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Osteopontin-a Alters Glucose Homeostasis in Anchorage-Independent Breast Cancer Cells*

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Abstract

Invasive breast tumor cells generate three splice variants of the metastasis gene osteopontin, while non-invasive breast cells express only the unspliced form or no osteopontin at all. One role for osteopontin in tumor progression is the support of anchorage-independence. Here we show that the full-length gene product, osteopontin-a, induces a gene expression profile that is associated with tissue remodeling and directed movement/sprouting. This occurs via signals through STAT1 and STAT3 to snlycero-3-phosphocholine. Osteopontin-a upregulates the levels of glucose in breast cancer cells, likely through STAT3 and its transcriptional targets apolipoprotein D and IGFBP5. The splice variants osteopontin-a and osteopontin-c may synergize, with each form activating signal transduction pathways that are distinct from the other. The elevated glucose is used by osteopontin-c dependent signals to generate chemical energy (Shi et al. manuscript submitted). The splice variant-specific metabolic effects of osteopontin add a novel aspect to the pro-metastatic functions of this molecule.

Keywords

cancer biology; metabolic regulation; glucose; cytokine action; metastasis

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Conflict of Interest Statement

The corresponding author is the founder and CEO of MetaMol Theranostics. He is an inventor on the following issued or pending patents:

Issued

Yang X-F, Weber GF, Cantor HI (inventors). Bcl-x, a novel Bcl-x isoform, and uses related thereto. US patents 6,472,170 (2002) and 7,160,986 (2007).

Ashkar S, Cantor H, Glimcher MJ, Weber GF (inventors). Methods and compositions for modulating immune responses. Australian patent 243575500 (2004).

Weber GF (inventor). Peptide sequence that promotes tumor invasion. US Patent 7,807,790 B2 (2009).

Weber GF (inventor). Peptide sequence that promotes tumor invasion. European Patent 1 949 109 B1 – Germany, England, France, Sweden (2012).

Pending

Weber GF (inventor). Peptide sequence that promotes tumor invasion. (US Patent 12/854,011 pending).

John Hurley, Mana Mirza, Gary Pestano, Elizabeth Shaughnessy, Kristie Vanpatten, Georg F. Weber (inventors). Grading breast cancer using osteopontin-c. (PCT patent US2008/080162 pending).

1. INTRODUCTION

The gene *spp1* (encoding osteopontin) mediates progression by various types of cancer. In humans, there exist two osteopontin splice variants with deletions of exon 4 (referred to as osteopontin-c) or 5 (called osteopontin-b) [1,2]. We have previously shown that the splice variant osteopontin-c is uniquely expressed in breast cancers, but not in normal breasts. In contrast, the full-length form osteopontin-a is present in both breast cancers and non-transformed breast tissue [3,4]. Osteopontin-c is never expressed without the full-length form osteopontin-a. It is not known whether the two splice variants have opposing, additive or synergistic functions.

While healthy epithelial cells undergo apoptosis consecutive to losing contact with their substratum, anchorage-independent survival is an essential characteristic of metastasizing cells [5]. We have found previously that the splice variant osteopontin-c is a more potent enhancer of anchorage-independent expansion than osteopontin-a [3]. It is possible that osteopontin-a can play a dual role of either being pro-adhesive (i.e. anti-metastatic) or being mildly supportive of anchorage-independent expansion (which is prometastatic). Osteopontin-a, but not osteopontin-c, contains exon 4, which may cause protein cross-linking and cell adhesion, in effect exerting an osteopontin-a-specific anti-metastatic effect under certain microenvironmental conditions. When in solution, osteopontin-a has anti-apoptotic, pro-survival properties via engagement of its cognate receptors [6], which may aid cancer progression. There is evidence [3] that osteopontin-a and osteopontin-c transduce differential signals in breast tumor cells, however the exact molecular pathways are unknown. Here we map a pathway associated with anchorage-independence that is selectively induced by osteopontin-a, but not by osteopontin-c. Osteopontin-a elevates cellular glucose levels, which may supply the energy source for osteopontin-c mediated anti-apoptosis. The results indicate that osteopontin-a and osteopontin-c may synergize in supporting the survival of circulating tumor cells.

2. EXPERIMENTAL PROCEDURES

Reagents

Poly-HEMA was purchased from Sigma-Aldrich, PpYLKTK-mts and a scrambled control peptide was obtained from Calbiochem.

