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Growth of v-src-Transformed Cells in Serum-Free Medium Through the Induction of Growth Factors

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Abstract

The v-src oncogene is one of only two oncogenes capable of transforming mouse embryo fibroblasts (MEFs) lacking the *IGF-1R* gene (R-cells). R-/v-src cells grow robustly in the absence of serum, suggesting the hypothesis that they may produce one or more growth factors that would sustain their ability to proliferate in serum-free condition. Using proteomic approaches on serum-free conditioned media derived from v-src-transformed cells, we have identified two growth promoting factors: osteopontin and proliferin. Subsequent experiments have indicated that osteopontin plays a prevalent role in promoting growth of v-src-transformed cells in serum-deprived condition.

v-src is the oncogenic version of the c-src cellular gene, that readily transforms cells in culture (colony formation in soft agar) and in xenotransplants. v-src is one of only two oncogenes capable of transforming R-cells, which are MEFs originated (Sell et al., 1993) from mice with a targeted disruption of the *type 1 insulin-like growth factor receptor* genes (Efstratiadis, 1998). R-MEFs are refractory to transformation by a number of cellular and viral oncogenes but promptly transform if the IGF-1R is re-introduced (reviewed in Baserga et al., 2003). R-/vsrc cells are not only transformed, but grow in the absence of serum (serum-free medium, SFM), while the cellular gene *c-src* cannot transform R-cells nor make them grow in SFM (Valentinis et al., 1997). We have recently focused on the ability of v-src to induce cell proliferation in serum-free medium (Valentinis et al., 1997; Sun and Baserga, 2008) and we have hypothesized that v-src may induce in cells the expression of a growth factor or factors, hypothesis also supported by the observation that R-/v-src cells grow better in SFM at high density than when plated in sparse cultures (see below).

To test this hypothesis, we examined by mass spectrometry the serum-free conditioned media (SFCM) produced by v-src-transformed cells (using their non-transformed counterparts as controls) for the presence of novel growth factors. Two growth factors are

consistently present in SFCM of v-src transformed cells but not in SFCM of control cells: osteopontin (OPN) and proliferin (PLF). Their presence in the SFCM of v-src-transformed cells was confirmed by Western immunoblots of the conditioned.

OPN is a phosphoprotein secreted by transformed, malignant cells, that plays a role in growth of metastases (Wai and Kuo, 2008) and whose promoter is activated by v-src (Tezuka et al., 1996). Proliferin (also called PRL2c) belongs to the prolactin family of growth factors, and is a growth factor in its own (Wilder and Linzer, 1989).

Using shRNA approaches, purified recombinant proteins, and appropriate antibodies, our experiments indicate that while both OPN and PLF are expressed and secreted by v-src-transfected cells, OPN plays a more prevalent role in the regulation of cell proliferation. Collectively, these results support the hypothesis that increased OPN secretion in MEFs-/v-src cells support their ability to grow in the absence of serum.

Materials and Methods

Tissue culture and transfections

Cells expressing v-Src (R508 and BT20) were generated by co-transfecting the expression vector pMv-src with the pRSVneo plasmid (to confer resistance to neomycin), at a molar ratio of 20:1, using Fugene transfection reagent (Roche, Indianapolis IN) at a DNA/reagent ratio of 1:3. Transformants were selected in 800 µg/ml G418 sulfate (Gibco, Life Technologies, Grand Island, NY). Parental and v-src transformed cells were cultured in 10% serum unless tested in serum-free medium (SFM).

Mass spectrometry

The technique is a standard one, which we have already described in other occasions (Drakas et al., 2005).

Western blots of conditioned media

Ultracentrifugation centrifugal devices (molecular weight cut-off: 9 K) were used to concentrate CM two- or fourfold. Equal volumes of samples were analyzed by Western immunoblot as described (Dalmizrak et al., 2007).

Proliferation assays

Cells were plated onto 35 mm dishes at 40,000–50,000 cells/dish and grown in DMEM containing 10% FBS for 24 h. The medium was removed, cells washed three times in PBS and incubated for 72 h in SFM with or without purified OPN at 2–10 µg/ml, and in various conditioned media (see text). Cell proliferation was assessed by cell counts with a hemocytometer. All growth experiments were carried out in triplicate.

