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Anchorage-independent growth is a characteristic of transformed cells, however, we do not fully understand the mechanisms which allow cells to grow in the absence of adhesion and spreading. We have shown that partial depletion of fibronectin from serum reduces colony efficiency suggesting that fibronectin is required for colony growth. SP1 cells express a number of β_1 integrins on the cell surface, the most abundant being the fibronectin receptor $\alpha_5\beta_1$. A monoclonal antibody to $\alpha_5\beta_1$ (BMA5) inhibited adhesion to fibronectin by 90%. Colony growth of SP1 cells is positively influenced by fibronectin and negatively by collagen type I. An SP1 cell clone, Cl-12-H, adhered well to collagen type I and expressed $\alpha_2\beta_1$, and grows more efficiently in agar. In contrast the clone Cl-24-L did not adhere to collagen, express $\alpha_2\beta_1$ or grow efficiently in agar. Cl-12-H cells grow more efficiently than Cl-24-L or SP1 cells in serum free media. This growth can be inhibited by suramin, suggesting an autocrine loop. HGF receptor on SP1 cells was not reduced under anchorage-independent conditions, however, receptor phosphorylation was reduced but can be increased by addition of exogenous HGF.

These results suggest that ECM-integrin interactions and growth factors are required for anchorage-independent growth of SP1 cells.

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Ron Saulnier Aug 25th/95

PI - Signature Date

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INTRODUCTION

Mortality in breast cancer is primarily due to metastasis, the dissemination of tumor cells from the primary tumor to distant sites. Once in distant organs, the carcinoma cells grow in a tight spherical nodule, disturb the normal histology and eventually the function of the organ. A property of metastatic cells is that they have acquired the ability to grow anchorage-independently. They have lost the requirement for cell adhesion and spreading to proliferate as required by their non-malignant cell counterpart. During malignant transformation, the microenvironment of the cells is altered. These changes include upregulation or downregulation of ECM proteins as well as the corresponding integrin receptors (1,2). ECM proteins regulate many cellular functions such as cell adhesion, shape, migration and differentiation (3). A number of ECM proteins including fibronectin, laminin, collagen, and proteoglycan have been demonstrated in tumor spheroids however the function of these ECM proteins in tumor spheroids is not known (4,5).

Integrins are transmembrane heterodimers composed of an α and a ß subunit non-covalently bound together. They mediate adhesion to the ECM and some cell-cell interactions (6-8), and are involved in a number of biological processes such as cell adhesion and spreading, migration, invasion and matrix remodelling (9,10).

Although there is a general reduction of integrin receptors on transformed cells, the pattern of expression is variable in each tumor and may vary considerably within a single tumor (1). The functions of $ß_1$ integrins are being investigated, but there still remain many questions as to the role of individual integrin receptors in the various stages of tumor progression.

Zutter *et al.* (11) found that expression of $\alpha_2\beta_1$ in the murine mammary cell line Mm5MT, which does not express endogenous $\alpha_2\beta_1$, but does express the $\alpha_1\beta_1$ collagen receptor, results in increased differentiation of these cells. Pignatelli *et al.* (12) also demonstrated a reduction of $\alpha_2\beta_1$ integrin on human mammary carcinomas, while D'Ardenne *et al.* (13) found a uniform positive staining for $\alpha_2\beta_1$ in both benign and malignant breast epithelium. Positive staining was often seen in the myoepithelial cells (14). In contrast, both chemical and viral transformation of a human osteosarcoma cell line (HOS) caused an upregulation in $\alpha_2\beta_1$ expression (15).

In CHO cells, over-expression of a transfected α_5 integrin gene inhibits anchorage-independent growth and tumorigenicity (16), thus the amount of α_5 expressed is critical. Overexpression is growth inhibitory in some cell types, whereas other cells require low levels of $\alpha_5\beta_1$ integrin for proliferation in response to growth factors (17). This apparent paradox may be resolved with the recent finding that expression of $\alpha_5\beta_1$ without ligation induces pathways leading to decreased cellular proliferation while ligation reverses this signal and induces cell proliferation (18). Pena *et al.* have shown that canine non-metastatic malignant mammary tumors express $\alpha_5\beta_1$ integrin, while expression on metastatic malignant tumors was reduced but there were some tumors which still express considerable amounts of $\alpha_5\beta_1$ (19). This data shows that there is variability in $\alpha_5\beta_1$ expression on tumor cells and its function still remains unknown.

Integrin cytoplasmic domains are very small, containing only 25-50 amino acids. None of the integrins described contain kinase domains or enzymatic activity, however it is now well established that integrin receptors are coupled to intracellular signaling pathways via

tyrosine kinases (reviewed in 20,21). The early events after integrin ligation are aggregation of integrins and the cytoskeletal proteins paxillin, talin, tensin, vinculin, and α-actinin to focal adhesion contacts (22).

A number of proteins including pp125 ^{FAK} (24), paxillin (25), pp60^{src} (26), MAP kinase (27), and other known proteins (28,29) have been demonstrated to be tyrosine phosphorylated after integrin ligation. Some of these signaling molecules are also used by growth factor receptors, suggesting a cooperative or competitive interaction between ECM and growth factor receptors. McNamee *et al.* (30) have shown that PIP_2 content was increased after adhesion to ECM and have suggested a cooperative interaction between adhesion and growth factor responsiveness. These results, primarily performed on adherent cells, indicate that specific integrin molecules generate intracellular signals which regulate different cell functions. However, very little is known about whether specific ECM-integrin interactions can regulate growth factor responsiveness and anchorage-independent growth. We are using a murine mammary carcinoma cell line, SP1 which can grow anchorage-independently similar to in vivo tumor nodules to examine ECM interactions that regulates growth factor responsiveness of these cells.

Our preliminary results suggest that both ECM (FN) and growth factors (HGF and TGF-ß) are required to promote anchorage-independent growth. Therefore, we need to look at the cooperative interactions between adhesion-mediated signals and growth factor signals.

RESULTS

I. Fibronectin fibrils are present and required for SP1 colony growth.

We have previously demonstrated that fibronectin (FN), primarily organized as fibrils, is present in SP1 cell colonies (Appendix I). A 70 Kda amino-terminal fragment of FN containing the matrix assembly domain can inhibit fibril formation and reduce colony growth when added to the agar culture (Appendix I). SP1 cells cultured on plastic secrete and organize both native and soluble fibronectin into fibrils. The addition of the 70 Kda amino-terminal fragment of FN was also able to inhibit FN fibril formation on SP1 cells on coverslips (Appendix I).

Using FBS partially depleted of FN (Sigma), we observed that colony growth is reduced, but is partially reconstituted by adding exogenous bovine plasma FN (Appendix I). We have further confirmed that a reduction in FBS fibronectin reduces colony growth. Immunoprecipitation of FN from the FBS using a polyclonal antibody (Telios) reduced colony growth and is also partially reconstituted by adding exogenous bovine plasma FN. No components required for growth were removed from the serum by protein A sepharose pretreatment or by IgG preclearing, suggesting that FN was the important component (Figure 1). Immunoprecipitates were analysed by 6% SDS-PAGE and silver stained to show that fibronectin had been removed from the serum (data not shown). Experiments are underway to ensure that other components that were important for growth of SP1 colonies were not co-precipitated with FN.

II. SP1 cells adhere to fibronectin via $\alpha_5\beta_1$.

In order to understand how the ECM affects cell growth we must determine which integrin receptors are being used to adhere to specific ECM proteins. SP1 cells express high levels of the $\alpha_5\beta_1$ fibronectin receptor as well as moderate levels of $\alpha_v\beta_3$ vitronectin receptor. There are lower levels of the $\alpha_3\beta_1$ and trace amounts of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ collagen/laminin receptors (manuscript in preparation). There was very little difference in integrin expression between SP1 cells cultured on plastic and anchorage-independently, except for $\alpha_2\beta_1$ which shows a slight upregulation in colonies compared to cells on plastic (Figure 2).

SP1 cells express a number of integrin receptors for fibronectin, however, $\alpha_5\beta_1$ is the most abundant FN receptor. Using a monoclonal antibody BMA5 directed to the murine α_5 subunit (gift from B. Chan) we were able to inhibit adhesion to fibronectin by greater than 90%. An IgG control or the antibody GoH3 which recognizes the laminin binding integrin α_6 subunit did not significantly inhibit adhesion to fibronectin. (Figure 3). These results indicate that the primary fibronectin receptor on SP1 cells is $\alpha_5\beta_1$.

III. Collagen inhibits SP1 colony growth in agar.

Our next task was to investigated the ability of purified ECM proteins to promote anchorage-independent growth of SP1 cells under limiting concentrations of serum (1%). None of the purified ECM proteins were able to significantly increase colony growth. However, there was a slight increase with FN (10μg/ml) and LM (10μg/ml) (Figure 4). There was also a dramatic decrease in colony growth with both collagen types I and IV. When FN or LM was combined with collagen, there was also a reduction in colony growth showing a

dominant negative effect of collagen (Figure 4). The inhibitory effect of collagen was also seen when SP1 colonies were grown in 7% FBS (Figure 4, inset). These results suggest that ECM proteins alone are not sufficient to permit optimal colony growth, other mitogenic factors are also required.

IV. HGF receptor phosphorylation is reduced under anchorage-independent conditions.

We have previously demonstrated that HGF is a major mitogen for SP1 cells under anchorage conditions, and that HGF and HGF receptor are co-expressed on SP1 cells (31). SP1 cells show continuous growth in serum-free medium consistent with an autocrine loop (31). Under anchorage-independent conditions, no growth occurs in serum-free medium, however, addition of exogenous HGF stimulates anchorage-independent growth of SP1 cells (Appendix I). HGF receptor expression was not reduced under anchorage-independent conditions (Figure 5B). However, tyrosine phosphorylation of HGF receptor was reduced under anchorage-independent conditions, but was restored in the presence of exogenous HGF suggesting a shift from an autocrine to a paracrine loop under anchorage-independent conditions (Figure 5A). Addition of suramin did not affect receptor levels or phosphorylation under these conditions.

These experiments will be pursued further using BrdU incorporation to analyze cell cycle changes in response to ECM proteins or HGF under anchorage-independent conditions.

V. An SP1 cell clone, Cl-12-H, cells grow efficiently in agar in serum-free media and expresses $\alpha_2\beta_1$

Since SP1 cells are clonaly heterogenous, we isolated clones from SP1 cells and tested them for their ability to grow under anchorage-dependent and anchorage-independent conditions. We selected two clones; Cl-12-H which grows very well in agar and Cl-24-L which does not grow well in agar. The parent SP1 cells and the two clones had similar growth rates when cultured in 7% FBS on tissue culture plastic. However, in serum free conditions, the Cl-12-H clone grew much better than either SP1 or the Cl-24-L clone (Figure 6). The growth of all three cell lines in serum-free medium could be inhibited by suramin (500μg/ml) (Figure 7). These results suggest that the increase in growth of Cl-12-H cells in serum-free conditions is a result of exogenously secreted factors. Experiments are underway to determine whether HGF which is secreted by SP1 cells on plastic, is differentially secreted by Cl-24-L and Cl-12-H clones under adherent conditions.

We have examined the HGF receptor status on SP1 cells and the two clones. Expression of HGF receptor and tyrosine phosphorylation is similar on all three cell lines, when cultured on tissue culture plastic (Figure 8). Since HGF receptor is constitutively phosphorylated equally on both clones under adherent conditions, other components are likely required for growth of Cl-12-H cells. Experiments are underway to determine whether Cl-12-H cells secrete more HGF or whether activation of HGF receptor on the two clones is differentially affected under anchorage-independent conditions.

We also found that the adhesive profile of the Cl-12-H and Cl-24-L clones was very similar on fibronectin, laminin, vitronectin and osteopontin (data not shown). However the

Cl-12-H clone adhered and spread on collagen but the Cl-24-L clone did not (Figure 9A-C). We also found that the Cl-12-H clone expressed higher levels of the $\alpha_2\beta_1$ integrin (Figure 10). We will therefore investigate whether $\alpha_2\beta_1$ is required for HGF-mediated proliferation of Cl-12-H cells.

We tested the ability of fibronectin to promote colony growth and collagen type I to inhibit colony growth of Cl-12-H and Cl-24-L cells. Interestingly collagen type I was able to inhibit colony growth of SP1 cells but not of the Cl-12-H clone, which binds to collagen, suggesting that collagen is permitting growth signals in Cl-12-H cells, likely via the $\alpha_2\beta_1$ integrin, while inhibiting growth signals on the parent SP1 cells which do not have collagen receptors (Figure 11). We have previously shown that fibronectin can promote colony growth of SP1 cells and we now show that it also promotes colony growth of Cl-12-H cells. Neither fibronectin nor collagen could affect growth of Cl-24-L cells which did not grow well in agar (Figure 11).

Conclusion

Our results have demonstrated several key events in the anchorage-independent growth of a murine mammary carcinoma cell line. We have demonstrated that fibronectin which is abundant and organized in microfibrils, is necessary but not sufficient to permit growth of SP1 cells in agar. The results provide evidence that the ECM in spheroids plays an active role in cell growth and that cells, although in a spherical morphology, do adhere to the ECM generating intracellular signals. There is a reduction in fibronectin secretion by SP1 cells cultured in an anchorage-independent manner suggesting that an exogenous source of fibronectin is required for colony growth (Appendix I). We have also demonstrated that SP1 cells express HGF receptor and secrete HGF in an autocrine loop when cultured on plastic. Experiments are underway to demonstrate whether SP1 cells secrete similar quantities of HGF in an anchorage-independent manner.

