Size-dependent regulation of Snail2 by hyaluronan: Its role in cellular invasion

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Hyaluronan (HA) induces changes in cellular behavior that are crucial during both embryonic development and cancer progression. However, the biological effects of varying sizes of HA and the signal transduction mechanisms that these polymers may activate remain unclear. In this study, we demonstrate that pulse stimulation of mouse embryonic fibroblasts with high-molecular-weight (HMW) HA, but not HA of lower molecular sizes, leads to increases in Snail2 protein, which are dependent on NFκB activity. Involvement of CD44, the main HA receptor, in these responses was determined by use of a CD44 blocking antibody and CD44 siRNA. Both the blockade and silencing of CD44 significantly abrogates the increases in nuclear factor kappaB (NFκB) activity and Snail2 protein following HMW-HA stimulation. Furthermore, we show that HMW-HA induces cellular invasion and that inhibition of CD44, Snail2, or NFκB significantly decreases this response. These studies elucidate a novel HA/Snail2 functional connection through CD44 and NFκB that is important for the induction of cellular invasion and is dependent on HA size.

Keywords: CD44/cellular invasion/hyaluronan/NFκB/Snail2

Introduction

Hyaluronan or hyaluronic acid (HA) is a linear glycosaminoglycan comprising repeating disaccharide units of glucuronic acid and N-acetylg glucosamine. This relatively simple molecule is a critical structural component of the extracellular matrix, but it also acts as a signaling initiator, thus eliciting significant changes in cellular behavior. In vertebrates, HA is produced by hyaluronan synthases and exported into the extracellular space as a high-molecular-mass polymer (~1 × 10⁶ Da) (Philipson et al. 1985; Ng and Schwartz 1989). However, HA can be cleaved by hyaluronidases into small oligosaccharides which have been shown to exert distinct biological activity from that of their high-molecular-weight counterparts (West et al. 1985; West and Kumar 1989; Spicer and Tien 2004). This functional difference between HAs of varying molecular masses is a matter of controversy as many studies show opposing results in regard to which type of HA can bring about cellular changes (McKee et al. 1997; Kim et al. 2007). These discrepancies may be due to differences in experimental settings, purity of HA reagents (McDonald and Camenisch 2003), and the possibility of diverse responses to HA depending on the cell type.

Interactions between HA and its principal cell surface receptor, CD44 (Aruffo et al. 1990) can lead to the activation of intracellular signaling pathways which mediate changes in cell morphology, proliferation, migration, and invasion (Bourguignon et al. 1997; Ouhtit et al. 2007). These effects on cellular behavior play a crucial role during normal developmental processes as well as during cancer progression. CD44 is a single-spanning transmembrane glycoprotein that is expressed in a wide variety of tissues (Screaton et al. 1992). Alternative splicing of the CD44 gene gives rise to many CD44 isoforms which bind HA with different affinities (Lesley et al. 1993; Dougherty et al. 1994). The ability of CD44 to bind HA appears to be regulated not only by structural variations in the CD44 extracellular domain but also by posttranslational modifications, such as phosphorylation and glycosylation (Bartolazzi et al. 1996; Naor et al. 1997). One of the proposed signaling effectors following the HA/CD44 interaction in cancer cells is the transcriptional regulator nuclear factor kappaB (NFκB) (Kim et al. 2007). However, it remains to be elucidated whether HA can activate NFκB during normal physiological processes such as embryonic development. NFκB is involved in cell survival, immune function, epithelial-to-mesenchymal transition (EMT), and cellular invasion (Bourguignon et al. 1997; Bachelder et al. 2005; Wu and Kral 2005; Vasko et al. 2007). This transcription factor is present in the cytosol complexed to an inhibitory kappaB (IκB) monomer. Signals that induce NFκB activity cause the phosphorylation of IκB and subsequent dissociation of the inhibitory complex thereby allowing free NFκB to translocate to the nucleus and activate target genes. Elevated NFκB expression has been associated with colon and breast cancer progression (Huber et al. 2004; Simianonaki et al. 2007). Additionally, NFκB is actively expressed during early embryonic development and its inhibition leads to the abnormal formation of cardiac structures such as the valves and septum (Hernandez-Gutierrez et al. 2006). Furthermore, although the mechanisms by which NFκB becomes activated during development are poorly understood, overexpression of NFκB in frog embryos has been shown to increase the transcription of many genes, including Snail family members (Zhang et al. 2006).

