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# Prostatic Epithelium Inhibiting Factor (PEIF): Organ Specificity and Production by Prostatic Fibroblasts

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**Summary.** The effects of prostate fibroblast conditioned medium on two prostate epithelial cell lines (PC-3, LNCaP) and on two non-prostatic cell lines (MCF-7, K562) was investigated. As prostate fibroblast conditioned medium exerts its main effect on DNA synthesis, $^3$H thymidine incorporation was monitored to measure factor activity. Conditioned media of all prostatic fibroblast lines investigated were inhibitory for PC-3, LNCaP and MCF-7. Conditioned medium of prostatic fibroblasts was clearly stimulatory for K562. Prostate specificity of production of PEIF was demonstrated by the fact that conditioned medium from skin fibroblasts proved to be stimulatory for PC-3. Inhibitory activity from conditioned medium as well as from a BPH homogenate was precipitated by 33–67% ammonium sulfate. These partly purified fractions were respectively five and ten times as active as "crude" conditioned medium. The physical nature of PEIF (protein or macroglycolipid) as well as the possible function (as a signal messenger between stroma and epithelium) is discussed.

**Key words:** Prostate, Inhibiting factor, Chalone.

## Introduction

The active interaction between stromal and epithelial components in the prostate and especially the dominant role of the prostatic stroma in mediating morphogenetic processes has been proven by numerous in vivo recombination experiments [2–4].

Stromal cells from several different tissues are known to produce both mitogenic [1, 17] and cell growth inhibiting factors [8, 16–19]. In a previous paper [7, 13] we reported the direct in vitro effect of prostatic fibroblasts on prostatic epithelial cell lines observed after plating fibroblasts on the bottom of a culture dish and growing epithelial cells in agarose above. A very clear inhibition of clonal growth of epithelial cells was seen, and since in that system no cell to cell contact between the two types of cells was possible, we concluded that, at least in tissue culture, prostatic fibroblasts shed an inhibitory factor. Further efforts were concentrated mainly on conditioned medium (CM) of prostatic fibroblasts (PF) and the inhibiting factor(s) present therein. The inhibitory effect of CM is prominent at the level of DNA synthesis, so that a modified micro-$^3$H thymidine ($^3$HdT) incorporation test [14] proved to be most convenient to assay prostatic epithelium inhibiting factor (PEIF) activity.

In at least one reported case it was shown that inhibiting activity can copurify with mitogenic activity [8]. Since there are several reports about the occurrence of a mitogenic factor in benign prostatic hyperplasia (BPH) [11, 15], investigation of the effect of a partially purified BPH extract [15] on prostatic epithelial cells also seemed relevant.

## Materials and Methods

### *Cell Lines*

Two human prostatic carcinoma cell lines, growing in vitro, were used, namely PC-3 [6] and LNCaP, which was kindly provided by Dr. Horoszewicz [5]. In addition, the mammary carcinoma cell line MCF-7 and the myeloid leukemia cell line K562 were used. Prostatic fibroblasts were isolated from BPH, prostatic carcinoma (PC) and normal prostate (NP) tissue, removed at surgery or at autopsy. Non-genital skin fibroblasts were grown from a normal skin biopsy. The preposition "PA" refers to fibroblasts originating from BPH, "PC" from prostatic carcinoma and "NP" from normal prostate. All fibroblast lines, including those from PC and BPH, had a finite life span in vitro (about 50 population doublings). Therefore none of these fibroblast lines were considered to be cell lines in the strict sense.

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*Cell Culture*

The cell lines used were routinely cultured and passaged at 37 °C in a humidified incubator in an atmosphere of 5% $CO_2$ in air. The culture media used were: RPMI 1640 (Gibco) + 10% FCS (Boehringer) for MCF-7 and LNCaP, RPMI 1640 + 5% FCS for K562 and MEM (Eagles', Gibco) + 10% FCS for PC-3. All media were supplemented with glutamine and antibiotics.

*Preparation of Conditioned Medium and Ammonium Sulfate Precipitates*

Fibroblasts of prostatic or skin origin were cultured in MEM + 10% FCS and grown to confluency. Cells of the 4th to 8th passage after initial isolation were used. Confluent cultures were fed with fresh medium every 3 days. The medium conditioned by the fibroblasts was collected, cell debris was spun down, and the conditioned medium was stored at −80 °C for later use. Ammonium sulfate precipitates of CM were prepared by adding slowly saturated ammonium sulfate solution (pH 7.6) to thawed and pooled CM until the desired percentage of saturation was reached. After mixing for one hour the precipitate was spun down at 1,088 x g for 30 min in a Beckman J21B centrifuge with a JA20 rotor. The precipitate was dissolved in water and extensively dialysed against 10 mM phosphate buffer pH 7.6. The supernatant was brought to a higher ammonium sulfate concentration by the same procedure and/or stored at −80 °C for later use.