Cell lines, DNA constructs and transfection

MCF-7 cells and their stable transfectants, were grown in α -MEM with insulin and 10% fetal bovine serum [3]. The constructs for expression of constitutively active and dominant negative STAT3 were obtained from Dr. Robert Arceci, Johns Hopkins University, and subcloned into pCDNA3.1/hygro(+). Genes cloned into this vector are expressed under the control of the CMV promoter. Sequence fidelity and accurate reading frame were verified by DNA sequencing analysis. MCF-7 cells were transfected by the Fugene reagent (FuGENE 6, Roche) and stable clones were selected by hygromycin.

Immuno-blot assay

For the analysis of secreted osteopontin, serum-free cell culture supernatant was collected from each transfectant. 40 μ l of supernatant per sample were electrophoresed on 10% SDS-polyacrylamide mini-gels with non-reducing sample buffer. For the analysis of intracellular osteopontin, the cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Nadeoxycholate, 0.1% sodium dodecyl sulfate). Cell lysates at equal amounts of protein (20 μ g/lane) were electrophoresed on reducing 10% SDS-

polyacrylamide gels. The separated proteins were transferred to PVDF membranes and probed with antibody O-17 (Assay Designs Inc.) to osteopontin and antibodies to STAT3 and phospho-STAT3 (Cell Signaling Technology) or (for transfected STAT constructs) with antibody to the Flag-tag (Cell Signaling). The expression levels of all transfected genes were confirmed every time after thawing and initiation of culture.

Protein-DNA array

Nuclear extracts were prepared from MCF-7 vector, MCF-7 OPNa, and MCF-7 OPNc cells and DNA binding was assessed with the Protein/DNA array I (Panomics). The procedure followed the manufacturer's instructions. Various time exposures were taken with radiography films, and the resulting densities were quantified with the imaging software Metamorph.

Analysis of growth rates

For the investigation of cell growth rates, each cell line was plated at 5000 cells/well in 24-well plates. Daily, five wells per group were harvested by trypsinization and the cell numbers were determined in a Coulter™ Z-Series Counter. At each time point, the cell numbers from the five wells of the various groups of transfectants were analyzed for statistically significant differences by the Wilcoxon-Mann-Whitney test and the t-test, accepting a probability of error of less than 5%.

Soft agar colony formation

1×10^5 cells per 60-mm dish were plated in triplicates with a top layer of 0.3% agar (BACTO Agar, Difco, Detroit, MI) and a bottom layer of 0.5% agar (both in α -MEM). Every other day, 0.4 ml of medium was supplemented and the plates were examined microscopically for growth. After one week, photographs were taken at high and low magnification and the surface area of all clones in five fields was measured with the imaging software Metamorph [7].

Deadherence in poly-HEMA

To assess the osteopontin splice variant-induced protection from cell death or cell cycle arrest through deadhesion, we plated cells on 0-2.0 $\mu\text{l}/\text{mm}^2$ poly(2-hydroxyethyl methacrylate) (poly-HEMA) plates for 24-72 h [8,9]. At the indicated time points, we harvested them for gene expression analysis by RNASeq or metabonomic analysis by nuclear magnetic resonance (NMR).

Metabonomics

We measured changes in cell physiology under de-adhesion conditions from MCF-7 transfectants in poly-HEMA. The extracted cells were processed for metabolomics analysis by 600 MHz NMR. The data were subjected to multivariate statistical analysis using Amix 3.9 (Bruker Biospin, Billerica, MA). NMR spectra were manually binned into buckets (after removal of regions subject to imperfect water suppression), and total intensity normalization was applied after binning. Initially, unsupervised principal component analysis (PCA) was performed without considering the class information. Statistical significance analysis of the loadings data was performed using Amix 3.9 (Bruker Biospin, Billerica, MA) based on a published procedure [10,11], except that a Kruskal-Wallis test was used instead of the Mann-Whitney U test for non-parametric analysis of datasets that were not normally distributed. The bucketing parameters are identical to those used for PCA. This approach has been successfully applied to identify changes in metabolites in breast cancer cells [10,12].