Snail2 (formerly Slug) is a vertebrate-specific member of the Snail superfamily of zinc-finger transcription factors. Snail2 promotes EMT during both embryonic development and tumor progression (Castro Alves et al. 2007; Eastham et al. 2007) and also plays an important role in cell migration, proliferation, and protection from apoptosis (Barrallo-Gimeno and Nieto 2005). Despite both HA and Snail2 being associated with similar
physiological and pathological events, no functional connection between these molecules has been established to this point.

Although HA is able to bind CD44 in fibroblast cells (Kothapalli et al. 2007; David-Raoudi et al. 2008; Mitsui et al. 2008), it has been shown that this HA/CD44 interaction can lead to significant HA degradation and CD44 internalization after 5 h of stimulation with HA (Culty et al. 1992). To circumvent the possibility of decreased signaling due to receptor turnover, here we perform a 30-min pulse stimulation with HA, followed by washes and a 24-h incubation period prior to measuring changes in cellular behavior.

In this study, we demonstrate that high-molecular-weight (HMW) HA induces NFκB activity and Snail2 expression in mouse embryonic fibroblasts and that this response is mediated by the CD44 receptor. We also show that pulse stimulation with HMW-HA promotes cellular invasion and that CD44, NFκB, and Snail2 are important effectors of this response. Similar experiments utilizing medium-, low-, and ultra-low-molecular-weight HA (MMW-HA, LMW-HA, and UMW-HA, respectively) did not show any changes in cellular invasion, proliferation, or NFκB signaling. These results indicate that, in fibroblast cells, the biological response to HA is dependent on the size of this polymer, with HMW-HA being the most relevant form.

Results

**HMW-HA induces NFκB activity in a CD44-dependent manner**

In initial experiments, mouse embryonic fibroblasts (NIH-3T3 cells) were transiently transfected with NFAT, SRE, AP-1, and NFκB-alkaline phosphatase reporter constructs to screen for activation of these transcription factors by HA stimulation. We observed a robust induction of NFκB activity but no significant increases in the activities of NFAT, SRE, or AP-1 following stimulation with HMW-HA (150 μg/mL) (Figure 1). Thus, HMW-HA appears to selectively mobilize NFκB activity. Next, we assessed whether the endogenous HA coat surrounding NIH-3T3 cells may affect the ability of exogenous HA to bind the cell surface and activate intracellular proteins. For this, cells transfected with NFAT, SRE, AP-1, and NFκB-alkaline phosphatase reporter constructs were incubated with hyaluronidase prior to the addition of exogenous hyaluronan. As shown in Figure 1, destruction of the hyaluronan-containing pericellular matrix by treatment with hyaluronidase did not significantly change the ability of exogenous HA to induce NFκB activity.

To further investigate the effect of HMW-HA on NFκB activation, cells were transiently transfected with the NFκB–SEAP reporter vector, treated with varying concentrations of HMW-HA for 30 min, and incubated for 24 h. Similar experiments were conducted using MMW-HA, LMW-HA, and UMW-HA to determine the effect of different sizes of HA on NFκB activity. While treatment with smaller HA sizes did not induce any changes in NFκB activity (Figure 2A), cells stimulated with HMW-HA exhibited a dose-dependent significant increase in NFκB activity when compared with the untreated group (Figure 2B).

Because CD44 has been described as the principal receptor for HA (Aruffo et al. 1990), we evaluated its role in the induction of NFκB by HMW-HA. Blocking of the CD44 receptor in NIH-3T3 cells using the CD44 antibody KM201 suppressed the induction of NFκB activity by HMW-HA (Figure 2B). These results were further validated by silencing of CD44 in our fibroblasts using CD44 small interfering RNA (siRNA) and then determining NFκB activity in response to HMW-HA. As shown in Figure 2B, cells transfected with CD44 siRNA exhibited no change in NFκB activity following HMW-HA stimulation. The specific silencing ability of the CD44 siRNA was verified by real-time PCR (Figure 2C). This demonstrates that CD44 is essential to the stimulation of NFκB activity by HMW-HA.