We have shown that HGF receptor phosphorylation is decreased under non-adherent compared to adherent conditions, and that exogenous HGF stimulates HGF receptor phosphorylation on these cells. These results suggest a shift to paracrine HGF stimulation under anchorage-independent conditions. We hypothesize that the Cl-12-H clone which grows well in agar will show a sustained level of HGF receptor phosphorylation and that the presence of ECM which facilitate this phosphorylation. McNamee *et al.* (30) have demonstrated that adhesion to ECM increases the PIP_2 content of cells making signaling by growth factors more efficient. It is possible that such a phenomena also exists in colonies. To test this hypothesis we will treat non-adherent cells with both FN and HGF and determine whether there is more growth compared to controls. BrdU incorporation will be used to assess the stages of cell cycle involved.

The selection of the clones will be of valuable assistance in understanding the role of ECM-integrin interactions in anchorage-independent growth. We have demonstrated that the Cl-12-H clone which grows well in agar expresses elevated levels of $\alpha_2\beta_1$ integrin and binds and spreads on collagens type I and IV. Collagen type I inhibits SP1 colony growth in a concentration-dependent manner from 2.5 - 10 μg/ml, suggesting that the inhibitory effect is through obstruction of a permissive matrix as opposed to generation of negative growth signals after integrin ligation. In contrast addition of collagen to Cl-12-H cells increases colony growth. The mechanism by which collagen inhibits colony growth is unknown however since SP1 cells are a heterogenous population of cells and express few receptors for collagen and do not bind to it, it is likely a mechanical obstruction rather than a negative signal generated from integrin-ECM interactions is involved. These results suggest that it is not necessarily a particular ECM protein which facilitates anchorage-independent growth of SP1 cells but interactions between an ECM protein and its particular integrin receptor. It appears that when placed in agar, a selection occurs for the cells expressing elevated levels of $\alpha_2\beta_1$ receptor.

Since other integrin profiles are unchanged it suggests that the $\alpha_2\beta_1$ integrin may have a role in anchorage-independent growth of SP1 cells. To test this hypothesis we will transfect the α_2 subunit in SP1 and Cl-24-L cells and determine whether the increased expression of $\alpha_2\beta_1$ is contributing to anchorage-independent growth in the presence of collagen.

In summary, there are still a number of questions to investigate such as (1) do Cl-12-H cells have an autocrine HGF loop under anchorage-independent conditions; (2) Is the $\alpha_2\beta_1$ integrin required for anchorage-independent growth and; (3) do the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ cooperate with growth factor signaling pathways to promote anchorage-independent growth.

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Figure 1. Partial depletion of fibronectin from FBS reduces colony growth in Agar.

FBS was either untreated (UT), treated with protein A sepharose (PAS) to remove some endogenous IgG and none specific binding proteins, then precleared with rabbit anti-mouse IgG (IgG) and protein A sepharose. Serum fibronectin was removed by immunoprecipitation with Rabbit anti-bovine antibody (-FN) and added back at 10 μg/ml (-FN+FN). 2500 SP1 cells were plated in 0.36% agar over a 0.6% agar layer in 5ml plates. The cultures were allowed to grow at 37°C for 10-14 days. The plates were then fixed with methanol, stained with Giemsa and counted manually. Experiments were done with 4 samples per group.

Figure 2. SP1 cells cultured on agar and plastic have similar integrin profiles.

SP1 cells were cultured on tissue culture plastic in RPMI supplemented with 7% FBS or on a layer of 0.6% agar in RPMI 1640 with 7% FBS for 24 h. The cells were harvested, cell surface-labelled with ^{125}I. The cells were lysed in RIPA lysis buffer and the integrins immunoprecipitated with anti-integrin antibodies. The immunoprecipitates were analysed by 7.5% SDS-PAGE. The gel was dried and autoradiographed. Antibodies used were: polyclonal anti-ß1 (K. Rubin); polyclonal anti-α2 cytoplasmic (B. Chan); polyclonal anti-α3 cytoplasmic (H. Hynes); polyclonal anti-α5 cytoplasmic (R. Hynes); GoH3 mouse monoclonal α6 supernatent (A. Sonnenberg) and polyclonal anti-αv cytoplasmic (E. Ruoslahti).
(P) cells cultured on tissue culture plastic. (A) cells cultured on agar for 24 h.

Figure 3. BMA5 antibody inhibits SP1 cell adhesion to fibronectin.

96 well tissue culture plates were precoated with 5μg/ml fibronectin or BSA (5μg/ml) overnight at 4°C. The wells were washed with PBS and blocked with 2.0 mg/ml BSA in RPMI for 2 h at 37°C. $3x10^4$ cells were plated in the presence of (a) no antibody (b) rabbit anti-mouse IgG (c) GoH3 (d) BMA5 antibody. After 30 min the unadherent cells were removed by washing with PBS. The adherent cells were fixed in 3.7% paraformaldehyde and stained with 1% toluidine in a solution of 1% Na Borate. The adsorbance was measured at 570 nM in an ELISA plate reader.

Figure 4. Collagen inhibits colony growth of SP1 cells in both 1% and 7% FBS.

The Agar assay was performed as described in Figure 1. ECM proteins were added to the agar at a concentration of 10 μg/ml in cultures containing either 1% or 7% FBS.

Figure 5. HGF receptor phosphorylation is reduced under anchorage-independent conditions.

SP1 cells were prestarved overnight in RPMI 1640 medium. 6 h prior to harvesting some SP1 cells were harvested and plated on non-tissue culture plastic coated with BSA. 30 min prior to harvest some groups were treated with 500μg/ml suramin or 20 ng/ml HGF. Cells were harvested and lysed in 1% NP40 lysis buffer, immunoprecipitated with anti-Met and analysed by 6.0% SDS-PAGE and transferred to nitrocellulose followed by western blotting. Panel A was immunobloted with anti-phosphotyrosine antibody. Panel B was immunobloted with anti-Met IgG. The proteins were visualized by ECL.

Figure 6. Cl-12-H cells grow better in serum-free medium than SP1 and Cl-24-L cells.

$2x10^4$, SP1, Cl-12-H, and Cl-24-L cells were seeded in 24 well plates in serum free medium or medium supplemented with 7% FBS. After 24 h ^{3}H-thymidine was added to the cultures and incubated for another 24 h. The cells were then harvested using a cell harvester and the counts analysed in a scintillation counter. Experiments were done using 4 samples per group.

Figure 7. Suramin inhibits growth of SP1, Cl-12-H, and Cl-24-L cells in serum-free medium.

Growth assay was performed as described in Figure 6 with the addition of 500μg/ml suramin was added at the same time that the ^{3}H-thymidine was added.

Figure 8. There is no difference in HGF receptor tyrosine phosphorylation between clones.

SP1 cells were prestarved overnight in RPMI 1640 medium. Cells were harvested and lysed in 1% NP40 lysis buffer, immunoprecipitated with anti-Met and analyzed by 6.0% SDS-PAGE and transfered to nitrocellulose. Membranes were blocked with 4% BSA for 1.5 h, washed then incubated with the primary and secondary antibodies. The proteins were visualized using ECL. Panel A was immunoblotted with anti-met. Panel B was immunobloted with anti-phosphotyrosine antibody.

Lane 1 - SP1
Lane 2 - Cl-12-H
Lane 3 - Cl-24-L

Figure 9. Cl-12-H clone adheres to collagen and Cl-24-L clone does not.

Cell adhesion assay was performed as described in Figure 3 with collagen type I coated at the indicated concentrations Panel A demonstrates the adhesive profile of Cl-12-H and Cl-24-L cells on collagen types I and IV. Panel B and C are photographs of Cl-12-H and Cl-24-L cells on 10 μg/ml collagen type I, respectively.

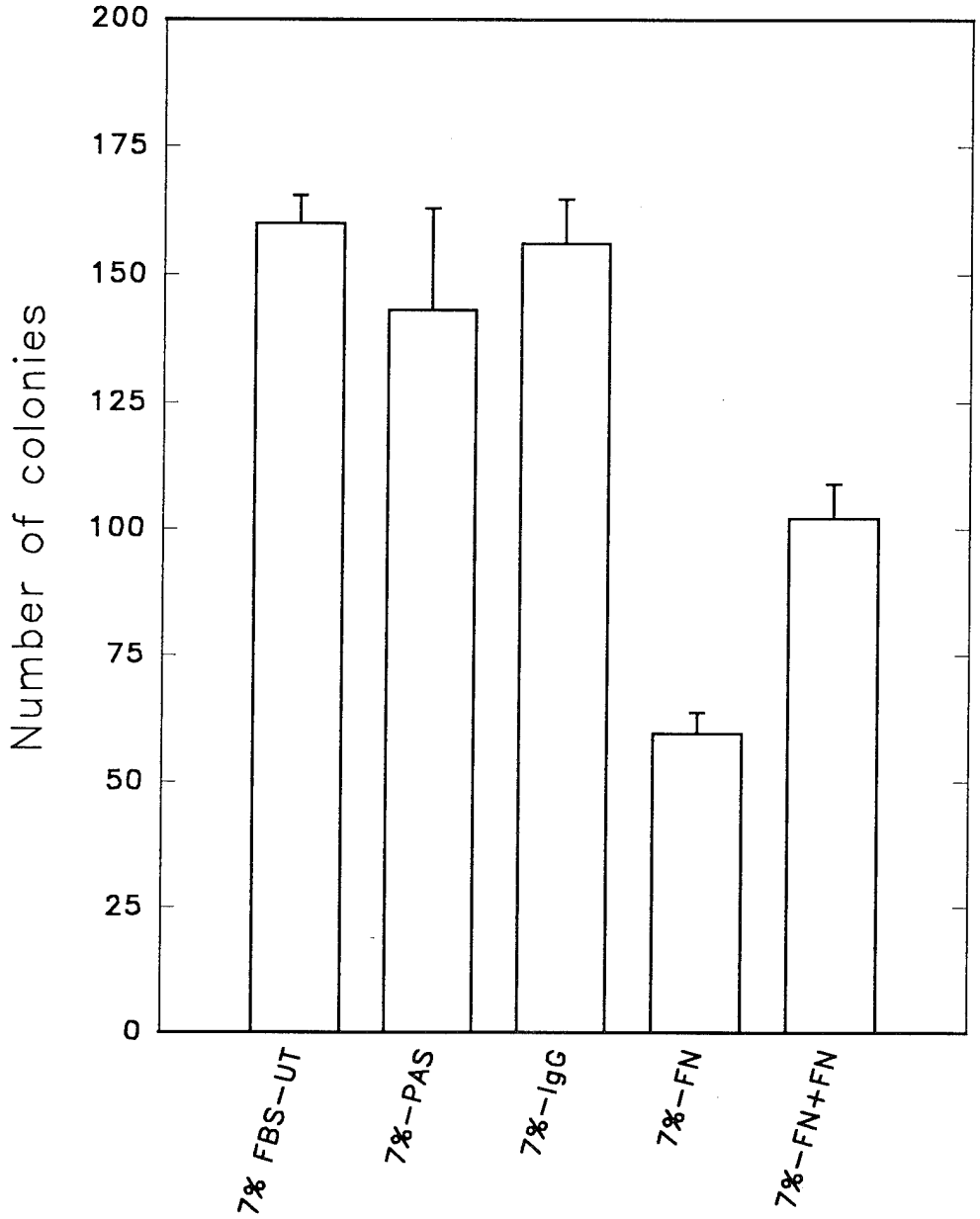
Figure 10. Cl-12-H cells express more $\alpha_2\beta_1$ than Cl-24-L.

Cl-12-H and Cl-24-L cells were labelled with a biotinylation reagent (Amersham) to biotinylate all cell surface proteins. The cells were washed in PBS and lysed in RIPA lysis buffer. The integrins were immunoprecipitated with the corresponding antibodies and resolved by 7.0% SDS-PAGE. The proteins were transfered to nitrocellulose and a western blot performed using a Streptavidin-HRP conjugate for detection. The proteins were visualized using ECL.

Figure 11. Fibronectin and collagen promote anchorage-independent growth of Cl-12-H cells.

The agar assay was performed as described in Figure 1. ECM proteins were added at concentrations of 2.5, 5.0 and 10μg/ml.