RNASeq

The Ovation RNA-Seq system (NuGen) was used to initiate amplification at both 3' end as well as randomly throughout the transcriptome in the sample. 100 ng of total RNA with RIN < 5.0, was converted into a library of template molecules suitable for subsequent cluster generation and sequencing by Illumina HiSeq. Total RNA was reverse transcribed and converted to double stranded cDNA with a unique DNA/RNA heteroduplex at one end. Nugen's Ribo-SPIA technology was used for isothermal amplification resulting in the rapid generation of cDNA with a sequence complementary to the original mRNA. The cDNA was then double stranded, fragmented to 200 bp using Covaris S2, and a sequencing library generated using Illumina's TruSeq DNA Sample Prep Kit V2 according to standard protocols. The cDNA library was enriched by a limited number of 10 PCR cycles, validated using an Agilent 2100 Bioanalyzer, and quantitated using the Quant-iT dsDNA HS Kit (Invitrogen). Two individually indexed cDNA libraries were pooled and sequenced on Illumina HiSeq to get a minimum of 90 million reads. Libraries were clustered onto a flow cell using Illumina's TruSeq SR Cluster Kit v2.5 and sequenced 50 cycles using TruSeq SBS Kit -HS on HiSeq. The obtained sequence reads were aligned to the genome by using the standard Illumina sequence analysis pipeline.

3. RESULTS

Osteopontin-a and osteopontin-c may synergize to support anchorage independence

We first tested, whether the effects of osteopontin-a and osteopontin-c on anchorage independence can be combined. Double-transfected MCF-7 cells showed larger clone formation in soft agar than MCF-7 cells transfected with osteopontin-a or osteopontin-c alone (Figure 1). While a mixture of singly transfected MCF-7 OPNa and MCF-7 OPNc cells formed clones of identical size to MCF-7 OPNc transfectants (not shown), the doubly transfected cells expressing osteopontin-a and osteopontin-c formed significantly larger clones, likely reflecting an additive or synergistic autocrine effect.

Osteopontin-a supports anchorage independence through a STAT3 pathway

We previously described signaling events that are selectively triggered by osteopontin-c to support anchorage independence [3]. Reanalysis of the microarray data from MCF-7 cells transfected with osteopontin-a, osteopontin-c, or vector grown in soft agar identified 16 genes that are selectively altered by osteopontin-a but not by osteopontin-c (Supplement S1). To gain further mechanistic insight, we analyzed the differential transcription factor activation in transfected MCF-7 cells with a protein/DNA array. While both osteopontin-a and osteopontin-c induced DNA binding by ARE, DNA binding by STAT1 and STAT3 were selectively activated by osteopontin-a. NFATc DNA binding was selectively induced by osteopontin-c (Table 1). Of note, several of the gene products identified to be increased selectively by osteopontin-a in Figure S1 transduce signals that depend on the activities of STAT3 or STAT1. They include the glutamate receptor [13,14], Wnt/Wingless [15], and Notch/Jagged [16,17].

Our molecular results suggested that STAT1 and STAT3 are important in mediating osteopontin-a dependent functions of breast epithelial cells. We set out to confirm the role of STAT3 selectively in osteopontin-a induced, but not in osteopontin-c induced soft agar clone formation. In initial experiments, we added a cell-permeable peptide inhibitor of STAT3 (PpYLKTK-mts) to the soft agar plates. This inhibitor, but not a control peptide, selectively suppressed the osteopontin-a induced clone formation without altering the osteopontin-c effect and without affecting the baseline clone size of MCF-7 cells (Figure 2A). We then transfected DNA constructs that modulate STAT3 activity into MCF-7 cells expressing osteopontin-a, osteopontin-c, or control vector. Again, the suppression of STAT3

activity (by the dominant negative STAT3 construct) selectively inhibited the osteopontin-a dependent formation of clones. Constitutively active STAT3 was sufficient to significantly increase the clone size produced by MCF-7 vector cells (Figure 2B,C). The STAT3 effect was not due to an accelerated rate of cell cycle progression, as a proliferation assay showed no difference between MCF-7 OPNa cells co-transfected with dominant-negative STAT3 and MCF-7 OPNa cells co-transfected with control vector. Further, MCF-7 vector cells co-transfected with constitutively active STAT3 showed similar proliferation as MCF-7 vector cells co-transfected with control vector (Figure 2D).