The activation of NFκB by HMW-HA observed with the reporter assays was verified by immunofluorescence detection of NFκB in NIH-3T3 cells. Following stimulation with HMW-HA, the amount of NFκB (red) present in the nucleus was substantially increased as compared with the control, untreated cells (Figure 2D). Activation of the NFκB pathway was also confirmed by examining changes in the phosphorylation state of IKKa/β through western blot analysis. Previous studies have shown that IKKa/β phosphorylation is increased during the activation of the NFκB pathway (Baldwin 1996). As shown in Figure 2E, cell treatment with varying concentrations of HMW-HA (50–300 μg/mL) leads to an increase in the levels of phosphorylated IKKa/β. Similar to the effect on NFκB activity observed with NFκB–SEAP reporter assays, HMW-HA appeared to increase IKKa/β phosphorylation in a dose-dependent manner. Collectively, these observations show a selective and robust NFκB response to HMW-HA stimulation.

**CD44 and NFκB are required for the induction of Snail2 expression by HMW-HA**

In order to determine whether HA regulates Snail2 expression, we pulse treated cells with varying concentrations of HMW-HA, MMW-HA, LMW-HA, or UMW-HA for 30 min, incubated them for 24 h, and performed real-time PCR experiments. As shown in Figure 3A, MMW-HA, LMW-HA, and UMW-HA did not promote any changes in Snail2 mRNA. In contrast, HMW-HA significantly increased Snail2 mRNA in a dose-dependent manner and this induction was observed with the addition of HMW-HA at concentrations as low as 50 μg/mL (Figure 3B).

To examine the involvement of CD44 in the induction of Snail2 by HMW-HA, cells were treated with KM201, a functional blocking antibody to CD44 (Zheng et al. 1995), for 30 min...
CD44 is required for HMW-HA to induce NFκB activity. (A) NFκB–SEAP reporter activity was measured in cells following 30-min stimulation with MMW-HA, LMW-HA, or UMW-HA and 24-h incubation. (B) NFκB–SEAP reporter activity was measured in cells treated with HMW-HA in the absence (gray bars) or presence of a CD44 blocking antibody (clear bars), and following transfection with control siRNA (patterned bars) or CD44 siRNA (dark bars). The data represent the means ± SD of three independent experiments performed in triplicate (*P < 0.01). (C) The silencing of CD44 message by siRNA was verified by real-time PCR. (D) Cells left untreated (panel a) or stimulated with HMW-HA (300 μg/mL) for 30 min (panel b) were immunofluorescently stained with the anti-NFκB antibody (red) and bisbenzamide (blue). (E) Changes in the phosphorylation state of IKKα/β in NIH-3T3 cells following stimulation with HMW-HA were evaluated by western blotting. β-Actin was used as a loading control. (D) and (E) are representative of three independent experiments.

Prior to HMW-HA stimulation. Blocking of the CD44 receptor in these cells abolished the induction of Snail2 expression by HMW-HA (Figure 3B). Similarly, cells transfected with CD44 siRNA exhibited no change in Snail2 mRNA expression following stimulation with HMW-HA when compared to the control group (Figure 3B).

To validate the results obtained by real-time PCR, total Snail2 protein levels were assessed by western blotting following stimulation with HMW-HA. As shown in Figure 3C, treatment with HMW-HA substantially increased Snail2 protein levels in NIH-3T3 cells after 24 h. These results correlate with the observed increases in Snail2 mRNA expression which suggests that HMW-HA stimulates Snail2 at both the transcriptional and translational levels.

Next, we sought to determine the mechanism by which HMW-HA regulates Snail2 expression. As we have shown here, HMW-HA induces NFκB activity and in turn, this transcription factor has been shown to drive the expression of the Snail family member Snail1 (Barbera et al. 2004). Thus, we explored whether HMW-HA induction of Snail2 transcription is mediated by NFκB. For this, we treated cells with the specific NFκB inhibitor SN50 (2 μM) for 30 min prior to the addition of HMW-HA. As shown in Figure 3D, inhibition of NFκB repressed the induction of Snail2 expression in response to HMW-HA treatment. Together, these data indicate that both CD44 and NFκB are important mediators of HA-stimulated increases in Snail2 expression.