Knockdown by short hairpin RNA

For shRNA transfections, R508/vSrc cells were seeded on six-well plates 24 h before transfection, and grown to 50–70% confluency, in medium supplemented with 10% FBS. The shRNA plasmids used (Santa Cruz Biotechnology, Santa Cruz, CA), for both PLF and

OPN, consist of a pool of three expression constructs each encoding target-specific 19–25-nucleotide (plus hairpin) shRNAs. For optimal efficiency, the shRNA transfection reagent was used at a DNA/reagent ratio of 1:3. A scrambled shRNA sequence was used as control.

Western blots of OPN-stimulated cells

To test the effect of added OPN, cells were plated onto six-well plates, in growing medium containing 10% FBS. After 24 h, cells were washed in PBS three times and incubated in SFM for an additional 24 h, before experimentation with OPN. Whole cell lysates from control and treated cells were analyzed by Western blot.

Antibodies and reagents

The primary antibodies to detect OPN and PLF in conditioned media as well as in cell extracts were mouse monoclonal antibodies from Santa Cruz Biotechnology. The following primary antibodies were from Cell Signaling: rabbit mAb phospho-Stat3 (Tyr705) (D3A7), mouse Ab Stat3 (124H6), phospho-Akt (Ser473) (D9E) and Akt, both rabbit mAbs, and mouse mAb phospho-p44/42 MAPK (Thr202/Tyr204) (E10). Rabbit Ab anti-actin was from Sigma (St. Louis, MO). Goat anti-mouse and anti-rabbit IgG peroxidase conjugated secondary antibodies were from Calbiochem (Billerica, MA). Goat polyclonal antibody to OPN (ab11503) used to neutralize OPN in the CM was from Abcam (Cambridge, MA). Recombinant mouse OPN from a murine myeloma cell line was from R&D Systems (Minneapolis, MN). Elisa for human OPN was performed according to the manufacturer instruction following the AbCam protocol. PLF shRNA plasmid, OPN shRNA plasmid, control shRNA plasmid, and shRNA transfection reagent were from Santa Cruz.

Results

R-/v-Src cells secrete OPN and PLF in SFCM

Because R-/v-src cells grow robustly in the absence of serum (Valentinis et al., 1997), we tested the hypothesis that these cells may produce one or more growth factors that would sustain their ability to proliferate in serum-free condition. The SFCM (serum-free conditioned medium) of R-/v-Src cells and its control R-cells were analyzed by mass spectrometry. Several independent experiments showed that R-/vrc cells produced substantial amounts of OPN and PLF (PRL2c), which were absent in SFCM of R-cells (Table 1). PLF peptides were most frequent in SFCM of R-/v-Src cells, behind actin and ahead of collagen. The most frequent proteins (and other relevant proteins) in SFCM of R-/vrc and R-cells are given in Table 1. OPN and PLF are not present in SFCM of non-v-src-transformed R-cells. A third growth factor, granulin (epithelin) was also present, but in both SFCM (controls and v-Src-transformed cells) and at lower concentrations.

In order to confirm these results in additional cell models, we transfected the v-src plasmid in R508 cells, which are R-cells stably expressing IGF-1Rs (Rubini et al., 1997). R508 cells do not form colonies in soft-agar, but respond to IGF-I with one cycle of cell division (Reiss et al., 1998). Several clones were selected, most of which had a highly phosphorylated Stat3 (Fig. 1A), which is characteristic of cells transformed by v-src (Garcia and Jove, 1998; Bromberg and Darnell, 2000; Pukka and Silvennoinen, 2004). R508/v-src cells grew in the

absence of serum (Fig. 1B) as compared to parental 508 cells (first plate on the left). The CM of all these clones were examined by mass spectrometry and subsequently by Western blots.

Table 2 summarizes the findings of OPN and proliferin in the CM of R508/v-srcv-transfected cells. OPN is present in all clones. Proliferin is present in all newly produced v-src-transformed clones with the only exception of clone 1. These experiments strongly confirm our previous results and confirm that v-src expression induces osteopontin and proliferin expression.