Figure 1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7%-PAS
7%-IgG
7%-FN
7%-FN+FN

Figure 2

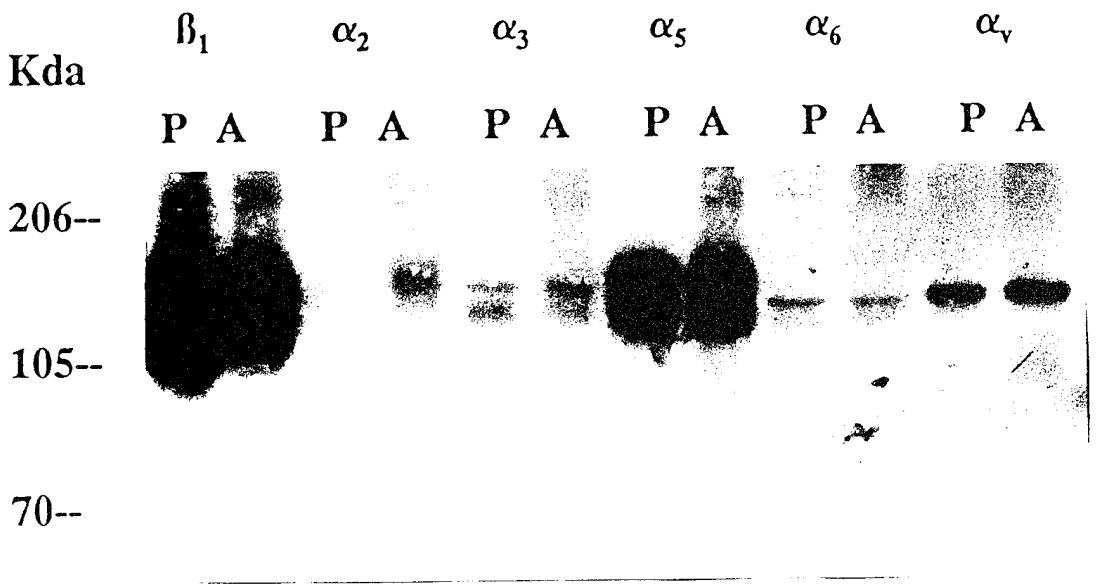

Figure 3

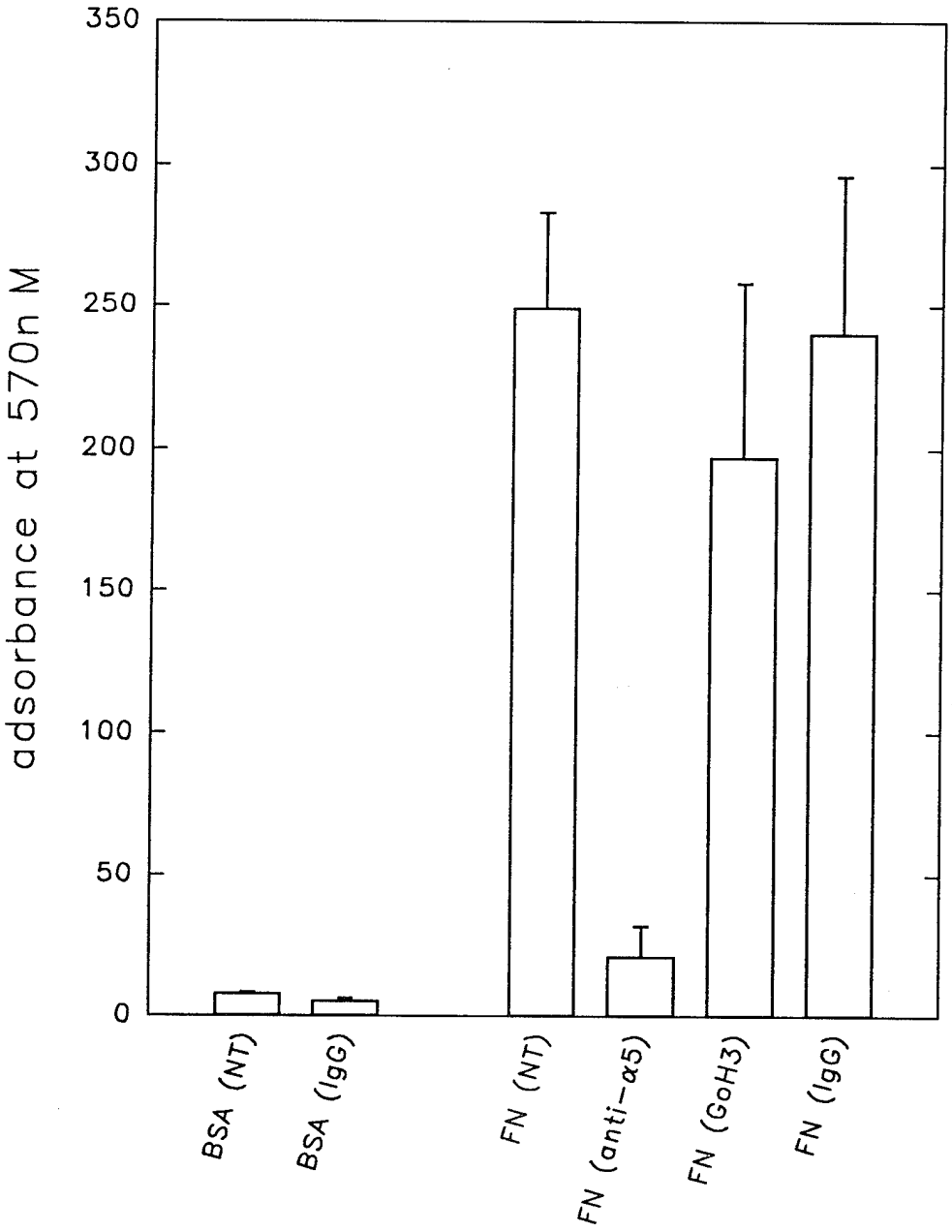
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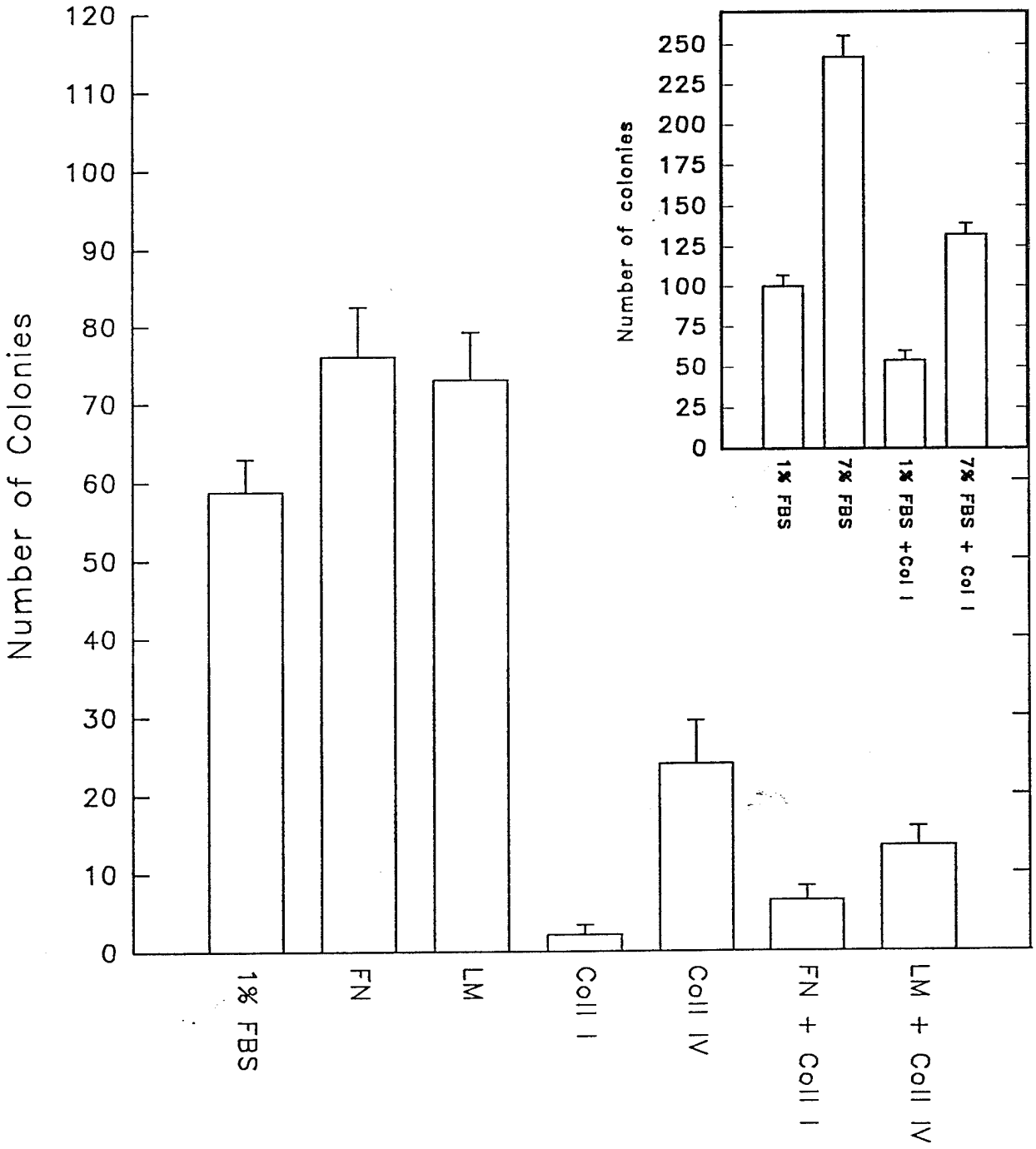
Figure 4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LM
Coll I
Coll IV
FN + Coll I
LM + Coll IV
Number of colonies
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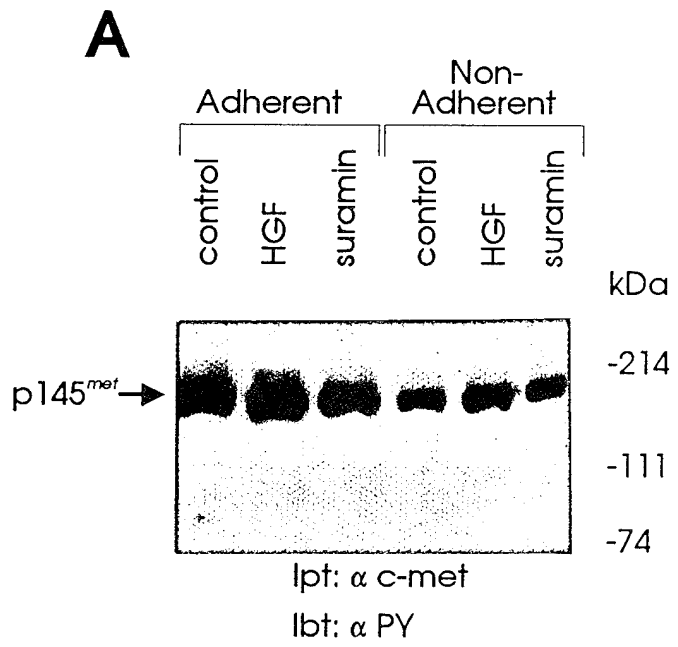
225
200
175
150
125
100
75
50
25
0
1% FBS
7% FBS
1% FBS + Coll I
7% FBS + Coll I

Figure 5


A
Adherent
Non-Adherent
control
HGF
suramin
control
HGF
suramin
kDa
p145met
-214
-111
-74
Ipt: α c-met
Ibt: α PY

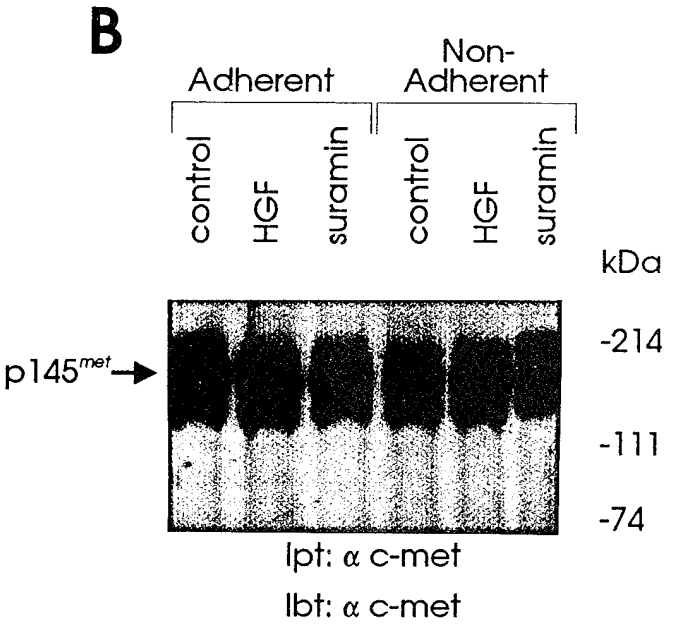

B
Adherent
Non-Adherent
control
HGF
suramin
control
HGF
suramin
kDa
p145met
-214
-111
-74
Ipt: α c-met
Ibt: α c-met

