically altered immortalized (established) cell lines. Swiss, BALB, and NIH lines (3T3, 3T6, and 3T12) as well as C3H 10T1/2, which are commonly used in studies of carcinogenesis and growth control, were derived in this way and exhibit near-tetraploid karyotypes with major chromosomal abnormalities (*1–4*). Because extended culture of some cell types has been achieved by replacing serum in the culture medium (*5–11*), we developed a serum-free approach to the culture of mouse embryo cells. These procedures allowed extended cell proliferation in the absence of detectable crisis or gross genomic alterations. Growth of cultures derived under these conditions was markedly inhibited by both serum and plasma, suggesting the presence of circulating inhibitors distinct from platelet-associated factors.

Cells from 16-day-old mouse embryos were cultured in nutrient medium supplemented with 10% calf serum (*1–4*) or in medium in which serum was replaced with insulin, transferrin, epidermal growth factor (EGF), high-density lipoprotein (HDL), and fibronectin. This medium was developed originally for the growth of the serum-derived, established C3H 10T1/2 and Swiss NIH 3T3 mouse embryo cell lines (*12, 13*) and will also support the growth, at least in the short term, of early passage mouse embryo cells cultured initially in serum. Growth of serum-derived cultures in this medium is enhanced by the addition of platelet-derived growth factor (PDGF). The medium formulation is related in nutritional and hormonal composition to other media developed for the growth of established lines of mouse embryo cells (*14, 15*).

Cultures carried in serum-containing media, in the presence or absence of additional supplementation with the above factors, underwent a well-defined crisis (Fig. 1). A single batch of unheated serum was used for the serum-containing cultures for the duration of the experiment. Similar results were obtained when the experiment was repeated with a different batch of serum or with heat-inactivated serum (56°C, 30 minutes). Cultures maintained in serum-free media grew exponentially without a significant time lag and represented a major portion of the population of embryo cells initially plated. Cell number in primary cultures 6 days after plating was three times higher in serum-free medium than in serum-containing medium. Cells of serum-free cultures appeared morphologically homogeneous upon light microscopic examination by the second or third passage and remained so upon long-term culture.

Cultures were derived in serum-free medium from both BALB/c and Swiss embryos. Initiation and long-term growth in the absence of serum was carried out with three independent Swiss mouse embryo cultures; similar results were observed in all cases.

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Cells spread poorly in low-density serum-free cultures, occasionally growing as detached clumps. At high density the cells exhibited elongated, bipolar, spindle-shaped morphology and organized into characteristic fibroblastic swirls. Swiss mouse embryo cells cultured for 200 generations in serum-free medium were nontumorigenic as measured by injection of 10^7 cells subcutaneously into 4- and 7-week-old male and female athymic Swiss mice. Mice were observed for 6 months. Post-crisis mouse embryo cells derived in serum-containing medium exhibited aneuploid karyotypes as expected, but cells cultured under serum-free conditions retained chromosome numbers in the diploid range (Fig. 2).

Karyotype analysis by Giemsa banding was carried out at the same time as chromosome counts. All of the metaphases from Swiss mouse embryo cultures in serum-containing medium contained one or more translocated marker chromosomes after 20 population doublings had occurred. Giemsa banding indicated that one marker was the result of a Robertsonian fusion involving chromosome 5 and an unidentifiable chromosome; the other consistently observed marker was a minute chromosome. No chromosomal abnormalities were identified in the serum-free derived cultures.

Translocations were detected in some clones derived from the serum-free Swiss mouse cultures, suggesting that cloning or freezing procedures may affect karyotype. One clone containing a translocation was examined further. This clone remained nontumorigenic and had a modal chromosome number of 40. Cultures derived in serum-free medium contained a small fraction of cells (4 to 7%) with karyotypes in the tetraploid range, similar to that reported for pre-crisis Swiss mouse embryo cells maintained in serum-containing media (*1*).

Mouse embryo cells derived in serum-free medium required EGF for survival (Fig. 3). The EGF requirement was also observed if MCDB 402, a formulation developed for Swiss 3T3 cells (*15*), was used as the basal nutrient medium. Individual omission of the other supplements resulted in decreased cell growth, but did not result in immediate cell death. Flow cytometric analysis indicated that cells maintained in the absence of EGF accumulated in the G1 phase of the cell cycle prior to loss of viability (*16*). The EGF requirement of mouse embryo cells in vitro suggests that this molecule or EGF-like factors (*17*) may play a critical role in fetal development. A comparison of biochemical properties suggests that EGF is not the survival factor identified previously for 3T3 cells (*18*).

