

Immunohistochemical expression of hyaluronic acid in the normal prostate, benign prostate hyperplasia and prostate carcinoma.

Anastasios Tahmatzopoulos¹, Eleni Papakonstantinou², Rodoula Kotakidou³,
Dimitrios Hatzihristou², George Karakiulakis^{2*}

¹*Departments of Urology, School of Medicine, Aristotle University*

²*Pharmacology, School of Medicine, Aristotle University*

³*Department of Pathology, Gennimatas Hospital, Thessaloniki Greece*

ABSTRACT: Hyaluronic acid (HA), a component of the extracellular matrix, is present in various tissues and tissue fluids. HA regulates cell adhesion and migration and it has been implicated in the progression of prostate cancer (PCa) as a molecule associated with the biological potential of PCa. The concentration of HA is elevated in several cancers, including bladder, colon, breast and Wilms' tumor. In this study, we compared the immunohistochemical expression of HA in the normal prostate, benign prostate hyperplasia (BPH) and prostate carcinoma. HA was immunohistochemically detected in 22 prostate tissues (6 histologically normal, 10 with BPH and 6 with PCa). Formalin fixed, paraffin-embedded sections were stained using an ABC method with biotinylated HA binding protein (B-HABP). Negative controls included sections incubated without B-HABP as well as sections incubated with *Streptomyces hyaluronidase*. In normal and BPH prostate glands staining was localized predominantly in the gland surrounding stroma, as well as in the fibrovascular core of the papillary projections of the glands. In prostate carcinoma samples the amount of HA in the stroma was markedly increased and staining was not localized around glandular structures but was diffuse throughout the stroma. There was a sharp diminution at the interface between tumor stroma and non-tumoral connective tissue. HA appears to be a supplementary tumor-associated marker. Insight gained in the mechanisms of increased production and hyaluronidase digestion of HA may eventually lead to new targets for pharmacological intervention in the treatment of PCa.

Key Words: Hyaluronic acid, Prostate cancer, Benign prostate hyperplasia.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is one of the most common disease processes affecting the aging male¹, while prostate cancer is the most common non-skin cancer among men worldwide² and the second leading cause of deaths from cancer in men in the United States and Europe³. Despite the prevalence and clinical implications of BPH and prostate cancer, surprising little is known about the pathophysiology of the diseases. Besides the efforts for pharmacotherapy and surgical treatment, there is considerable effort devoted

in detecting BPH and/or prostate cancer in the early stages, with curative intent, to decrease overall and disease specific morbidity and mortality⁴.

Besides hormones, cytokines and growth factors there is increasing evidence that molecules of the extracellular matrix (ECM) may be an alternative target to this end⁵. The ECM of the prostate contributes significantly in the structural framework of the organ⁶ and there is evidence that the ECM is involved in intercellular trafficking of steroid hormones and growth factors⁷ and in the regulation of epithelial cell proliferation⁸, indicating that BPH may be the consequence

Corresponding author: George Karakiulakis, 1st Department of Pharmacology, School of Medicine, Aristotle University, 541 24 Thessaloniki, Greece. e-mail: gkaraki@med.auth.gr

of alterations in ECM components affecting stromal - epithelial interactions.

ECM molecules, particularly glycoproteins, glycosaminoglycans (GAG) and proteoglycans, comprise a fibrous network that separates prostate stroma from epithelial elements. GAG molecules that is chondroitin and dermatan sulphates (CS, DS), hyaluronic acid (HA), heparin and heparan sulphate (HS), and keratan sulphate (KS)^{9,10} regulate cell proliferation, cell-to-cell interactions, as well as the action of growth factors. In pathologic situations, such as benign prostatic hyperplasia (BPH) and prostate cancer, GAG influence the diffusion rates of messenger molecules within and between tissues¹¹, affecting cell proliferation and/or migration¹²⁻¹⁴. GAG homeostasis in the prostate may be regulated by hormones because castration and androgen/oestrogen treatment significantly affect stromal deposition of prostate GAG¹⁵. With respect to individual GAG, DS has been reported to decrease¹⁶ and CS to increase cell proliferation^{15,16}. Increases in CS levels may be associated with hyperplastic changes of the prostate gland¹⁷⁻¹⁹, although we have previously shown that in patients with BPH the prostate content of CS is significantly enhanced, whereas DS is significantly reduced²⁰.