Figure 6

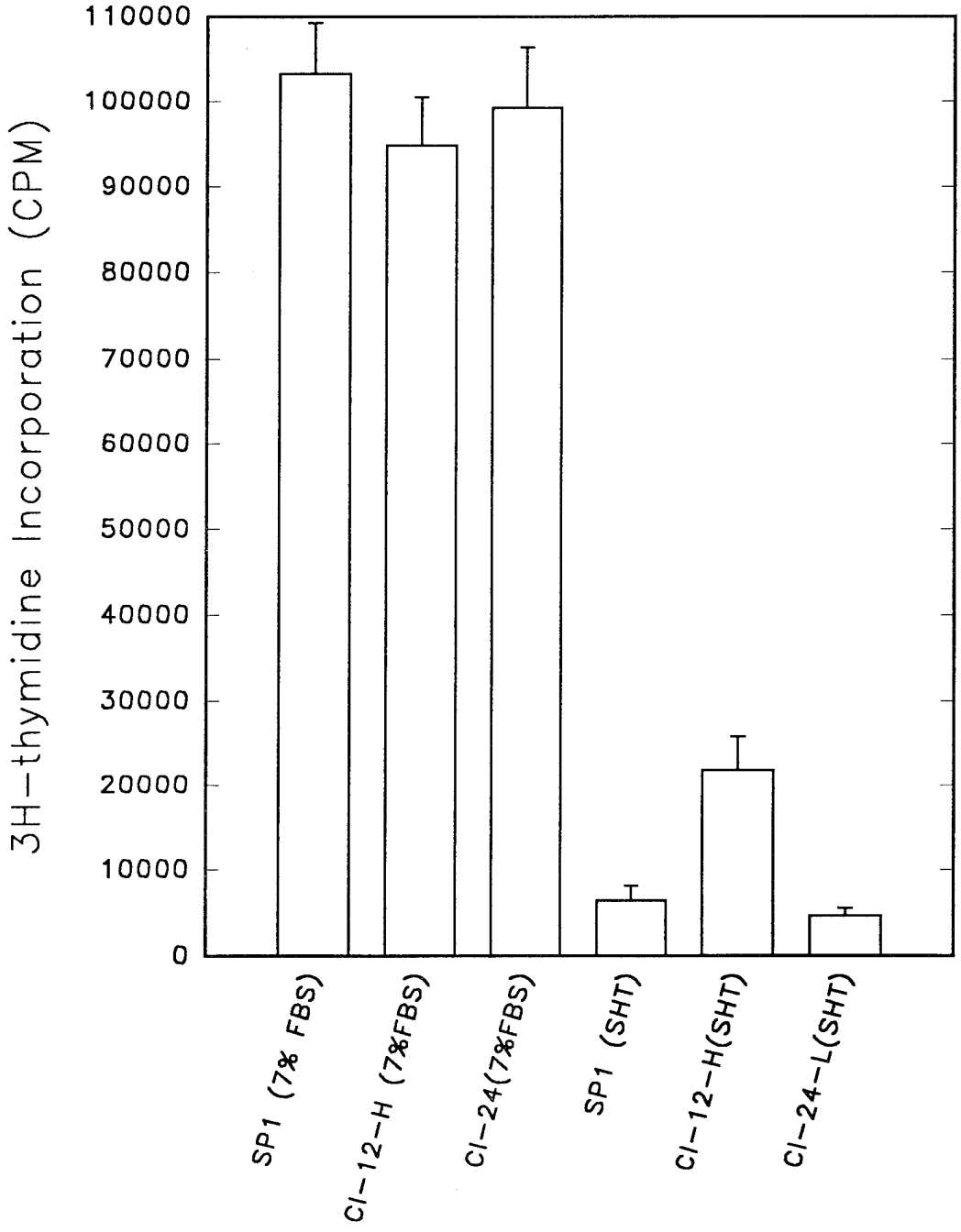

3H-thymidine Incorporation (CPM)
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
SP1 (7% FBS)
Cl-12-H (7%FBS)
Cl-24(7%FBS)
SP1 (SHT)
Cl-12-H(SHT)
Cl-24-L(SHT)

Figure 7

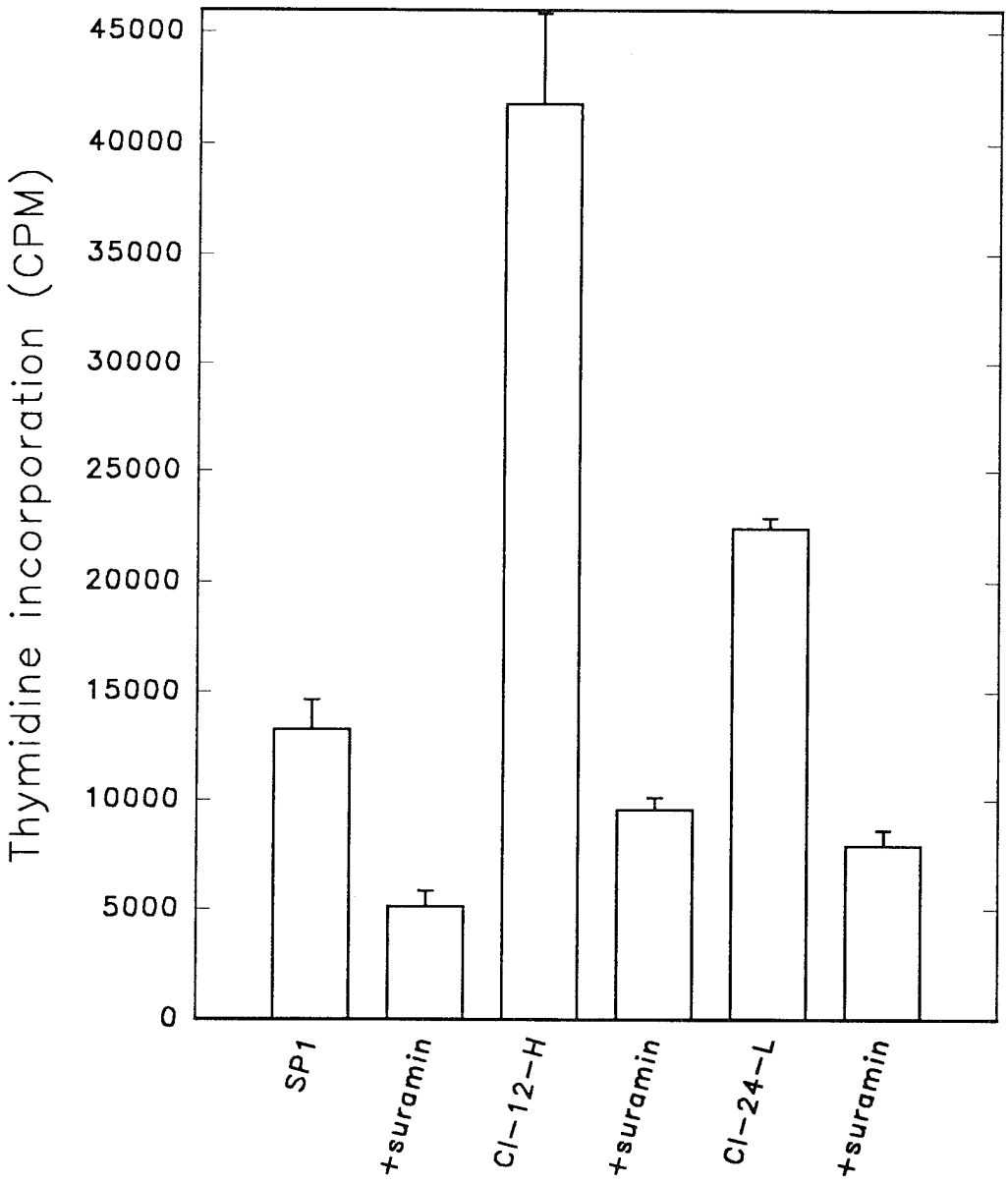
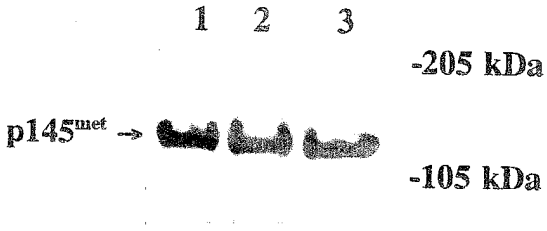

Thymidine incorporation (CPM)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
SP1
+suramin
Cl-12-H
+suramin
Cl-24-L
+suramin

Figure 8

A

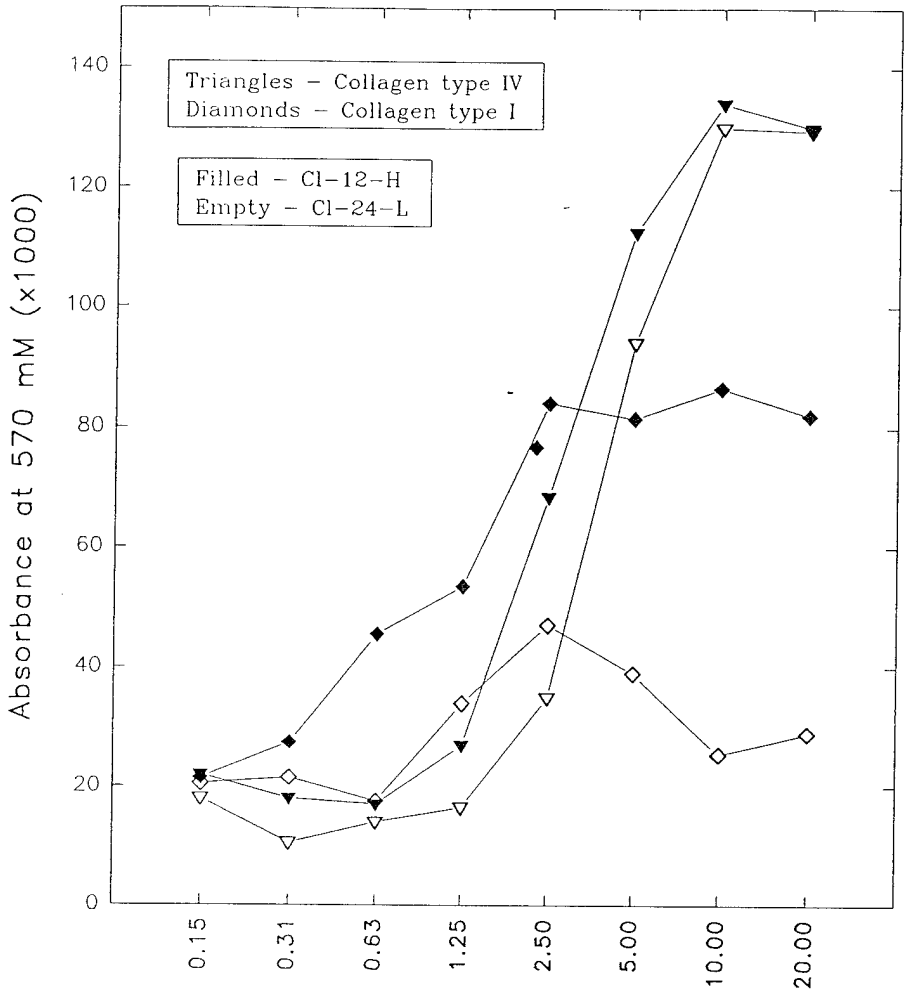
Blot: Anti- Met

B



Blot: Anti- PY

Figure 9


Triangles – Collagen type IV
Diamonds – Collagen type I
Filled – Cl-12-H
Empty – Cl-24-L
Absorbance at 570 mM (x1000)
0
20
40
60
80
100
120
140
0.15
0.31
0.63
1.25
2.50
5.00
10.00
20.00