Western blots of SFCM

We then examined the presence of OPN and proliferin in SFCM of R508/v-src transfected cells by Western immunoblots. Significantly, the presence of OPN and PLF in SFCM of v-Src transformed cells was confirmed in non-concentrated (1×) and concentrated (2× and 4×) media from R508/v-Src cells (Fig. 2), while both proteins were not detectable in fourfold concentrated media conditioned from R508 parental cells (Fig. 2). It is important to mention that all CM are serum-free and this is critical since PLF is induced by stimulation of cells in culture with 10% serum (Wilder and Linzer, 1989).

Effect of down-regulation of proliferin or OPN on growth of R508 cells

In order to assess the relative contributions of OPN and PLF on growth of R508/v-src cells in the absence of serum, we first used shRNA approaches to deplete endogenous OPN and PLF. Transfection of the respective shRNA into R508/v-Src cells resulted in a strong down-regulation of either OPN or PLF as compared to parental and scrambled shRNA-transfected cells (Fig. 3A) We then tested the SFCM derived from OPN- and PLF-depleted R508/v-Src and control cells for the ability to promote the growth of R508 parental cells. CM from v-Src transfected cells strongly enhanced the growth of R508 cells (Fig. 3B, lane 3) compared to SFM alone (Fig. 3B, lane 1) or CM from parental R508 cells (Fig. 3B, lane 2). Significantly, while PLF depletion had no major effect on proliferation (Fig. 3B, lane 4), OPN depletion severely reduced the ability of R508/v-Src-derived CM (Fig. 3B, lane 5) to induce cell growth of parental R508 cells. Collectively, these results suggest that OPN may play a more prevalent role than PLF in promoting growth of v-Src-expressing cells in the absence of serum.

Next, to confirm the role of osteopontin in cell proliferation, we compared the growth in SFM of R508 parental cells and R508/v-src clones 1 and 18, which express OPN but not proliferin, and both OPN and proliferin, respectively. Both R508/v-src clones 1 and 8 (Fig. 4A) showed significant growth after 72 h of incubation. However, there was no statistical difference between the two clones further suggesting that osteopontin is more critical than proliferin in promoting cell growth of v-src-transfected cells.

Finally, we tested cell growth of parental R508 cells in SFM supplemented solely with purified recombinant OPN, which supported proliferation of parental R508 cells at values very similar to CM from v-src expressing cells (Fig. 4 part B). In addition, targeting OPN with specific anti-osteopontin neutralizing antibodies (v-src CM(+)) severely suppressed the growth promoting effect of R508/v-src-derived CM (Fig. 4 part B).

The results from these experiments confirm a major role of OPN in promoting the growth of v-src-transformed cells in SFM.

Signaling pathways induced by media conditioned from V-src-expressing cells

In order to characterize the signaling pathways induced by v-src expression and OPN secretion in R508/v-src cells, we use Western immunoblots to detect the activation of MAPK and Akt, which are critical for mitogenesis of MEFs. While R508/ v-src showed a slight decrease in the level of ERK1/2 activation compared to parental R508 cells both in serum-free (-) and after serum stimulation (+) (Fig. 5A), v-src expression significantly increased Akt activation compared to parental cells in serum-free (-) and serum-containing media (+) (Fig. 5B). These results support therefore the hypothesis that Akt activation is the critical event in the regulation of cell growth in the absence of serum of v-src-expressing fibroblast cells.

Discussion

Our experiments show that expression of v-src induces secretion in SFM of two proteins absent from the SFM of cells that do not express v-src: OPN and PLF. v-src is a bona fide oncogene (see Introduction) and its induction of OPN and PLF in SFM can explain the ability of v-src-transformed cells to grow in SFM. Our experiments indicate that OPN is the major growth factor involved in the growth of v-src-transformed cells in SFM. We have in fact shown that: (1) v-src-transfected R508 cells expressing solely osteopontin grow in the absence of serum at comparable levels to v-src-transfected cells expressing both osteopontin and proliferin (2) down-regulation of OPN (but not of proliferin) by shRNA approaches inhibits growth of v-src-transformed cells in SFM (3) Recombinant osteopontin promotes cell proliferation of v-src-transfected cells (4) Anti-osteopontin neutralizing antibodies inhibits cell proliferation induced by media conditioned from v-src-transfected cells.