Among GAG, hyaluronic acid, which consists of repeating units of D-glucuronic acid and N-acetylglucosamine²¹, is of particular interest, since it has been shown that its synthesis is increased by cancer cells²² and to facilitate the proliferation and metastasis of cancer cells via its CD44 and RHAMM receptors²³⁻²⁶. Implantation of tumor cell lines that differ in their capacity to synthesize hyaluronic acid in mice indicated that the cell line with higher rate of hyaluronic acid synthesis gave significantly higher number of metastasis²⁷.

HA has been in focus for several clinical applications because it is an immunoneutral polysaccharide, found in abundance in the human body, and is essential in the pathophysiology of many cellular and tissue functions⁹⁻¹³. Chemically modified HA can be transformed into many physical forms, such as soft or stiff hydrogels, viscoelastic solutions, non-woven meshes, electrospun fibers, macroporous and fibrillar sponges, nanoparticulate fluids and flexible sheets for use in a range of preclinical and clinical settings²⁸. In

addition HA can be produced in stretches of varying molecular mass, apparently with different physiological functions⁹. Thus, endogenous levels of HA may be modified accordingly by administration or by intervening on the expression of enzymes involved in the homeostasis of HA⁹. HA synthases (termed HAS 1, HAS 2 and HAS 3) have distinct catalytic rates, resulting to HA products of different molecular mass: HAS 1 and HAS 2 synthesize relatively long stretches (2-4 x 10⁶ Da), associated with stress-induced conditions and wound healing, while HAS 3 polymerizes short stretches of disaccharide chains (0.4-2.5 x 10⁵ Da) that may be involved in activation of signal transduction⁹. HA is metabolized by hyaluronidases (HYAL), mainly by HYAL 1 and HYAL 2, present in various tissues⁹.

The aim of this study was to further clarify the involvement of hyaluronic acid in prostate pathology. We compared the immunohistochemical expression of hyaluronic acid in the normal prostate, BPH and prostate carcinoma. We found that the amount of hyaluronic acid in the stroma was markedly increased in prostate carcinoma samples indicating that hyaluronic acid may be a supplementary tumor-associated marker.

MATERIALS AND METHODS

Tissue sections: Twenty two prostate tissue samples were selected from the archives of the Department of Pathology, G. Gennimatas Hospital. The samples were fixed in formalin, embedded in paraffin, cut at 5µm and stained with haematoxylin and eosin for histological typing and grading. Six histologically normal prostate tissue samples from cystoprostatectomy specimens were included, as well as 10 benign prostatic hyperplasia samples. The results were compared to a group of 6 prostate cancer samples obtained during radical prostatectomy. The patients had not received hormonal therapy prior to radical prostatectomy.

Immunohistochemical staining

The sections were deparaffinized in xylene, rehydrated with graded alcohols and washed with PBS. Endogenous peroxidase was blocked with 0.5% H₂O₂ for 20 min and non-specific binding was blocked with 1% BSA in PBS for 30 min. The sections were incubated with biotinylated haluronic acid binding protein (B-

HABP, Seikagaku Corporation, Tokyo, Japan). The concentration of the protein was 2.5 $\mu\text{g/ml}$, diluted in PBS, and the incubation was carried out overnight at 4°C. The slides were then washed with PBS and treated with avidin-biotin-peroxidase (StreptABCComplex/HRP, Dako, Glostrup, Denmark) for 1h at room temperature according to the manufacturer's instructions. After further washing, the slides were treated with Sigma Fast (Sigma, St. Louis, USA) for color development according to the manufacturer's instructions. Finally, the preparations were washed in water, counterstained with Mayer's haematoxylin and finally dehydrated and mounted.

