# IRON SALTS AND TRANSFERRIN ARE SPECIFICALLY REQUIRED FOR CELL DIVISION OF CULTURED 3T6 CELLS

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ABSTRACT 3T6 Swiss mouse fibroblasts can be plated in medium without serum. Prostaglandin $F_2\alpha$, fibroblastic growth factor, epidermal growth factor and insulin stimulate DNA synthesis in medium containing vitamin $B_{12}$. A combination of these factors, however, does not stimulate cell division under our conditions. Iron salts and transferrin or low concentrations of serum are required to be concurrently present with the growth factors before cell division is observed.

The growth of established lines of nontransformed fibroblasts in tissue culture can be controlled by several defined macromolecular factors and hormones as well as serum (1). These factors (2-6) stimulate the rate of growth of relatively quiescent fibroblasts, by increasing the rate at which $G_1$ cells initiate DNA synthesis. The need for additional growth factors which may operate at other points in the cell cycle is obscured by the requirement of Swiss and BALB/c mouse 3T3 cell lines for low levels of serum (7) or bovine serum albumin (5) for viability.

Here we show that additions of fibroblast growth factor (FGF)(4), epidermal growth factor (EGF)(2) or prostaglandins $F_2\alpha$ ($PGF_2\alpha$)(6), each with insulin, to relatively quiescent 3T6 cells (8) in the absence of serum will stimulate the majority of the cells to synthesise DNA in 30 hours. However, the cells fail to divide under these conditions. Transferrin and iron salts can partially replace this mitogenic function of serum, but the presence of the mitogenic agents is required prior to the DNA synthetic phase for the occurence of cell division.

Abbreviations: $PGF_2\alpha$, prostaglandin $F_2\alpha$; FGF, fibroblastic growth factor; EGF, epidermal growth factor. DEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.





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Materials and Methods: Cell Cultures; Swiss mouse 3T6 fibroblasts (8) were maintained in Dulbecco's modified Eagle's medium (DEM) (9,10) containing 100 units/ml of penicillin, 100 µg/ml streptomycin and supplemented with 10% fetal calf serum. Subconfluent cultures were grown in 90 mm Nunc Petri dishes at 37°C equilibrated with 10% $CO_2$ in air and routinely monitored for the absence of mycoplasma contamination. DEM, normally contains 0.25 x $10^{-6}$ M $Fe(NO_3)$ (9,10). FGF, from bovine pituitary (4) and EGF from mouse submaxillary gland (2) were the generous gift of Dr. D. Gospodarowicz, Salk Institute and S. Cohen, Vanderbilt University, respectively. Prostaglandin $F_{2\alpha}$ was generously supplied by Dr. John Pike, Upjohn Company, Kalamazoo, Michigan 49001.

Conditions for resting cells in serum free medium: For determination of the initiation of DNA synthesis, 3T6 cultures wre plated in serum free medium. Subconfluent cultures from stock dishes were rinsed with 5 ml of (0.17 M NaCl; 0.005 M KCl; 0.01 M sodium phosphate (pH 7.0)) and then cultures were trypsinized by adding 2 ml of the same solution containing 100 µg/ml of crystalline trypsin for 1 minute at 37°C. The trypsin-solution was then removed and the cells were resuspended in 2.5 ml of DEM containing 0.1% calf serum and 200 µg/ml soybean trypsin inhibitor and collected by centrifugation (11). Cells were resuspended in 5 ml DEM, diluted 40 fold into DEM supplemented with 100 ng/ml of vitamin $B_{12}$ (12) and then plated in 2 ml of medium at 1.5 x $10^5$ cells in 30 mm dishes. Under these conditions mouse 3T6 cells grew about one cell division before becoming quiescent (11). For determinations of radioactively labeled nuclei the cells were pulsed continuously with 3 µCi of methyl-[$^3$H] thymidine, 1 µM, from 0 to 28 hours after additions. Subsequent conditions were as previously described (5,6).

Determination of DNA content was as described by Burton (13)

Determination of Cell Number was as previously described (5,6). The number of cells floating in the medium and not attached to the surface of the petri dishes was less than 10% of the number of cells in surface monolayer cultures. These numbers did not vary for any experimental point.

Reagents: Insulin, crystalline trypsin and soybean trypsin inhibitor were purchases from Sigma. Methyl-[$^3$H] thymidine was obtained from the Radiochemical Centre, Amersham, England.

