Tumor Microenvironment Modulates Hyaluronan Expression: The Lactate Effect

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Hyaluronan (HA) synthesis is a tightly regulated process and is partly controlled by the microenvironment (e.g., lactate concentration). Experimental evidence has indicated that the melanoma cells that synthesize large amounts of HA exhibit enhanced tumor cell growth and increased metastatic capacity compared to those expressing smaller amounts. Because most studies have examined HA expression on melanoma cells *in vitro*, we compared the patterns of HA expression by B16-F1 and B16-F10 melanoma cells *in vitro* and *in situ*. Cell surface HA expression was assessed with the HA-binding peptide Pep-1. B16-F1 melanoma cells showed significantly higher levels of Pep-1 binding compared with B16-F10 cells *in vitro*. On the other hand, expression levels of HA were comparable between B16-F1 and B16-F10 melanoma cells in cryostat sections. These results show that B16-F1 cells express high levels of HA *in vitro* and *in vivo*, while B16-F10 cells express high concentrations of HA only in the context of skin tumors. Finally, B16-F10 melanoma cells, but not B16-F1 cells, expressed high concentrations of HA after stimulation with lactate. We propose that components of the tumor microenvironment (e.g., lactate) can induce melanoma cells to express HA and thus acquire an aggressive phenotype.

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INTRODUCTION

Hyaluronan (HA) is an unbranched, nonsulfated glycosaminoglycan composed of repeating subunits of D-*N*-acetylglucosamine and D-glucuronic acid. Although originally considered an inert structural biopolymer, HA is now known to be involved in numerous biological activities, including: (a) fertilization (Sliwa, 1999; Zhuo *et al.*, 2001), (b) embryonic development (Camenisch *et al.*, 2000), (c) wound healing (Jameson *et al.*, 2005), and (d) leukocyte trafficking (Mummert *et al.*, 2000). HA has also been implicated in the progression of many types of cancer, including breast cancer (Aaltomaa *et al.*, 2002; Udabage *et al.*, 2005), prostate cancer (Wernicke *et al.*, 2003), and malignant melanoma (Zhang *et al.*, 1995; Karjalainen *et al.*, 2000).

HA is synthesized by the HA synthases (HAS) which utilize uridine diphosphate-glucuronic acid and uridine diphosphate-*N*-acetylglucosamine as substrates. Three HAS enzymes (designated as HAS1, HAS2, and HAS3) have been identified in humans and mice. These enzymes have distinct catalytic activities (HAS3 > HAS2 > HAS1) as well as their final products. HAS3 polymerizes short stretches of disaccharide chains, while HAS1 and HAS2 synthesize relatively long stretches (reviewed in Weigel *et al.*, 1997). Various stimuli (e.g., phorbol esters, tumor growth factor β, platelet-derived growth factor, retinoic acid, epidermal growth factor, and tumor growth factor α) have been shown to modulate HA synthesis in subsets of skin cells (fibroblasts and keratinocytes) (Tammi *et al.*, 1989; Agren *et al.*, 1995; Ogawa *et al.*, 1998; Pienimaki *et al.*, 2001).

Over the past several years, there has been significant interest in the functions of HA in tumor progression (reviewed in Toole, 2002). In fact, HA has been suggested to play several important roles in tumor biology, including: (a) tumor growth (Kosaki *et al.*, 1999; Xu *et al.*, 2003), (b) angiogenesis (Deed *et al.*, 1997; Trochon *et al.*, 1997; Savani *et al.*, 2001), (c) tumor cell invasion (Fieber *et al.*, 2004), and (d) metastasis (Zhang *et al.*, 1995). The concentration and distribution of HA may also be important for tumor cell behavior. For example, melanoma cells that express high concentrations of cell surface HA exhibit enhanced motility (Ichikawa *et al.*, 1999) and increased metastatic capacity (Zhang *et al.*, 1995) compared to those expressing smaller amounts.

Despite the potential importance of HA in tumor progression, most studies have examined HA expression *in vitro* and have not examined the impact of the tumor microenvironment on melanoma cell HA expression. As previous reports (Winkelhake and Nicolson, 1976) have shown that B16-F10 melanoma cells have a more malignant phenotype (i.e., enhanced metastatic potential and increased invasive properties) relative to B16-F1 melanoma cells, we compared their HA expression profiles *in vitro* and *in situ*. Herein, we report that B16-F1 melanoma cells expressed HA polymers on their

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Abbreviations: GFP, green fluorescent protein; HAS, HA synthase; HA'se, hyaluronidase; PE, phycoerythrin

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surfaces abundantly, while B16-F10 melanoma cells showed little, if any, HA expression *in vitro*. On the other hand, B16-F10 tumors were indistinguishable from B16-F1 tumors in terms of HA expression levels as well as HA distribution patterns *in situ*. As lactate is found in tumors at high concentrations and has been shown to stimulate HA synthesis by fibroblasts (Stern *et al.*, 2002), we investigated the potential of lactate to stimulate melanoma cells to synthesize HA. B16-F10 melanoma cells, but not B16-F1 melanoma cells, expressed soluble HA and cell surface HA in high concentrations when cultured in the presence of lactate. HA polymers were bound on both B16-F1 and B16-F10 melanoma cell surfaces at least partially by CD44. Finally, profiles of CD44 mRNA showed that lactate induced splice variant transcripts in B16-F10 melanoma cells, but not in B16-F1 cells. Our results may suggest that components in the tumor microenvironment (e.g., lactate) can stimulate subsets of melanoma cells to synthesize HA, alter CD44 expression and thus acquire an aggressive phenotype.

RESULTS

Expression of HAS mRNAs

We first examined the potential of B16-F1 and B16-F10 melanoma cells to express the mRNAs for HA synthesis (HAS1–3). Results of the RT-PCR showed that the mRNAs for all the three HAS (HAS1–3) were present, indicating the potential for both B16-F1 and B16-F10 melanoma cells to synthesize HA (Figure 1).