Osteopontin-a activates glucose and lipid signaling

Because cells cannot be cleanly extracted from soft agar, we used plating on poly-HEMA as an alternative assay for anchorage-independence. We first confirmed by Western blotting that osteopontin-a induces the activation of STAT3 (reflected in its phosphorylation) when cells are grown under these deadherent conditions (Figure 3A). We then prepared cell extracts after deadhesion on poly-HEMA for metabolomics according to NMR analysis. Osteopontin-a, but not osteopontin-c, upregulated the levels of glucose and sn-glycero-3-phosphocholine but downregulated the levels of lactate and taurine (Figure 3B,C). The effect is autocrine as it is reversed by a neutralizing antibody (Supplement S2).

An additional experiment with osteopontin-a/dominant-negative STAT3 double-transfected MCF-7 cells (versus MCF-7 OPN-a/vector) to examine the contribution by STAT3 in osteopontin-a signaling showed that the suppression of the STAT3 signal transduction intermediate reversed the upregulation of sn-glycero-3-phosphocholine and glucose as well as the downregulation of lactate by osteopontin-a. It also increased the levels of AMP, which may reflect a build-up due to compromised ATP synthesis (Figure 4).

Osteopontin-a activates the expression of a characteristic gene profile

For further insight into the osteopontin-a induced signaling in deadherent breast tumor cells, we performed RNASeq from MCF-7 transfectants grown on poly-HEMA. The gene ontology (GO) categories selectively upregulated by osteopontin-a are consistent with the physiologic role of osteopontin in tissue remodeling and directed movement/sprouting (Table 2). Individual genes upregulated by osteopontin-a compared to osteopontin-c and vector are shown in Supplement S3. The highly upregulated IGFBP5 and apolipoprotein D are STAT3 target genes [18-20]. Both are regulators of glucose homeostasis. When analyzed separately, osteopontin-a induced the expression of aldo-keto reductase family 1 members and UDP glucuronosyltransferase 1 family members over osteopontin-c. These genes are metabolic regulators, confirming the extensive metabolic effect of osteopontin-a signaling.

4. DISCUSSION

The upregulation of glucose by osteopontin-a is a striking result of this study. STAT3, IGFBP5, and apolipoprotein D, which are targets of osteopontin-a signaling, have been described to contribute to glucose homeostasis. Previously, these observations had been made in cell types and organs other than the breast [21-23] and had not been linked to osteopontin. Osteopontin signaling has been studied extensively. While various important aspects of signal transduction pathways have been elucidated, the finding of osteopontin-a dependent glucose regulation in breast cancer is novel (Figure 5). The distinct metabolic changes induced by the individual osteopontin splice variants, specifically osteopontin-a and osteopontin-c (Shi et al. manuscript submitted), add a new facet to the biological functions of this group of molecules. The importance of these mechanisms initially came to the forefront with the discovery of the osteopontin contributions to anchorage independence by cancer cells, which involve the osteopontin-c mediated upregulation of oxidoreductases [3].

The induction of anchorage independent survival and expansion, which is essential for the dissemination of cancer cells, may be achieved, in part, through a component of metabolic adjustment within the deadherent cells. The autocrine secretion of osteopontin variants mediates this effect.

Osteopontin has been shown to correlate with STAT3 induction [24,25] and to induce STAT3 activation [26,27]. We now have identified osteopontin-a as the relevant form of osteopontin for this activity, whereas osteopontin-c does not signal through pathways that involve STAT3. Hence, the two splice variants, osteopontin-a and osteopontin-c, induce qualitatively distinct autocrine signals in cancer cells. Multiple receptors have been identified for osteopontin, and it is likely that the splice variants have additional, yet unknown receptors, which are engaged by their different far N-terminal domains. Which of these receptors are responsible for the differential signal transduction will be subject to further research.

The role of sn-glycero-3-phosphocholine in osteopontin-a signaling is not fully clarified. As oxidized phospholipids are known to activate STAT3 signaling [28,29], sn-glycero-3-phosphocholine could act upstream as a STAT3 inducer. However, the suppression of sn-glycero-3-phosphocholine levels by dominant-negative STAT3 indicates that this metabolite is synthesized downstream of STAT3. We hypothesize a positive feedback loop, in which sn-glycero-3-phosphocholine is generated downstream of STAT3 but then further activates this signal transducer (see Figure 5).