Results and Discussion: $PGF_{2\alpha}$, EGF and FGF were added at 400, 10 and 50 ng/ml respectively, at twice the saturating concentration for the stimulation of DNA synthesis (2,3,5,6). Addition of these growth factors separately or with insulin, stimulate respectively 17.4%, 21.2% and 15.8%, or 65%, 77% and 80% of the cells to synthesize DNA after 28 hours. The combination of all three factors and insulin produce a similar stimulation as did one factor and insulin alone. When 3T6 cells were plated in DEM without vitamin $B_{12}$ then the factors failed to initiate DNA synthesis (12 and O'Farrell, M. K. et al., manuscript in preparation). Although the majority of the cells were synthesising DNA by 28 hours after all the hormones were added to the medium there was virtually no increase (about 10%), within experimental error, in the numbers of

Table 1. Stimulation of DNA Synthesis and Cell Division by Different factors.

| Additions | [$^3$H] labeled nuclei (%) | Cell No. x $10^{-5}$ |
|---|---|---|
| none | 1.8 ± 0.9 | 2.54 ± 0.18 |
| Insulin | 4.5 ± 0.5 | 2.50 ± 0.40 |
| $PGF_{2\alpha}$ | 17.4 ± 3.5 | 2.46 ± 0.20 |
| FGF | 21.2 ± 3.2 | 2.31 ± 0.02 |
| EGF | 15.8 ± 0.3 | 2.48 ± 0.08 |
| Insulin + $PGF_{2\alpha}$ | 64.0 ± 7.0 | 2.15 ± 0.06 |
| Insulin + FGF | 77.3 ± 0.6 | 2.76 ± 0.09 |
| Insulin + EGF | 69.2 ± 1.7 | 2.55 ± 0.23 |
| Insulin + FGF + EGF | 79 ± 9.0 | 2.91 ± 0.09 |
| Insulin + FGF + EGF + $PGF_{2\alpha}$ | 75.7 ± 3.5 | 2.84 ± 0.22 |
| 0.3% FCS | 27.6 ± 4.0 | 3.02 ± 0.10 |
| 0.3% FCS + Insulin + FGF + EGF + $PGF_{2\alpha}$ | 94.5 ± 2.0 | 4.74 ± 0.10 |
| 1.0% FCS | 89.8 ± 1.3 | 5.74 ± 0.37 |
| 1.0% FCS + Insulin + FGF + EGF + $PGF_{2\alpha}$ | 100 | 6.56 ± 0.21 |
| Transferrin + $FeSO_4$ | 1.5 ± 0.7 | 2.52 ± 0.04 |
| Transferrin + Insulin + FGF + EGF + $PGF_{2\alpha}$ | 77.1 ± 5.6 | 5.28 ± 0.48 |
| $FeSO_4$ + Insulin + FGF + EGF + $PGF_{2\alpha}$ | 76.5 ± 4.0 | 5.24 ± 0.09 |
| Transferrin + $FeSO_4$ + Insulin + FGF + $PGF_{2\alpha}$ | 79.2 + 3.0 | 6.24 ± 0.16 |
| 10% FCS | 100 | 12.01 ± 0.02 |

Cells were plated in DEM containing 100 ng/ml vitamin $B_{12}$. Additions of 400 ng/ml $PGF_{2\alpha}$, 10 ng/ml EGF, 50 ng/ml FGF, 50 ng/ml insulin, or FCS, 5 µg/ml human transferrin, and 3.6 x $10^{-6}$ M $FeSO_4.7H_2O$, were made. The fraction (%) of cells with radioactively-labeled nuclei after exposure to [$^3$H]thymidine from 0 until 28 hours and the cell numbers per 33 mm dish after 48 hours were recorded (5,6). Standard deviations in the means of the percentages of radioactively labeled nuclei and in cell numbers were recorded for triplicate samples. In medium without vitamin $B_{12}$ the labeling index in the same time period for any or all of the growth factors and insulin was less than 1.5%.

