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RON-expressing MCF-10A breast epithelial cells exhibit alterations of hyaluronan expression, promoting RON-mediated early adhesion events

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ABSTRACT

The receptor tyrosine kinase known as RON appears to play a role in the progression of human carcinomas, and is associated with a poor patient prognosis. Our current study demonstrates that RON expression in MCF-10A breast epithelial cells lead to an alteration of cell-surface hyaluronan compared to the parental cells. We found that hyaluronan was important for initial cell attachment to poly-D-lysine-coated coverslips, but did not contribute to the process of cell spreading. Previous data implied that the Src kinase was important for spreading but not the initial attachment of 10A cells, and here we demonstrate Src activation was also not necessary for hyaluronan production in these cells.

Introduction

The RON receptor tyrosine kinase (RTK) is expressed in several types of human cancers and may contribute to metastatic disease [1]. RON is normally activated by binding to its ligand, macrophage-stimulating protein (MSP) but its signaling can also be mediated by interaction with the hyaluronan (HA) receptors CD44 [2] and RHAMM (receptor for hyaluronan-mediated motility) [3]. Our recent work has shown that MSP-independent signaling of RON in MCF-10A breast epithelial cells resulted in an increase in cell spreading, survival, and migration compared to a parental cell line [4]. Although we demonstrated that activated Src kinase was required for these behaviors, it was not clear from our previous studies what might be acting upstream of Src to confer its constitutive activation in 10A/RON cells. One possibility was that RON mediated an increase of cell-surface HA availability, which could potentially drive Src activation via CD44 or RHAMM [5].

Hyaluronan is a glycosaminoglycan (GAG) consisting of glucoronic acid and *N*-acetylglucosamine disaccharides [6]. Although HA plays a structural role in normal tissues, it is also upregulated in inflammation, wound repair, and cancer [7]. HA is implicated in a number of different cancer types, including breast cancers, and its increased expression correlated with invasive cell behavior [8–10]. In human cancers, it is most likely the interactions of HA with CD44 and RHAMM that contribute to malignancy [6]. CD44 interacts with several RTKs, including c-Met [11], EGFR [12], and ErbB2 [13]. Through interactions with RTKs as well as several downstream effectors, such as Src kinase and MAPK, both CD44 and RHAMM mediate signaling that promotes proliferation, survival, and migration [6]. Blocking of HA binding, either by soluble HA peptides [14], antisense CD44 cDNA [15], soluble CD44 [16] or soluble RHAMM protein [17] leads to an attenuation of oncogenic behaviors, which suggests that HA might be a potential target in cancer treatment.

In our current study, we examined the contribution of HA to the MSP-independent oncogenic behaviors of 10A/RON cells. The increased adhesive properties of 10A/RON cells and their increased migratory potential can be explained by increased expression together with a change in distribution of HA over the cell surface compared to 10A/Vector cells. Src activity was not necessary for the expression of HA in MCF-10A cells and HA did not contribute to Src-dependent cell spreading.

Materials and methods

Materials. EGF was from Peprotech (Rocky Hill, NJ, USA). Biotinylated *Wisteria floribunda* agglutinin (bWFA) was kind gift from Dr. Joel Levine (Stony Brook University). Poly-D-lysine and bovine testes hyaluronidase were from Sigma–Aldrich (St. Louis, MO, USA). Hoechst 33342 was from Molecular Probes/Invitrogen (Eugene, OR, USA). Cy3/Streptavidin was from Jackson Immunolabs (West Grove, PA). Biotinylated HABP was from Northstar Bioproducts (East Falmouth, MA, USA). Antibodies for Western blots were

Abbreviations: CSPG, chondroitin sulfate proteoglycan; GAG, glycosaminoglycan; HA, hyaluronic acid or hyaluronan; HABP, hyaluronan binding protein; HAS, hyaluronan synthase; MSP, macrophage-stimulating protein; WFA, *Wisteria floribunda* agglutinin.

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anti-MAPK from Upstate Biotechnology, Inc. (Lake Placid, NY, USA) and anti-Src pY418 from BioSource (Camarillo, CA, USA). PP2 was from BioMol (Plymouth Meeting, PA, USA).

Cell culture. Cell culture materials were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). 10A/Vector and 10A/RON cells were cultured as described [18]. 10A/Vector and 10A/RON cells were generated as previously described [4].

Transwell migration assay. The migration assay was performed as described [4]. The cell suspension either with or without 300 U/ml HAase was pre-incubated for 1 h before 10 ng/ml EGF was added to the bottom chamber.

Attachment assays. Approximately 100,000 cells were allowed to attach to the poly-D-lysine-coated coverslips for 10 min. Attached cells were fixed and treated as described [4].

Immunoblotting. Western blots were performed as described [19] using a 9% SDS polyacrylamide gel.

Reverse-transcriptase PCR. RNA was isolated with RNeasy kit (Qiagen, Valencia, CA, USA), and reverse transcribed to cDNA using the Superscript II reverse transcriptase system (Invitrogen, Carlsbad, CA). HAS 1, 2, and 3 were detected using primers previously described [20]. CD44s was detected with previously described primers [21]. RHAMM was detected from primers published in [22]. The house-keeping gene (60S region of the human RPLP1 gene) that was used as a loading control was a kind gift from Dr. Edward Chan (Stony Brook University).