**HMW-HA promotes cellular invasion but not proliferation**

As cells can either “go or grow” but cannot perform both cellular functions at the same time, we sought to determine whether HA instructs mesenchymal cells to invade or proliferate. Cell growth in response to HA was assessed 24 h posttreatment using the MTT proliferation assay. As shown in Figure 4, fibroblasts treated with MMW-HA, LMW-HA, UMW-HA, or HMW-HA in the presence or absence of CD44 siRNA showed no difference in growth as compared to the controls. These results demonstrate that, regardless of molecular weight, HA does not affect mesenchymal cell proliferation.

To study the effect of HA on cellular invasion, we performed an in vitro three-dimensional invasion assay based on a modified Boyden chamber assay. Briefly, in a two-chamber system, collagen gels were cast in the upper chamber over an 8-μm pore membrane while the bottom chamber was filled with 20% FBS to induce chemotaxis. Cells labeled with CalceinAM were placed on top of the gel, pulse treated with different HA sizes for 30 min and allowed to invade for 24 h. While cells treated with MMW-HA, LMW-HA, or UMW-HA showed responses similar to the untreated controls (Figure 5A), cells stimulated with HMW-HA exhibited a significant increase in their invasive response (Figure 5B). However, invasion was inhibited in cells pretreated with the CD44 blocking antibody or transfected with CD44 siRNA (Figure 5B). These results show that HMW-HA, but not smaller sizes of HA, promotes cellular invasion and suggest an important role for CD44 in facilitating this response.
Mesenchymal cell invasion requires NFκB and Snail2

As we have shown that HMW-HA induces NFκB activity, we investigated whether the induction of cellular invasion by HMW-HA is dependent on NFκB. As depicted in Figure 5C, treatment of cells with SN50 previous to the addition of HMW-HA did not completely abolish the invasive response but substantially decreased it. This suggests that NFκB participates in the induction of cellular invasion by HMW-HA but other mechanisms may partially compensate for the loss of NFκB activity.

We investigated the role of Snail2 in the invasive response to HMW-HA by knocking down Snail2 in our cell line via siRNA technology. Specific silencing of Snail2 expression with Snail2 siRNA was verified by real-time PCR. Importantly, the siRNA to Snail2 is specific as it does not affect Snail1 mRNA levels (Figure 5D). As expected, cells transfected with control siRNA exhibited cellular invasion similar to untransfected cells following HMW-HA treatment. In contrast, cells with Snail2 knocked down by siRNA showed a significant decrease in HA-stimulated cellular invasion (Figure 5E). This shows that Snail2 is an important central mediator of cellular invasion stimulated by HMW-HA.

Discussion

HA is present in high levels around cells undergoing migration and invasion during embryonic morphogenesis, e.g., around neural crest cells migrating to form peripheral ganglia (Pratt et al. 1975; Peterson et al. 1993), around mesenchymal cells invading the primary corneal stroma (Koga et al. 2005) and around cardiac mesenchyme traveling into endocardial cushions during heart valve formation ( Schroeder et al. 2003). Moreover, mice lacking HA due to disruption of the Has2 gene exhibit severe cardiovascular defects which lead to a lethal embryonic phenotype (Camennisch et al. 2000). This indicates that HA influences a variety of morphogenetic processes and is necessary for proper embryonic development. In addition, HA plays an important role in cancer, where elevated levels of this polymer are associated with increased cellular invasion and tumor progression (Itano et al. 2002; Toole 2002). Although several signaling pathways downstream of HA have been identified, the wide range of biological responses affected by this molecule indicates that an even more complex array of signal transduction events is likely to take place. Here, we provide direct evidence for HMW-HA promoting mesenchymal cell invasive activity in a manner that is dependent on CD44 and partially dependent on NFκB and Snail2. This also highlights that, beyond its well-characterized prosurvival role, NFκB may also contribute to other responses such as cellular invasion during embryonic development.