Negative controls included sections incubated without B-HABP as well as sections incubated with 50 U/ml *Streptomyces hyaluronidase* (Seikagaku Corporation, Tokyo, Japan) for 3h at 37°C.

Image analysis: Densitometric scanning of hyaluronic acid staining was performed using a computer-assisted image analysis program (1D Image Analysis Software, Kodak Digital Science v.3.0, Eastman Kodak, Rochester, NY, USA). Results were quantified and a staining score was calculated based on the area of staining and staining intensity.

RESULTS

Six different normal prostates and ten benign prostatic hyperplasia specimens were used to determine the localization and distribution of hyaluronan in nonmalignant prostate tissue. Staining was localized predominantly in the stroma surrounding normal and hyperplastic prostate glands (Figure 1A), as well as in the fibrovascular core of the papillary projections in those glands (Figure 1B). The smooth muscular element of the prostatic stroma did not stain, in contrast to the fibrous element. Few (<1%) epithelial cells, basal as well as cuboidal, showed cytoplasmic staining. Control slides treated first with hyaluronidase did not stain, showing the specificity of the probe (data not shown).

In contrast to normal and hyperplastic prostate tissue, there was a marked increase in the amount of hyaluronan in cancerous prostate in the six samples studied. Hyaluronan was distributed throughout the tumor stroma with a rather sharp diminution at the interface between tumor stroma and nontumoral connective tissue (Figure 1C). In contrast to nonmalig-