Signal transduction has been a major research focus of molecular biology over the past decades. It has elucidated mechanisms, by which cells communicate environmental cues via receptors to their nuclei to induce adaptive changes in gene expression. Cell responses based on epigenetic mechanisms are less well understood. Signaling in cancer metastasis has been associated with directed cell migration (homing), penetration of tissue barriers (invasion), and more recently with survival of cells in the circulation (anti-anoikis). All of these functions are likely to have a strong gene expression-independent component. Our studies now show that the response of deadherent cells to pro-metastatic ligands, such as osteopontin splice variants, involves the adjustment, or skewing, of the metabolism. The alteration in glucose homeostasis, resulting from osteopontin-a signaling, may contribute to satisfying an increased energy requirement for the survival of cancer cells in circulation (see also Shi et al. manuscript submitted). It is likely that metabolic responses to environmental cues are more common in cell biology than has hitherto been recognized.

We have not investigated the roles of OPN-b in anchorage-independence of breast cancer. OPN-b is expressed at very low levels in breast tumor cells and the protein may be rapidly degraded in the proteasome [4]. While this splice variant can be produced in abundance by lung cancers [30], it is uncertain whether it has a role in breast biology or pathobiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW. cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). *Genomics*. 1990; 7:491–502. [PubMed: 1974876]
2. Saitoh Y, Kuratsu J, Takeshima H, Yamamoto S, Ushio Y. Expression of osteopontin in human glioma. Its correlation with the malignancy. *Lab Invest*. 1995; 72:55–63. [PubMed: 7837791]
3. He B, Mirza M, Weber GF. An osteopontin splice variant induces anchorage independence in human breast cancer cells. *Oncogene*. 2006; 25:2192–2202. [PubMed: 16288209]
4. Mirza M, Shaughnessy E, Hurley JK, Vanpatten KA, Pestano GA, He B, Weber GF. Osteopontin-c is a highly selective marker for breast cancer. *Int J Cancer*. 2008; 122:889–897. [PubMed: 17960616]
5. Weber GF. Molecular mechanisms of metastasis. *Cancer Lett*. 2008; 270:181–190. [PubMed: 18522865]
6. Weber, GF.; Adler, B.; Ashkar, S. Osteopontin in oxidative stress responses.. In: Ruffolo, RR., Jr; Feuerstein, GZ.; Hunter, AJ.; Poste, G.; Metcalf, BW., editors. *Inflammatory Cells and Mediators in CNS Diseases*. Harwood Academic Publishers; Amsterdam: 1999. p. 97-112.
7. Shen H, Weber GF. The osteopontin-c splice junction is important for anchorage-independent growth. *Mol Carcinog*. 2013 doi: 10.1002/mc.21997. [Epub ahead of print].
8. Folkman J, Moscona A. Role of cell shape in growth control. *Nature*. 1978; 273:345–349. [PubMed: 661946]
9. Valentini B, Morrión A, Peruzzi F, Prisco M, Reiss K, Baserga R. Anti-apoptotic signaling of the IGF-I receptor in fibroblasts following loss of matrix adhesion. *Oncogene*. 1999; 18:1827–1836. [PubMed: 10086337]
10. Goodpaster AM, Romick-Rosendale LE, Kennedy MA. Statistical significance analysis of nuclear magnetic resonance-based metabolomics data. *Anal Biochem*. 2010; 401:134–143. [PubMed: 20159006]
11. Goodpaster AM, Kennedy MA. Quantification and statistical significance analysis of group separation in NMR-based metabolomics studies. *Chemometrics Intel Lab Sys*. 2011; 109:162–170.
12. Watanabe M, Sheriff S, Lewis KB, Tinch SL, Cho J, Balasubramanian A, Kennedy MA. HMGA-targeted phosphorothioate DNA aptamers increase sensitivity to gemcitabine chemotherapy in human pancreatic cancer cell lines. *Cancer Lett*. 2012; 315:18–27. [PubMed: 22036895]
13. Aguilera P, Ortega A. Stat3 participates in the metabotropic glutamate signaling pathway in Bergmann glial cells. *Neurochem Res*. 1999; 24:981–986. [PubMed: 10478936]
14. Li H, Zhang Q, Zhang G. Signal transducer and activator of transcription-3 activation is mediated by N-methyl-D-aspartate receptor and L-type voltage-gated Ca^{2+} channel during cerebral ischemia in rat hippocampus. *Neurosci Lett*. 2003; 345:61–64. [PubMed: 12809989]
15. Yamashita S, Miyagi C, Carmany-Rampey A, Shimizu T, Fujii R, Schier AF, Hirano T. Stat3 Controls Cell Movements during Zebrafish Gastrulation. *Dev Cell*. 2002; 2:363–375. [PubMed: 11879641]
16. Kamakura S, Oishi K, Yoshimatsu T, Nakafuku M, Masuyama N, Gotoh Y. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. *Nat Cell Biol*. 2004; 6:547–554. [PubMed: 15156153]
17. Monsalve E, Perez MA, Rubio A, Ruiz-Hidalgo MJ, Baladron V, Garcia-Ramirez JJ, Gomez JC, Laborda J, Diaz-Guerra MJ. Notch-1 up-regulation and signaling following macrophage activation modulates gene expression patterns known to affect antigen-presenting capacity and cytotoxic activity. *J Immunol*. 2006; 176:5362–5373. [PubMed: 16622004]
18. Chapman RS, Lourenco PC, Tonner E, Flint DJ, Selbert S, Takeda K, Akira S, Clarke AR, Watson CJ. Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev*. 1999; 13:2604–2616. [PubMed: 10521404]
19. Kojima H, Kunitomo H, Inoue T, Nakajima K. The STAT3-IGFBP5 axis is critical for IL-6/gp130-induced premature senescence in human fibroblasts. *Cell Cycle*. 2012; 11:730–739. [PubMed: 22374671]