It is well established that OPN plays a significant role in growth of tumor cells (Sohdi et al., 2000; Weber, 2001; Rittling and Chambers, 2004; Robertson and Chellaiah, 2010), especially metastases (Rodrigues et al., 2007; Suzuki et al., 2007; Wai and Kuo, 2008) and interacts with growth factors and growth factor receptors (Maretzky et al., 2011). v-src is known to interact and stimulate the OPN promoter (Guo et al., 1995; Tezuka et al., 1996) and its presence clearly stimulates in our cells the secretion of OPN into CM. The down-regulation of OPN by shRNA against it, the effect of OPN addition to unconditioned SFM and the fact that v-src transformed cells that do not express PLF, but express OPN, still grow in SFM (clone 1), all clearly indicate that OPN is probably the major component of serum-free growth of v-src-transformed cells. The mechanisms(s) by which OPN may promote the growth of v-src transformed cells in SFM may include a direct action (see references above) or an interaction with low levels of progranulin production. All our cells expressed, though at low concentrations, the growth factor progranulin, expressed in similar amount in control cells. But OPN also induces the expression of growth factor receptors like the EGFR (Maretzky et al., 2011), interacts with adhesion molecules (Christensen et al., 2007) and is a biomarker in tumor progression and metastases (Wai and Kuo, 2008). Thus, OPN may

promote growth in SFM by stimulating the movement and the interactions of cells among themselves.

PLF was characterized originally by Linzer and Nathans (1984) and by Linzer et al. (1985). Wilder and Linzer (1989) identified it as a protein induced by stimulation of cells with 10% serum and shown to inhibit the differentiation of myogenic cell lines. Proliferin is known to be strongly induced by v-src (Paz et al., 2004), and we show here that it is secreted in the SF medium of v-src-transfected MEF. However, our results suggest that PLF may not be critical for the ability of v-src-transformed cells to grow in the absence of serum.

v-src has been shown to regulate the expression of ERKs (Stofega et al., 1997; Maretzky et al., 2011). Accordingly, our data have shown that CM of Src-transfected cells shown reduced ERK1/2 activation compared to media conditioned from parental cells. On the contrary, v-src-expressing cells have enhanced Akt activation, suggesting that the Akt pathway may play a critical role in regulating cell proliferation induced by v-src expression. In addition, Stat3 is strongly activated in v-src-transfected fibroblasts cells (Garcia and Jove, 1998) indicating that Stat proteins may constitute an additional pathway regulating v-src-dependent cell proliferation.

A clue to the transforming activity of v-src could be its well-established interaction with the IGF-1R, but R-cells do not have IGF-1Rs. We have looked at a second clue, that is, its interaction with a docking protein of the IGF-1R, the insulin receptor substrate-1 (IRS-1). In support of an IRS-1/v-src interaction is the finding that v-src cannot transform cells that do not express IRS-1, like BT-20 mammary cancer cells. Furthermore, an IRS-1 with mutations at the 2 tyrosines required for its binding to PI3K also fails to co-operate with v-src for transformation of BT-20 cells (Sun and Baserga, 2008). In addition, IRS-1 and v-src interact and this interaction results in tyrosine phosphorylation of IRS-1 (Dalmizrak et al., 2007). The IRS-1/v-src complex is accompanied by a constitutive phosphorylation of Stat3, which is likely mediated by PI3K.

Previous experiments suggest that IRS-1 interacts with and activates Stat3 (Sun and Baserga, 2008). OPN may stimulate the growth of v-src-transformed cells by activating IRS-1, which may be ultimately responsible for growth in SFM (Mardilovich et al., 2009). IRS-1 and v-src also interact in phosphotyrosine-dependent manner (Dalmizrak et al., 2007) and v-src cannot transform cells that do not express IRS-1, like BT-20 mammary cells. Furthermore, an IRS-1 with mutations at the 2 tyrosines required for its binding to PI3K also fails to co-operate with v-src for transformation of BT-20 cells (Sun and Baserga, 2008).

