Osteopontin is required for full expression of the transformed phenotype by the ras oncogene

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Summary  The secreted phosphoprotein osteopontin (OPN) is strongly associated with the process of neoplastic transformation, based both on its pattern of expression in vivo and in vitro and on functional analyses. We have used 3T3 cells derived from wildtype and OPN-deficient mice and transformed by transfection with oncogenic ras to assess the role of OPN in transformation in vitro and in tumorigenesis in vivo. There was no effect of an absence of OPN on the ability of the cells to undergo immortalization or to form morphologically transformed foci following ras transfection. Wildtype and OPN-deficient cell lines were established from such foci, and lines with similar ras mRNA levels selected for further analysis. Ras-transformed cell lines from both wildtype and OPN-deficient mice could form colonies in soft agar indicating that this process can occur in the absence of OPN. However, the ability of the OPN-deficient cell lines to form colonies was reduced as compared to wildtype cell lines. Tumorigenesis in syngeneic and nude mice was assessed for a subset of cell lines that formed colonies efficiently in soft agar. Cell lines unable to make OPN formed tumors in these mice much more slowly than wildtype cells, despite similar growth of the cells on plastic and in soft agar. Taken together, these results indicate that maximal transformation by ras requires OPN expression, and implicate increased OPN expression as an important effector of the transforming activity of the ras oncogene. © 2000 Cancer Research Campaign

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Changes in cell adhesion properties are hallmarks of the processes of tumorigenesis and metastasis. Indeed, normal cells depend on interaction with the extracellular matrix for growth, and the loss of this dependence is the aspect of cell transformation in vitro most closely correlated with tumorigenicity (Freedman and Shin, 1974). The integrin family of cell surface receptors mediates attachment of cells to the extracellular matrix (reviewed in Hynes, 1992; Juliano, 1994), and alterations in integrin binding or signaling properties are being increasingly recognized as important in tumorigenesis (Ruoslathi, 1997; Clezardin, 1998).

The secreted phosphoprotein osteopontin (OPN), ubiquitously expressed in body fluids, binds with high affinity to integrins of the α_v class (Liaw et al, 1995; Hu et al, 1995a; 1995b) including α_vβ_1, α_vβ_3, and α_vβ_5. Interaction of the protein with several β_integrins has also been reported recently (Smith et al, 1996; Denda et al, 1998; Bayless et al, 1998), as well as with a non-integrin cell surface receptor, CD-44 (Weber et al, 1996; Katagiri et al, 1999).

In vivo, OPN expression is increased in a variety of pathologies (reviewed in Rittling and Denhardt, 1999), where it may act as a macrophage chemoattractant (Singh et al, 1990; Giachelli et al, 1998), or as a repressor of induced nitric oxide synthase (iNOS) levels (Hwang et al, 1994; Rollo et al, 1996); this activity may be especially relevant in pathologies involving ischaemia, in which nitric oxide (NO) production is thought to be an important mediator of tissue damage (Goligorsky et al, 1997; Noiri et al, 1999). In vitro, the protein has cell attachment activity (Somerman et al, 1987; Chambers et al, 1993; Senger et al, 1994), and stimulates migration of several cell types, including endothelial cells (Senger et al, 1996) and smooth muscle cells (Liaw et al, 1995).

There is extensive evidence derived from observations made both in vitro and in vivo linking elevated osteopontin expression and transformation/tumorigenesis. The protein was originally characterized as transformation-associated due to its increased expression in transformed cell lines (Senger et al, 1983); indeed, a wide variety of transformed cells in culture express much higher levels of the protein than their normal counterparts (Senger et al, 1988). These observations have been extended in that among transformed cells, the highest levels of expression were found in the most metastatic cell lines (Chambers et al, 1992a). OPN is particularly highly expressed in ras-transformed cells (Chambers et al, 1990), and ras has a direct effect on OPN transcription, via a ras-stimulated transcription factor that enhances transcription of the OPN gene (Guo et al, 1995). OPN is also highly expressed in tumors in vivo. In papillomas and carcinomas induced by DMBA/TPA initiation/promotion in mouse skin, OPN expression levels correlate with tumor stage (Craig et al, 1990). In mammary tumors arising in transgenic mice expressing ras and/or myc specifically in mammary gland, OPN expression is dramatically increased at both the message and protein level over levels in the corresponding normal mammary gland (Rittling and Novick, 1997). OPN is typically overexpressed in human tumors (Brown et al, 1994; Bellahcene and Castronovo, 1995; Hirota et al, 1995; Tuck et al, 1998), although the source of the protein in these malignancies can be either tumor cells or tumor-associated macrophages (Brown et al, 1994; Casson et al, 1997).