Expression of HA by melanoma cells *in vitro*

B16-F1 and B16-F10 melanoma cells transfected with green fluorescent protein (GFP) were grown in Lab-Tek chambers and stained for HA 24 hours later using biotinylated Pep-1 as our probe. Previous studies have shown that Pep-1 is a HA-specific probe (Zmolik and Mummert, 2005). As shown in Figure 2, Pep-1 showed significant binding to the surfaces of B16-F1 melanoma cells (Figure 2a), but not B16-F10 melanoma cells (Figure 2b). Binding of Pep-1 to B16-F1 melanoma cells was specific for HA, as shown by the dramatic reduction in Pep-1 binding following pretreatment with *Streptomyces* hyaluronidase (HA'se) (Figure 2a). Furthermore, we failed to detect binding of the scrambled peptide control to B16-F1 and B16-F10 melanoma cells (Figure 2a and b), showing the specificity of the Pep-1 amino acid sequence for molecular interaction with HA.

Expression of HA by melanoma tumors *in situ*

As described in the Introduction, a number of studies have suggested a role for HA in the growth and metastasis of tumor cells *in vitro* and *in vivo*. In order to test the impact of the

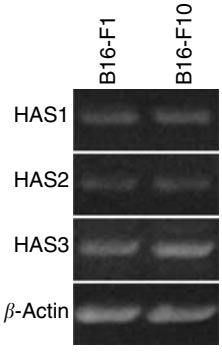

Figure 1. Expression of mRNA for HAS enzymes. B16-F1 and B16-F10 melanoma cells were examined for the expression of HAS1–3 mRNAs by RT-PCR. Data shown are the ethidium bromide-stained PCR products separated on a 1% agarose gel.

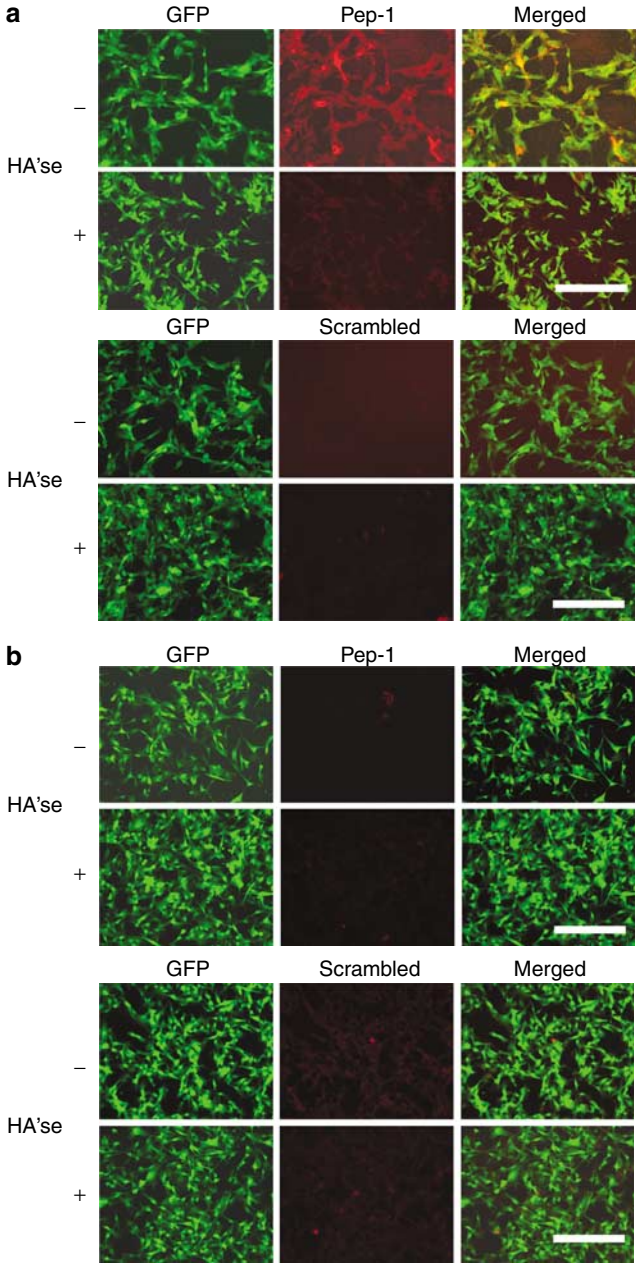

Figure 2. Expression of HA on the surfaces of melanoma cells. (**a**) B16-F1 and (**b**) B16-F10 melanoma cells were fixed in paraformaldehyde and stained by anti-green fluorescent protein (GFP), Pep-1, and the scrambled control. Some samples were pretreated with *Streptomyces* HA'se to show HA specificity. Images are of original magnification × 200 (Bar = 100 μm).

tumor microenvironment on HA synthesis by melanoma cells, we next examined the expression of HA *in situ*.

Cryostat sections of B16-F1 melanoma tumors showed high concentrations of HA associated with the surfaces of tumor cells. Pep-1 binding to tumors was reduced significantly by pretreating tissue sections with *Streptomyces* HA'se, showing HA specificity. Once again, we failed to detect staining with the scrambled peptide control, showing the requirement for the Pep-1 amino acid sequence for HA binding (Figure 3a). In contrast to results obtained *in vitro*, Pep-1 showed dramatic binding to B16-F10 melanoma tumors that was comparable with the staining intensities obtained for B16-F1 tumors (Figure 3b). These results show that B16-F1 melanoma cells constitutively expressed HA polymers *in vitro* and *in situ*, while B16-F10 melanoma cells expressed high concentrations of HA only in the context of the tumor microenvironment. Hence, the local environment of some tumor cell types may induce HA synthesis.

It has been previously shown that soluble factors and physical interactions between melanoma cells and fibroblasts can augment HA synthesis (Knudson *et al.*, 1984; Edward, 2001). Thus, it is possible that HA synthesized by fibroblasts may be responsible for the HA content that we observed *in situ*. To further examine this possibility, we evaluated HA synthesis by B16-F1 and B16-F10 cells when cultured in the absence and presence of mouse fibroblasts. Briefly, tumor cells were grown in an anchorage-independent manner to form three-dimensional cell layers termed spheroids. Importantly, spheroids mimic *in vivo* tumor growth in regard to pH (Avarez-Pérez *et al.*, 2005), oxygen tension, and nutrient gradients (Walenta *et al.*, 2000). We found that B16-F1 and B16-F10 melanoma cells synthesized comparable concentrations of HA ($\sim 20\,\mu g/10^4$ cells). Multicellular B16-F10 tumor spheroids containing fibroblasts produced only slightly more HA than B16-F10 spheroids lacking the fibroblast component ($22.2 \pm 0.3\,\mu g/10^4$ cells vs $20.8 \pm 0.6\,\mu g/10^4$ cells, respectively; $P < 0.05$). Similarly, B16-F1 multicellular spheroids produced slightly more HA than B16-F1 spheroids alone, though the difference was insignificant ($20.4 \pm 0.6\,\mu g/10^4$ cells vs $20.0 \pm 2.3\,\mu g/10^4$ cells, respectively; $P > 0.05$). These results show the potential of the B16 melanoma cells themselves to synthesize most of their own HA under conditions in the tumor microenvironment.