In conclusion, our experiments indicate that OPN is most likely the growth factor used by v-src-transformed cells to grow in SFM. The mechanism may involve the activation and tyrosine phosphorylation of IRS-1 by v-src (Dalmizrak et al., 2007; Sun and Baserga, 2008).

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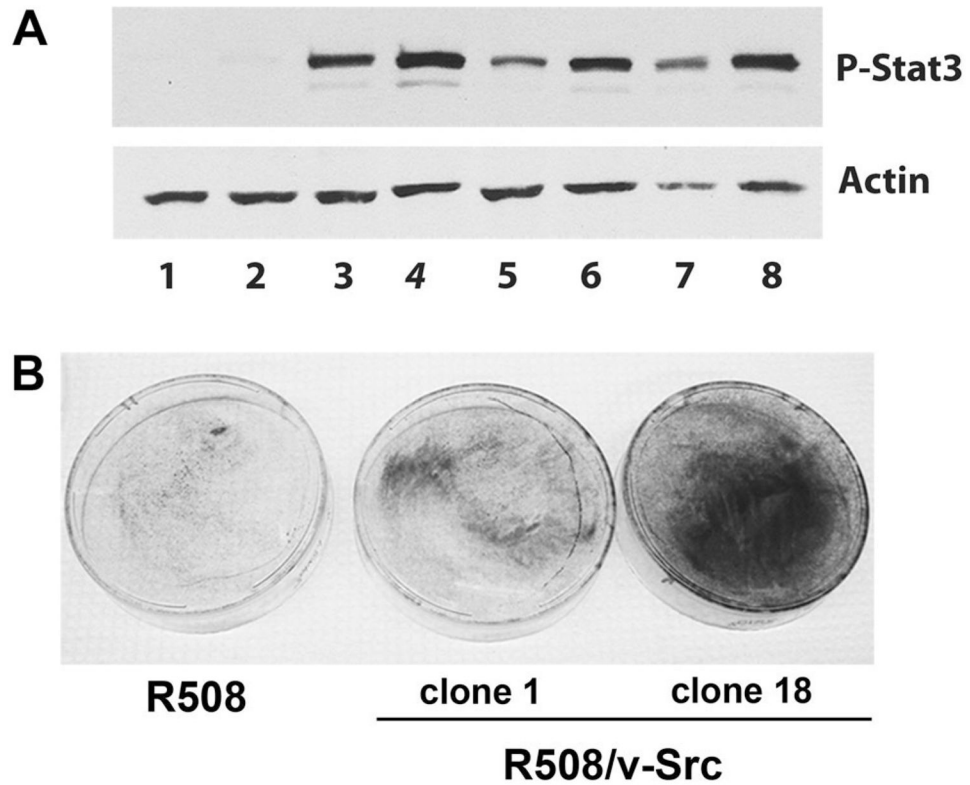


Fig. 1. Phosphorylation of Stat3 in v-src-transformed cells in serum-free medium. Phosphorylation was determined by using an antibody that recognized only a phosphorylated Stat3 at tyrosine 705. Parental R508 cells are used as controls (lane 1), or after transfection with an empty vector (lane 2). All other lanes (lanes 3–8) are R508 clones transfected with v-src (A). Growth of R508/v-src cells in absence of serum, as compared to parental R508 cells: v-src clones 1 and 18 are shown (B).

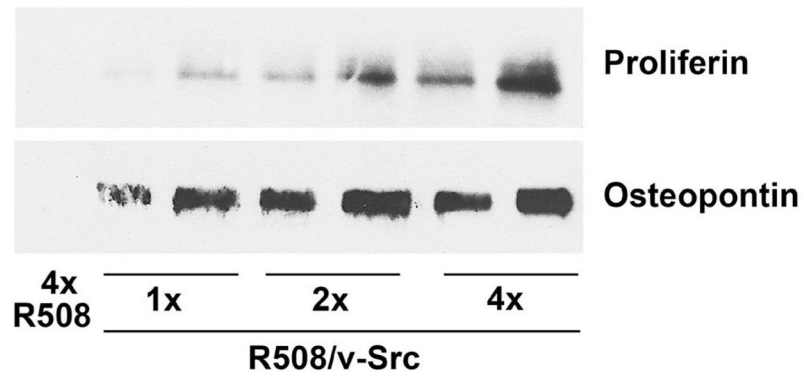


Fig. 2.