B

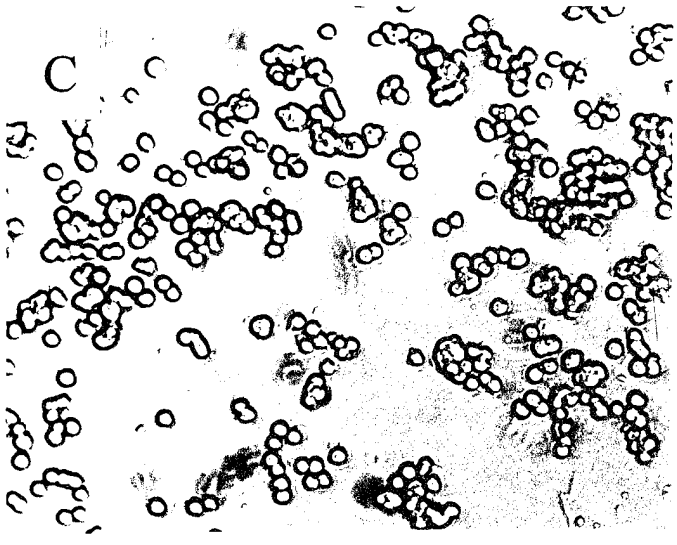

C

Figure 10

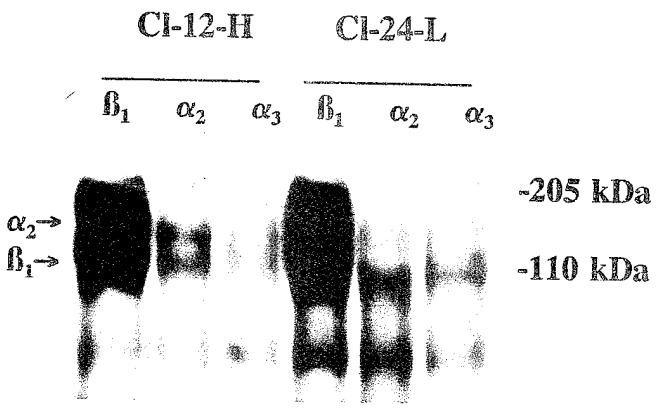
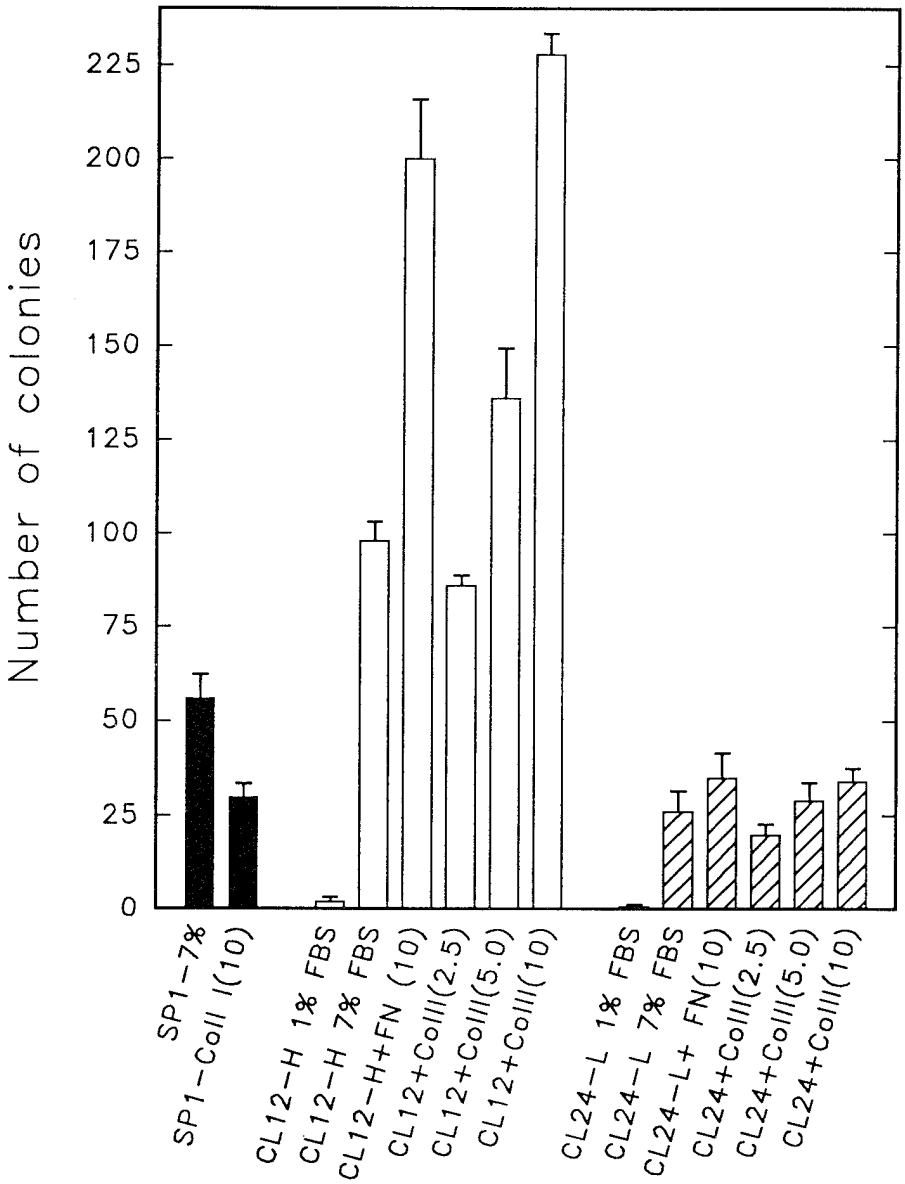


Figure 11


Number of colonies
0
25
50
75
100
125
150
175
200
225
SP1-7%
SP1-Coll I(10)
CL12-H 1% FBS
CL12-H 7% FBS
CL12-H+FN (10)
CL12+CoIII(2.5)
CL12+CoIII(5.0)
CL12+CoIII(10)
CL24-L 1% FBS
CL24-L 7% FBS
CL24-L+ FN(10)
CL24+CoIII(2.5)
CL24+CoIII(5.0)
CL24+CoIII(10)

APPENDIX I

FIBRONECTIN FIBRILS AND GROWTH FACTORS STIMULATE ANCHORAGE-INDEPENDENT GROWTH OF A MURINE MAMMARY CARCINOMA: POSSIBLE ROLE IN STROMAL-TUMOR INTERACTIONS

BY

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Running heading: Extracellular matrix in -tumor interactions

ABSTRACT

Stromal cells are important regulators of mammary carcinoma growth and metastasis. We have previously shown that a 3T3-L1 adipocyte cell line secretes hepatocyte growth factor (HGF) which stimulates proliferation of a murine mammary carcinoma (SP1) in monolayer cultures (DNA and Cell biology 13, 1189-1897, 1994). We now examine the role of growth factors and the extracelular matrix protein, fibronectin, in stimulation of anchorage-independent growth of SP1 cells. Purified transforming growth factor ß stimulated significant colony growth in soft agar cultures; whereas HGF had a lesser effect. Analysis by confocal microscopy revealed that carcinoma cell colonies contained extracellular microfibrils composed of fibronectin. Partial depletion of fibronectin from 7% FBS/agar cultures reduced the number of colonies; colony growth could be recovered by adding back exogenous fibronectin. Addition of the 70 kDa amino-terminal fragment of fibronectin, which inhibits fibronectin fibril formation, abrogated growth of carcinoma cell colonies. These findings indicate that deposition of extracellular fibronectin fibrils is necessary but not sufficient for anchorage-independent growth of SP1 mammary carcinoma cells; growth factors are also required. Carcinoma cells had less fibronectin mRNA and secreted less fibronectin protein under anchorage-independent compared to anchorage-dependent conditions, as determined by northern blotting and immunoprecipitation analysis. Thus, both growth factors (HGF and TGF-ß) and fibronectin, secreted by adipocytes, may be important regulators of paracrine stimulation by stromal cells of anchorage-independent growth of mammary carcinoma cells.

INTRODUCTION

The mammary stroma, made up primarily of adipocytes and fibroblasts, is an important regulator of growth and development of normal and malignant epithelial cells in the breast [1-6]. Stromal cells, especially adipocytes, are in direct contact with epithelial stem cells in the terminal end buds of the mammary gland and are required for hormone responsiveness and tissue-specific differentiation of epithelial cells [reviewed in 2]. At puberty, estrogen delivers signals via stromal cells to the end bud. These signals lead to increased expression of transforming growth factor-α peptide and of epidermal growth factor receptor in epithelial cells of the terminal end bud, followed by ductal growth and side-branching [7,8]. *In vitro*, primary mammary adipose tissue fragments [9-11] and an adipocyte cell line 3T3-L1 [11-13] can stimulate growth of normal and malignant mammary epithelial cells. However, the signalling mechanisms involved in mammary stromal-epithelial interactions are not known.

Two growth factors, keratinocyte-derived growth factor [14,15], and hepatocyte growth factor (HGF) [16] are produced by mammary stromal cells and can stimulate ductal growth of mammary epithelial cells *in vitro*; HGF receptor is frequently upregulated in breast cancer [17]. In addition, the ECM basement membrane protein, laminin, has been shown to stimulate differentiation of normal breast epithelium without growth [18], and to stimulate growth of primary breast carcinoma cells without differentiation [19]. Fibronectin (FN), though not normally expressed in basement membrane, is up-regulated in some malignancies [20,21] and can promote a growth response to certain growth factors [22,23]. Thus, both growth factors and extracellular matrix (ECM) are considered to be important paracrine regulators of mammary epithelial growth.

We have developed a murine mammary carcinoma (SP1) model to examine mammary stromal interactions in tumor growth and invasiveness [5,9]. *In vivo*, SP1 cells grow subcutaneously as a benign lesion with no metastasis. When SP1 cells are injected into the mammary fat pad or co-injected with adipose tissue into subcutaneous sites, tumor growth is augmented and pulmonary metastases frequently occur. *In vitro*, we have shown that a 3T3-L1 adipocyte cell line secretes HGF which is mitogenic for SP1 cells in monolayers [24]. In addition, 3T3-L1 adipocytes secrete latent forms of transforming growth factor-ß1 (TGF-ß1) and TGF-ß2 which, upon activation, inhibit growth of SP1 cells in monolayer cultures [25]. Thus, adipocytes, which are a dominant mammary stromal cell type, secrete both growth factors [24,26] and ECM proteins [27] and may be important regulators of the stromal environment in breast cancer.

In the present report, we examined the effect of HGF and TGF-ß, both secreted by adipocytes, on anchorage-independent growth (i.e. without cell spreading) of SP1 cells in agar cultures, a phenotype which is characteristic of malignant cells. TGF ß stimulated significant colony growth, whereas HGF had a lesser effect. Since TGF-ß can stimulate expression of ECM proteins which could affect cell growth [36], we examined the possible role of ECM in SP1 colony growth. Colonies grew as tightly adhesive spheroids with prominent extracellular FN fibrils. We therefore examined the requirement for FN fibrils in the colony growth of SP1 cells, and the possible source of FN in SP1 colonies. The results suggest that both growth factors (HGF and TGF-ß) and ECM (FN) may be important paracrine regulators of anchorage-independent growth of SP1 cells.

MATERIALS AND METHODS

Media: Maintenance medium for SP1 tumor cell cultures was RPMI 1640 (GIBCO), with 20 mM L-glutamine (GIBCO) and 7% fetal bovine serum (FBS, Flow Laboratories). For some experiments, RPMI 1640 was supplemented with 10^{-5} M sodium selenite, 3 X 10^{-3} M hydrocortisone and 15 μg/ml transferrin (referred to as serum-free medium). For 3T3-L1 pre-adipocytes, maintenance medium was DMEM (GIBCO) supplemented with 10% FBS and 20 mM L-glutamine. Differentiation medium was DMEM supplemented with 10% FBS, 10 μg/ml insulin (Sigma), and 0.25 μM dexamethasone (Sigma). In some experiments, FBS prescreened for reduced level of FN (Sigma, Catalog No. F-2013) was used.

SP1 tumor cell line: SP1 is a mammary adenocarcinoma which arose spontaneously in an 18 month old CBA/J female retired breeder obtained from Jackson Laboratories. The histological and ultrastructural characterization of the original SP1 tumor as an infiltrating ductal carcinoma and its growth properties have been described previously [28]. A cell line was established *in vitro* and frozen down to maintain stock. Cells were kept in culture for no more than 3 months before thawing out fresh stocks. All tumor cell lines were tested periodically for mycoplasma [28].

3T3-L1 adipocyte differentiation: A 3T3-L1 pre-adipocyte cell line and a derivative fibroblast cell line 3T3-C2 were obtained from Dr. Howard Green (Harvard Medical School, Boston, MS, USA) [29]. 3T3-L1 pre-adipocytes undergo differentiation to adipocytes under certain culture conditions [24]. Briefly, 3T3-L1 pre-adipocytes are grown to 80% confluence in T175 tissue

culture flasks in maintenance medium. Differentiation medium is added for 48 hours and then replaced with dexamethasone-free differentiation medium (i.e. DMEM with 10 μg/ml insulin and 10% FBS). Complete differentiation occurs after an additional 5-7 days.

Collection of conditioned medium (CM): Serum-free medium (30 ml) was added to T175 tissue culture flasks containing SP1 cell, or 3T3-L1 pre-adipocyte or adipocyte monolayers which were pre-washed 2x with PBS and 1x with serum free medium. After 24 h, the medium was removed, centrifuged at 2000 rpm in an IEC clinical centrifuge for 20 min to remove any cellular debris, and frozen at -70°C in 10 ml aliquots. In some experiments, CM was acid-treated as follows: pH was lowered to 2.8 by dropwise addition of 1 N HCl. After 30 min at 4°C, the pH was readjusted to 7.2 with 1 N NaOH.

Assay for colony-forming cells: For stromal-tumor cell co-cultures, feeder layers of induced and uninduced 3T3-L1 adipocytes and the non-adipocyte sister clone, 3T3-C2, were prepared in 60 mm tissue culture plates (NUNC). A liquid solution (42°C) of 1.2% Bactoagar (Difco Lab.) was mixed (1:1) with 2x DMEM, supplemented to yield the final concentrations indicated of L-glutamine (20 mM) and FBS (0%, 0.1%, 1% or 7%). Each mixture was layered onto tissue culture plates with the appropriate feeder layers and allowed to set. A 0.36% Bactoagar solution with DMEM and FBS (as above) was similarly prepared, mixed with 1 x 10^3 SP1 cells/2.5 ml and layered on top of the 0.6% agar (2.5 ml/plate). Controls of SP1 cells on plastic with and without agar were used. Plates were incubated at 37°C and 10% CO_2 for 10-12 days.

SP1 colonies were stained with Geimsa stain, and counted manually. Experiments without feeder cells were carried out under the same conditions except RPMI instead of DMEM medium was used.

Cell adhesion assay: The quantitation of cell adhesion has been described previously [23]. Briefly, a plate-based assay was used in which ECM proteins were coated on 96-well tissue culture plates (Linbro) for 48 h at 4°C. Remaining protein-binding sites on the plate were blocked with 0.1% BSA. Plates were washed three times in PBS and cells (10^5/well) were added in DMEM with 0.1% BSA. Cells were incubated at 37°C for 45 min. The plates were washed and the adherent cells remaining were stained with coomassie blue and quantitated by measuring optical density at a wavelength of 270 nm in an ELISA plate reader.