*Preparation of BPH Extract*

Ammonium sulfate precipitates from a BPH homogenate were prepared according to the method of Story et al. [15] for prostatic growth factor. A BPH tissue homogenate was brought first to 33% saturation, the precipitate was spun down at 1,088 x g for 30 min and the supernatant was brought to 67% saturation. After centrifugation, the pellet obtained between 33 and 67% saturation was dissolved in 10 mM phosphate buffer pH 7.6, dialysed and stored at −80 °C.

*Measurement of $^3$HdT Incorporation*

The method described by Shrivastav et al. [14] was followed to monitor DNA synthesis by $^3$H thymidine incorporation. The method was adapted to the present experimental set up in the following way: the optimum cell concentration and the best incubation time (prerequisites: high incorporation by control cells, exponentially growing cells) were determined separately for each cell line. When these parameters were defined the experiments were designed as follows: 96 wells microtiter plates were pipetted with 150 µl cell suspension per well in complete medium. Plates were incubated overnight to allow attachment of cells (not for K562, which grows in suspension). After 24 h the plates were centrifuged at 300 x g for 10 min and the medium was removed by suction. Subsequently test media were added to a total of 150 µl/well. Plates were put back in the incubator for the selected optimal incubation time. Then 1.5 µCi [5'-$^3$H]-thymidine (Amersham, UK, specific activity 15 Ci/mmol) per well was added and a final incubation time of 16 h followed. Incorporation of $^3$HdT was stopped by the addition of an excess of unlabelled thymidine. Cells were loosened from the surface by 2.5% trypsine (not for K562) and precipitated by 10% tri-chloroacetic acid. Precipitated radioactive DNA was collected on filter paper, solubilised in Soluene-350 (Packard, Belgium) and counted in a scintillation spectrometer after addition of scintillation fluid (Instagel from Packard + 1% v/v acetic acid and 0.1% w/v butylated hydroxytoluene).

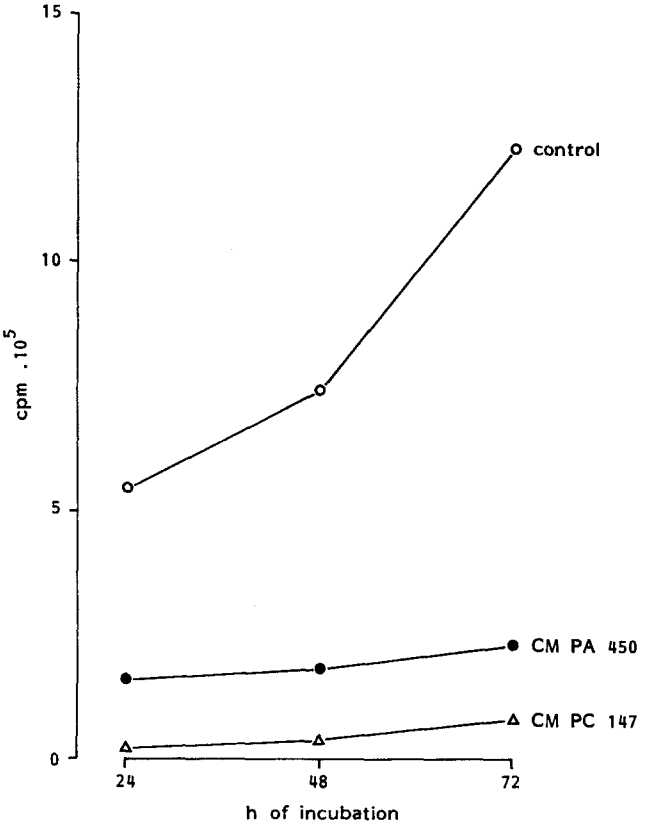


Fig. 1. $^3$HdT incorporation of PC-3, 5.10$^3$ cells per well at day 0. Medium was added as described in Materials and Methods. ○—○ MEM + 10% FCS, ●—● MEM + 10% FCS, conditioned by PA450, △—△ MEM + 10% FCS, conditioned by PC147. Each medium was tested in 12 wells

*Inhibition Ratio*

In all $^3$HdT incorporation experiments controls were used which consisted of cells incubated in fresh medium (control 100%) and in 50% fresh medium + 50% phosphate buffered saline (PBS) (control 50%). Control 50% was taken to correct for eventual exhaustion of medium constituents in the tested CM. CM was tested undiluted (150 µl on test cells; CM 100%) and at 50% (75 µl CM, 75 µl fresh medium). Inhibition ratio = $\frac{\text{control 50\%} - \text{CM 50\%}}{\text{CM 50\%} - \text{CM 100\%}}$.