A series of functional studies links OPN causally to tumorigenesis. Experiments in which OPN mRNA level was reduced in...
already transformed cells indicated that the protein is important in transformation. Thus, transfection of different antisense OPN constructs or ribozymes into transformed, tumorigenic cells resulted in impaired anchorage-independent growth, as well as tumor-forming ability in vivo (Gardner et al, 1994; Su et al, 1995; Feng et al, 1995; Behrend et al, 1994). Even more compelling are results showing that transfection of OPN expression constructs into cells producing benign tumors converts them to a malignant phenotype (Oates et al, 1996; Chen et al, 1997).

In order to more clearly define the role OPN plays in transformation in vitro and tumorigenesis in vivo, we have examined these processes in cells derived from mice with a targeted disruption of the OPN gene (Rittling et al, 1998). The results presented here indicate that while anchorage-independent growth and tumor formation can occur in the absence of OPN, these phenotypes, in particular tumor formation in vivo, are substantially enhanced in wildtype, OPN-expressing cells as compared to OPN deficient ones. Thus, OPN is required for full expression of the transformed phenotype.

**MATERIALS AND METHODS**

**Preparation and immortalization of mouse embryo fibroblasts**

Mouse embryo fibroblast (MEF) cells were prepared as described (Rittling, 1996). Briefly, mouse embryos were removed from the uterus of 12–16-day pregnant mice, minced finely with scissors and digested with 0.125% trypsin. The cells obtained were plated on 150 mm tissue culture dishes at a density of 1 × 10^7 in DMEM (Gibco, Grand Island NY, USA) supplemented with 10% FBS (Gemini Bio-Products, Calabasas, CA, USA). For the first and second passages, cells were plated on 100 mm tissue culture dishes at a density of 1 × 10^6; in later passages, cells were plated at 3T3 density (8 × 10^5) on 100 mm dishes. For immortalization, cells were passed every three days and plated at 8 × 10^5 on 100 mm dishes (Rittling, 1996). The numbers of cells plated (No), and the numbers of cells after three days of growth (N) were recorded, and the ratio (N/No) was calculated as a growth-rate indicator. Cells were considered to be immortalized when the ratio N/No became higher than 3.0.

**Mice**

Wildtype and OPN-deficient mice were housed and bred separately under specific pathogen-free conditions as described (Rittling et al, 1998). Embryos were obtained from homozygous females, either wildtype or OPN-deficient, that had been mated to males of the same genotype. Mice used for embryo collection were in the 129 × C57BL/6 F2 genetic background.

**Tetracycline-inducible cell lines**

OPN-deficient 3T3 cells were transfected sequentially with pTet-Off (pUHD-15-1) (Gossen and Bujard, 1992), and with OPN under the control of the Tet operator, and the clones expressing the highest level of OPN selected for further analysis. The resulting clonal lines were transfected with pSV-Ha-ras, and foci were picked. Transformed cells from these foci were plated in soft agar at 10^5 cells per 35 mm well, in the presence or absence of 2 μg ml⁻¹ tetracycline.

**Transfection and selection**

Plasmid DNA (pSV-Ha-ras: 20 μg per 100 mm dish, 10 μg per 60 mm dish) was introduced into cells by the calcium phosphate precipitation method, followed by glycerol shock (Malyankar et al, 1994). Foci, identified as clumps of piled-up cells against a background monolayer, were picked after incubation for 2 weeks with medium containing 3% or 1% FBS, to select for cells with reduced growth factor requirements. Transfection efficiency was assessed by cotransfection with a β-galactosidase expression construct.

**Growth curves**

Cells were plated in 12-well dishes at 2.7 × 10^4 cells per well. Each day for 6 days, cells were removed from triplicate wells and counted, and the results plotted. Cell number at 3 days after plating, towards the end of the exponential growth phase, is reported.

**Northern and western blotting**

RNA preparation, analysis, and probes were as described (Rittling and Novick, 1997). Western blotting for OPN was as in (Rittling et al, 1998b) and (Rittling and Feng, 1998), using antisum 732, developed in the OPN-deficient mice against mouse osteopontin (Kowalski et al, unpublished). For quantification of ras protein levels, cells were scraped into RIPA buffer with protein inhibitors, and the protein concentration determined. Equal amounts of protein (25–35 μg) were separated on 12% SDS-PAGE and blotted. The blots were reacted with anti-H-ras antibody F235 (Santa Cruz Biotechnology, non-cross reactive with N-ras and K-ras) at 1 μg ml⁻¹.