Western blots of SFCM from parental 508 cells (lane 1) and R508/vsrc cells. Antibodies to proliferin and osteopontin. The SFCM of parental cells' SFCM was concentrated 4x, the v-src transformed cells were concentrated 1x, 2x, or 4x before staining with antibodies.

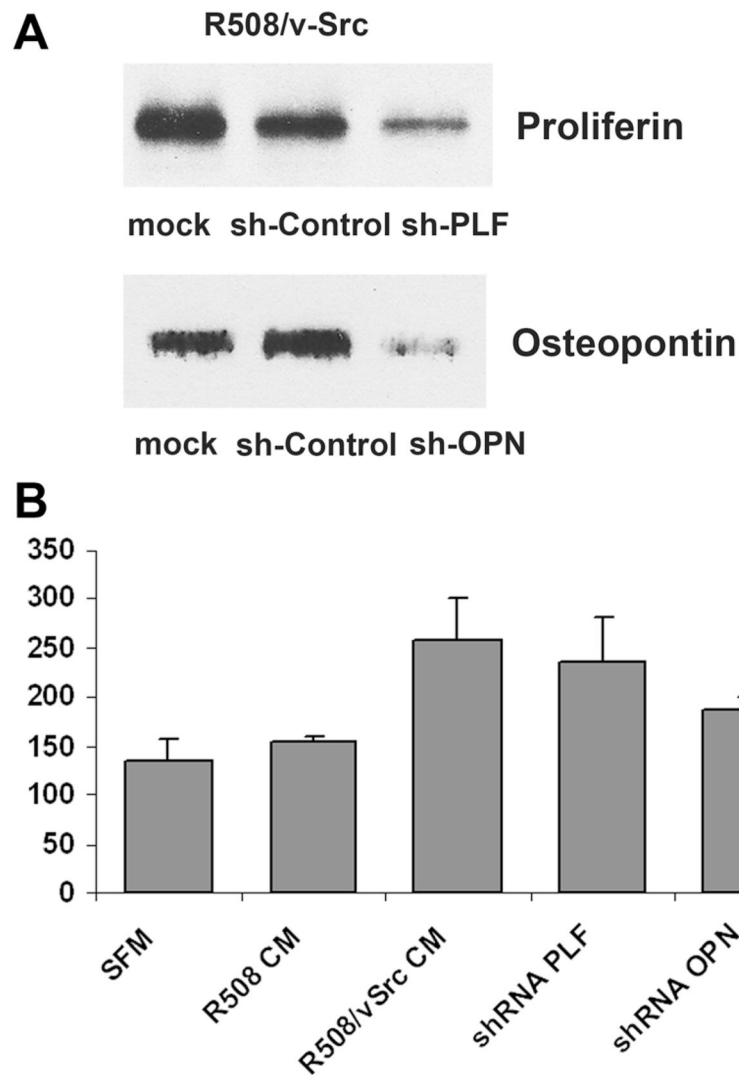


Fig. 3. Effect of down-regulation of proliferin and osteopontin on the growth of parental R508 and R508/v-src cells. Part A: R508/v-src cells were treated first with shRNA against either OPN or Proliferin, which caused down-regulation of the respective protein (scrambled shRNA was used as control). In part B, R508/v-src cells were then tested for growth in the indicated media (sfm is serum-free medium, CM is medium conditioned by R508 cells, R508/vsrc CM is conditioned medium from v-src transformed R508 cells, PLF is proliferin and OPN is osteopontin). CM from v-src transformed cells causes growth of R508 cells. R508/v-src cells grow when they are down-regulated with shRNA to PLF but much less when treated with shRNA to OPN.