Independent studies have implicated HA in the malignant EMT process, and cell proliferation and migration using in vivo approaches as well as cancer cell lines ( Bourguignon et al. 1997; Ouhtit et al. 2007). On the other hand, similar studies utilizing embryonic cells such as NIH-3T3s have yielded controversial results that may however be explained by differences in HA concentrations. While experiments using HMW-HA doses as low as 10 μg/mL have failed to detect any biological response in NIH-3T3 cells (Tzircotis et al. 2005), other studies employing the same cell line have reported significant changes in cellular behavior by treating it with HMW-HA concentrations as...
CD44-dependent manner. In a mesenchymal cell type, HMW-HA mobilizes NFκB in the cytosol. Together, these data show that, in a mesenchymal cell type, HMW-HA mobilizes NFκB in a CD44-dependent manner.

Interestingly, some studies utilizing different cell types such as chondrocytes, macrophages, and lymphoma cells suggest that hyaluronan oligosaccharides and not HMW-HA activate NFκB (Noble et al. 1996; Alaniz et al. 2004; Ohno et al. 2006). One possible explanation for these incongruities is that HAs of differing molecular weights may exert distinct physiological effects that are cell-type and context specific. Another possibility is that stimulation with HMW-HA over long periods of time may lead to degradation of the polymer and CD44 internalization (Culty et al. 1992) thus interfering with the propagation of intracellular signals. For this reason, we employed a different approach in which cells were pulse stimulated with HA for 30 min and incubated for 24 h previous to the measurement of parameters, thereby decreasing the possibility of signal blockade due to receptor turnover.

Several lines of evidence suggest that Snail family members are activated by NFκB during both embryonic development and tumor metastasis (Zhang et al. 2006; Criswell and Arteaga 2007), although the extracellular signals that regulate this pathway remain unknown. Thus, we investigated whether Snail2 is a transcriptional target of NFκB during HA signaling. Here we demonstrate that HMW-HA activates Snail2 gene transcription, resulting in a significant increase in Snail2 mRNA and protein levels. However, inhibition of either CD44 or NFκB completely abolishes this increase in Snail2. This suggests that HMW-HA induces Snail2 expression in a CD44- and NFκB-dependent manner.

In NIH-3T3 cells, HMW-HA significantly induces cellular invasion, with up to 5-fold increases during dose-dependent responses when compared to untreated cells. However, fibroblasts in which CD44 has been blocked or silenced fail to undergo invasion following stimulation with HMW-HA, which suggests that no other cell surface receptor on fibroblasts compensates for the loss of CD44 in vitro.

Because recent studies have shown that NFκB is overexpressed in highly invasive cancer cells (Vasko et al. 2007; Jethwa et al. 2008), we sought to determine whether HA stimulates cellular invasion via induction of the NFκB/Snail2 pathway during embryonic development. Our results show that pharmacological inhibition of NFκB or silencing of the Snail2 gene substantially decreases the invasive response of embryonic fibroblasts following HMW-HA stimulation but does not fully suppress it. This indicates that there is significant redundancy by NFκB-independent pathways which may also be activated by HMW-HA to promote fibroblast cellular invasion.

To our knowledge, this study is the first to demonstrate a functional connection between HMW-HA and the activation of a CD44/NFκB/Snail2 pathway to promote cellular invasion. But, how does this relate to the current models of HA-dependent signaling? One of the proposed mechanisms for cellular invasion following HA stimulation involves activation of c-Src kinase by CD44. Active c-Src kinase then phosphorylates cortactin which interacts with F-actin to promote polymerization and rearrangement of the actin cytoskeleton (Tehrani et al. 2007). These changes allow for the formation of filopodia and invadopodia which facilitate cellular invasion (Bryce et al. 2005; Artym et al. 2006). Another HA-dependent mechanism includes activation of the Tiam1/Rac1 pathway, which also regulates cytoskeleton function. Interestingly, NFκB can be activated by both Src kinase and Rac1 following multiple stimuli (Frost et al. 2000; Cox et al. 2006; Kwei et al. 2006); therefore, it is likely that HA may
signal through either one or both of these signaling effectors to induce NFκB activity during cellular invasion. However, further studies are necessary in order to establish these connections and to determine which compensatory mechanisms contribute to HA-stimulated cellular invasion.