All figures are in dpm so the ratio is without dimension. The ratio takes into account exhaustion of the tested CM and expresses the inhibiting activity present. For instance when inhibition cannot be abolished by adding fresh medium (the difference between CM 100% and CM 50% is small), the resulting ratio will be large. This indicates a large amount of inhibiting activity present in the tested CM. When slight or even no activity is present in the tested CM, the ratio will be small or approaching zero, resulting from a small difference between CM 50% and CM 100% and/or between control 50% and CM 50%. A negative ratio expresses stimulation of DNA synthesis.

## Results

*Effects of Conditioned Media on DNA Synthesis*

Representative $^3$HdT incorporation curves are shown in Fig. 1. When PC-3 cells were cultured for 72 h in fresh

**Table 1.** Optimal cell concentrations for each cell line ($5.10^3$ cells/well for PC-3, $4.10^4$ cells/well for LNCaP and $2.10^3$ cells/well for MCF-7 and K562) were incubated with conditioned media of the fibroblasts cell lines indicated under CM. DPF = dog prostatic fibroblasts; H1 = non-genital skin fibroblasts. All media were tested in: 6 wells 100% CM, 6 wells 50% CM. Ratio = Inhibition ratio as calculated according to the formula explained in Materials and Methods

Inhibition of prostatic and non-prostatic cell lines

| Cell line | CM | Ratio |
|---|---|---|
| PC-3 | PA486 | 0.69 |
| | PA450 | 0.37 |
| | PC147 | 3.09 |
| | NP1 | 3.10 |
| | H1 | −0.75 |
| | DPF | −0.30 |
| LNCaP | PA486 | 5.2 |
| | NP1 | 0.98 |
| | LNCaP | −0.11 |
| MCF-7 | PA486 | 0.48 |
| K562 | PA486 | −1.0 |
| | NP1 | −3.94 |

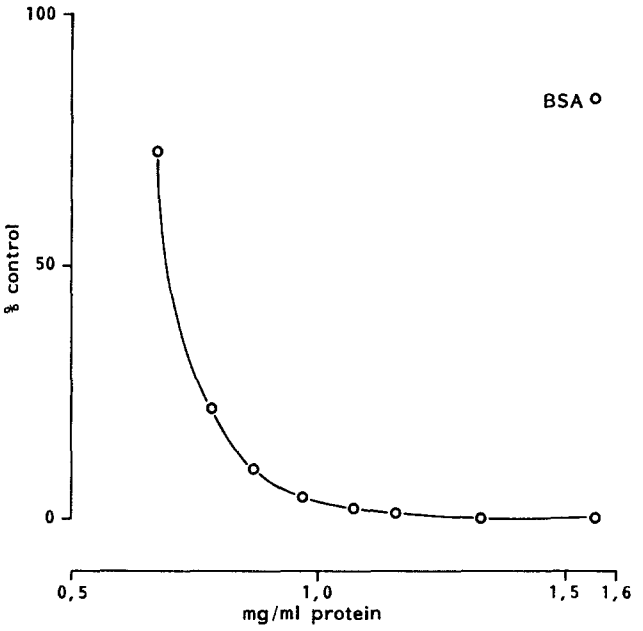


**Fig. 2.** Concentration dependent inhibition of $^3$HdT incorporation of PC-3 by BPH extract. Incorporation of radioactivity is expressed as % of the control wells, consisting of cells in MEM + fosfate (P)-buffer. BPHE and bovine serum albumine (*BSA*) were also dissolved in P-buffer

**Table 2.** 1, 2 or 4 mg/ml (final concentration in fresh medium) was added to $5.10^3$ PC-3 cells/well and incubated for 72 h prior to the radioactive pulse. Incorporated $^3$HdT was counted and dpm's were calculated using the external standard channels ratio. Results are expressed as % of the control