20. Shen L, Tso P, Woods SC, Sakai RR, Davidson WS, Liu M. Hypothalamic apolipoprotein A-IV is regulated by leptin. *Endocrinol.* 2007; 148:2681–2689.
21. Inoue H, Ogawa W, Ozaki M, Haga S, Matsumoto M, Furukawa K, Hashimoto N, Kido Y, Mori T, Sakaue H, Teshigawara K, Jin S, Iguchi H, Hiramatsu R, LeRoith D, Takeda K, Akira S, Kasuga M. Role of STAT-3 in regulation of hepatic gluconeogenic genes and carbohydrate metabolism in vivo. *Nat Med.* 2004; 10:168–174. [PubMed: 14716305]
22. Gleason CE, Ning Y, Cominski TP, Gupta R, Kaestner KH, Pintar JE, Birnbaum MJ. Role of insulin-like growth factor-binding protein 5 (IGFBP5) in organismal and pancreatic beta-cell growth. *Mol Endocrinol.* 2010; 24:178–192. [PubMed: 19897600]
23. Jiménez-Palomares M, Cózar-Castellano I, Ganfornina MD, Sánchez D, Perdomo G. Genetic deficiency of apolipoprotein D in the mouse is associated with nonfasting hypertriglyceridemia and hyperinsulinemia. *Metabolism.* 2011; 60:1767–1774. [PubMed: 21632073]
24. Wang Y, Chen B, Shen D, Xue S. Osteopontin protects against cardiac ischemia-reperfusion injury through late preconditioning. *Heart Vessels.* 2009; 24:116–123. [PubMed: 19337795]
25. Wu Y, Jiang P, Lin Y, Chen S, Lin N, Li J. Expression of phosphorylated-STAT3 and osteopontin and their correlation in melanoma. *J Huazhong Univ Sci Technol Med Sci.* 2009; 29:246–250. [PubMed: 19399415]
26. Behera R, Kumar V, Lohite K, Karnik S, Kundu GC. Activation of JAK2/STAT3 signaling by osteopontin promotes tumor growth in human breast cancer cells. *Carcinogenesis.* 2010; 31:192–200. [PubMed: 19926637]
27. Zheng Y, Wang Z, Deng L, Yuan X, Ma Y, Zhang G, Gantier MP, Liu JP, Shen L, Xu D. Osteopontin promotes inflammation in patients with acute coronary syndrome through its activity on IL-17 producing cells. *Eur J Immunol.* 2012; 42:2803–2814. [PubMed: 22711477]
28. Yeh M, Gharavi NM, Choi J, Hsieh X, Reed E, Mouillesseaux KP, Cole AL, Reddy ST, Berliner JA. Oxidized phospholipids increase interleukin 8 (IL-8) synthesis by activation of the csrc/signal transducers and activators of transcription (STAT)3 pathway. *J Biol Chem.* 2004; 279:30175–30181. [PubMed: 15143062]
29. Gharavi NM, Alva JA, Mouillesseaux KP, Lai C, Yeh M, Yeung W, Johnson J, Szeto WL, Hong L, Fishbein M, Wei L, Pfeffer LM, Berliner JA. Role of the Jak/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids in vitro and in atherosclerosis in vivo. *J Biol Chem.* 2007; 282:31460–31468. [PubMed: 17726017]
30. Hartung F, Weber GF. RNA Blood levels of osteopontin splice variants are cancer markers. *SpringerPlus.* 2013; 2:110. [PubMed: 23543917]