**Colony formation in soft agar**

Anchorage-independent growth assays were performed using 6-well plates (Freedman and Shin, 1974). Experiments were done in triplicate. Each well was coated with 2 ml of a base layer containing 0.6% agar, 1 × DMEM, 10% FBS. Subconfluent cells were collected in 10 ml of medium, counted and suspended in DMEM containing 10% FBS, 0.3% agar and 1 ml of the mixture containing 10^5 cells was plated over the 2 ml base layer in each well (three wells total for each cell line). After 14 days incubation with twice-a-week feeding (1 ml each time per dish of DMEM, 0.3% agar and 10% FBS), colonies (consisting of about 20 cells or more) in 10 high-power fields per dish were counted, and total colonies per dish calculated.

**Tumor formation**

Subconfluent, rapidly growing cells were collected and suspended in PBS at 1 × 10^6 cells per ml, and 0.5 ml of this cell suspension was injected subcutaneously in the upper dorsal region of 3–4 syngeneic (129 × C57Bl/6 Fl, wildtype) and/or nude mice. Tumor size was measured with calipers every second day until the tumors reached 20% of the weight of the animal. Tumor volume was calculated according to the following formula: 4/3π (l-1) (w-1)^2, the volume of an oblote ellipse, where l is the long dimension of the tumor, and w is the width, or short dimension, in mm: 1 mm was subtracted from each measurement to compensate for the
thickness of the skin. In cases where results from only two mice are shown, a third animal in the experiment was sacrificed prior to 20 days, usually because the tumor had grown beyond 20% of the weight of the animal. For metastasis, 10^5 cells in 0.1 ml of PBS were injected via the lateral tail vein of syngeneic F1 mice, and the mice were sacrificed after 4 weeks. Lungs were excised, fixed and cut into 1–2 mm slices prior to embedding. Several lung slices were embedded in a single block, and tumor size in the resultant H+E stained sections determined by measurement of the projected image. Mice were housed in microisolator cages in an AALAC-approved animal facility, and all procedures were approved by the Rutgers University Institutional Review Board – Animal Care and Facilities Committee.

RESULTS

Establishment of immortal and transformed cell lines

Primary embryo fibroblasts were prepared from 13–15-day pregnant females. Initial experiments in which these primary cells were transfected with a mutant ras construct together with an activated p53 construct indicated that in both wildtype and OPN-deficient mice, the rate of focus formation was too low to be useful. Therefore, spontaneously immortalized cell lines were derived by continuous passage according to the 3T3 protocol. Three independent mouse embryo fibroblast (MEF) cultures from both wildtype and OPN-deficient mice were obtained, and passed to obtain immortal cell lines. While there is considerable variability in the rate at which different cell lines pass through crisis (Rittling, 1996), there was no consistent difference between the wildtype and OPN-deficient cells (Figure 1), nor was there any consistent difference in the average growth rate of the wildtype and OPN-deficient cell lines (data not shown). Previous work has shown that
Four sets of cell lines (wildtype and OPN-deficient) were selected for further analysis. From six independent immortal cell lines (three of each genotype) a total of 19 wildtype and 17 OPN-deficient ras-transformed cell lines were selected for further analysis.

OPN protein levels in the primary, immortal, and transformed cells were monitored by western blotting of media conditioned by the different cells. While the primary wildtype cells secrete readily detectable OPN, the level is increased about 10-fold in the corresponding immortal cells, and increased still further, another sevenfold, after ras transfection (Figure 3). No OPN was detected in media conditioned by OPN-deficient cells. While the increase in OPN secretion after ras transfection was expected, and confirms that ras is active in these transformed cells, these results indicate that immortalization can also increase the amount of OPN secreted by cells.

Analysis of transformation in vitro

Following selection and expansion of the ras transformed cell lines, ras, cathepsin L, and OPN mRNA levels were analysed and normalized to β-actin mRNA levels. Cathepsin L expression has been shown in some cases to be, like OPN, induced by activated ras (Chambers et al, 1992b), and so the level of this mRNA was used as a means of assessing ras activity in the individual cell lines. Figure 4 illustrates these data for a single set of cell lines: two additional sets were similarly analysed. Following normalization to β-actin mRNA levels, neither ras nor cathepsin L mRNA levels were found to differ significantly between the wildtype and OPN-deficient clones.