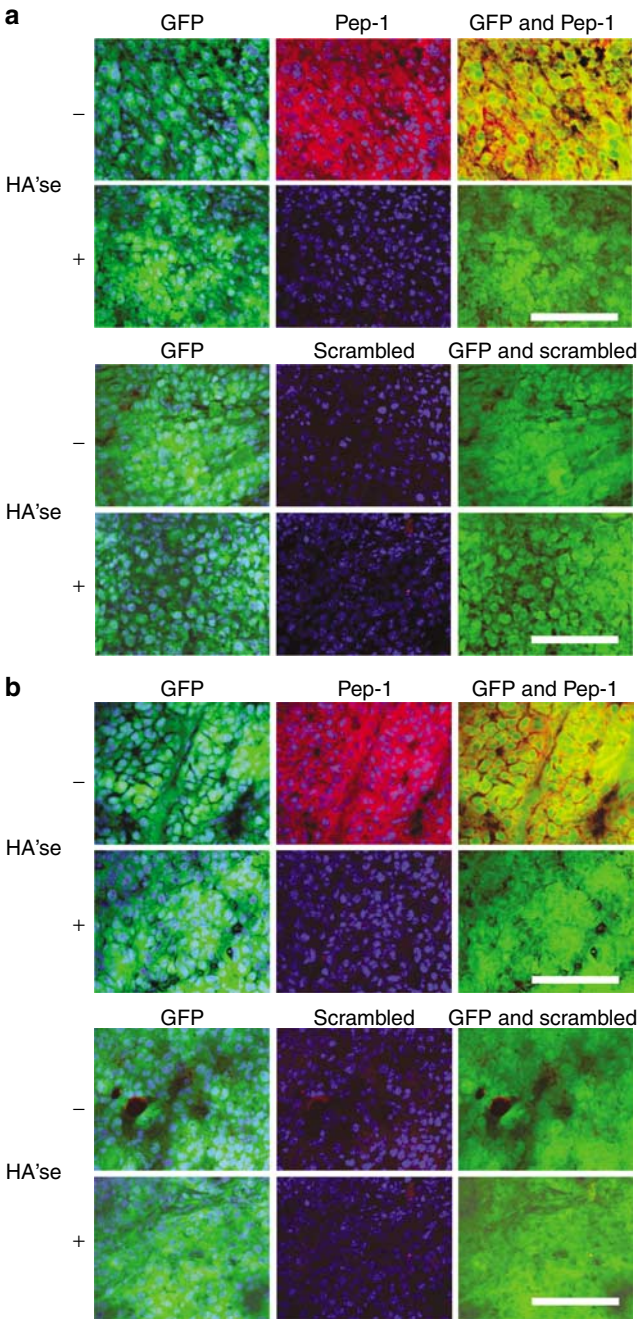

Figure 3. HA expression profiles in melanoma tumors. (**a**) Cryostat sections of B16-F1 and (**b**) B16-F10 tumors in mouse ear skin were stained with anti-GFP, Pep-1, and the scrambled control. Nuclei of cells (blue) were stained with the Hoechst 33258 dye. Some samples were pretreated with *Streptomyces* HA'se to show HA specificity. Images are of original magnification × 400 (Bar = 100 μm).

Lactate production by melanoma cell monolayers, tumors, and spheroids

Tumors produce lactate under both aerobic and hypoxic conditions. Moreover, lactate has been shown to stimulate some cell types to synthesize HA (Stern *et al.*, 2002). Thus, we tested if B16-F1 and B16-F10 melanomas produced lactate *in vitro* and *ex vivo*. First, we measured concentrations of lactate in the supernates of B16-F1 and B16-F10 cultures. A lactate dehydrogenase assay showed that lactate concentrations were comparable between B16-F1 (19 μg/ml) and B16-F10 (14 μg/ml) melanoma cells cultured as monolayers. Next, we measured lactate concentrations in tumors *ex vivo*. Differences between the sizes of B16-F1 melanoma tumors (191 ± 33 mg) and B16-F10 melanoma tumors (211 ± 88 mg) were insignificant ($P > 0.05$). Both B16-F1 and B16-F10 tumors showed significantly higher concentrations of lactate compared to normal skin (Figure 4a). On the other hand, B16-F10 tumors contained significantly higher concentrations of lactate compared with B16-F1 tumors ($P < 0.01$, Figure 4a).

As lactate concentrations determined *ex vivo* most likely include lactate produced by stromal cells and contaminating skin cells in addition to the lactate production by melanoma cells, we next evaluated lactate produced in spheroid cultures of B16-F1 and B16-F10 melanoma cells. B16-F1 and B16-F10 spheroids both secreted lactate (Figure 4b). However, B16-F10 spheroids secreted significantly more lactate than B16-F1 spheroids ($P<0.01$). These results are consistent with the notion that B16-F10 cells produce more lactate than B16-F1 melanoma cells in the context of the tumor microenvironment.

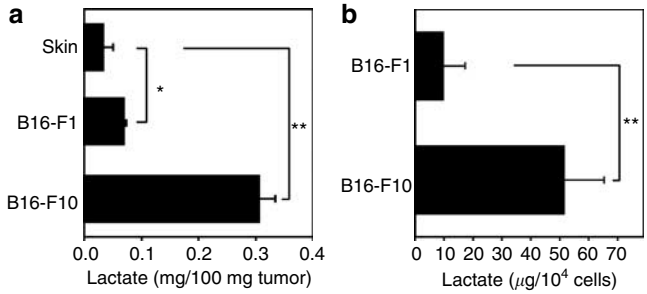

Figure 4. Lactate concentrations in melanoma tumors and spheroids. (**a**) Normal skin, B16-F1 tumors, and B16-F10 tumors were harvested from perfused C57BL/6 mice and the lactate contents determined with a lactate dehydrogenase assay. (**b**) B16-F1 and B16-F10 melanoma cells were grown as spheroids and lactate contents in the culture supernates determined with a lactate dehydrogenase assay. Values are expressed as means ± SD from triplicate samples. Asterisks indicate statistically significant differences (*$P<0.05$; **$P<0.01$) assessed by the Student's *t*-test.