Figure 1. Soft agar colony formation

Singly or doubly transfected MCF-7 cells were plated under anchorage-independent conditions. After 7 days, representative clones were photographed in 5 fields, such that 1 field was in the center of the plate and 4 fields covered each quadrant, and there were at least 2 clones in focus per field. The surface areas of all clones per plate were measured (in relative units, mean value for vector = 100%) with the imaging software Metamorph. This resulted in at least 20 measurements of clone areas per group. Because these areas follow a log-normal distribution, the numbers were converted to their logarithms so that the conventional t-test was applicable (after confirmation of equal variance between groups). * indicates $p < 0.05$ of the labeled bar compared to the bar to its left.

Figure 2. Soft agar clone formation by osteopontin-a, but not osteopontin-c depends on STAT3

The cells were plated in soft agar, medium was added every other day, and clone size was evaluated on day 8 or 10. **A**) MCF-7 transfectants with osteopontin constructs were plated in soft agar and were treated with 40 μ M (final concentration) of a cell-permeable STAT3 inhibitory peptide or control peptide with the exchange of medium every other day. **B**) Western blot of doubly transfected MCF-7 cells showing the expression of Flag-tagged STAT3 constructs and osteopontin. **C**) MCF-7 cells expressing osteopontin constructs were stably transfected with dominant-negative STAT3 or control vector. MCF-7 pCR3.1 cells were also transfected with constitutively active STAT3. The double-transfectants were compared for their ability to form clones in soft agar. dn = transfected with dominant negative STAT3, CA = transfected with constitutively active STAT3. **D**) Proliferation assay of MCF-7 OPNa double-transfected with vector or dominant-negative STAT3, as well as MCF-7 vector double-transfected with constitutively active STAT3 or vector control. V = vector, A = osteopontin-a.

Figure 3. Osteopontin-a supports anchorage-independence through distinct metabolic changes

A) Osteopontin-a induces STAT3 phosphorylation in poly-HEMA. Western blot of cell lysates from MCF-7 transfectants after plating on poly-HEMA. The top panel shows the expression of osteopontin in serum-free cell culture supernatants from the same cells. **B)** Pair wise comparison of the metabolic profiles induced by osteopontin-a versus vector control (top panel) and by osteopontin-a versus osteopontin-c (bottom panel). For osteopontin-c versus vector see Shi et al. (manuscript submitted). The graphs on the left show the score plots of principal component analysis with osteopontin-a samples in black and vector (top graph) or osteopontin-c (bottom graph) samples in blue. The asterisks indicate the centers of the corresponding clusters with the same color. The black lines connecting the asterisks represent the group distance (Mahalanobis distance). The graphs on the right are the loading plots. The colors indicate significant buckets after statistical analysis. The red squares are filtered by Bonferroni correction and the green triangles are corrected by Benjamini-Hochberg false discovery rate. **C)** Summary of metabolites changed by osteopontin-a versus vector control. For osteopontin-c versus osteopontin-a and osteopontin-c versus vector see Shi et al. (manuscript submitted). The table shows ppm values (indicating the middle values of the peaks), concentration estimates as the average values of 6 replicates, the standard deviations for six replicates of osteopontin-a (A) or vector (V); and the fold change for comparisons between osteopontin-a and vector control. Negative numbers indicate that the metabolite is higher in vector controls than in osteopontin-a transfected cells. The p-values are based on Bonferroni correction (italic) or Benjamini/Hochberg false discovery rate (non-italic).