The ability of the transformed cell lines to grow under anchorage-independent conditions was tested by seeding 10^4 cells in 35 mm wells in 0.3% agar in complete medium with 10% FCS. After 2 weeks, colonies containing more than 20 cells were counted. The OPN-deficient cells were able to form colonies in soft agar under these conditions, and in several cell lines colonies were formed with as high efficiency as the wildtype cell lines (Figure 4), and the morphology of the colonies was similar (data not shown). However, when the number of colonies formed per 10^4 cells plated was averaged over all the cell lines analyzed (19 wildtype and 17 OPN –/–), the wildtype cell lines were found to form nearly twice as many colonies as the OPN-deficient cells (Figure 5), despite considerable variability in the numbers of colonies formed by the individual cell lines. To confirm this observation, ras transformed OPN –/– cell lines were developed that expressed OPN under the control of a tetracycline-repressible promoter (Gossen and Bujard, 1992). In these cells OPN expression is reduced about 10-fold in the presence of tetracycline. Growth of these cells in soft agar, but not on plastic, was reduced in the presence of tetracycline by about 20–30% (Figure 5), confirming that OPN under certain circumstances can facilitate anchorage-independent growth of cells.

Analysis of tumorigenesis in vivo

The ability of transformed cells to form colonies in soft agar is the parameter that most closely correlates with tumor forming ability in vivo (Freedman and Shin, 1974). Accordingly, the ability of several of the wildtype and OPN-deficient cell lines to form tumors after injection subcutaneously into mice was assessed. Four sets of cell lines (wildtype and OPN-deficient) were selected...
that could form colonies efficiently in soft agar and that had similar ras mRNA and protein levels (Figure 6, Table 2). These cells were collected, resuspended in PBS and injected subcutaneously into nude or syngeneic mice. The mice used for these injections were wildtype as there are at present no syngeneic OPN-deficient hosts for these 129 C57Bl/6 F2 cells. Every second day after the tumors became palpable, the tumor size was measured with calipers. In every case the wildtype cells formed tumors significantly earlier than did the OPN-deficient cell lines (Figure 7) despite comparable growth of the cells in vitro both on plastic and in soft agar (Table 2). Similar results were obtained with both syngeneic and nude mice (Table 2). The main defect observed in the OPN –/– cells was in the lag-time before tumor formation could be detected: once the tumors were formed, the growth rate of the OPN –/– and wildtype tumors was similar (Figure 7). Thus OPN is required for efficient initiation of tumor growth in vivo in this system.

One pair of these cell lines was tested for the ability to form metastases in lungs following intravenous injection into syngeneic mice. The OPN-deficient cells were found to form fewer metastases, (5.5 ± 1.8 metastases per lung section for wt vs 1.2 ± 1.0 for OPN –/– cells; n = 3, P < 0.05), and those metastases were much smaller than those formed by WT cells (the mean cross-sectional area of the largest metastatic tumor examined in each section was 0.79 ± 0.42 mm² for wt and 0.162 ± 0.16 mm² for OPN –/– (n = 9; P < 0.01)).

Table 2 Growth characteristics and tumour formation of different cell lines with high efficiency of growth in soft agar. The indicated cell lines were injected subcutaneously into mice. At the same passage, the growth rate of the cells on plastic and in soft agar was measured.

| Cell line | Genotype | Growth on plastic × 10⁴ | Growth in agar | Tumour size (syngeneic) mm³ | n | Tumour size (nude) mm³ |
|-----------|----------|-------------------------|--------------|---------------------------|---|------------------------|
| 279–3–7   | +/+      | 4.5 ± 0.1               | 772 ± 64     | 3848 ± 2636               | 2 | 2576 ± 667*            |
| 279–3–12  | +/+      | 3.1 ± 0.4               | 793 ± 110    | 2354 ± 3203               | 2 | 2851 ± 758*            |
| 275–3–2   | +/+      | 6.5 ± 0.5               | 753 ± 70     | 1206 ± 237*               | 3 | 2946 ± 5353            |
| 275–1–4   | +/+      | 1.6 ± 0.2               | 628 ± 35     | 1151 ± 778*               | 3 | nd                     |
| 247–3–8   | +/-      | 2.2 ± 0.0               | 955 ± 203    | 137 ± 106                 | 3 | 287 ± 156*             |
| 247–3–12  | +/-      | 1.9 ± 0.3               | 1342 ± 239   | 335 ± 264                 | 2 | 673 ± 489*             |
| 277–3–4   | +/-      | 4.6 ± 0.0               | 810 ± 122    | 46 ± 64*                  | 3 | 372 ± 253              |
| 277–1–11  | +/-      | 2.8 ± 0.4               | 1502 ± 343   | 44 ± 62*                  | 3 | nd                     |

* Growth curves were constructed for each cell line as described in Methods. Total cell number per well at 3 days, when the cells were still in exponential growth, is reported. Numbers represent the average of three determinations ± SD. 10⁴ cells were plated in 0.3% agar, and the total number of colonies present at 14 days determined. Numbers represent the average of three determinations ± SD. 5 × 10⁴ cells were injected subcutaneously into mice, and tumour size determined after 20 (*) or 21 days. The average total tumour size ± SD for three or four mice is shown, n represents the number of mice used. Where n = 2, results from individual animals are shown.