Inhibiting activity of $(NH4)_2SO_4$ precipitates of PFCM

| % $(NH4)_2SO_4$ prec. | mg/ml | dpm | % control |
|---|---|---|---|
| 0 | – | 585,000 | 100 |
| 40–50 | 2 | 580,000 | 99 |
| | 4 | 475,000 | 81 |
| 50–60 | 1 | 485,000 | 83 |
| | 2 | 15,000 | 3 |
| 60–75 | 2 | 600,000 | 103 |
| | 4 | 515,000 | 88 |

MEM, DNA synthesis rose from day 1 to day 3. At day 4, wells were confluent and as a consequence incorporation levelled off (not shown). When PC-3 cells were incubated in 100% CM collected from either BPH-(PA450) or PC fibroblasts (PC147) instead of in fresh medium $^3$HdT incorporation remained at a low level and rose only marginally with time. We chose the 72 h time point for subsequent experiments, because at that time differences in $^3$HdT incorporation were most substantial.

Table 1 shows the effect of CM from PF (PA486, PA450, PC147, NP1, DPF) as well as skin fibroblasts (H1). It is clear that although PC-3 and LNCaP were inhibited to different extents by the same CM's, in principle all CM's from PF inhibited both the prostatic epithelial cell lines. As CM of skin fibroblasts gave a negative ratio, we conclude that the factor produced by skin fibroblasts has a stimulatory effect on prostatic epithelial cell lines. CM from LNCaP had no effect, indicated by the very low (negative) inhibition ratio. Incubation with CM of dog prostatic fibroblasts (DPF) also resulted in a slightly negative ratio, therefore CM of DPF had a slight stimulating effect on PC-3 cells. CM of PF also had a distinct effect on non-prostatic cell lines. The effect of CM from PA486 was inverse; it was inhibitory for MCF-7 but was clearly stimulatory for K562.

### *Ammonium Sulfate Precipitation of PFCM and BPH Tissue Homogenate*

0–40%, 40–50%, 50–60% and 60–75% ammonium sulfate precipitates were made from 92 ml of pooled PFCM. After three rounds of recrystallization and subsequent dialysis the preparations were dissolved in fresh complete medium to a final concentration of 1, 2 and 4 mg protein/ml. As can be read from Table 2, 2 mg/ml protein from the 50–60% batch (total yield 6.0 mg protein) proved to be most active in inhibiting DNA synthesis of PC-3. It was concluded that the inhibiting factor was precipitated by 50–60% ammonium sulfate.

**Table 3.** The effect of various medium supplementations on the $^3$HdT incorporation by PC-3 cells

| Supplements | BPHE[c] (μg/ml) | % control |
|---|---|---|
| – | – | 100 |
| EGF[a] | – | 94 |
| Insulin[b] | – | 98 |
| EGF + Insulin | – | 87 |
| – | 1,330 | 0.5 |
| – | 1,560 | 0.1 |
| EGF | 1,560 | 0.6 |
| Insulin | 1,560 | 0.8 |
| EGF + Insulin | 1,560 | 0.7 |

[a] EGF = epidermal growth factor, 10 ng/ml
[b] Insulin 5 μg/ml
[c] BPHE = BPH extract. Each medium was tested in 12 wells. Control medium was MEM + 10% FCS

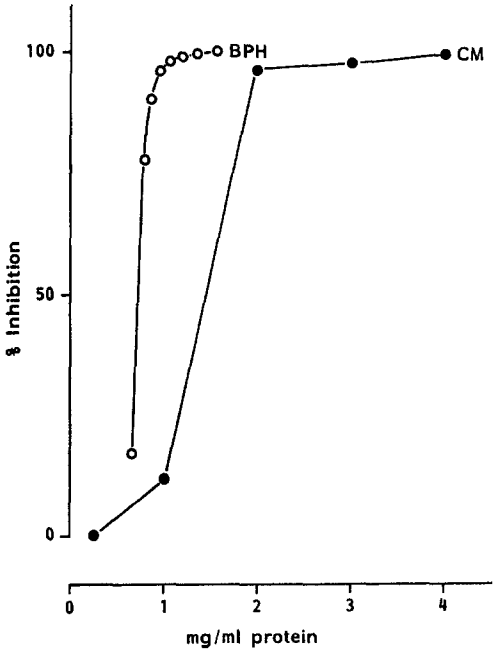


**Fig. 3.** Comparison of inhibition of PC-3 by BPH- and CM ammonium sulfate precipitate. *BPH* = 33–67% ammonium sulfate precipitate from BPH; *CM* = 50–60% ammonium sulfate precipitate from pooled CM; *mg/ml protein* = final concentration in the test medium. Controls as in Fig. 2