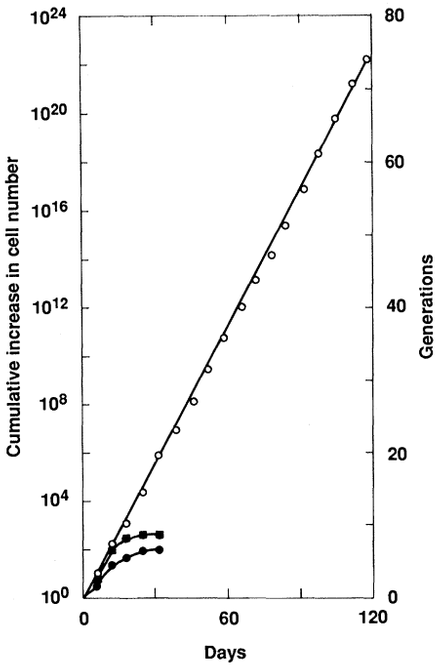

Fig. 1. Growth of BALB/c mouse embryo cells upon successive transfer in serum-containing or serum-free media. Cultures were grown in (●), medium supplemented with 10% calf serum (Gibco); (○), serum-free medium supplemented with bovine insulin (10 μg/ml; Sigma), human transferrin (25 μg/ml; Sigma), EGF (50 ng/ml; Collaborative Research), human HDL (20 μg/ml; Meloy) on flasks precoated with human fibronectin (20 μg/ml; Meloy) as described (*23*), except without the use of bovine serum albumin; (■), cultures containing both the serum-free supplements and 10% calf serum. Mouse embryo cells were prepared as described (*1*). Cultures were initiated at 10^5 cells per 25-cm^2 flask, medium was changed 3 or 4 days after plating, and cells were passaged 6 or 7 days after plating. Cells remained subconfluent under these circumstances. Serum-free cultures were passaged with the use of trypsin and trypsin inhibitor (*6*). Cell number per flask was determined on the first day after plating and at passage by counting suspensions of trypsinized cells in a Coulter particle counter and by hemocytometer. Three or more replicate flasks were initiated at each passage; cell number per flask for replicates varied less than 10% from the average. Nutrient medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (Gibco) containing 10 n*M* sodium selenite, sodium bicarbonate (1.2 g/liter), 15 m*M* Hepes buffer, penicillin (200 IU/ml), streptomycin (200 μg/ml), and ampicillin (25 μg/ml) (*6*).

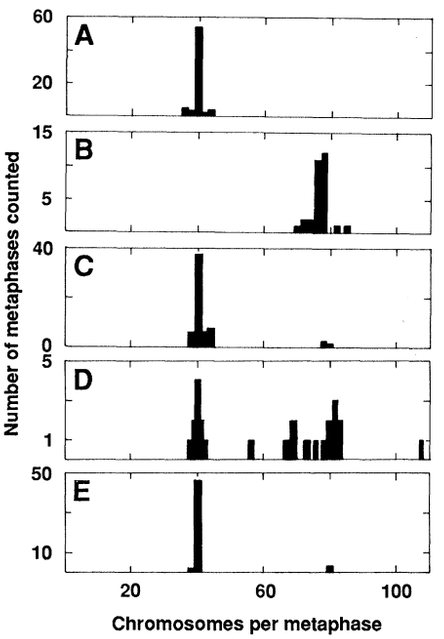

Fig. 2. Karyotype of mouse embryo cell cultures. (**A**) Swiss mouse embryo cells cultured in medium containing 10% calf serum for two population doublings in vitro; 25-hour doubling time (primary culture). (**B**) Swiss mouse embryo cells cultured in medium containing 10% calf serum for 20 population doublings; 23-hour doubling time (post-crisis culture). (**C**) Swiss mouse embryo cells cultured in serum-free medium for 200 population doublings; 20-hour doubling time. (**D**) BALB/c mouse embryo cells cultured in medium containing 10% calf serum, seven population doublings, 30-hour doubling time. (**E**) BALB/c mouse embryo cells cultured in serum-free medium for 70 population doublings; 24-hour doubling time. BALB/c cultures and Swiss cultures in serum-free medium were initiated and passaged as described in Fig. 1. Swiss cultures in serum-containing medium were initiated and passaged as described previously for the derivation of Swiss 3T3 lines (*1*). Swiss cells in serum-free medium were maintained initially in the presence of human PDGF (1 unit/ml; Collaborative Research). PDGF stimulation of cell growth in the presence of the other supplements was minimal, and addition of PDGF was discontinued at 60 population doublings.

Mouse embryo cells derived in serum-free medium were capable of growing to very high densities (Fig. 3). Under these conditions the cells were smaller than at lower densities, forming multilayered piles and aggregates. It has been shown previously that fibroblastic cultures can be grown to high cell densities if adequate nutritional conditions are maintained (*19*). The apparent decrease in growth rate of serum-free mouse embryo cultures at high cell density was the result of both increased cell loss due to depletion of nutrients and accumulation of cells in G1 due to rapid depletion of EGF. Survival of the cultures at very high cell densities was precarious because of rapid depletion of EGF.

Addition of serum to the culture medium led to inhibition of growth; supplementation of serum-containing medium with the growth-stimulatory factors used in the serum-free medium marginally improved cell growth (Fig. 3). Inhibition of cell proliferation was reversible upon replacement of serum-containing medium with the serum-free medium. Flow cytometric analysis indicated that cells in serum-containing medium accumulated in the G1 phase of the cell cycle.