cells meassured at 48 (Table 1), 65, 93 or 114 hours (Fig. 1). Additions of 0.3% (FCS) alone caused only a small increase in the labeling index (28%) and in cell numbers after 48 hours (19%), but when added with the hormones it increased cell numbers by about 60% to 70%, which corresponded more closely to the original fraction of cells synthezising DNA (Table 1). Addition of 1% FCS alone more than doubled the number of cells and together with hormones gave an additional 14% increase. In controls the DNA content of quiescent 3T6 cell cultures with or without the addition of FGF, EGF, $PGF_2\alpha$, insulin

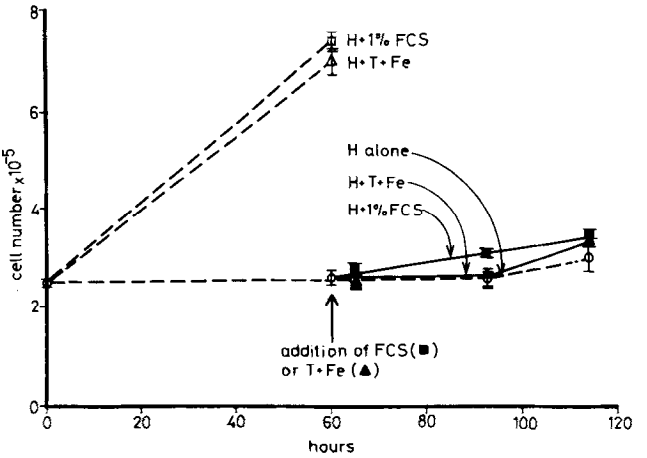


Fig. 1. Changes in Cell Numbers with Time after the Addition of Transferrin and $FeSO_4$ or Serum. Cells were set up as described in Methods. After 4 days 50 ng/ml FGF, 10 ng/ml EGF, 500 ng/ml $PGF_2\alpha$ and 50 ng/ml insulin were added (H) and after 60 hours the following additions were made: 5 μg/ml transferrin and 4 x $10^{-6}$M $FeSO_4$. (H+T+Fe) (—▲—); 1% FCS ( H + 1% FCS) (—□—); or no additions (H alone) (- -0- -). The means of the cell number ± standard deviations were then recorded for triplicate cultures at the stated times. Control cultures with growth factors and insulin together with transferrin and $FeSO_4$ (- -△- -) or together with 1% FCS (- -□- -) added at the start and isolated 60 hours later are also shown.

was 20.8 ± 1.5 or 13.1 ± 1.3 μg of DNA per $10^6$ cells measured 48 hours after the additions (13), confirming that the stimulated cells had nearly twice the amount of DNA. When these hormonally stimulated cells were removed by trypsinisation and replated in medium containing 20% FCS, the viability was less than 5% as measured by the fraction of original cells which formed colonies after 1 week. The cells without hormonal stimulation were adjudged greater than 90% viable by the above criterion.

When the growth factors and insulin were added concurrently with iron salts (14-fold over the concentration normally in DEM) or transferrin (5 μg/ml) (14) an appreciable increase in cell number was observed (Table 1). Other components in serum were without effect (14). When added separately with the growth factors and insulin, transferrin or $FeSO_4$ stimulated cell division in the physiological concentration ranges, 0.1-5 μg/ml (1-50 x $10^{-9}$ M)(15)

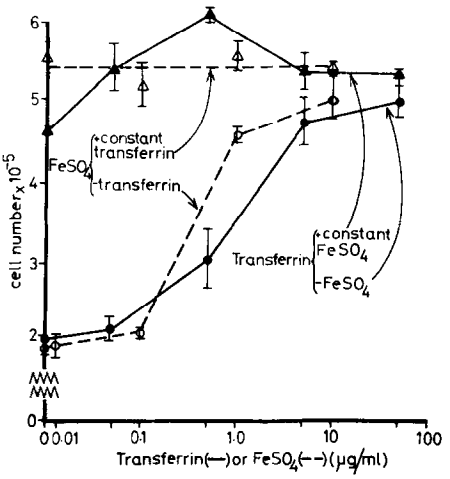


Fig. 2. Effect of Different Transferrin and Iron Concentrations on Cell Division. 3T6 cell cultures were set up as described in table 1 in DEM + 100 ng/ml vitamin $B_{12}$. After 4 days 50 ng/ml FGF, 10 ng/ml EGF, 500 ng/ml $PGF_2\alpha$, and 50 ng/ml insulin were added together with either varying concentrations (μg/ml) of human transferrin (———) without (●) or with 1 μg/ml $FeSO_4$ (4 x $10^{-6}$ M) (▲) or varying concentrations (μg/ml) of $FeSO_4.7H_2O$ (- - - -) without (○) or with 5 μg/ml transferrin (Δ). Means of cell numbers per 33 mm dish ± standard deviations were recorded after a further 52 hours for triplicate samples.