Induction of HA synthesis by exogenous lactate

Based on our findings that B16-F10 melanoma cells express HA in the tumor microenvironment (where concentrations of lactate are high) but not *in vitro* (where lactate concentrations were relatively low), we hypothesized that the high lactate concentrations present in tumors stimulated B16-F10 melanoma cells to synthesize HA. To test this concept, we examined the impact of lactate to stimulate B16-F1 and B16-F10 melanoma cells to synthesize HA. Synthesis of HA polymers by melanoma cells was first assessed based on the uptake of ^{3}H-glucosamine. As shown in Figure 5a, B16-F1 melanoma cells synthesized similar concentrations of HA even when cultured in the presence of 20 mM lactate ($P>0.05$). By contrast, B16-F10 melanoma cells synthesized significantly more HA when cultured in the presence of 20 mM lactate ($P<0.05$). These results suggest that B16-F10 cells, but not B16-F1 melanoma cells, can be induced to synthesize HA polymers upon exposure to lactate. Next, we cultured B16-F1 and B16-F10 melanoma cells in Lab-Tek

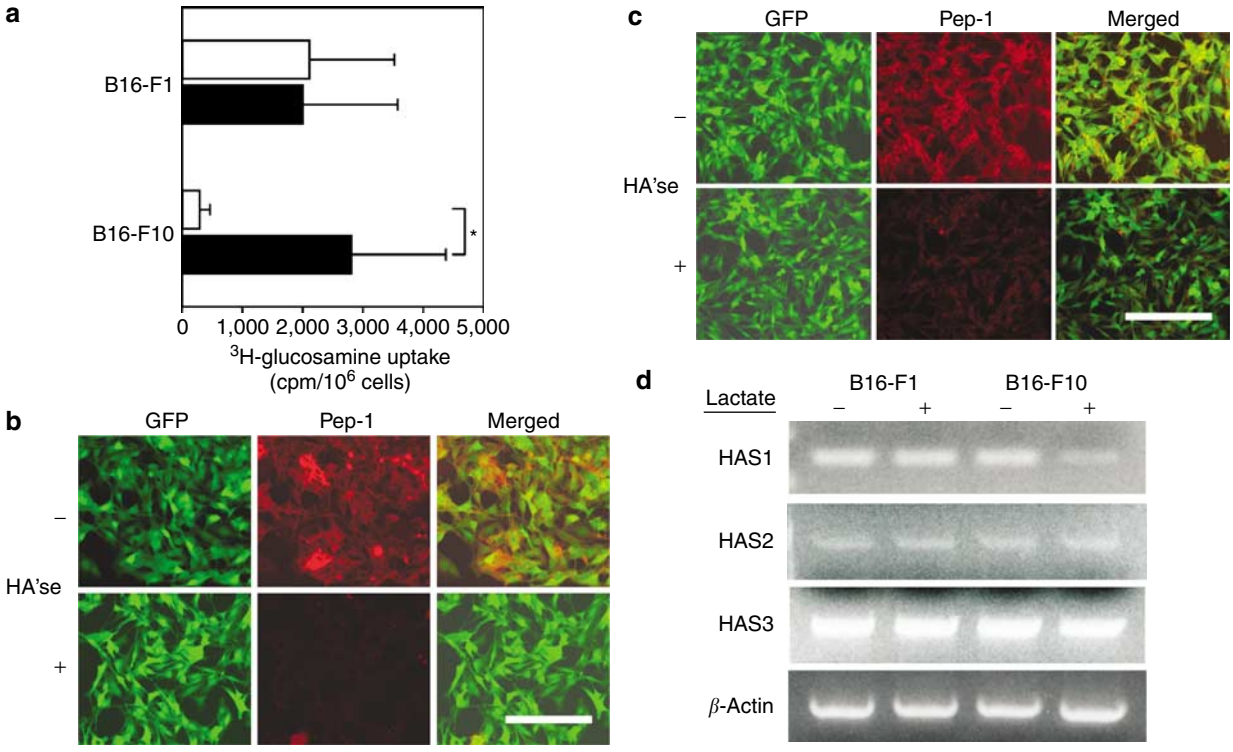

Figure 5. Impact of lactate on HA synthesis by melanoma cells. (**a**) B16-F1 and B16-F10 melanoma cells were metabolically labeled with ^{3}H-glucosamine in medium alone (open bars) or medium containing 20 mM lactate (closed bars). The data shown are the amounts of *Streptomyces* HA'se-sensitive radioactivities in culture supernates. Values are expressed as means ± SD from triplicate samples. Statistically significant differences are indicated by an asterisk (*$P<0.05$) assessed by the Mann–Whitney *U*-test. (**b**) B16-F1 and (**c**) B16-F10 melanoma cells were fixed in paraformaldehyde and stained by anti-GFP and Pep-1. Some samples were pretreated with *Streptomyces* HA'se to show HA specificity. Images are of original magnification × 400 (Bar = 100 μm). (**d**) B16-F1 and B16-F10 melanoma cells were cultured with and without lactate and examined for the expression of HAS1–3 mRNAs by RT-PCR. Data shown are the ethidium bromide-stained PCR products separated on a 1% agarose gel.

chambers containing graded concentrations of lactate. B16-F10 melanoma cells, but not B16-F1 melanoma cells, expressed HA in a dose-dependent manner (data not shown), with the highest HA expression at 20 mM lactate (Figure 5b). These results show that lactate can stimulate B16-F10 melanoma cells to express HA. Importantly, the pH of the culture media with 20 mM lactate was similar to the pH of culture media without the addition of lactate (pH 6.97 ± 0.03 vs 7.05 ± 0.03, respectively). Next, we examined the potential of lactate to induce surface expression of HA in human WM-115 and WM-266-4 melanoma cells. Both WM-115 and WM-266-4 expressed low concentrations of HA. Moreover, HA expression was variable, with not all of the melanoma cells expressing this polymer. Unlike B16-F10 melanoma cells, we failed to detect surface expression of HA after lactate stimulation in either WM-115 or WM-266-4 cells (data not shown). These results show that not all melanoma cells express HA in response to induction by lactate.