Immunofluorescence staining: SP1 colonies were collected by pipetting and then dried onto glass slides. For two-dimensional cultures, SP1 cells were cultured in serum-free medium on glass coverslips coated with 0.1 M poly-L-lysine (Sigma). Dried cells were fixed with paraformaldehyde (2%) and treated with Triton X-100 (0.03%) to allow permeation of antibodies. Cells were blocked with 1% BSA, and stained with one of the following ECM-specific primary antibodies: polyclonal rabbit anti-mouse FN antiserum (1:100 dilution, GIBCO), rabbit anti-mouse vitronectin antiserum (1:50 dilution) (M. Pera, Oxford University, Oxford, England), goat anti-human collagen type IV IgG (25 μg/ml) (Southern Biotechnologies), rabbit anti-mouse laminin B1/B2 chain antiserum (1:400) (UBI), and monoclonal rat anti-mouse tenascin IgG (0.25 μg/ml) (provided by Dr. P. Ekblom, Uppsala University, Uppsala, Sweden). Primary antibodies were detected by the appropriate FITC-conjugated secondary antibodies: goat

anti-rabbit IgG, rabbit anti-goat IgG, and sheep anti-rat IgG (Cappel Laboratories). Fluorescence was viewed with a Leitz Aristoplan microscope equipped with epi-illumination with a mercury 100 watt lamp, or with a Meridian confocal microscope equipped with an argon laser. T-MAX 400 Kodak film was used.

mRNA extraction and northern analysis: The Micro-Fast Track system (InVitrogen) was used to extract poly $(A)^+$ RNA from cultured cells or tissues. mRNA was subjected to denaturing electrophoresis on a 1.4% agarose gel, then transferred to Zeta-Probe Membranes (Bio-Rad). The hybridization method used was a modification of that of Sambrook *et al.* [30]: Blots were pre-hybridized for 4 hours at 42°C in standard hybridization solution (50% formamide, 4x Denhardt's solution, 0.25 x SSC, 0.1 mg/ml sheared herring testes DNA, 1% SDS, 5 mM EDTA, 50 mM NaH_2PO_4). ^{32}P-labelled probes were prepared by a nick translation method (Nick Translation Kit, GIBCO) and hybridized with the membrane for 24 hours at 42°C in standard hybridization solution. Membranes were washed for 15 min at room temperature with each of the following: 2x SSC/0.1% SDS, 0.5x SSC/0.1% SDS and 0.1x SSC/0.1% SDS. Autoradiograms were scanned on a laser densitometer (Molecular Dynamics). Volume analysis for specific bands was performed and normalized with values for GAPDH mRNA determined by the same methods in the same lane on the same membrane. A cDNA probe (821 bp) corresponding to two type III homology domains in the N-terminal region of the mature form of murine FN was obtained from Dr. P. Ekblom (unpublished), and a cDNA probe (1.1 kb) for the murine laminin ß1 chain [31] was obtained from Dr. K. Rubin.

Immunoprecipitation and western blot analysis: CM (10 ml) from SP1 or adipocyte cell lines were concentrated in Centricon 30 microconcentrating tubes (Amicon) in a Beckman Rotor 20.1 at 5000 rpm, 20 min, 4°C. Concentrated CMs (each 1 ml) were immunoprecipitated with polyclonal rabbit anti-FN antiserum (2 μl) overnight at 4°C, and then with 50 μl 10% protein-A-sepharose (Pharmacia) for 3 hours. Precipitates were collected by centrifugation at 9000 x g for 15 minutes. Samples were eluted from beads with 50 μl Laemmli buffer at 4°C for 30 min. Samples were boiled for 5 min and subjected to 7% SDS-PAGE. The proteins were transferred overnight (30 mA / 4°C) in Towbin transfer buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% methanol), onto nitrocellulose membrane (BioRad). The membranes were blocked with 3% BSA in 0.05% Tween-TBS. FN was probed first with rabbit anti-FN antiserum (0.5 μg/ml) and then with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Amersham; 1:1000 dilution). The ECL system (Amersham) was used to detect the proteins [32].

In some experiments, SP1 cells, on plastic and in agar, were biosynthetically labelled with 100 μCi/ml ^{35}S-methionine (ICN) in methionine-free RPMI medium (GIBCO) with 7% FBS dialysed against PBS. After a 5 hour incubation followed by a 2 hour pulse with ^{35}S-methionine, cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 μg/ml leupeptin, 2 μl/ml aprotinin, and 0.1 μl/ml 4-amidinophenylmethanesulfonyl fluoride (APMSF) (Sigma)). FN and laminin were sequentially immunoprecipitated from cell lysates and concentrated CM using the corresponding polyclonal rabbit anti-mouse antisera (2 μl/ml) and 10% protein-A-sepharose (Pharmacia). Samples were run on a 7% SDS-PAGE under reducing conditions. The gel was amplified, dried, and the autoradiogram was developed after a 2 day exposure.

RESULTS

TGF-ß and HGF stimulate anchorage-indepndent growth of SP1 cells: Our preliminary results showed that 3T3-L1 adipocyte monolayers can stimulate anchorage-independent growth of SP1 cells in soft agar cultures (data not shown). Previously, we have shown that adipocytes secrete HGF [24] and latent forms of TGF-ß1 and TGF-ß2 [25], which are activated by acid-treatment [33]. Since both HGF [34] and TGF-ß [35,36] have been shown to stimulate colony growth of certain tumor cells, we tested the ability of HGF and TGF-ß to stimulate SP1 colony growth in soft agar cultures. The results show that TGF-ß strongly stimulated colony growth, whereas HGF stimulated colony growth to a lesser extent (Figure 1). No additive effect was observed when both growth factors were added together to cultures (not shown). Thus, TGF-ß and HGF promote SP1 colony growth.

Extracellular fibronectin (FN) fibrils are present in SP1 colonies: SP1 colonies in agar grew as tight spheroids (Figure 1, insert). This morphology suggests the presence of adhesive proteins that promote close cell contact [37]. We therefore examined the presence of ECM proteins in SP1 colonies grown on adipocyte monolayers (not shown) and in 7% FBS/agar cultures (Table 1, Figure 2f, 2g). In both conditions, extracellular FN was detected in SP1 colonies whereas very little cytoplasmic FN was expressed, as determined by confocal microscopy. The presence of FN fibrils was evident, particularly in 7% FBS/agar cultures (Figure 2g). In contrast, SP1 cells in monolayer cultures revealed a perinuclear punctate pattern of cytoplasmic FN as well as some membrane-associated FN as determined by immunofluorescence staining (Figure 2b, 2c). Punctate and diffuse expression of vitronectin and, to a lesser extent, laminin were also observed in SP1 cells in both colonies and monolayers. Tenascin (Figure 2d, 2h) and collagen type IV (Table I) were not detected in either monolayers

or colonies of SP1 cells. FN is therefore a major ECM substrate in SP1 colonies.

FN fibrils are required for SP1 colony growth: We have previously shown that FN can enhance the proliferative response of SP1 cells to certain growth factors (e.g. basic fibroblast growth factor) in monolayer cultures [23]. To determine the role of FN in the growth of SP1 colonies, we examined the ability of SP1 cells to form colonies in cultures with 7% FBS partially depleted of FN in the absence of any feeder cells. Under these conditions, SP1 colony growth was greatly reduced, but could be recovered by adding exogenous FN (5-10 μg/ml) (Figure 3). Addition of FN alone to cultures with 1% FBS only slightly stimulated cell growth (not shown); thus FN is necessary but not sufficient for optimal colony growth of SP1 cells.

To examine the role of FN fibrils in the growth of SP1 colonies, we tested the affect of the 70 kDa amino-terminal FN fragment, which has been shown to inhibit FN fibril formation [38], on the growth of SP1 colonies in 7%/agar cultures. At concentrations of 10 and 20 μg/ml of the N-terminal fragment of FN, we observed a marked inhibition of FN fibril formation (Table 2). The results show that colony growth was inhibited by increasing concentrations of the 70 kDa amino-terminal FN fragment (Figure 4A). SP1 cells did not bind directly to substratum consisting of the 70 kDa FN fragment (Figure 4B); nor did the 70 kDa FN fragment compete for binding of SP1 cells to native FN substratum (not shown). Thus the observed inhibition of growth with the 70 kDa amino-terminal fragment of FN occurred indirectly, most likely via inhibition of FN fibril formation.

FN mRNA and protein are expressed in SP1 cells but are down-regulated under anchorage-independent conditions: The absence of cytoplasmic FN in SP1 cells in colonies compared to SP1 cells in monolayers (Figure 2) suggests that FN synthesis is reduced under non-adherent

conditions. We therefore determined the steady state levels of FN and laminin mRNA and secreted protein in SP1 cells in monolayers (anchorage-dependent) and in colonies (anchorage-independent) in 7% FBS cultures (Figure 5A). Both FN and laminin mRNA were abundantly expressed in SP1 cells on monolayers. In contrast, expression of FN mRNA was significantly reduced in SP1 colonies; whereas the level of laminin and GAPDH mRNA remained unchanged. Only trace levels of collagen types I and IV mRNA were detected in SP1 monolayers or colonies, whereas all four ECM mRNAs were present in adipocytes (not shown).

Both FN and laminin proteins were detected in immunoblots of CM from monolayers of SP1 cells (data not shown). To examine the direct effect of cell adhesion on FN and laminin production, we assessed protein synthesis and secretion of FN and laminin by SP1 cells in short term (5 h) suspension (non-adherent) and monolayer (adherent) cultures with 7% FBS. Synthesis of ^{35}S-methionine-labelled FN in SP1 cells was reduced 10-fold within 5 h following incubation of cells under non-adherent compared to adherent conditions (Figure 6A). Likewise, SP1 cells in suspension secreted 13-fold less FN than SP1 cells in monolayers (Figure 6B). Synthesis and secretion of laminin was also reduced under non-adherent conditions.

DISCUSSION

Growth factors and ECM proteins are important regulators of stromal cell interactions that affect normal and malignant mammary epithelial growth. Using defined culture conditions with serum, we have demonstrated a role of two growth factors, TGF-ß and HGF, as well as the the ECM protien, FN, in the anchorage-independent growth of SP1 cells.

Both HGF and latent TGF-ß are present in 3T3-L1 adipocyte conditioned medium (CM) and have been shown previously to differentially affect SP1 cell growth in monolayer cultures [24,25]. HGF stimulated growth [24], whereas TGF-ß, activated by acid-treatment, inhibited SP1 cell growth in monolayers. We now show that purified TGF-ß strongly stimulated SP1 cell colony growth; HGF stimulated growth to a lesser extent. Together, these results suggest that TGF-ß and HGF are important paracrine regulators of stromal interactions that affect carcinoma growth. However, interactions with other components secreted by stromal cells or SP1 tumor cells (e.g. proteinases or ECM proteins) may also modulate colony growth. Earlier reports, that TGF-ß in combination with other growth factors (e.g. platelet-derived growth factor and basic fibroblast growth factor) stimulate anchorage-independent growth of certain tumor cells [34,35], corroborate these findings. Other effects of TGF-ß on tumor microenvironment, such as increased synthesis of extracellular matrix proteins [36], and corresponding integrin receptors [36,39], may provide a growth advantage which overrules any direct anti-proliferative effects of TGF-ß [25].

Partial depletion of FN in 7% FBS-supplemented cultures reduced the number of colonies, and colony growth could be recovered by addition of exogenous FN. This finding suggests that FN is limiting for SP1 growth under these conditions. This contention is further supported by the observation that FN mRNA level and FN synthesis and secretion were markedly down-regulated in SP1 cells under anchorage-independent compared to anchorage-

dependent conditions. Thus, SP1 cells appear to be dependent primarily on paracrine sources of FN during colony growth in agar cultures (i.e. from FBS or adipocyte feeder cells).

The finding that laminin and GAPDH mRNA levels are the same in SP1 monolayers and colonies indicates that the reduction in FN mRNA is related not simply to differences in growth rate. These results also suggest that both transcriptional and translational regulatory processes are involved in regulation of FN synthesis and secretion in SP1 cells. In contrast, Dalton *et al.* [40] have shown at least 50-fold reduction in FN secretion in non-adherent compared to adherent fibroblasts, while the level of mRNA and protein synthesis remained the same. In the latter system, post-translational mechanisms affecting FN assembly and secretion are likely to be involved.

Extracellular FN fibrils were present in abundance in SP1 colonies and inhibition of FN fibril formation with the 70 kDa amino-terminal fragment of FN abolished colony growth. The 70 kDa FN fragment did not bind directly to SP1 cells or inhibit binding of SP1 cells to FN substratum; this result is consistent with earlier reports [38,41] that the 70 kDa amino-terminal FN fragment contains the matrix assembly site but not the cell-attachment site and blocks growth of NRK fibroblasts in response to TGF-ß [42]. These results suggest that the deposition of, and adhesion to, FN may be important events in anchorage-independent growth of SP1 cells. Extracellular FN fibrils detected *in vitro* (also called superfibronectin [43]) have greatly enhanced adhesive properties compared to soluble FN [44], and are the predominant form of FN in tissues [38]. FN may therefore stimulate growth of SP1 cells directly [45], or act synergistically with certain growth factors [19,20].