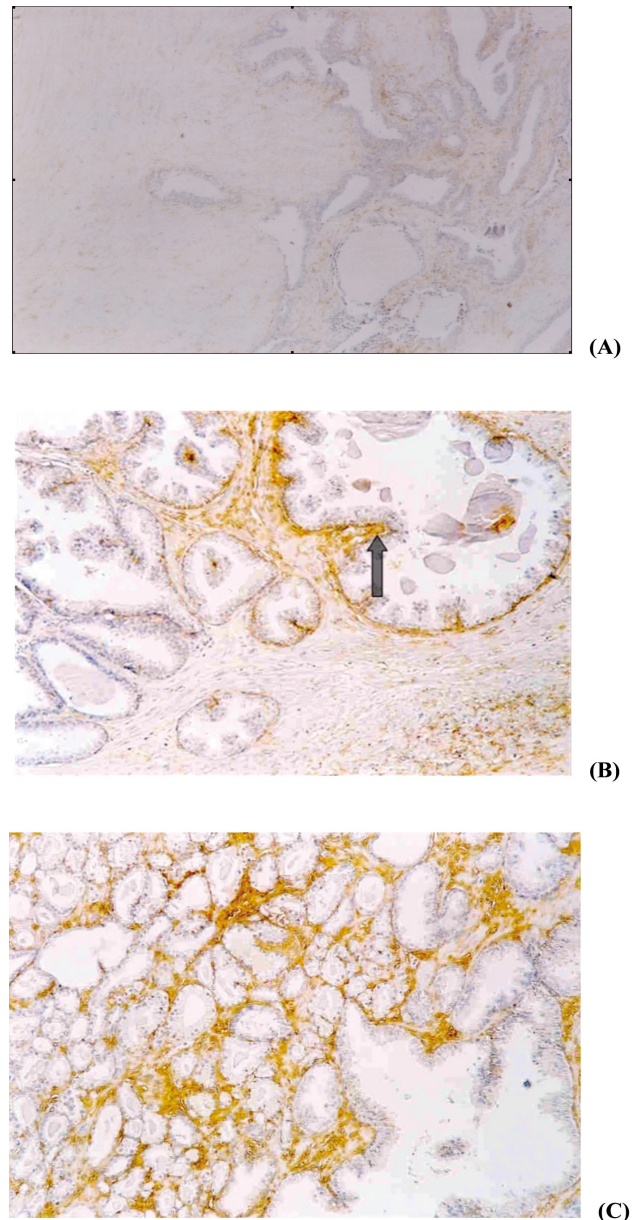


Figure 1. (A) Localization and distribution of hyaluronic acid in nonmalignant prostate tissue. Staining was localized predominantly in the stroma surrounding normal prostate glands. (250x). (B) Localization and distribution of hyaluronic acid in hyperplastic prostate glands. Staining was localized in the stroma surrounding BPH prostate glands, but predominantly in the fibrovascular core of the papillary projections (black arrow) of those glands. (250x). (C) Localization and distribution of hyaluronic acid in cancerous prostate. In this Gleason 3 prostate cancer specimen, hyaluronic acid is distributed throughout the tumor stroma with a rather sharp diminution at the interface between tumor stroma and nontumoral connective tissue. In contrast to nonmalignant tissue, staining in tumor samples was diffuse throughout the stroma and not predominantly localized around glandular structures. (250x).

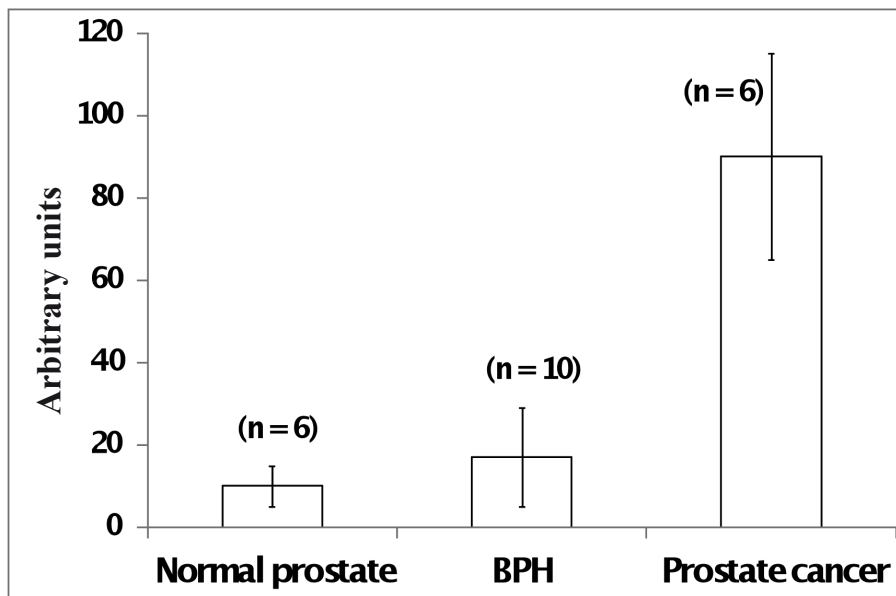


Figure 2. Immunohistochemical hyaluronic acid expression in normal, BPH and cancerous prostate glands. Staining score (vertical axis) was calculated based on the immunohistochemically positive staining area as well as staining intensity. Mean values \pm SEM for n samples are shown.

nant tissue, stromal staining was diffuse throughout the stroma and not predominantly localized around glandular structures. The presence of few (<1%) scattered malignant cells that showed cytoplasmic staining was noted.

Image analysis indicated that in prostate cancer specimen, hyaluronic acid was immunohistochemically overexpressed by as much as five-fold as compared to BPH or normal prostate (Figure 2), while the difference between BPH and benign prostate was non significant.

DISCUSSION

There were no statistical differences observed in the content of hyaluronic acid between normal prostate and BPH. In BPH, staining was localized in the stroma surrounding the glands, but predominantly in the fibrovascular core of the papillary projections of those glands. In contrast, there was considerable increase (5x) in hyaluronic acid staining in samples from prostates with cancer. In the latter case, staining was diffuse throughout the stroma and not predominantly localized around glandular structures.