In summary, we have elucidated a previously unknown signaling mechanism by which HMW-HA promotes cellular invasion in embryonic cells, which includes activation of Snail2 through CD44 and NFκB. The induction of invasive responses in these cells is dependent on HA size and may also be dependent on the temporal regulation of HA and CD44 as suggested by the pulse treatment experiments. Nevertheless, cellular invasion is a complex process and additional studies are required to identify other effectors of HA signaling and to determine the integration of these molecules to promote changes in cellular behavior. Comprehension of the signaling mechanisms underlying HA-induced cellular invasion is imperative not only to the understanding of diseases such as congenital heart defects but also to the development of more efficacious treatments against cancer.

Materials and methods

Reagents
SN50 cell permeable NFκB inhibitor and hyaluronidase from *Streptomyces hyalurolyticus* were purchased from Calbiochem (San Diego, CA). The rat antimouse CD44 blocking antibody (clone KM201), which directly blocks HA binding to CD44 (Zheng et al. 1995), was obtained from Southern Biotech (Birmingham, AL). Vectors pNFκB–SEAP, p-CMVβ, pTAL–SEAP, and pSEAP2 were purchased from Clontech (Mountain View, CA). HMW-HA (900–1200 kDa; average mass of 980 kDa; catalog GLR002), MMW-HA (90–150 kDa; average mass of 132 kDa; catalog GLR004), and LMW-HA (15–40 kDa; average mass of 31 kDa; catalog GLR001) were manufactured by LifeCore Biomedical Inc. (Chaska, MN). UMW-HA (2.3 kDa, HA12), which consists of 12 monosaccharides in length, was obtained from Associates of Cape Cod Inc. (East Falmouth, MA). All HA sizes were produced by microbial fermentation of *Streptococcus pyogenes*. Stock
solutions were prepared by reconstituting HA in sterile water, boiling for 30 min at 100°C to inactivate any contaminants, and addition of Dulbecco’s modification of Eagle’s medium (DMEM).

**Cell culture**

NIH-3T3 mouse fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% FBS and 1% antimycotic/antibiotic solution (penicillin, streptomycin, and amphotericin) (Mediatech Inc.). All cell cultures were maintained in an atmosphere of 5% CO₂ at 37°C in a humidified incubator.

**NFB activity assays**

NFB activity was determined by transfecting cells with a pNFβ-SEAP reporter vector expressing a secreted form of placental alkaline phosphatase (SEAP) driven by four NFβ consensus sequences in tandem (Clontech). Cells were cotransfected with β-galactosidase expression vector (p-CMVβ) to normalize the SEAP activity to the β-galactosidase activity. The vehicle vector pTAL-SEAP and the pSEAP2 expression vector were used as negative and positive controls, respectively. Twenty-four hours posttransfection, cells were serum starved, treated with HA at indicated doses for 30 min, and washed with PBS, followed by a serum-free 24-h incubation period. The cell supernatant was collected for SEAP assay and cell lysates were prepared for assaying the β-galactosidase activity. Alkaline phosphatase and β-galactosidase activity levels were detected using the Great EscAPE(™) SEAP chemiluminescence kit and the luminiscent β-gal detection kit, respectively, according to the manufacturer’s instructions (Clontech). For some experiments, transfected cells were treated with hyaluronidase (30 U/mL) for 1 h at 37°C and washed twice with PBS prior to the addition of exogenous HA.

**Western blotting**

Total cellular lysates were prepared from cells and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After the electrobolt transfer onto polyvinylidene difluoride membrane and blocking in 3% BSA, primary antibodies against pKKα/β (Cell Signaling Technology, Danvers, MA), Snail2 (Cell Signaling Technology), or β-actin (ABR, Golden, CO) were used followed by secondary antibodies. Detection was performed using Super Signal West Pico substrate (Pierce, Rockford, IL).

**Immunofluorescence**

Cells grown and treated on glass cover slips were fixed with paraformaldehyde, permeabilized with Triton X-100, and blocked with BSA. Cells were then incubated with the antibody against NFβ (Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h and antirabbit-594 secondary antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing with PBS, bisbenzamide (Molecular Probes) was added for 5 min. Fluorescent images were taken with an Olympus inverted microscope through appropriate fluorescence filters using a 60 × 1.2 NA objective. Z stacks of images were collected using an RT microscope system (Applied Precision, Issaquah, WA) and deconvolved using the SoftWorx program.