The requirement for FN fibrils for SP1 colony growth suggests a partial anchorage-dependence, i.e. attachment without cell spreading. Similarily, direct ligation of $\alpha 5 ß 1$ integrin, a major FN receptor, with anti-$\alpha 5$ antibody was shown to stimulate growth of human melanoma

cells in suspension [45]. Studies with K562 cells showed that RGD-containing peptides can stimulate cyclin A activity and anchorage-independent growth [46]. In NIH-3T3 fibroblasts, over-expression of cyclin A leads to complete loss of anchorage-dependence [47]. Thus cell adhesion is a type of "competence" signal which may have different effects depending on cell type and ECM microenvironment; tumor cells may undergo a stepwise loss of the adhesion requirement for growth. SP1 cells appear to have lost the cell spreading requirement, but still require cell-substrate attachement and growth factor signals.

In summary, our results show that, under defined culture conditions, purified TGF-ß and HGF stimulated significant colony growth. FN fibrils were also necessary but not sufficient for SP1 colony growth. Thus, paracrine regulation of SP1 colony growth by stromal cells may occur via both growth factors (HGF and TGF-ß) and ECM (FN) components. TGF-ß and HGF may affect expression/secretion of FN thereby supporting colony growth; or these growth factors may induce signal transduction events, such as generation of cAMP [48], resulting in colony growth without any ECM requirement. Activation of latent TGF-ß secreted by adipocytes could occur via proteinases from tumor or stromal cells; this possibility is currently being investigated. Similar paracrine mechanisms may affect growth factor and ECM composition at the tumor site, and thereby regulate tumor growth *in vivo*. Future work will involve identifying the growth factor receptor and ECM/integrin signalling pathways involved.

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Table 1

Detection of extracellular matrix proteins by indirect immunofluorescence in SP1 cells, 3T3-L1 pre-adipocytes and 3T3-L1 adipocytes in monolayer cultures, and SP1 colonies in agar

ECM[a]	SP1 cell monolayer	SP1 colonies	3T3-L1 pre-adipocytes	3T3-L1 adipocytes
Fibronectin	+[b]	+[c]	+	+
Vitronectin	+	+	-	+
Laminin	trace	+	+	+
Collagen IV	-	-	+	+
Tenascin	-	-	-	-

Legend:

a) ECM proteins were detected with the following primary antibodies: polyclonal rabbit anti-FN antiserum (1:100); goat anti-collagen type IV IgG (25 μg/ml); rabbit anti-laminin antiserum (1:400); rabbit anti-vitronectin antiserum (1:50), and monoclonal rat anti-mouse tenascin IgG (0.25 μg/ml). Primary antibodies were detected with appropriate FITC-conjugated secondary antibodies: goat anti-rabbit IgG (10 μg/ml), goat anti-rat IgG (25 μg/ml), and donkey anti-goat IgG (7 μg/ml). "+" indicates presence of ECM protein; "-" indicates absence of ECM protein.

b) Staining was predominantly intracellular.

c) Staining was predominantly extracellular.

TABLE 2

INHIBITION OF FIBRONECTIN FIBRIL FORMATION IN SP1 MONLAYER CULTURES WITH THE 70 KDa AMINO-TERMINAL FRAGMENT OF FIBRONECTIN

Native Fibronectin[a]	70 KDa fibronectin fragment[a]	Proportion (%) of cells with fibrils
10 μg/ml	0	87.9 $\pm$ 4.7
10 μg/ml	10 μg/ml	23.8 $\pm$ 1.5
10 μg/ml	20 μg/ml	19.9 $\pm$ 4.5

Legend:

(a) SP1 cells (8×10^4) were subcultured in Nunc coverslip culture dishes with 1 ml of 0.5% FBS/RPMI culture medium containing native fibronectin and the 70 KDa fibronectin fragment at the concentrations indicated.

(b) After 24 h, cells were washed with serum-free RPMI and fixed in a 1% paraformaldehyde/RPMI solution (20 min). Cells were then wahsed and stained with rabbit anti-fibronectin IgG and FITC-conjugated goat anti-rabbit IgG as described in Materials and Methods. Fibril formation was visualized using a Meridian confocal microscope, and the proportion of total cells with fibrils was determined manually by counting 250-300 cells per group. Results are expressed as the average of two independent experiments $\pm$ range.

FIGURE LEGENDS

Figure 1: Effect of purified growth factors on SP1 colony growth: SP1 cells (1 X 10^3/plate) were mixed with 0.36% agar and layered onto 0.6% agar in 1% FBS/RPMI medium. Cultures were supplemented with control medium (no growth factors), HGF (10 ng/ml) or TGF-ß (0.2 ng/ml). After 10-12 days, plates were stained with Giemsa stain and blue colonies were counted. Results are expressed as the mean number of colonies per plate ± S.D. Control medium received no growth factors. Figure 1B shows an example of an SP1 colony in agar.

Figure 2: Immunofluorescence analysis of FN expression in SP1 colonies and monolayers: SP1 colonies or SP1 cells grown on glass coverslips were dried and fixed as described in Materials and Methods. Fixed cells were permeated with Triton-X 100 (0.03%), blocked with 1% BSA, and stained with rabbit anti-FN antiserum (1:100) and FITC-conjugated goat anti-rabbit IgG. (a) Phase contrast photograph of cells on coverslips; (b) same field as (a) stained with rabbit anti-FN antiserum (arrow indicates region of punctate perinulcear staining); (c) SP1 cells in monolayer stained with anti-FN antiserum at higher magnification; (d) SP1 cells in monolayer stained with rat anti-tenascin IgG (0.25 μg/ml); (e) bright field of SP1 colony; (f) same field as (e) stained with anti-FN antiserum and viewed by confocal microscopy; (g) SP1 colony stained with anti-FN antiserum and viewed by confocal microscopy at higher magnification (arrow indicates extracellular FN fibril); and (h) SP1 colonies stained with anti-tenascin IgG. Bar indicates scale of 10 nm.

Figure 3: Effect of partial depletion and reconstitution of FN on SP1 colony growth in 7% FBS cultures: SP1 cells (1 x 10^3 cells per plate) were cultured in RPMI supplemented with standard FBS, or FBS partially depleted of FN, at concentrations of 1% and 7%. In some cultures with 7% FBS partially depleted of FN, FN (5 and 10 μg/ml) was added back. Colonies were assayed after 10-12 days as described in Figure 1. Results are expressed as the mean number of colonies per plate $\pm$ S.D.

Figure 4A: Effect of 70 kDa amino-terminal fragment of FN on SP1 colony growth: SP1 cells were grown as colonies with 7% FBS as described in Materials and Methods. Under these conditions colonies grow independently of adipocyte monolayers. The 70 kDa amino-terminal fragment of FN was added at the concentrations indicated. Plates were incubated at 37°C for 10-12 days, fixed, and quantitated as described in Figure 1. Results are expressed as the mean number of colonies per plate $\pm$ S.D.

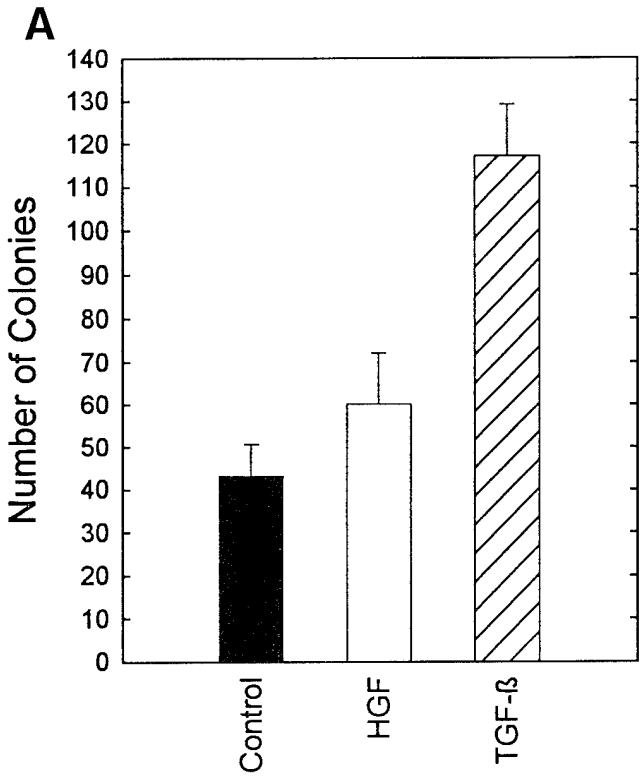
Figure 4B: Comparison of adhesion of SP1 cells to native FN and to the 70 kDa amino terminal FN fragment: Linbro 96-well non-tissue culture plates were coated with BSA, FN and the 70 kDa amino-terminal FN fragment at 20 μg/ml. The plates were incubated at 4°C for 48 h. The excess matrix was removed with PBS, and the wells were blocked with DMEM containing 2.5 mg/ml BSA for 2 h at 37°C. SP1 cells were added at a concentration of 3 x 10^4 cells per well in DMEM containing 2.5 mg/ml BSA. The cells were incubated at 37°C for 45 min. Unattached cells were removed with PBS and the remaining cells were fixed with 2% paraformaldehyde containing brilliant coomassie blue for 24 h. Absorbency (270 nm) was measured in an ELISA plate reader. Results are expressed as the mean absorbency of 3 wells $\pm$ S.D.

Figure 5: Analysis of FN and laminin mRNA in SP1 cell colonies and monolayers: mRNA was prepared from SP1 cell monolayers or colonies and analyzed by northern blotting as described in Materials and Methods. The blot was probed with cDNA against FN and laminin B1 chain. The upper bands correspond to each ECM mRNA as indicated by arrows: Left, FN (8.0 kb); right, laminin B1 (9.5 kb). The lower band in all lanes is GAPDH mRNA (1.3 kb). The autoradiograms were scanned on a laser densitometer and the level of each mRNA was expressed relative to GAPDH mRNA.

Figure 6) Analysis of FN synthesis and secretion by SP1 cells under adherent and non-adherent conditions.

Panel A: SP1 cells were cultured under adhesive or non-adhesive conditions in methionine-free RPMI with 7% FBS (dialysed against PBS) for 5 h at 37°C. During the final 2 h, ^{35}S-methionine (100 μCi/ml) was added to each culture. Cells were harvested, washed, lysed, and immunoprecipitated with rabbit anti-FN antiserum (Panel A) or rabbit anti-laminin antiserum (Panel B), as described in Materials and Methods. Immunoprecipitates were resuspended in sample buffer and subjected to 7% SDS-PAGE under reducing conditions. The gel was amplified, dried and exposed for 2 days. Immunoprecipitates were from: lane 1, biosynthetically labelled adherent cells; lane 2, conditioned medium from cells in lane 1; lane 3, biosynthetically labelled non-adherent cells; lane 4, conditioned medium from cells in lane 3. The autoradiograms were scanned on a laser densitometer and the level of each protein band relative to lane 1 is indicated on the lower axes.

Figure 1


A
Number of Colonies
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Control
HGF
TGF-ß