This observation may be associated with the biology of cancer in the prostate gland and is in good

correlation with previous reports regarding hyaluronic acid and prostate or other tissues. In this respect it has been reported that high levels of hyaluronic acid in the stroma of prostate cancer are associated with low differentiation, high mitotic activity and metastasis²⁹. Furthermore, hyaluronic acid has been shown to interfere with the distribution and absorption of antitumor agents³⁰ and hyaluronidase enhanced the penetration of the chemotherapeutic doxorubicin in lung and laryngeal squamous carcinoma cell lines³¹. It has also been reported that hyaluronic acid may inhibit immune responses³², while small fragments of hyaluronic acid (3 to 16 disaccharides) promote angiogenesis³³ and a hexasaccharide enhances endothelial cell migration and lumen formation of new vessels³⁴.

Although hyaluronic acid has not been shown to offer any advantages over other markers for prostate cancer, it appears that this GAG may be proved to be a supplementary tumor-associated marker. Insight gained in the mechanisms of controlling the homeostasis of hyaluronic acid via increased production and hyaluronidases or hyaluronic acid synthases may eventually lead to new targets for pharmacological intervention in the treatment of human prostate cancer.

Διαφορική έκφραση του υαλουρονικού οξέος μεταξύ φυσιολογικού προστάτη, καλοήθους υπερπλασίας και προστατικού καρκινώματος.

Αναστάσιος Ταχματζόπουλος¹, Ελένη Παπακωνσταντίνου², Ροδούλα Κωτακίδου³,
Δημήτριος Χατζηχρήστου¹, Γεώργιος Καρακιουλάκης²

¹Ουρολογική κλινική, Ιατρική Σχολή,

²Εργαστήριο Φαρμακολογίας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

³Παθολογοανατομικό Εργαστήριο Νοσοκομείου «Γ. Γεννηματάς»

ΠΕΡΙΛΗΨΗ: Το υαλουρονικό οξύ (HA) είναι ένα βασικό συστατικό της εξωκυττάριας θεμέλιας ουσίας το οποίο, μεταξύ άλλων, ρυθμίζει σημαντικές κυτταρικές λειτουργίες, όπως η προσκόλληση και η μετανάστευση των κυττάρων. Η συγκέντρωση του HA αυξάνεται σε ορισμένα είδη καρκίνου, συμπεριλαμβανομένων των καρκίνων της ουροδόχου κύστης, του παχέος εντέρου, του μαστού και του όγκου του Wilms. Στον καρκίνο του προστάτη το HA έχει ενοχοποιηθεί ως μόριο που σχετίζεται με το βιολογικό δυναμικό του εν λόγω καρκίνου. Το HA ανιχνεύτηκε ανοσοϊστοχημικά σε 22 δείγματα προστατικού ιστού (6 ιστολογικά φυσιολογικά, 10 με ΚΥΠ και 6 με καρκίνωμα του προστάτη). Χρησιμοποιήθηκαν τομές παραφίνης μονιμοποιημένες σε φορμαλίνη στις οποίες εφαρμόστηκε η ανοσοϊστοχημική μέθοδος ABC με βιοτινυλιωμένη πρωτεΐνη δέσμησης του HA (B-HABP). Για τον έλεγχο της χρώσης χρησιμοποιήθηκαν τομές επωασμένες χωρίς B-HABP καθώς και τομές επωασμένες με *Streptomyces hyaluronidase*. Η χρώση ήταν εντοπισμένη κατά κύριο λόγο στο στρώμα που περιέβαλλε τους φυσιολογικούς και υπερπλαστικούς προστατικούς αδένες καθώς και στον αγγειοσυνδετικό μίσχο των θηλωδών προσεκβολών στους αδένες αυτούς. Η ποσότητα του HA στο στρώμα των καρκινικών εστιών ήταν σημαντικά αυξημένη, ενώ η χρώση στις περιοχές αυτές ήταν μάλλον διάχυτη σε όλο το στρώμα και όχι κυρίως εντοπισμένη γύρω από τους αδενικούς σχηματισμούς. Παράλληλα, υπήρχε σαφής μετάπτωση της χρώσης στα όρια μετάβασης από καρκινικό στρώμα σε μη καρκινικό συνδετικό ιστό. Η διαφορική κατανομή του HA μεταξύ φυσιολογικού προστάτη, καλοήθους υπερπλασίας και προστατικού καρκινώματος υποδεικνύει ότι το βιομόριο αυτό μπορεί να αποδειχθεί χρήσιμο στην κατανόηση των μοριακών μηχανισμών που σχετίζονται με την παθοφυσιολογία του προστάτη αδένου.