Figure 5 Colony-forming ability in soft agar of wildtype and OPN-deficient ras-transformed cell lines, and in cells with a tetracycline-responsive OPN construct, grown with and without tetracycline. +/- = average colony formation per 10⁴ cells of 19 wildtype cell lines; –/– = average colony formation per 10⁴ cells of 15 OPN-deficient cell lines; +/- = colony formation of 10⁴ OPNtet cells in the absence of Tet (expressing OPN); +Tet = colony formation of 10⁴ OPNtet cells in the presence of Tet (reduced OPN expression). Shown is the mean +/- SEM, P < 0.05.

Figure 6 ras protein level in wildtype and OPN-deficient cells used for tumour formation. Cell lysates were prepared from each cell line shown in Table 2, and equal amounts of protein separated on 12% SDS Page gels. Ras protein was detected with antibody F235. The designation and genotype of each cell line is shown above the lanes. Cell line 275-1-4 expresses very low levels of ras, and may be derived from spontaneously transformed cells.

Figure 7 Growth of tumours in nude mice after injection of wildtype cell line 279-3-7 (solid symbols) or OPN-deficient cell line 247-3-8 (open symbols). 5 × 10⁴ cells in PBS were injected subcutaneously into the dorsal area of 3–4 mice. Tumour size was measured every other day, and tumour volume calculated as described in Methods. Each line represents results from a single mouse. Results for a representative pair of cell lines are shown – other cell lines gave similar results, as shown in Table 2.
Osteopontin is required for maximal transformation

DISCUSSION

The development of mice deficient for osteopontin expression (Rittling et al., 1998; Liaw et al., 1998) has provided a unique system in which to compare directly the transformed properties of otherwise very similar cells in the presence and absence of osteopontin expression. The work presented here is important in that it provides an uncomplicated analysis of the role of OPN in multiple aspects of cellular transformation. Our results confirm and extend the long-held idea that OPN is important in the transformation process, in that the transformed phenotype was attenuated in the absence of OPN. Thus we observed lower rates on average of colony formation in soft agar, and dramatically reduced rates of primary tumor and metastasis growth in syngeneic and nude mice. The only aspect of transformation for which we did not detect a reduction in the OPN-deficient cells was in the process of focus formation: both the wildtype and osteopontin-deficient cells formed foci efficiently after ras transformation. This phenotype, focus formation, most likely represents a loss of contact inhibition of growth resulting from the expression of oncogenic ras, and we conclude that OPN, and by deduction also the integrins to which OPN binds in these cells, do not play a major role in this process. We also did not detect an effect of osteopontin on the process of spontaneous immortalization. This process requires an alteration in the cells, probably a mutation (Rittling, 1996) that enables the cells to overcome the growth restraints of senescence.

Our results indicate that the effects of OPN expression on anchorage-independent growth are complex. While antisense experiments suggested that OPN was necessary for the formation of colonies in soft agar (Su et al., 1995; Gardner et al., 1994), we clearly show here that OPN-deficient cell lines can form colonies efficiently in soft agar, indicating that OPN is not absolutely required for this process in ras-transformed cells. However, our data show that OPN can enhance anchorage-independent growth. Thus, OPN is not strictly required for anchorage-independent growth, but can enhance such growth in some cell lines. Anchorage-independent growth in ras-transformed cells probably results from stimulation by activated ras of signaling pathways that mediate both growth factor and integrin-initiated signals (reviewed in Schwartz, 1997). For example, MAPK activation by growth factors in normal cells requires adhesion through integrins (Renshaw et al., 1997), while oncogenic ras stimulates MAPK independently of both growth factor and integrin ligation to allow anchorage-independent growth. The binding of soluble OPN to integrins such as the $\alpha_\beta_1$, may stimulate the signal throughput of these pathways, even in the presence of activated ras, thereby enhancing anchorage-independent growth, much as transformed cells that are growth factor independent show higher rates of growth in the presence of growth factors.