**Real-time PCR**

Total RNAs were extracted from cultured cells using the RNASAT60 reagent (Tel-test, Friendswood, TX) according to the instructions provided by the manufacturer. RNA concentration was determined on a Thermo Spectronic (Rochester, NY) Biotome3 UV spectrophotometer by the 260 nm absorbance. cDNA synthesis was conducted using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions.

Quantitative real-time PCR was performed on a Lightcycler® 2.0 instrument (Roche Applied Science) using the Lightcycler® Taqman Master Mix (Roche Applied Science). The gene RPS7 was used for normalization. All samples were analyzed in triplicate and a calibrator and negative control were employed for each assay. Cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression was calculated using the 2−ΔΔCT method. The primer sequences are as follows: RPS7, forward, 5′-AGC TGG TCT TCA TTG CT-3′, and reverse, 5′-CTG TCA GGG TAC GGC TTC TG-3′; and Snail2, forward, 5′-GAT CTG TGG CAA GGC TTT CT-3′, and reverse 5′-ATT GCA GTG AGG GCA AGA GA-3′. All oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). The gene-specific probes were obtained from the Universal Probe Library (Roche Applied Science).

**Proliferation assays**

Cells were seeded into 96-well culture plates at a density of 2.0 × 10⁴ cells/well and routinely cultured for 24 h. Next, cells were serum starved for 1 h, followed by 30-min treatment with HA at 0–300 μg/mL and a 24-h incubation period. Cell proliferation was assessed 24 h after stimulation with HA using the Vybrant® MTT cell proliferation assay kit (Molecular Probes) according to the manufacturer’s indications.

**Invasion assays**

The invasive potential of mouse embryonic fibroblasts upon HA stimulation was determined by using a transwell chamber system with 8 μm pore polyester membrane inserts (Corning Inc., Corning, NY). Collagen was neutralized to pH 7.4 with a buffer containing 10× M199 and 2.2% sodium bicarbonate. The collagen was allowed to polymerize on top of the membrane at room temperature for 30 min. Next, DMEM + 20% FBS was added to the lower chamber as a chemoattractant. Cells were fluorescently labeled with CalceinAM (BD Biosciences) and plated on top of the collagen layer at a density of 2.0 × 10⁵ cells per insert in serum-free DMEM. After 30 min treatment with HA at 50–300 μg/mL, cells were washed with PBS and incubated in serum free DMEM for 24 h. Following incubation, transwell inserts were removed from the plate containing 20% FBS in DMEM and positioned in a plate with 2 mM EDTA in PBS for 15 min. Invasion was quantified by measuring fluorescently labeled cells that crossed the polyester membrane and were detached into the EDTA solution. Fluorescence was determined...
with a plate reader at 538 nm (Spectramax Gemini, Molecular Devices, Sunnyvale, CA).

siRNA experiments
siRNA against Snail2, CD44, and control siRNA (siRNA-A) were purchased from Santa Cruz Biotechnology. NIH-3T3 cells grown to 50% confluence in six-well plates were transfected with 4 μg of siRNA using XtremeGene siRNA transfection reagent (Roche Molecular Systems, Alameda, CA) according to the manufacturer’s instructions. Following transfection, cells were incubated for 48 h in a medium containing 10% FBS prior to their use in different experiments.

Statistical analysis
Two sample Student’s t-tests were performed as appropriate using the Stata 9.0 data analysis and statistical software (StataCorp LP, College Station, TX). The level of significance was established at P < 0.01.

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Conflict of interest statement
None declared.

Abbreviations
DMEM, Dulbecco’s modification of Eagle’s medium; EMT, epithelial-to-mesenchymal transition; HA, hyaluronan; Has2, hyaluronan synthase 2; HMW-HA, high-molecular-weight hyaluronan; IκB, inhibitory kappaB; LMW-HA, low-molecular-weight hyaluronan; MMW-HA, medium-molecular-weight hyaluronan; NFκB, nuclear factor kappa B; pIkkα/β, phosphorylated IκB kinase α/β; SEAP, secreted embryonic alkaline phosphatase; siRNA, small interfering RNA; UMW-HA, ultra-low-molecular-weight hyaluronan.

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