37 grams of BPH tissue were homogenized and processed for preparation of ammonium sulfate precipitates. The 33–67% precipitate was extensively dialysed against 10 mM phosphate buffer pH 7.6 and contained 58.55 mg total protein. Figure 2 shows the concentration-dependent inhibition of PC-3 by the extract. When 1560 μg/ml bovine serum albumin (BSA) was applied, there was virtually no inhibiting effect, which excludes an aspecific effect of high protein concentrations in the culture medium. We conclude that also BPH tissue contains an inhibiting factor, precipitated by 33–67% ammonium sulfate.

*Growth Factors and BPHE*

It is known that the effect of certain inhibiting factors can be abolished by addition of growth factors [1]. We tried to ascertain whether EGF and Insulin alone or together could (partly) undo the inhibiting effect of BPHE. Table 3 shows that with concentrations of growth factors which are optimal for responsive cells [12] we could neither stimulate PC-3 cells without BPHE nor reverse the effect of BPHE when present. Only a very slight stimulation of $^3$HdT incorporation was found in the presence of EGF + Insulin. This, however, could easily be accounted for by the normal variability of incorporation.

*Activity of Inhibiting Fractions*

From a comparison of the inhibition curves of ammonium sulfate precipitates from PFCM and BPHE (Fig. 3) we drew several conclusions: a) both curves are saturation curves; b) both curves are very steep, which means that total inhibition of DNA synthesis is reached within a narrow concentration range; c) BPHE is about two times as concentrated as PFCM. When we compared the activity of the inhibiting fractions that were discussed in this paper and state an arbitrary activity coefficient which is 1 at 50% inhibition with untreated PFCM, we calculated from Fig. 3 that the 50–60% precipitate of PFCM had a coefficient of 5 and is consequently 5 times as active as CM. Applying the same coefficient BPHE was 10 times as active (or pure) as "crude" CM.

## Discussion

The results presented in this paper prove the presence of DNA synthesis inhibiting activity in partially purified BPH homogenate as well as in conditioned medium of prostatic fibroblasts. Inhibition of prostatic epithelial cell lines was established within 24 h of incubation. Inhibitory activity was released by all prostatic fibroblast lines tested, but neither by other human fibroblasts (skin) nor by prostate fibroblasts from another species (dog). However, it cannot be ruled out that the CM of DPF had a specific inhibitory effect on dog epithelial cells. So at present we conclude that production of PEIF is probably organ specific and perhaps is also species specific. There seemed to be no clear prostate specificity with respect to the effect on target cells, as suggested by the inhibitory effect on prostate carcinoma cells as well as on breast carcinoma cells, as opposed to the very clear stimulatory effect on myeloid cells. It is feasible, however, that in tissues that have a structure that is similar to that of prostate (glandular cells arranged in acini supported by stromal components) analogous factors may exist. The inhibitory effect of CM from PF on MCF-7 fits in with this assumption. The stimulatory effect of PFCM on K562 was not considered contradictory

to the rest of the results, since K562 originated from an unrelated source.

From the ammonium sulfate precipitation experiments it was clear that the inhibiting activity precipitated in the 33–67% ammonium sulfate range. The observation that the 40–50% fraction of CM produced very little DNA synthesis inhibiting activity (Table 2) can be explained by the fact that in that concentration range there was precipitation of most of the serum proteins. This could have masked the inhibiting effect of PEIF.

It seemed that PEIF had no apparent relationship with the action of growth factors like EGF and Insulin (Table 3). We could not exclude the possibility that EGF and Insulin were able to abolish the inhibiting effect of BPHE and CM of PF when added to other cells that were principally responsive to these growth factors.

As to the physical nature of the factor, we are not sure whether PEIF should be considered to have a polypeptide structure. From previous experiments [7, 13] we know that PEIF has a molecular weight (MW) between 10 and 20 kD, which is quite similar to that of other growth modulating factors [1, 16, 18], thus PEIF could be a (small) protein. The heat-stability of PEIF was, however, surprisingly unlike that of most polypeptides. Upon incubation for 1 h at 60 °C nearly 100% of the inhibiting activity was retained. In this respect PEIF closely resembled the epidermal G1-chalone, investigated by Marks and coworkers [9, 10]. This factor is also stable against heat as well as proteolytic enzymes, it is amphipathic, very tissue specific and it has an estimated MW of 10 kD. These properties correspond with that of a macroglycolipid rather than a protein. In the case of PEIF more particular experiments would be necessary to distinguish the nature of PEIF. PEIF is likely to have a specific function in prostate tissue, because it is present in vivo as well as in vitro and has a definite effect on prostate epithelial cells. Speculating on the nature of the in vivo function, and extrapolating from what we know at present, it seems possible that PEIF could act as a sort of signal messenger between the stromal and the epithelium compartment of the prostate. The presence of these messengers has been postulated by Cunha et al. [2] as part of a general concept of regulating mechanisms in the prostate gland.