Figure 4. Suppression of osteopontin-a signaling by dominant-negative STAT3

A) Western blot confirmation of osteopontin-a secretion in serum-free cell culture supernatant and expression of the transfected STAT3 construct in the lysate. Actin served as a loading control. **B)** Differences in metabolites (expressed as -fold change) between adherent MCF-7 OPNa dnSTAT3 cells and MCF-7 OPNa pCDNA3 cells (vector control). Negative numbers indicate that the metabolite is higher in MCF-7 OPNa pCDNA3 cells. The MCF-7 transfectants were plated at 1×10^6 cells per 25cm^2 flask for two days. The cells were then harvested and frozen at -80°C until extraction and metabolite analysis by NMR. The p-values are based on Bonferroni correction (italic) or Benjamini/Hochberg false discovery rate (non-italic). A = osteopontin-a, dnSTAT3 = dominant-negative STAT3.

Figure 5. Model for synergism between osteopontin splice variants in deadherent cells
 Osteopontin-a induces signal transduction that increases the cellular glucose levels. Osteopontin-c (Shi et al. manuscript submitted) utilizes the glucose to activate the mitochondrial respiratory chain and the hexose monophosphate shunt (hms) to provide the chemical energy required for anoikis escape.

Table 1
Induction of DNA binding proteins by osteopontin variants

MCF-7 cells transfected with OPNa, OPNc, or vector were plated in complete medium for 7 h to allow for adhesion. The medium was replaced with serum-free medium. After additional 13 hours, nuclear extracts were made and analyzed in the Protein/DNA array I (Panomics). The numbers represent relative density units.

	OPN-a	OPN-c	vector
NFATc	0.092	0.952	0.000
Stat3	0.979	0.014	0.000
Stat1	1.127	0.000	0.000
ARE	0.966	1.472	0.028

GO categories induced by osteopontin-a

MCF-7 cells were plated on poly-HEMA before RNA extraction and gene expression analysis with RNASEq. The table shows gene ontology categories that are significantly affected by osteopontin-a. FDR = false discovery rate.

Table 2

category ID	description	number of genes	zScore	p-value	FDR
GO:0045780	positive regulation of bone resorption	10	38.546	0	0
GO:0046852	positive regulation of bone remodeling	10	38.546	0	0
GO:0048668	collateral sprouting	9	40.678	0	0
GO:0048670	regulation of collateral sprouting	7	46.119	0	0
GO:0048681	negative regulation of axon regeneration	5	54.615	0	0
GO:0070571	negative regulation of neuron projection regeneration	6	49.853	0	0
GO:0048679	regulation of axon regeneration	12	35.259	1.25E-272	1.24E-269
GO:0070570	regulation of neuron projection regeneration	12	35.259	1.25E-272	1.24E-269
GO:0046697	decidualization	17	29.681	6.84E-194	6.02E-191
GO:0034105	positive regulation of tissue remodeling	17	29.528	6.37E-192	5.05E-189
GO:0001893	maternal placenta development	21	26.689	3.15E-157	2.27E-154
GO:0048640	negative regulation of developmental growth	22	25.980	4.14E-149	2.74E-146
GO:0045124	regulation of bone resorption	23	25.536	3.94E-144	2.40E-141
GO:0046850	regulation of bone remodeling	24	24.996	3.37E-138	1.91E-135
GO:0033280	response to vitamin D	26	23.925	8.38E-127	4.43E-124
GO:0031103	axon regeneration	28	23.172	4.39E-119	2.17E-116
GO:0010977	negative regulation of neuron projection development	28	23.006	2.04E-117	9.51E-115
GO:0007566	embryo implantation	29	22.669	4.50E-114	1.98E-111
GO:0031102	neuron projection regeneration	33	21.336	2.61E-101	1.09E-98
GO:0050840	extracellular matrix binding	33	21.163	1.04E-99	4.13E-97
GO:0034103	regulation of tissue remodeling	34	20.962	7.33E-98	2.77E-95
GO:0050771	negative regulation of axonogenesis	34	20.929	1.45E-97	5.23E-95
GO:0045453	bone resorption	38	19.832	7.94E-88	2.74E-85
GO:0061387	regulation of extent of cell growth	45	18.120	1.11E-73	3.66E-71
GO:0010811	positive regulation of cell-substrate adhesion	45	18.078	2.38E-73	7.54E-71