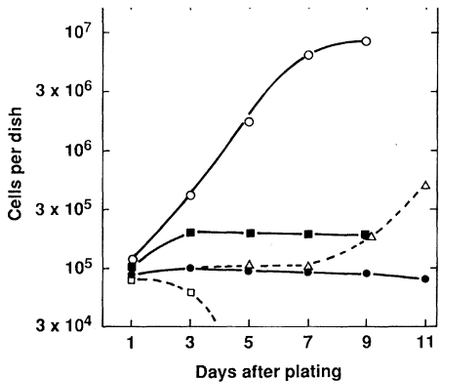

Fig. 3. Effect of medium supplements on growth of mouse embryo cells that were derived in serum-free medium. Cultures were grown in (○), serum-free medium with supplements as described in Fig. 1; (●), medium supplemented with 10% calf serum; (■) medium containing both the serum-free supplements and 10% calf serum; (□), serum-free medium with all supplements as described in Fig. 1 except EGF; (△), medium supplemented with 10% calf serum for the first 3 days followed by a change to serum-free medium with the supplements. Swiss mouse embryo cells initiated and grown for approximately 200 generations in serum-free medium were used for the experiment. Similar results were obtained with BALB/c mouse embryo cells that had been derived in serum-free medium. Cells were plated at 10^5 per 35-mm dish. Cell number was determined at the indicated times after plating by counting cell suspensions in a Coulter particle counter; individual values varied less than 10% from the average. All cells in the serum-free plates from which EGF was omitted were dead by day 5.

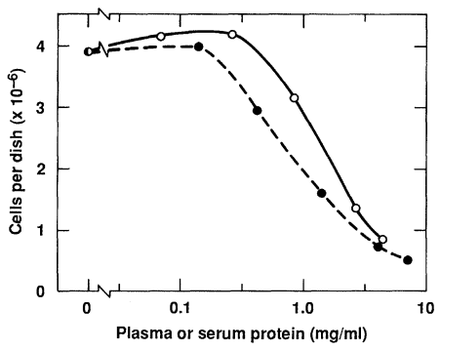

Fig. 4. Growth inhibition of serum-free–derived mouse embryo cells by plasma or serum. Cells were plated in serum-free medium supplemented as described in Fig. 1 in the presence of the indicated concentrations of calf serum (●) or plasma (○). Cell number was determined 6 days later by counting cell suspensions in a Coulter particle counter; individual values varied less than 10% from the average. Serum and platelet-free plasma used for this experiment were derived from a single donor bleed. Plasma was defibrinated before use as described (*24*) and serum was similarly treated. Cells were plated at 10^5 per 35-mm dish. Swiss mouse embryo cells initiated and grown for approximately 200 generations in serum-free medium were used for the experiment. Similar results were obtained with serum-free–derived BALB/c mouse embryo cells. Assay of calf plasma indicated less than 2 pg of transforming growth factor–β per milligram of plasma protein.

Both calf serum and platelet-free plasma were effective at inhibiting cell growth (Fig. 4). In addition to the inhibitory activity of plasma factors, platelet-derived factors (*10, 20*) also may be inhibitory for serum-free–derived mouse embryo cells, since the inhibitory activity per milligram of protein was somewhat higher for serum than for plasma. Inhibitory activity was detected in six commercial lots of calf serum, all of which were capable of supporting the growth of 3T3 mouse embryo cell lines established by conventional procedures.

Our work indicates that mouse embryo cells exhibiting the characteristics of pre-crisis cultures can be maintained on a long-term basis if serum is replaced by hormonal and nutritional supplements to the basal nutrient medium. Although the precise relationship of the cell type in the cultures derived in serum-free medium to those derived in serum-containing media remains to be resolved, several lines of evidence suggest that the serum-free–derived cells may be of mesodermal origin, like serum-derived lines. Under appropriate conditions the cells derived in serum-free medium were capable of expressing classical fibroblastic morphology. In addition, malignant cells derived from serum-free cultures by calcium phosphate–mediated oncogene transfection formed undifferentiated sarcomas in athymic mice (*21*).

The phenomena of crisis (or senescence) of diploid rodent embryo cells that have been maintained in serum-supplemented media and the transfection-induced immortalization or establishment of genomically altered lines are being actively studied. Inhibition of growth by serum or plasma factors and selection of cells unresponsive to these factors may contribute to the processes of crisis and immortalization under conventional culture conditions. Although we found it possible in serum-free medium to carry out extended culture of mouse embryo cells without an obvious crisis, human fibroblasts have been reported to undergo senescence in serum-free medium at approximately the same number of population doublings as serum-grown cultures (*22*). These results may reflect differences in species or in serum-free medium formulations. In any case, mouse embryo cells in serum-free media may provide a stable model system for the identification and study of growth inhibitors and examination of the mechanisms by which cells become hyperploid, malignantly transformed, or otherwise genomically aberrant.

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