To investigate the possibility that lactate altered HA synthesis in B16-F10 melanoma cells by regulating transcription of the HAS genes, we compared the mRNA profiles of HAS1, HAS2, and HAS3 in B16-F1 and B16-F10 melanoma cells. We found that the mRNA profiles of HAS1–3 were similar between B16-F1 and B16-F10 melanoma cells even after stimulation with lactate (Figure 5d). These results suggest that lactate does not alter transcription of the HAS genes.

Finally, as a first step in identifying other factors that could induce melanoma cells to synthesize HA, we have investigated the role of retinoic acid in stimulating HA expression in B16-F10 melanoma cells *in vitro*. Retinoic acid is known to stimulate HA synthesis possibly by regulating the *HAS2* gene (Prehm, 1980; Saavalainen *et al*., 2005). However, we found that retinoic acid did not induce the expression of HA in B16-F10 melanoma cells *in vitro* (data not shown).

Potential role of CD44 to serve as a HA receptor on melanoma cells

CD44 is a major cell surface receptor for HA and has been shown to retain HA on the surfaces of some cell types (Nandi *et al*., 2000). Moreover, CD44 may play a role in regulating HA synthesis on melanoma cell surfaces (Lüke and Prehm, 1999). Thus, we investigated the potential of CD44 to serve as a receptor for HA on B16-F1 and B16-F10 melanoma cells. As shown in Figure 6a, expression levels of CD44 were comparable between B16-F1 and B16-F10 tumors *in situ*. These results show that CD44 remained expressed by melanoma cells in the context of the tumor microenvironment. Next, we cultured B16-F1 and B16-F10 melanoma cells in media containing different concentrations of lactate. Expression levels of CD44 were not altered at any of the tested concentrations of lactate, as measured by FACS (Figure 6b). As shedding of CD44 from the surfaces of cells results in decreased FACS staining, we interpreted these results to suggest that lactate does not induce cleavage of CD44 from the surfaces of B16-F1 or B16-F10 melanoma cells (Bazil and Horejsi, 1992; Annabi *et al*., 2005). Next, B16-F1 and B16-F10 melanoma cells were cultured in the presence of 20 mM lactate in Lab-Tek chambers. As shown in Figure 6c, CD44 signals (red) and HA signals (blue) significantly overlapped (purple in the merged images). These results strongly suggest that CD44 serves as a receptor for retaining HA on the surfaces of B16-F1 and B16-F10 melanoma cells.

CD44 is a family of glycoproteins produced by expression of variant exons and post-translational modifications (reviewed in Zhou *et al*., 1999). As molecular interaction of HA with CD44 isoforms generated by the expression of splice variants may impact the behavior of tumor cells *in vivo*, we examined the CD44 profiles of B16-F1 and B16-F10 melanoma cells after lactate stimulation. Both B16-F1 and B16-F10 melanoma cells expressed the standard CD44 isoform when cultured in medium alone. On the other hand, lactate stimulation of B16-F10 melanoma cells resulted in the appearance of bands for CD44 splice variants in addition to the standard CD44 isoform. Lactate did not alter the mRNA profile of CD44 in B16-F1 melanoma cells (Figure 6d). These results may suggest that lactate generates different CD44 isoforms in B16-F10 cells that could potentially contribute to the malignant phenotype of this melanoma cell line.

DISCUSSION

HA is a high-molecular-weight glycosaminoglycan expressed by many different tumor cell types, including malignant melanomas. Far from an inert structural biopolymer, HA also has multiple roles in tumor progression, including tumor growth and metastasis. As HA expression is a tightly regulated process, it is important to determine how the tumor microenvironment impacts the synthesis of HA by melanoma cells. Identification of factors in the tumor microenvironment that stimulate HA synthesis may represent targets for therapeutic intervention or serve as markers for prognosis.

We have investigated the expression levels and distribution patterns of HA by B16-F1 and B16-F10 melanoma cells *in vitro* and *in situ*. Our results show that B16-F1 melanoma cells express HA polymers *in vitro* and in the context of the tumor microenvironment. By contrast, B16-F10 melanoma cells expressed high concentrations of HA *in situ* but not *in vitro*. Based on these observations, we conclude that the tumor microenvironment can stimulate some melanoma cell types to express HA and thus acquire an aggressive phenotype. Alternatively, these results could be interpreted to suggest that HA is not a marker for aggressive tumor cell behavior, because both aggressive (B16-F10) and relatively non-aggressive (B16-F1) melanoma cells expressed comparable concentrations of HA *in vivo* and after stimulation with lactate *in vitro*. We propose that HA expression by itself may not be sufficient for malignant cell behavior. Instead, overexpression of HA coupled with molecular interaction with the appropriate CD44 isoform may be required for HA-mediated tumor cell behavior. For example, Bourguignon *et al*. (2003) found that a variant form of CD44 (CD44v3) binds to HA, leading to activation of phosphatidylinositol-3-kinase and Akt signaling, thus promoting the tumor cell phenotype of breast tumor cells. Interestingly, we found that lactate results in the expression of variably spliced mRNAs for CD44 in B16-F10 melanoma cells. Based on these results, we propose that lactate induces HA synthesis and alters the