The most pronounced effect of a lack of OPN that we observed was in the ability of the OPN-deficient transformed cells to form tumors. For these experiments, a subset of ras-transformed cell lines was selected that had similar ras levels and formed colonies efficiently in soft agar. While the OPN-deficient clones were able to form tumors in syngeneic or nude mice, they did so at a substantially slower rate than the wildtype cells. Thus, OPN clearly can enhance tumor formation in vivo. It is important to note here that the recipients for these tumor cell injections were wildtype mice, so OPN could have been produced by the stromal cells in the vicinity of the injection of the OPN-deficient cells. Thus host-derived OPN may have contributed to the ultimate ability of the OPN-deficient cells to form tumors. Indeed, we have found (Wu, Feng and Rittling, manuscript in preparation) that tumors arising from the OPN-deficient cells contain measurable amounts of OPN, which may have been supplied by infiltrating macrophages (Brown et al., 1994; Casson et al., 1997) or other sources.

We have considered several hypotheses to explain the mechanism of OPN's ability to enhance tumor formation. It is unlikely that OPN is acting in an autocrine manner to stimulate growth of the tumor cells themselves since these cells already show efficient anchorage-independent growth. Alternatively, high level OPN expression could protect cells from apoptosis in the first few days following tumor cell injection. A role for the OPN-binding integrins $\alpha_\beta_3$ and $\alpha_\beta_1$ (in preventing apoptosis (or anoikis (Frisch and Ruoslahti, 1997))) of transformed cells has previously been demonstrated (Montgomery et al., 1994; Zhang et al., 1995). Denhardt and Chambers (1994) have proposed that OPN, by virtue of its ability to inhibit iNOS activity, protects tumor cells from attack by cytotoxic cell in vivo, and this may be a possible mechanism of action of OPN in stimulating tumor growth. The possible conflicting role of macrophages in OPN-dependent tumorigenesis has been discussed recently (Crawford et al., 1998). Finally, OPN has been shown to stimulate migration of endothelial cells (Liaw et al., 1995), particularly in association with VEGF (Senger et al., 1996). Possibly, expression of OPN in the injected cells is required for optimal angiogenesis. This idea is supported by the observations that ligation of the $\alpha_\beta_3$ integrin protects endothelial cells against apoptosis (Brooks et al., 1994a; Strömblad et al., 1996) and stimulates angiogenesis (Brooks et al., 1994b), and that surface-bound OPN can protect endothelial cells from apoptosis (Scatena et al., 1998). Work is in progress to distinguish these possibilities.

Experiments with transformed cells are hampered by the well-known heterogeneity of such cells, making it difficult to make direct comparisons between different transformed cell lines. Our analysis of a large number of cell lines has allowed us to develop a consensus of the effects of OPN in vitro, despite considerable variability among individual cell lines. The results with tumor formation, however, were less variable, in that every cell line tested showed a slower rate of tumor growth. Thus, it may be that the effects of OPN are more pronounced in the whole animal than in culture, possibly reflecting an important role for this protein in mediating cellular interactions.

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REFERENCES

Bayless KA, Meininger GA, Scholtz JM and Davis GE (1998) Osteopontin is a ligand for the $\alpha_\beta_3$ integrin. J Cell Sci 111: 1165–1174
Bellreng E, Craig AM, Wilson SM, Denhardt DT and Chambers AF (1994)
Bellahcène A and Castronovo V (1995) Increased expression of osteonectin and osteopontin expression. British Journal of Cancer (2000) 83(2), 156–163
Brooks PC, Clark RAF and Cheresh DA (1994a) Requirement of vascular integrin $\alpha_\beta_3$ for angiogenesis. Science 264: 569–571

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Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G and Cheresh DA (1994) Inhibin α,β, antagonists promote 1 regression by inducing apoptosis of angiogenic blood vessels. *Cell* 79: 1157–1164

Brown LF, Papadopoulos-Sergiou A, Berse B, Manseau EL, Tognazzi K, Perruzzi CA, Dvorak HF and Senger DR (1994) Osteopontin expression and distribution in human carcinomas. *Am J Pathol* 145: 610–623

Cain AE, Wilson SM, McCoy IA, O’Malley FY, Oncetti H, Tsao MS and Chambers AF (1997) ras mutation and expression of the ras-regulated genes osteopontin and cathepsin L in human esophageal cancer. *Int J Cancer* 72: 739–745

Chambers AF, Denhardt DT and Wilson SM (1990) RAS-transformed NIH3T3 cells, selected for metastatic ability in chick embryos, have increased proportions of p21-expressing cells and are metastatic in nude mice. *Invasion Metastasis* 10: 225–230