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## References

1. Böhmer FD, Lehmann W, Schmidt HE, Langen P, Grosse R (1984) Purification of a growth factor for Ehrlich ascites mammary carcinoma cells from bovine mammary gland. Exp Cell Res 150:466–476
2. Cunha GR, Chung LWK, Shannon JM, Taguchi O, Fujii H (1983) Hormone-induced morphogenesis and growth: Role of mesenchymal-epithelial interactions. Res Prog Horm Res 39: 559–598
3. Cunha GR, Fujii H, Neubauer BL, Shannon JM, Sawyer L, Reese BA (1983) Epithelial-mesenchymal interactions in prostatic development. I. Morphological observations of prostatic induction by urogenital sinus mesenchyme in epithelium of the adult rodent urinary bladder. J Cell Biol 96: 1662–1670
4. Cunha GR, Sekkingstad M, Meloy BA (1983) Heterospecific induction of prostatic development in tissue recombinants prepared with mouse, rat, rabbit and human tissues. Differentiation 24:174–180
5. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Ming Chu T, Mirand EA, Murphy GP (1983) LNCaP model of prostatic carcinoma. Cancer Res 43:1809–1818
6. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW (1979) Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 17:16–23
7. Koenig JJ, Romijn JC, Schroeder FH (1986) Inhibition of the clonal growth of prostate carcinoma cells by a factor, produced specifically by prostatic fibroblasts. (Submitted for publication)
8. Levine AE, Hamilton DA, Yeoman LC, Busch H, Brattain MG (1984) Identification of a tumor inhibitory factor in rat ascites fluid. Biochem Biophys Res Commun 119:76–82
9. Marks F, Richter KH (1984) A request for a more serious approach to the chalone concept. Br J Dermatol 111 Suppl 27: 58–63
10. Marks F, Richter KH (1986) The epidermal G1-chalone: A tissue specific "negative growth factor" in skin. Abstract nr. 3004, 14th International Cancer Congress Budapest, August 21–27, 1986
11. Nishi N, Matuo Y, Muguruma Y, Yoshitake Y, Nishikawa K, Wada F (1985) A human prostatic growth factor (hPGF): Partial purification and characterization. Biochem Biophys Res Commun 132:1103–1109
12. Keehan WL, Adams PS, Rosser MP (1984) Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen on normal rat prostate epithelial cells in serum-free, primary cell culture. Cancer Res 44:1998–2010
13. Oosterwijk-Koenig JJ, Romijn JC, Schroeder FH (1985) Modulation by prostatic fibroblasts of the clonal growth of a prostatic carcinoma cell line (PC-3) in vitro. Prostate 6:459–460
14. Shrivastav S, Bonar RA, Stone KR, Paulson DF (1980) An in vitro assay procedure to test chemotherapeutic drugs on cells from human solid tumors. Cancer Res 40:4438–4442
15. Story MT, Jacobs SC, Lawson RK (1984) Partial purification of a prostatic growth factor. J Urol 132:1212–1215
16. Strobel-Stevens JD, Lacey JC Jr (1981) Further evidence for an inhibitor of proliferation elaborated by normal human fibroblasts in culture: Partial characterization of the inhibitor. J Cell Phys 106:201–207
17. Szaniawska B, Majewski S, Kaminski MJ, Noremberg K, Swierz M, Janik P (1985) Stimulatory and inhibitory activities of lung conditioned medium on the growth of normal and neoplastic cells in vitro. J Natl Cancer Inst 75:303–306
18. Wu K, Pope JH, Ellem KAO (1985) Inhibition of growth of certain human tumour cell lines by a factor derived from human fibroblast-like cell lines. I. Demonstration by mixed culture and by use of cell washings. Int J Cancer 35:477–482
19. Wang JL, Hsu Y (1986) Negative regulators of cell growth. Trends Biochem Sci 11:24–26

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