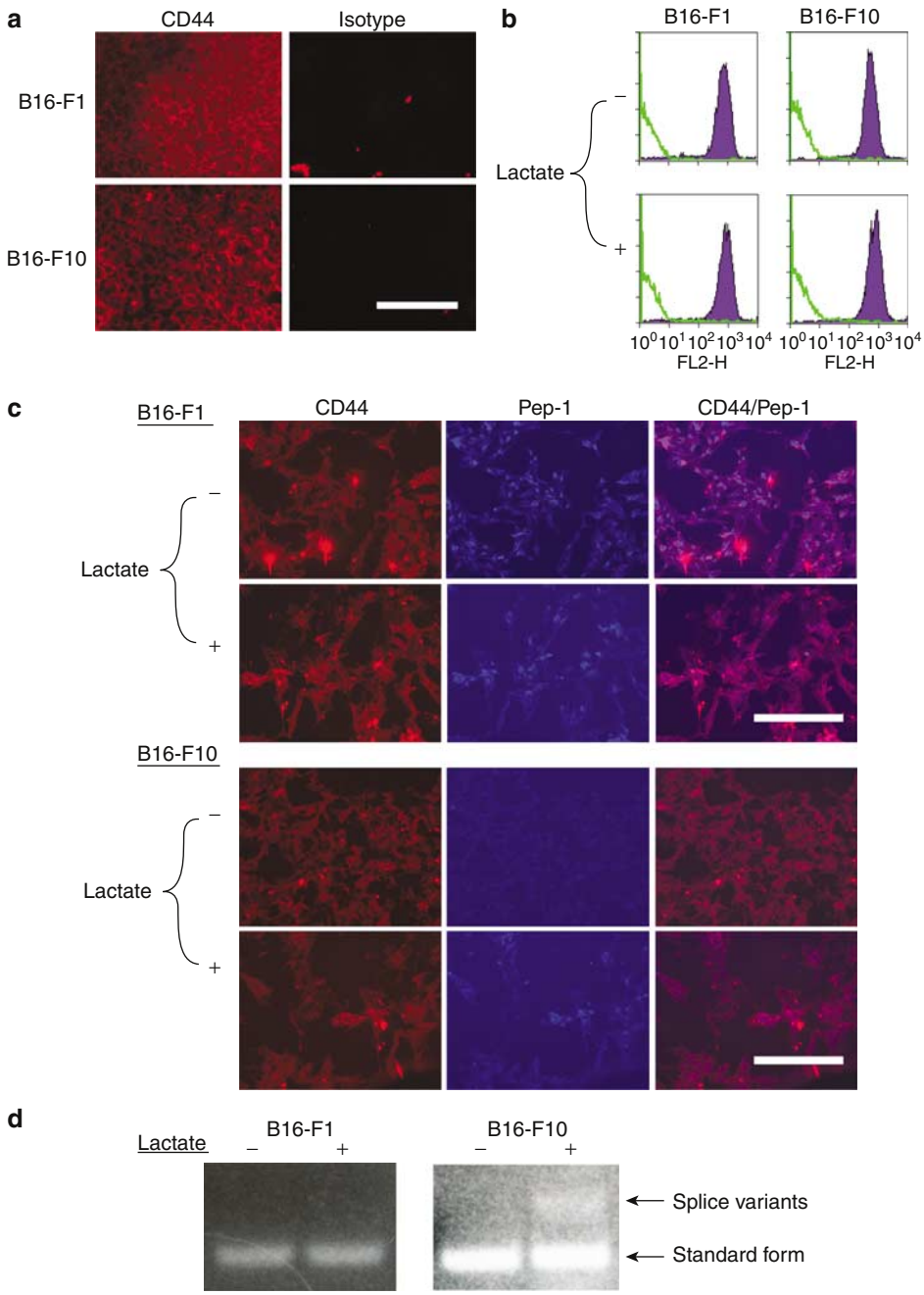

Figure 6. Effect of lactate on CD44 expression by melanoma cells. (**a**) Cryostat sections of B16-F1 and B16-F10 tumors established in mouse ear skin were stained with anti-CD44 or isotype control antibodies. Images are of original magnification × 400 (Bar = 100 μm). (**b**) B16-F1 and B16-F10 melanoma cells were grown in medium alone or lactate containing medium. After 16 hours, cells were stained with anti-CD44 (closed histograms) or isotype control (open histograms) antibodies and subjected to FACS. Results are shown for 20 mM lactate. (**c**) B16-F1 and B16-F10 melanoma cells were fixed in paraformaldehyde and stained by anti-CD44 and Pep-1. Images are of original magnification × 400 (Bar = 100 μm). (**d**) B16-F1 and B16-F10 melanoma cells were treated with 20 mM lactate and CD44 transcripts evaluated by RT-PCR using a primer pair to simultaneously detect the standard CD44 isoform and splice variants. The upper band corresponds to one or more CD44 splice variants, while the lower band is the standard CD44 isoform.

expression of CD44 isoforms in B16-F10 melanoma cells, thus leading to their aggressive behavior. We are currently cloning these splice variants into vectors to learn their molecular identities by sequencing.

What factor(s) stimulate melanoma cells to express HA in the context of the tumor microenvironment? As a first attempt to identify a factor that stimulates melanoma cells to express HA, we tested the possibility that lactate induces melanoma cells to synthesize HA *in vitro*. Our rationale for choosing lactate was based on the following: (a) the content of lactate in tumors is known to be enhanced due to hypoxia and/or the Warburg effect (Brizel *et al.*, 2001), and (b) lactate has been

shown to stimulate some cell types (e.g., fibroblasts) to synthesize HA (Stern *et al.*, 2002). We first tested lactate concentrations in B16-F1 and B16-F10 tumors and found that both tumor types contained enhanced concentrations of lactate relative to normal mouse skin. Interestingly, B16-F10 tumors contained significantly more lactate than B16-F1 tumors. Thus, the lactate content of melanoma tumors may be determined by the melanoma cell types that compose the tumor. Addition of exogenous lactate to B16-F10 cultures, but not B16-F1 cultures, stimulated melanoma cells to synthesize soluble HA polymers as well as express high concentrations of surface-associated HA. Cell surface retention of HA was at least partially mediated by CD44 in both B16-F1 and B16-F10 melanoma cells, as assessed under fluorescence microscopy. These results show that some melanoma cell types (i.e., B16-F10 but not B16-F1) initiate HA synthesis in response to lactate. As HA expression has been reported to confer an aggressive phenotype to tumor cells, it is tempting to speculate that lactate-responsive melanoma cells would in turn correlate with enhanced tumor progression. In support of this concept, Brizel *et al.* (2001) have reported that a high concentration of lactate in tumors is correlated with an increased risk of metastasis in head-and-neck cancer. On the other hand, our results with WM-115 and WM-266-4 melanoma cells shows that lactate does not enhance HA expression in all melanoma cell types. Therefore, lactate may promote aggressive tumor cell behavior in an HA-independent fashion in some melanoma cells.