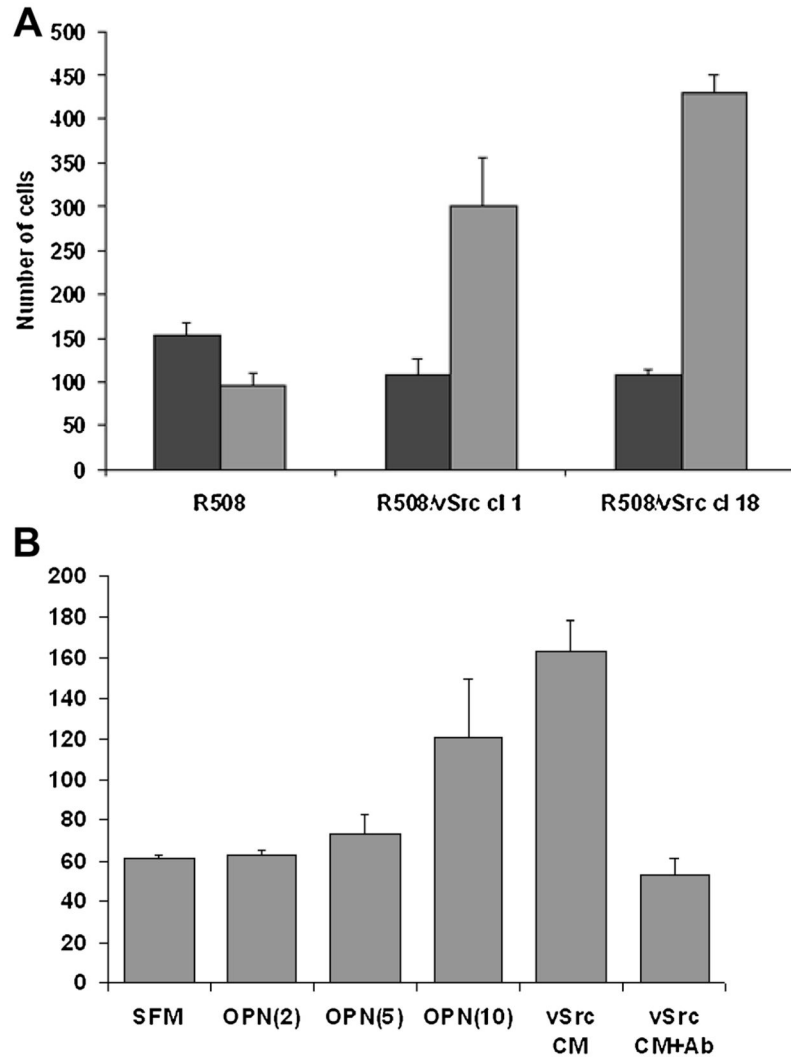


Fig. 4. Effect of Osteopontin on the growth of R508/v-src cells. Part A: R508 cells and their clones 1 and 18 were grown in SFM, and their growth assessed at day 3. Both clones 1 (no proliferin) and 18 (both proliferin and OPN) grow in SFM, while parental R508 cells do not. Part B: effect of Osteopontin and Osteopontin antibodies on the growth of R508 cells. In the first four lanes, OPN was added at increasing concentrations to SFM of R508 cells, and it caused growth at the highest concentrations (10 g/ml). In the last two lanes, R508 cells were grown in SFM of v-src-transformed cells that caused growth of R508 cells. Addition of an antibody to OPN inhibited their growth (+, last lane).