Chambers AF, Behrend EI, Wilson SM and Denhardt DT (1992a) Induction of expression of osteopontin (OPN, secreted phosphoprotein) in metastatic, ras-transformed NIH 3T3 cells. *Anticancer Res* 12: 43–48

Chambers AF, Hota C and Prince CW (1993) Adhesion of metastatic, ras-transformed NIH 3T3 cells to osteopontin, fibronectin, and laminin. *Cancer Res* 53: 701–706

Chen H, K Y, Oates AJ, Barracough R and Rudland PS (1997) Isolation of and effectors for metastasis-inducing DNAs from a human metastatic carcinoma cell line. *Oncogene* 14: 1581–1588

Clearkin P (1998) Recent insights into the roles of integrins in cancer metastasis. *Cell Mol Life Sci* 54: 531–548

Craig AM, Bowden GT, Chambers AF, Spearman MA, Greenberg AH, Wright JA, McLeod M and Denhardt DT (1990) Secreted phosphoprotein mRNA is induced during multistage carcinogenesis in skin and correlates with the metastatic potential of murine fibroblasts. *Int J Cancer* 46: 133–137

Crawford HC, Matrisian LM and Liaw L (1998) Distinct roles of osteopontin in host defense against host defenses: Osteopontin (OPN) as a shield against attack by cytotoxic host cells. *J Cell Biochem* 58: 48–51

Feng B, Rollo EE and Denhardt DT (1995) Osteopontin (OPN) may facilitate metastasis by protecting cells from macrophage NO-mediated cytotoxicity: evidence from cell lines down-regulated for OPN expression. *Clin Exp Metastasis* 13: 453–462

Friedman VH and Shin SI (1974) Cellular tumourigenicity in nude mice: correlation of p21-expressing cells and are metastatic in nude mice. *Invasion Metastasis* 10: 225–230

Gardner HAR, Berse B, Manseau EL, Tognazzi K, Perruzzi CA, Dvorak HF and Senger DR (1994) Osteopontin expression and distribution in human carcinomas. *Am J Pathol* 145: 610–623

Gardner HAR, Berse B, Manseau EL, Tognazzi K, Perruzzi CA, Dvorak HF and Senger DR (1994) Osteopontin expression and distribution in human carcinomas. *Am J Pathol* 145: 610–623

Goligorsky MS, Noiri E, Kessler H and Romanov V (1997) Therapeutic potential of cathepsin L in human esophageal cancer. *Cancer Res* 57: 701–706

Hu DD, Hoyer JR and Smith JW (1995) Calcium suppresses cell adhesion to osteopontin by attenuating binding affinity for integrin α3β1. *J Biol Chem* 270: 487–496

Hu DD, Lin ECK, Kovach NL, Hoyer JR and Smith JW (1995b) A biochemical characterization of the binding of osteopontin to integrins α1β1, and αvβ3. *J Biol Chem* 270: 26232–26238

Hwang SM, Lopez CA, Heck DE, Gardner CR, Laskin DL, Laskin JD and Denhardt DT (1994) Osteopontin inhibits induction of nitric oxide synthase gene expression by inflammatory mediators in mouse kidney epithelial cells. *J Biol Chem* 269: 711–715

Hynes RO (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69: 11–25

Juliano RL (1994) Signal transduction by integrins and its role in the regulation of tumour growth. *Cancer Metastasis Rev* 13: 25–30

Katagiri YU, Sleeman J, Fuji H, Hirrelich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF and Uede T (1999) CD-44 variants but not CD-44a cooperate with beta-1 containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic-acidic, thereby stimulating cell motility and chemotaxis. *Cancer Res* 59: 219–226

Liaw L, Skinner MP, Rains EW, Ross R, Cheresh DA, Schwartz SM and Giachelli CM (1995) The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins: role of α5β1 in smooth muscle migration to osteopontin in vitro. *J Clin Invest* 95: 713–724

Liaw L, Birk DE, Baillas CB, Whitiss JS, Davidson JM and Hogan BLM (1998) Altered wound healing in mice lacking a functional osteopontin gene (spp1). *J Clin Invest* 101: 1468–1478

Malyankar UM, Rittling SR, Connor A and Denhardt DT (1994) The mitogen-regulated protein/profalin transcript is degraded in primary mouse embryo fibroblasts but not 3T3 nuclei: altered RNA processing correlates with immortalization. *Proc Natl Acad Sci USA* 91: 335–339