Λέξεις Κλειδιά: Υαλουρονικό οξύ, Καρκίνος προστάτη, Καλοήθης υπερπλασία προστάτη.

REFERENCES

- Berry SJ, Coffey DS, Walsh PC. The development of human benign prostatic hyperplasia with age. *J Urol* 1984;132:474-9.
- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58:71-96.
- Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010;60:277-300.
- Lin K, Lipsitz R, Miller T, Janakiraman S. Benefits and harms of prostate-specific antigen screening for prostate cancer: an evidence update for the U.S. Preventive Services Task Force. *Ann Intern Med* 2008;149:192-9.
- Kyprianou N, Isaacs JT. Expression of transforming growth factor- β in the rat ventral prostate during castration-induced programmed cell death. *Mol Endocrinology* 1989;3:1515-22.
- Udayakumar TS, Jeyaraj DA, Rajalakshmi M, Sharma RS. Culture of prostate epithelial cells of the rhesus-monkey on extracellular matrix substrate: influence of steroids and insulin-like growth factors. *J Endocrinol* 1999;162:443-50.
- Kassen AE, Sensibar JA, Sintich SM, Pruden SJ, Kozlowski JM, Lee C. Autocrine effect of DHT on FGF signaling and cell proliferation in LNCaP cells: role of heparin/heparan degrading enzymes. *Prostate* 2000;44:124-32.
- McConnell. Epidemiology, etiology, pathophysiology and diagnosis of benign prostatic hyperplasia. In: Walsh PC, Wein AJ, Retik AB, Vaughan EDJr, editors. *Campbell's Urology* (7th ed.). Philadelphia: W.B. Saunders Co; 1998;1429-52.
- Papakonstantinou E, Karakioulakis G. The 'sweet' and