What mechanisms account for lactate-induced HA synthesis by B16-F10 cells but not B16-F1 melanoma cells? Results of the RT-PCR showed that mRNAs for HAS1–3 were expressed in both B16-F1 and B16-F10 melanoma cells even after stimulation with lactate. Therefore, differences in HA synthetic capacity between these two melanoma cells due to gain or loss of biosynthetic enzymes appear unlikely. More importantly, B16-F10 melanoma cells expressed cell-associated and soluble HA polymers at concentrations that were comparable with B16-F1 melanoma cells after stimulation with lactate. Lüke and Prehm (1999) have shown that the regulation of the HA synthetic machinery is highly complex and is at least partially controlled by CD44 proteolysis and dissociation of nascent HA chains from the cell membrane. Thus, one possible explanation for our results is that lactate induced proteolysis of CD44 from the surfaces of B16-F10 cells, triggering HA synthesis. However, levels of CD44 expressed by B16-F10 melanoma cells were not significantly altered after stimulation with lactate, as shown by FACS. A number of CD44 isoforms can be generated (due to expression of splice variants or post-translational modifications), which differ markedly in their molecular interaction with HA (reviewed in Zhou *et al.*, 1999). For example, phosphorylation of CD44 can reduce HA-binding activity and may promote HA synthesis (Lüke and Prehm, 1999). Thus, lactate may induce expression of a CD44 variant, which initiates HA synthesis in B16-F10 melanoma cells via altered molecular interaction with HA polymers. Our RT-PCR results showed that B16-F10 melanoma cells expressed splice variants of CD44 transcripts after lactate treatment. Finally, enzymatic digestion of HA from the membrane surface has been reported to enhance rates of HA synthesis (Philipson *et al.*, 1985; Larnier *et al.*, 1989). Therefore, a third possibility is that lactate stimulates the expression of HA'ses in B16-F10 melanoma cells. HA'se-mediated digestion of the low levels of HA constitutively expressed by B16-F10 melanoma cells could then trigger deposition of newly synthesized HA on the surfaces of melanoma cells, as well as secretion of soluble HA polymers. Interestingly, Formby and Stern (2003) have recently shown that lactate significantly increased mRNAs for two known HA'ses (HA'se-1 and -2) and suggested that lactate may augment HA synthesis by digesting cell-associated HA.

We should note that the tumor milieu is complex in terms of cellular content (tumor cells, interstitial fibroblasts, and infiltrating cells of the immune system), growth factors, and extracellular matrix components. Thus, it is possible that factors other than lactate contribute to HA expression by melanoma tumors *in vivo*. For example, Edward *et al.* (2005) has recently shown that melanoma cells grown on contracted collagen lattices, but not on tissue culture plates, express high concentrations of HA *in vitro*. Collagen is found in the microenvironment of some melanoma tumors (Brown *et al.*, 2003), and thus may play a role in stimulating melanoma cells to synthesize HA.

In conclusion, we have shown that the tumor microenvironment can stimulate some melanoma cell types to synthesize HA. Lactate, a major metabolite in melanoma tumors, is a potential candidate for stimulating a subset(s) of melanoma cells to synthesize HA in the context of skin tumors. Our results provide a technical and conceptual framework to further investigate the effect of the tumor microenvironment on HA synthesis by melanoma cells as well as other tumor cell types.

MATERIALS AND METHODS

Animals and cell lines

C57BL/6 mice (6–8 weeks) were obtained from breeding colonies maintained at the University of Texas Southwestern Medical Center. All animal experiments were approved by the Institutional Review Board at the University of Texas Southwestern Medical Center. The B16-F1 and B16-F10 murine melanoma cells and WM-115 and WM-266-4 human melanoma cells were purchased from American Type Culture Collection (Manassas, VA) and maintained as suggested by the supplier. Finally, NS47 mouse fibroblasts were maintained as described previously (Xu *et al.*, 1995).

Preparation of enhanced green fluorescent protein melanoma cells

The pHRGFP-1 vector (Stratagene, Inc., La Jolla, CA) containing the humanized recombinant *GFP* gene was cloned as a *Bam*HI/*Eco*RI fragment into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA). B16-F1 and B16-F10 melanoma cells were transfected with the GFP/pcDNA3.1 vector using the SuperFect reagent (Qiagen, Valencia, CA) as described by the manufacturer. Transfected cells were selected in medium containing hygromycin (500 μg/ml) and fluorescent cells collected by FACS. B16-F1 cells and B16-F10 melanoma cells expressing GFP were used for all experiments.

Generation of spheroids

B16-F1 and B16-F10 melanoma spheroids were generated similarly as in a report described before (Walker *et al.*, 2004). Briefly, 4×10^3 melanoma cells in a volume of 200 μl were cultured on top of solidified agar (0.75% wt/vol) in 96-well plates for 9 days. For multicellular spheroids, melanoma cells were mixed in a 3:1 ratio of B16 cells to fibroblasts and cultured on agar as above.

RT-PCR analyses

Total RNA was isolated from melanoma cells and the mRNA reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the directions from the manufacturer. Primers and reaction conditions for detecting HAS1–3 were as described previously (Mummert *et al.*, 2002). In order to detect CD44 splice variants, we used primers flanking variant exons 1–10 as described previously (Yu *et al.*, 1996).

Peptide synthesis

Pep-1 (GAHWQFNALTVR) or the scrambled peptide control (WRHGFALTAVNQ) was synthesized by Invitrogen using standard fMOC chemistry as described previously (Zmolik and Mummert, 2005). Briefly, an amidated and biotinylated lysine residue was included at the C-terminus of the linker sequence (GGGS). Stock solutions were prepared by dissolving peptides in dH_2O to 1 mg/ml and were stored at −20°C.

Immunocytochemistry

B16-F1 and B16-F10 cells expressing GFP were grown in wells of Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) at 37°C and 5% CO_2 for 24 hours. After washing three times in PBS, cells were fixed with 4% paraformaldehyde for 30 minutes at 4°C. Fixed cells were blocked with PBS containing 5% BSA and 5% normal goat serum for 1 hour at room temperature before permeabilizing with 0.1% Triton X-100 for 15 minutes at room temperature. GFP was detected by incubating cells with goat anti-GFP antibody (BD Pharmingen, San Diego, CA; 1:200 dilution) overnight at 4°C, followed by a 1-hour incubation at room temperature with fluorescein 5 isothiocyanate (FITC)-conjugated rabbit anti-goat antibody (Zymed Laboratories, South San Francisco, CA; 1:200 dilution). For detection of cell surface HA, fixed cells were incubated with biotinylated Pep-1 (5 μg/ml) or the biotinylated scrambled peptide control (5 μg/ml) for 1 hour at 37°C before permeabilization with Triton X-100. Bound peptides were detected with phycoerythrin (PE)-conjugated streptavidin diluted 1:200 for 1 hour at room temperature. Some samples were digested with *Streptomyces* HA'se (Sigma Aldrich, St Louis, MO) at 100 U/ml or mock treated with enzyme buffer alone (100 mM sodium acetate, pH 5.0) overnight at 4°C before adding biotinylated peptides to assess HA specificity.