or 0.1-1 μg/ml (0.4-4 x $10^{-6}$ M) respectively (16). There was no additional effect on the increases in cell numbers when saturating concentrations of $FeSO_4$ were mixed with transferrin, or vice versa (Fig. 2). The maximum number of cells produced with saturating concentrations of transferrin and $FeSO_4$ were approximately the same as those produced by 1% FCS in the same time period (Table 1), although only half the number produced with 10% FCS (Table 1). This suggested that iron salts and transferrin could only partially replace serum. Without the growth promoting factors and insulin, 5 μg/ml of transferrin and 4 x $10^{-6}$ M $FeSO_4$ were without effect on DNA synthesis and cell division (Table 1). The ability of iron salts to stimulate 3T6 cell division with the growth factors and insulin was relatively specific, 4 x $10^{-6}$ M $ZnSO_4$, $CuSO_4$ were without effect although $FeCl_3$ at the same concentration was as effective as $FeSO_4$ (Table 2).

Table 2. Effect of Different Ions on DNA Synthesis and Cell Division.

| Additions | Radioactively labeled nuclei % | Cell number x $10^{-5}$ |
|---|---|---|
| Growth Factors + Insulin + $FeSO_4$ | 73.0 ± 1.0 | 4.74 ± 0.09 |
| Growth Factors + Insulin + $ZnSO_4$ | 70.0 ± 5.0 | 2.04 ± 0.12 |
| Growth Factors + Insulin + $CuSO_4$ | 78.0 ± 6.0 | 2.02 ± 0.16 |
| Growth Factors + Insulin + $FeCl_3$ | 73.0 ± 2.0 | 5.00 ± 0.42 |

Growth factors EGF, FGF, $PGF_{2\alpha}$, insulin were added as in Table 1 with the following salts at 4 x $10^{-6}$ M: zinc sulphate ($ZnSO_4$), cupric sulphate ($CuSO_4$), ferrous sulphate ($FeSO_4$), ferric chloride ($FeCl_3$). The means of the percentage of cells with radioactively-labeled nuclei after 26 hours and cell numbers after 52 hours were recorded for two sets of parallel triplicate cultures.

If the entire combination of growth factors and insulin was added to the cells then about 80% of the cells were synthesising DNA by 28 hours. The subsequent addition of either 1% FCS or saturating concentrations of transferrin and $FeSO_4$ after a total of 60 hours failed to increase the numbers of cells significantly at 65, 93 hours and only small increases (10% to 13%) were observed at 114 hours (Fig. 2). The same results were obtained when 0.3% FCS, 1% FCS or transferrin and $FeSO_4$ were added after 20 and 40 hours instead of 60 hours (not shown). In contrast, cultures which contained 1% FCS or $FeSO_4$ and transferrin from the start more than doubled the number of cells by 60 hours (Fig. 2).

Previous results have suggested that "conditioned medium" can be separated into two activities on the basis of heat lability, one initiating DNA synthesis and one promoting BHK cell division (17). In 3T3 cells semi-purified FGF, hydrocortisone, and insulin, appreciably stimulated DNA synthesis in serum-free medium but failed to promote cell division. Addition of 0.25% serum at this stage increased the number of monolayer cells within two hours (17). The 3T3 cells, however, were gradually losing viability (18) and detaching from the petri dish, and hence the addition of 0.25%

serum may be restoring an attachment or survival factor rather than promoting cell division. In contrast, under our serum-free conditions there is a requirement for a serum component which can be partially replaced by transferrin or iron salts to allow the 3T6 cells to proceed from the DNA synthetic phase into cell division. However, whether the hormonally stimulated cells are arrested near the completion of the S phase or in the $G_2$-M phases requires further elucidation. The presence of these components, however, is required prior to the DNA synthetic phase and not during later stages of the cell cycle. This suggests that the nontransformed fibroblasts outside the $G_1$ phase are refractory to permissive components which are required during the remainder of the cell cycle, and that their presence is obligatorily required during $G_1$, otherwise cell death ensues.

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