- 'bitter' involvement of glycosaminoglycans in lung diseases: pharmacotherapeutic relevance. *Br J Pharmacol* 2009;157:1111-27.
10. Papakonstantinou E, Roth M, Karakiulakis G. Isolation and characterization of glycosaminoglycans from human atheromatous vessels. *Methods Mol Med*. 2001;52:123-36.
 11. Nishimura M, Yan W, Mukudai Y, Nakamura S, Nakamasu K, Kawata M, et al. Role of chondroitin sulfate-hyaluronan interactions in the viscoelastic properties of extracellular matrices and fluids. *Biochim Biophys Acta* 1998;1380:1-9.
 12. Papakonstantinou E, Karakiulakis G, Eickelberg O, Perruchoud AP, Block LH, Roth M. A 340 kDa hyaluronic acid secreted by human vascular smooth muscle cells regulates their proliferation and migration. *Glycobiology* 1998;8:821-30.
 13. Papakonstantinou E, Roth M, Block LH, Mirtsou-Fidani V, Argiriadis P, Karakiulakis G. The differential distribution of hyaluronic acid in the layers of human atheromatic aortas is associated with vascular smooth muscle cell proliferation and migration. *Atherosclerosis* 1998;138:79-89.
 14. Mogyorosi A, Ziyadeh FN. Increased decorin mRNA in diabetic mouse kidney and in mesangial and tubular cells cultured in high glucose. *Am J Physiol* 1998;275:F827-32.
 15. Terry DE, Clark AF. Glycosaminoglycans in the three lobes of the rat prostate following castration and testosterone treatment. *Biochem Cell Biol* 1996;74:653-8.
 16. Passi A, Albertini R, Campagnari F, De Luca G. Modifications of proteoglycans secreted into the growth medium by young and senescent human skin fibroblasts. *FEBS Lett* 1997;402:286-90.
 17. De Klerk DP. The glycosaminoglycans of normal and hyperplastic prostate. *Prostate* 1983;4:73-81.
 18. Iida S, Suzuki K, Matsuoka K, Takazono I, Shimada A, Inoue M, et al. Analysis of glycosaminoglycans in human prostate by high-performance liquid chromatography. *Br J Urol* 1997;79:763-9.
 19. Cardoso LE, Falcao PG, Sampaio FJ. Increased and localized accumulation of chondroitin sulphate proteoglycans in the hyperplastic human prostate. *BJU Int* 2004;93:532-8.
 20. Goulas A, Hatzichristou DG, Karakiulakis G, Mirtsou-Fidani V, Kalinderis A, Papakonstantinou E. Benign hyperplasia of the human prostate is associated with tissue enrichment in chondroitin sulphate of wide size distribution. *Prostate* 2000;44:104-10.
 21. Papakonstantinou E, Kouri FM, Karakiulakis G, Klagas I, Eickelberg O. Increased hyaluronic acid content in idiopathic pulmonary arterial hypertension. *Eur Respir J* 2008;32:1504-12.
 22. Knudson W, Biswas C, Toole BP. Interactions between human tumor cells and fibroblasts stimulate hyaluronate synthesis. *Proc Natl Acad Sci USA* 1984;81:6767-71.
 23. Ichikawa T, Itano N, Sawai T, Kimata K, Koganehira Y, Saida T, et al. Increased synthesis of hyaluronate enhances motility of human melanoma cells. *J Invest Dermatol* 1999;113:935-9.
 24. Miyake H, Hara I, Okamoto I, Gohji K, Yamanaka K, Arakawa S, et al. Interaction between CD44 and hyaluronic acid regulates human prostate cancer development. *J Urol* 1998;160:1562-6.
 25. Hall CL, Turley EA. Hyaluronan: RHAMM mediated cell locomotion and signaling in tumorigenesis. *J Neurooncol* 1995;26:221-9.
 26. Herrlich P, Morrison H, Sleeman J, Orian-Rousseau V, König H, Weg-Remers S, et al. CD44 acts both as a growth- and invasiveness-promoting molecule and as a tumor-suppressing cofactor. *Ann N Y Acad Sci* 2000;910:106-18; discussion 118-20.
 27. Zhang L, Underhill CB, Chen L. Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. *Cancer Res* 1995;55:428-33.
 28. Burdick JA, Prestwich GD. Hyaluronic acid hydrogels for biomedical applications. *Adv Mater*. 2011 23:H41-56.
 29. Lipponen P, Aaltomaa S, Tammi R, Tammi M, Agren U, Kosma VM. High stromal hyaluronan level is associated with poor differentiation and metastasis in prostate cancer. *Eur J Cancer* 2001;37:849-56.
 30. Höbarth K, Maier U, Marberger M. Topical chemoprophylaxis of superficial bladder cancer with mitomycin C and adjuvant hyaluronidase. *Eur Urol* 1992;21:206-10.
 31. Kohno N, Ohnuma T, Truog P. Effects of hyaluronidase on doxorubicin penetration into squamous carcinoma multicellular tumor spheroids and its cell lethality. *J Cancer Res Clin Oncol* 1994;120:293-7.
 32. McBride WH, Bard JB. Hyaluronidase-sensitive halos around adherent cells. Their role in blocking lymphocyte-mediated cytotoxicity. *J Exp Med* 1979;149:507-15.
 33. West DC, Kumar S. The effect of hyaluronate and its oligosaccharides on endothelial cell proliferation and monolayer integrity. *Exp Cell Res* 1989;183:179-96.
 34. Banerjee SD, Toole BP. Hyaluronan-binding protein in endothelial cell morphogenesis. *J Cell Biol* 1992;119:643-52.