In order to assess surface expression of CD44, cells were fixed in paraformaldehyde as above, blocked with 5% BSA, and incubated for 1 hour at room temperature with PE-conjugated anti-CD44 monoclonal antibody IM7 (BD Pharmingen) or PE-conjugated isotype control (BD Pharmingen) (both diluted 1:100 in 5% BSA). Next, cells were permeabilized with 0.1% Triton X-100 and GFP expression assessed with goat anti-GFP antibody exactly as described above. To assess the molecular interaction of CD44 with HA, fixed cells were incubated with biotinylated Pep-1 or biotinylated scrambled peptide control as described above. Bound peptides were detected with Alexa Fluor 350-conjugated streptavidin (Molecular Probes (Invitrogen), Eugene, OR) diluted 1:500 for 1 hour at room temperature. Some samples were digested with *Streptomyces* HA'se (100 U/ml) or mock treated with enzyme buffer alone (100 mM sodium acetate, pH 5.0) overnight at 4°C before adding biotinylated peptides to assess HA specificity.

Immunohistochemistry

Melanoma cells expressing GFP were inoculated subcutaneously into the ears (10^6 cells/ear) of C57BL/6 mice. When tumors reached ~3 mm in diameter mice were killed, the ears embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), snap frozen in liquid N_2, and cryostat sections prepared (8 μm thick). Cryostat sections were fixed for 30 minutes at 4°C in 4% paraformaldehyde, followed by blocking for 15 minutes in 0.1 M glycine at room temperature. After washing the slides with PBS, tissues were blocked for an additional 1 hour at room temperature with 5% BSA and 5% normal goat serum in PBS. Tissues were permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature and incubated overnight at 4°C with goat anti-GFP diluted 1:50. After washing the slides in PBS, samples were incubated with FITC-conjugated rabbit anti-goat antibody diluted 1:50 and incubated for 1 h at room temperature. Finally, the nuclei of cells were stained with Hoechst 33258 (Molecular Probes) as described by the manufacturer. In order to detect HA associated with tumor cell surfaces, samples were incubated with biotinylated Pep-1 (5 μg/ml) or the biotinylated scrambled peptide control (5 μg/ml) for 1 hour at room temperature before permeabilization. Bound peptides were detected with PE-conjugated streptavidin diluted 1:200. Some samples were digested with *Streptomyces* HA'se (100 U/ml) or mock treated with enzyme buffer alone (100 mM sodium acetate, pH 5.0) before adding biotinylated peptides to assess HA specificity.

Estimation of lactate concentrations in tumors and spheroids

Melanoma tumors established in the abdominal skin of C57BL/6 mice (grown to ~6 mm in diameter) were excised after first perfusing the animals. Tumors were snap frozen in liquid N_2, ground to a powder using a mortar and pestle, and suspended in 1 ml of PBS. After centrifugation to remove insoluble material, the concentrations of lactate were determined with a lactate dehydrogenase assay (R-BiopharmAG, Darmstadt, Germany). Concentrations of lactate were calculated exactly as described by the manufacturer and expressed as milligrams of lactate per 100 milligrams of tumor weight.

To estimate the concentrations of lactate secreted by spheroids, we collected supernates from day 9 cultures. Lactate concentrations in the culture supernates were determined using the lactate dehydrogenase assay as described above. To normalize lactate concentrations with respect to cell numbers, B16-F1 and B16-F10 spheroids were dispersed by incubating in trypsin/EDTA for 5 minutes at 37°C and cells counted under microscopy with a hemacytometer. Results were expressed as micrograms of lactate per 10^4 cells.

HA production by spheroids

Supernates from B16 melanoma spheroids or from multicellular spheroids (composed of melanoma cells and fibroblasts) were collected from day 9 cultures. The concentration of HA polymers was determined using the biotinylated hyaluronic acid binding

protein assay as described before (Kongtawelert and Ghosh, 1990). hyaluronic acid binding protein was purchased from Seikagaku Corp. (Tokyo, Japan) and biotin labeled with *N*-hydroxysuccinimido biotin (Pierece Chemical Co., Rockford, IL) as described by the manufacturer.

Impact of lactate on HA synthesis

To assess the impact of lactate on HA synthesis, B16-F1 and B16-F10 melanoma cells were cultured in media containing 20 mM lactate (Sigma Aldrich) and ^{3}H-glucosamine (Amersham Biosciences, Piscataway, New Jersey) at 1 μCi/ml. Culture supernates were collected 20 hours later and digested for 5 hours with bovine pancreatic protease (30 U/ml) at 37°C. After boiling, half of each sample was digested for 16 hours with *Streptomyces* HA'se (10 U/ml), while the other half was mock treated with buffer alone (100 mM sodium acetate, pH 5.0). Samples were again boiled and glycosaminoglycans precipitated by adding an equal volume of 2% cetylpyridinium chloride/0.04 M NaCl in the presence of carrier HA (100 μg/ml). Pellets were dissolved in 4.0 M guanidine-HCl, precipitated with ethanol, and radioactivities measured in a β-counter. Finally, to examine the effect of lactate on HA expression, we grew B16-F1 and B16-F10 melanoma cells in Lab-Tek chambers with media containing graded doses of lactate (2.5, 10, and 20 mM). Melanoma cells were fixed and stained for surface-associated HA moieties as described above.

Impact of lactate on CD44 expression

To test the impact of lactate on the expression of CD44, B16-F1 and B16-F10 melanoma cells were cultured in media containing graded concentrations of lactate (2.5, 10, and 20 mM). After 20 hours, cells were harvested and incubated with PE-conjugated anti-CD44 monoclonal antibody IM7 or PE-conjugated isotype control (both antibodies were diluted 1:1000) in PBS containing 1% fetal calf serum. Antibodies were incubated with cells on ice for 30 minutes, washed in PBS containing 1% fetal calf serum, and subjected to FACS.

Statistics

Groups were compared pairwise using either the two-tailed Student's *t*-test or the two-tailed Mann–Whitney *U*-test. Differences between groups were considered significant for $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Effect of lactate on HA synthesis by human melanoma cells.

Figure S2. Impact of retinoic acid on HA expression by B16-F10 melanoma cells.

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