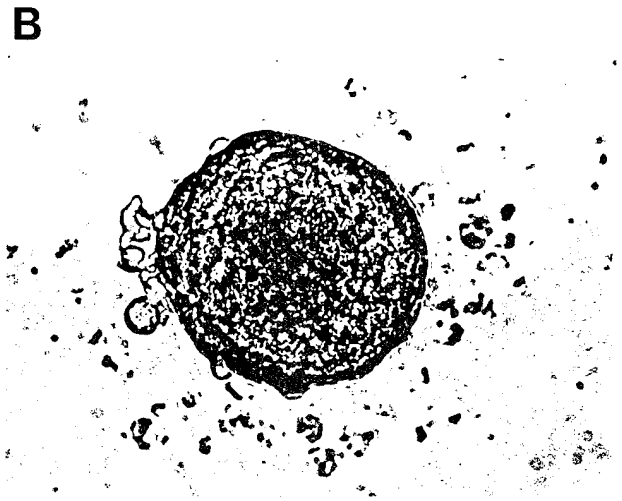

B

FIGURE 2

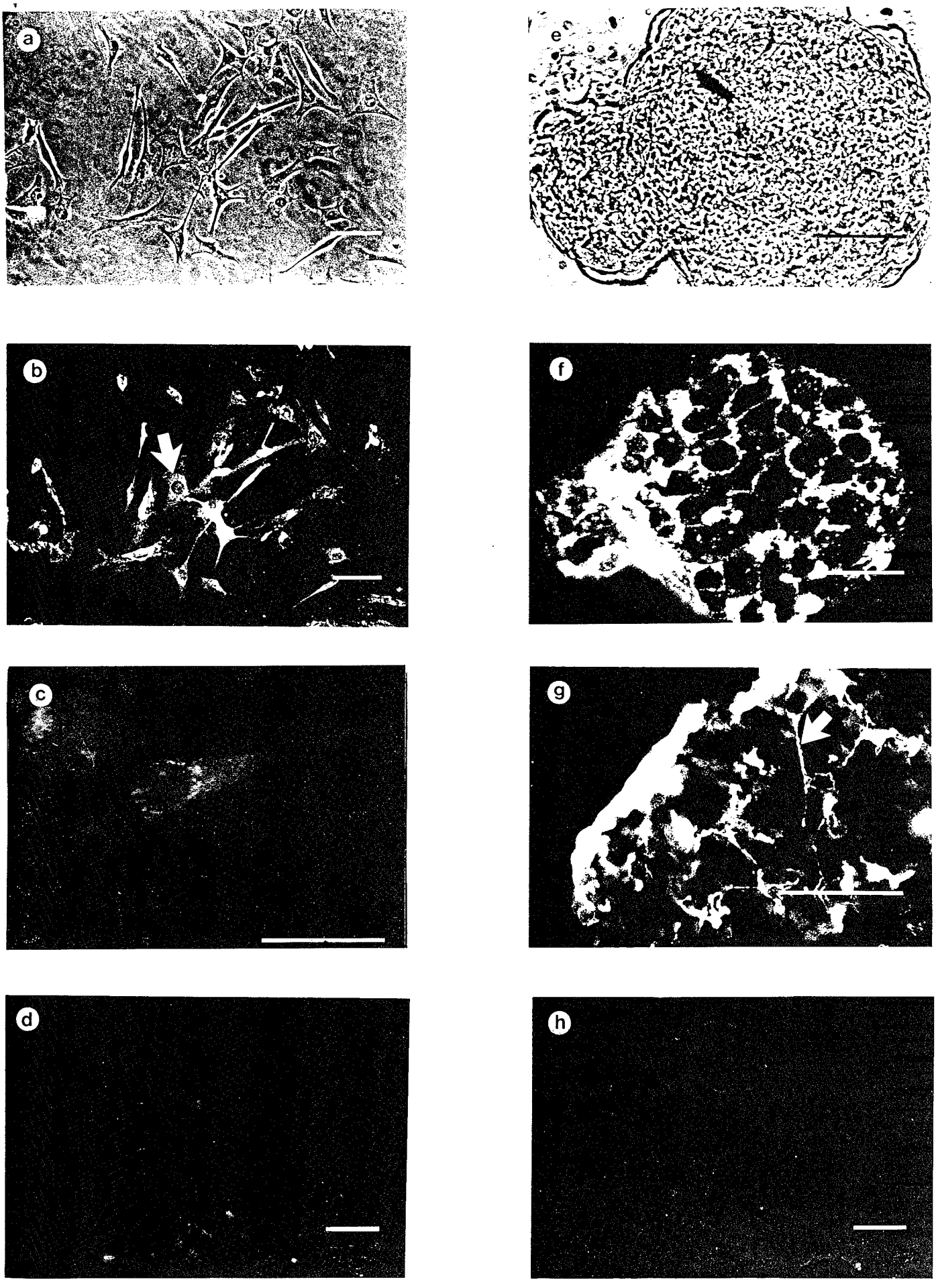


Figure 3

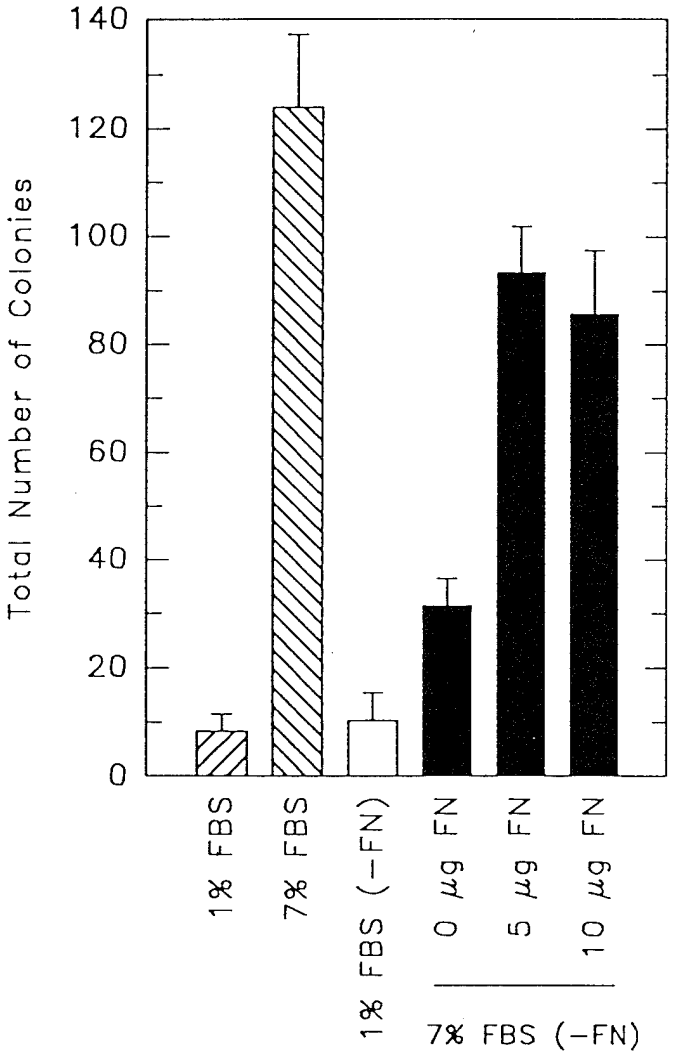
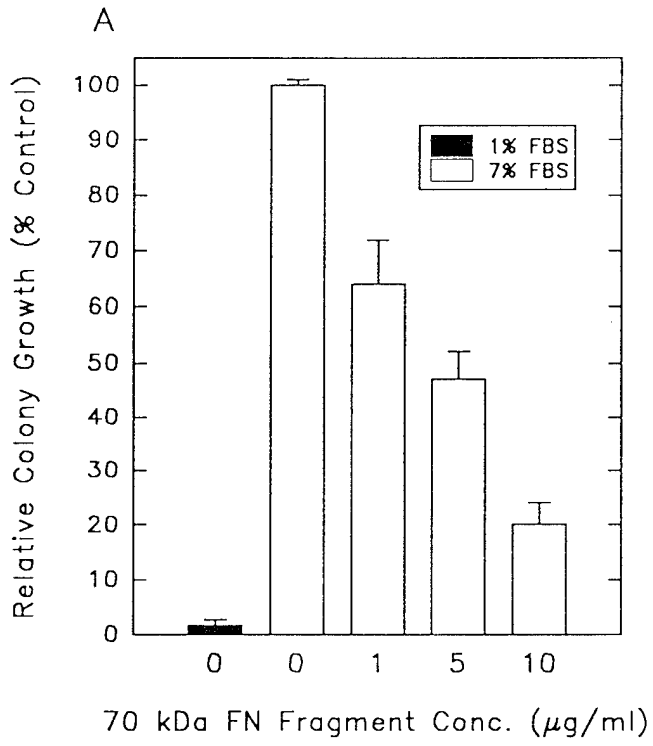

Total Number of Colonies
140
120
100
80
60
40
20
0
1% FBS
7% FBS
1% FBS (−FN)
0 μg FN
5 μg FN
10 μg FN
7% FBS (−FN)

Figure 4


A
Relative Colony Growth (% Control)
100
90
80
70
60
50
40
30
20
10
0
1% FBS
7% FBS
0
0
1
5
10
70 kDa FN Fragment Conc. (μg/ml)

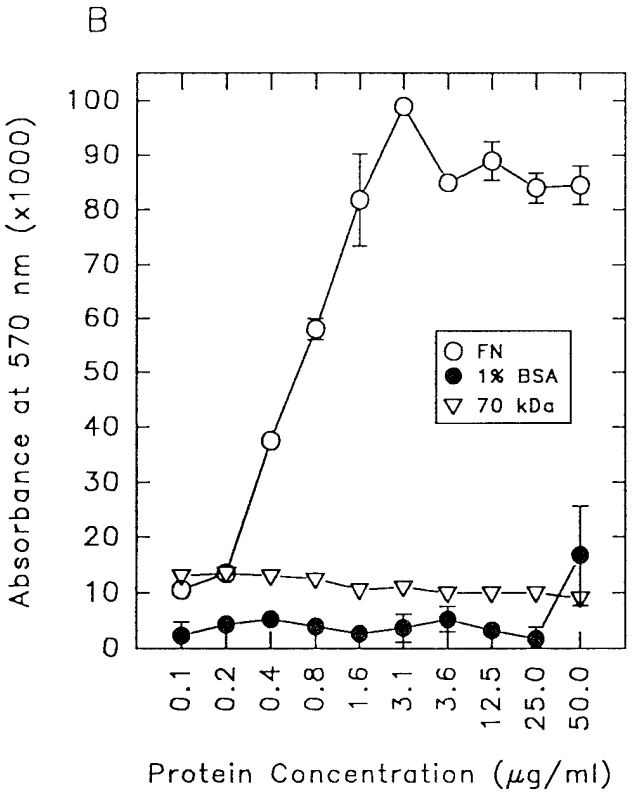

B
Absorbance at 570 nm (x1000)
100
90
80
70
60
50
40
30
20
10
0
FN
1% BSA
70 kDa
0.1
0.2
0.4
0.8
1.6
3.1
3.6
12.5
25.0
50.0
Protein Concentration (μg/ml)

Figure 5

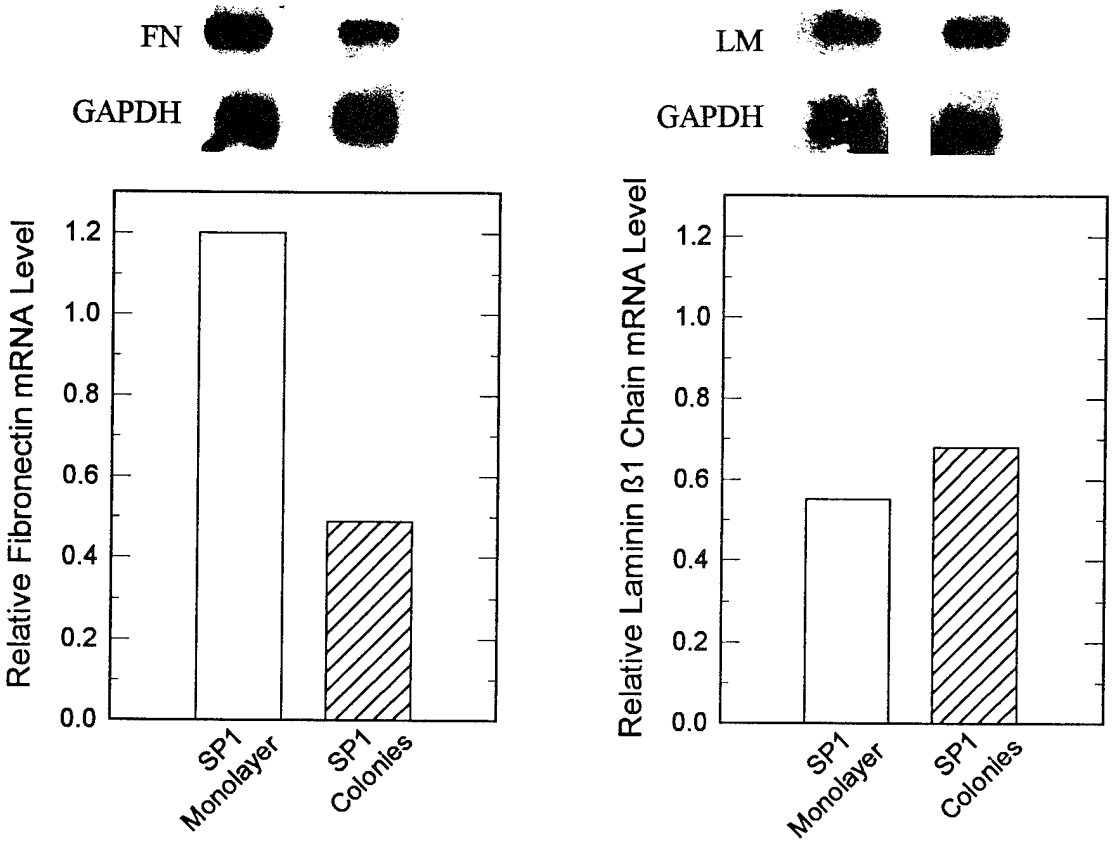
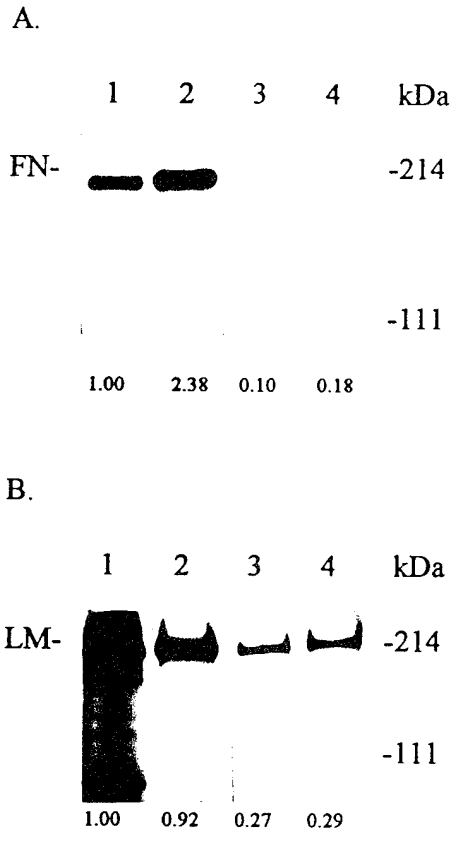

FN
GAPDH
Relative Fibronectin mRNA Level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SP1 Monolayer
SP1 Colonies
LM
GAPDH
Relative Laminin ß1 Chain mRNA Level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SP1 Monolayer
SP1 Colonies

Figure 6


A.
1
2
3
4
kDa
FN-
-214
-111
1.00
2.38
0.10
0.18
B.
1
2
3
4
kDa
LM-
-214
-111
1.00
0.92
0.27
0.29