Montgomery AM, Reisfeld RA and Cheresh DA (1994) Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three- dimensional dermal collagen. *Cancer Res* 54: 8856–8860

Nori E, Dickman K, Miller F, Romanov G, Romanov V, Shaw R, Rittling SR, Denhardt DT and Goligorsky MS (1999) Acute renal ischemia in mice with a targeted disruption of the osteopontin gene: link to nitric oxide-induced injury. *Kidney Int* 56: 74–83

Oates AJ, Barracough R and Rudland PS (1996) The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. *Oncogene* 13: 97–104

Renshaw MW, Ren XD and Schwartz MA (1997) Growth factor activation of MAP kinase requires cell adhesion. *EMBO J* 16: 5592–5599

Rittling SR (1996) Clonal nature of spontaneously immortalized 3T3 cells. *Exp Cell Res* 229: 7–13

Rittling SR and Novick KW (1997) Osteopontin expression in mammary gland development and tumorigenesis. *Cell Growth Differ* 8: 1061–1069

Rittling SR and Feng F (1998) Detection of mouse osteopontin by western blotting. *Biochem Biophys Res Commun* 258: 287–292

Rittling SR and Denhardt DT (1999) Osteopontin (OPN) function in pathology: lessons from OPN-deficient mice. *Experimental Nephrology* 7: 103–113

Rittling SR, Matsumoto KN, McKee MD, Nance A, An XR, Novick KE, Kowalski AJ, Noda M and Denhardt DT (1998) Mice lacking osteopontin show normal development and bone structure but display altered osteoclast formation in vitro. *J Bone Miner Res* 13: 1101–1111

Rollo EE, Laskin DL and Denhardt DT (1996) Osteopontin inhibits nitric oxide production and cytotoxicity by activated RAW 264.7 macrophages. *J Leukoc Biol* 59: 139: 575–578

Sanger DR, Asch BR, Smith BD, Perruzzi CA and Dvorak HF (1983) A secreted phosphoprotein marker for neoplastic transformation of both epithelial and fibroblastic cells. *Nature* 302: 714–715

Sanger DR, Perruzzi CA, Gracey CE, Papadopoulos-A and Tenen DG (1988) Secreted phosphoproteins associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. *Cancer Res* 48: 5770–5774

Sanger DR, Perruzzi CA, Papadopoulos-Sergiou A and Van De Water L (1994) Adhesive properties of osteopontin: regulation by a naturally occurring thrombin-cleavage in close proximity to the GRGDS cell-binding domain. *J Biol Chem* 269: 565–574

Sanger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Perruzzi CA and Detmar M (1996) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the α5β1 integrin, osteopontin, and thrombin. *Am J Pathol* 149: 293–305
Osteopontin is required for maximal transformation

Singh RP, Patarca R, Schwartz I, Singh P and Cantor H (1990) Definition of a specific interaction between the early T lymphocyte activation 1 (Eta-1) protein and murine macrophages in vitro and its effect upon macrophages in vivo. *J Exp Med* **171**: 1931–1942

Smith LL, Cheung HK, Ling LE, Chen J, Sheppard D, Pytela R and Giachelli CM (1996) Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by \( \alpha_v\beta_1 \) integrin. *J Biol Chem* **271**: 28485–28491

Somerman MJ, Prince CW, Saut J, Foster RA and Butler WT (1987) Cell attachment activity of the 44 kilodalton bone phosphoprotein is not restricted to bone cells. *Matrix* **9**: 49–54

Strömblad S, Becker JC, Yebra M, Brooks PC and Cheresh DA (1996) Suppression of p53 activity and p21waf1/cip1 expression by vascular cell integrin \( \alpha_v\beta_3 \) during angiogenesis. *J Clin Invest* **98**: 426–433

Su L, Mukherjee AB and Mukherjee BB (1995) Expression of antisense osteopontin mRNA inhibits tumour promoter-induced neoplastic transformation of mouse JB6 epidermal cells. *Oncogene* **10**: 2163–2169

Tuck AB, O’Malley FP, Singhal H, Tonkin KS, Kerkvliet N, Saad Z, Doig GS and Chambers AF (1998) Osteopontin expression in a group of lymph node negative breast cancer patients. *Int J Cancer* **79**: 502–508

Weber GF, Ashkar S, Glimcher MJ and Cantor H (1996) Receptor-ligand interaction between CD44 and osteopontin (ETA-1). *Science* **271**: 509–512

Zhang Z, Vuori K, Reed JC and Ruoslahti E (1995) The \( \alpha_v\beta_1 \) integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci USA* **92**: 6161–6165