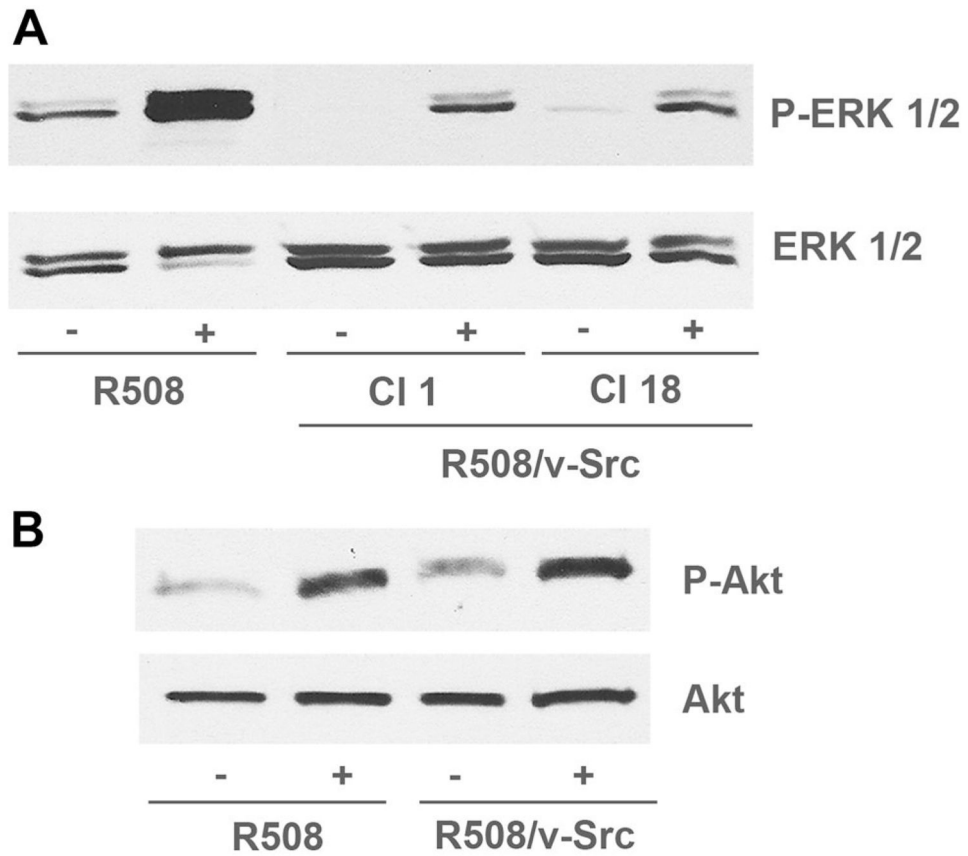


Fig. 5. Part 5A shows the activation of ERK in R508 cells and R508/ v-src cells, clone 1 and 18, after 10 min stimulation with 10% serum. Part 5B shows activation of Akt in R508 cells and R508/v-src cells (only clone 18), after 10 min stimulation with 10% serum.

TABLE 1

Mass spectrometry of SFCM of R- and R-/v-src cells

R	Score	R-/v-Src	Score
Collagen	1072	Actin	650
Actin	366	PRL2C3	641
HMG1	141	Vimentin	516
Galectin	131	Enolase	387
Granulin	112	Cathepsin	313
Vimentin	97	Collagen	195
Cathepsin	57	Granulin	154
Annexin	53	Osteopontin	103

R-cells are MEFs generated from mouse embryos with a targeted disruption of the IGF-1 receptor genes (Sell et al., 1993; Efstratiadis, 1998).

TABLE 2

Mass spectrometry of R508 and R-508/v-src cells

R508	Score	V-Src clone1	Score	V-Src clone5	Score
Collagen	1176	Collagen	542	Collagen	875
Actin	256	Osteopontin	509	Osteopontin	770
Galectin	147	Procollagen	532	Procollagen	543
Enolase	210	Cadherin	120	Actin	186
Vimentin	153	Cathepsin	116	PRL2C2	96
HMG1	89	Granulin	112	Cadherin	92
Cathepsin	61	TIMP2	69	Granulin	89
Granulin	42	Vimentin	38	TIMP1	144

R508/V-Src								
Protein	R508	CI 1	CI 3	CI 5	CI 6	CI 12	CI 16	CI 18
Osteopontin	0	509	438	770	371	664	339	450
PRL2C2	0	0	191	96	155	149	168	240

R508 cells are R-cells stably transfected with and expressing 18×10^3 IGF-I receptors/cell (Rubini et al., 1997). This table summarizes the presence or absence of osteopontin and proliferin in SFCM of R508/v-src cells and parental R508 cells.