Fluorescence studies. To label cells with biotinylated *W. floribunda* agglutinin (bWFA), cells were fixed with 4% paraformaldehyde at room temperature for 15 min, incubated with 1:100 bWFA in 1% BSA for 90 min at room temperature, then 1:5000 Cy3-streptavidin for 40 min at room temperature. To label cells with biotinylated HA-binding protein (bHABP), cells were grown on coverslips for 48 h, fixed with 100% Methanol at −20 °C for 20 min and blocked in 3% BSA for 1–2 h. BHABP (1:200) was added to the cells overnight and was detected by incubation with 1:5000 Cy3-streptavidin for 1 h. DNA was labeled with Hoechst and coverslips were mounted with Prolong Gold (Molecular Probes). The images were captured with an Axiovert 200 M microscope (Zeiss, Thornwood, NY, USA) using a 63× oil DIC lens and the images were analyzed using the Axiovision software (Zeiss).

Results and discussion

Our earlier studies indicated that 10A/RON cells also exhibited increased cell adhesion to poly-D-lysine-coated coverslips and rapid cell spreading in comparison to 10A/Vector cells [4]. Supplementary Fig. S1 demonstrates that following 30 and 150 min of attachment to tissue culture plates, 10A/RON cells still exhibited increased attachment compared to 10A/Vector cells. We considered whether the difference in attachment between the two cell types could be explained by the degree of attraction between positively charged lysines on the coverslips and negative charges covering the cell surface. We reasoned that 10A/RON cells could have a greater overall negative charge on their cell surface as the result of increased extracellularly deposited GAGs, such as chondroitin sulfate proteoglycans (CSPGs) and/or hyaluronan (HA). These molecules are highly negatively charged and both are known to be involved in cell adhesion to the extracellular matrix (ECM) [6,23]. To determine if 10A/Vector and 10A/RON cells express these glycoproteins, we labeled the cells with *W. floribunda* agglutinin (WFA), a lectin that binds *N*-acetylgalactosamine residues on chondroitin sulfate chains found on some proteoglycans [24]. We examined whether there was a change in either expression levels or distribution of CSPGs between 10A/Vector and 10A/RON cells. After allowing cells to attach to poly-D-lysine-coated coverslips for 10 min, as previously described in [4], cell-surface CSPGs were labeled with bWFA. As shown in Fig. 1A, both 10A/Vector and 10A/RON cells appeared to express similar amounts of CSPGs on the cell surface. It was not possible to accurately determine whether there were small quantitative differences of cell-surface CSPGs with this assay. The CSPGs were differentially distributed depending on how much cell spreading occurred. 10A/RON cells exhibited increased CSPG-containing microspikes than 10A/Vector cells (Fig. 1A). This pattern of CSPG localization is similar to that shown previously for the distribution of actin after attachment (Fig. 3B and [4]). The quantity of CSPGs on the cell surface does not appear to play a direct role in cell spreading, as we noted that cells exhibiting a more dense concentration of CSPGs may not necessarily have a larger cell span (Fig. 1B).

HA forms the extracellular framework to which other GAGs, including CSPGs, are structurally bound. When HA is cleaved into fragments by hyaluronidase (HAase) [6], this so-called "glue" no longer holds the GAGs in place [25]. To determine if CSPGs on the cells' surface might be responsible for the increased adhesion of 10A/RON cells, we repeated the attachment assay in the presence of HAase, which removed both extracellular CSPGs and HA. Pre-incubation with 300 U/ml of HAase for 10 min before allowing the cells to attach to the coverslip nearly completely abrogated attachment in both 10A/Vector and 10A/RON cells (Fig. 1C), implying that HA and/or other GAGs in the ECM are required to mediate early attachment of 10A cells.

An increase in expression or a change in cell-surface availability of negatively charged HA could promote faster anchoring of cells onto positively charged lysine residues. HA expression between 10A/Vector and 10A/RON cells was compared by labeling cells with biotinylated HA-binding protein (bHABP) after culturing for 48 h. As shown in the 3-D (Fig. 2A) and 2-D images (Fig. 2B), both cell types are positive for HA-staining but 10A/RON cells exhibited changes in HA expression and cell-surface distribution compared to 10A/Vector cells. Although this is evident in 3-D images (Fig. 2A) it is probably best shown in 2-D images (Fig. 2B) in which the cell nuclei have been counter-stained blue. Denser and more widespread extracellular bHABP staining in 10A/RON cells obscures the nuclei. This is not the case in the 10A/Vector cells, which generally have less extracellular bHABP staining. To address the specificity of the staining we treated the cells with Haase to remove extracellular HA. Treatment of live cells with 300 U/ml HAase for 1 h before fixation, permeabilization and subsequent bHABP labeling removed nearly all of the extracellular HA, but left the intracellular stores of HA intact. 10A/RON cells have a slightly larger internal supply of HA than 10A/Vector cells, as can be seen in Fig. 2A. The role of intracellular HA is still unclear [26].

To determine if HA is localized to microspikes after immediate adhesion in the same distribution as CSPGs and actin (Fig. 3B and C), we allowed the cells to attach for 10 min, which is sufficient time for spreading of the 10A/Ron cells. Unlike CSPGs and actin (Fig. 3B and C), we found that HA was not found in microspike structures when cells are allowed to attach to the coverslip for 10 min (Fig. 3A). However, it was again evident that 10A/RON cells secreted more HA than 10A/Vector cells onto the coverslip, which was visualized as increased staining of punctuate deposits of bHABP staining in the 10A/Ron cells. The punctuate nature of the staining is best seen in the magnified image (Fig. 3A, right panel).

Since 10A/RON cells appear to have more HA over their extracellular cell surface than 10A/Vector cells, we hypothesized that 10A/RON cells generate increased levels of hyaluronan synthase (HAS) gene expression which could potentially lead to a greater amount of HA secretion at the cell membrane. To this end, we used reverse-transcriptase PCR to detect HAS 1, HAS 2, and HAS 3 genes in 10A/Vector and 10A/RON cells. For comparison, we assessed the levels of HAS genes in the invasive breast cancer cell line, MDA-231, as these cells are known to exhibit increased

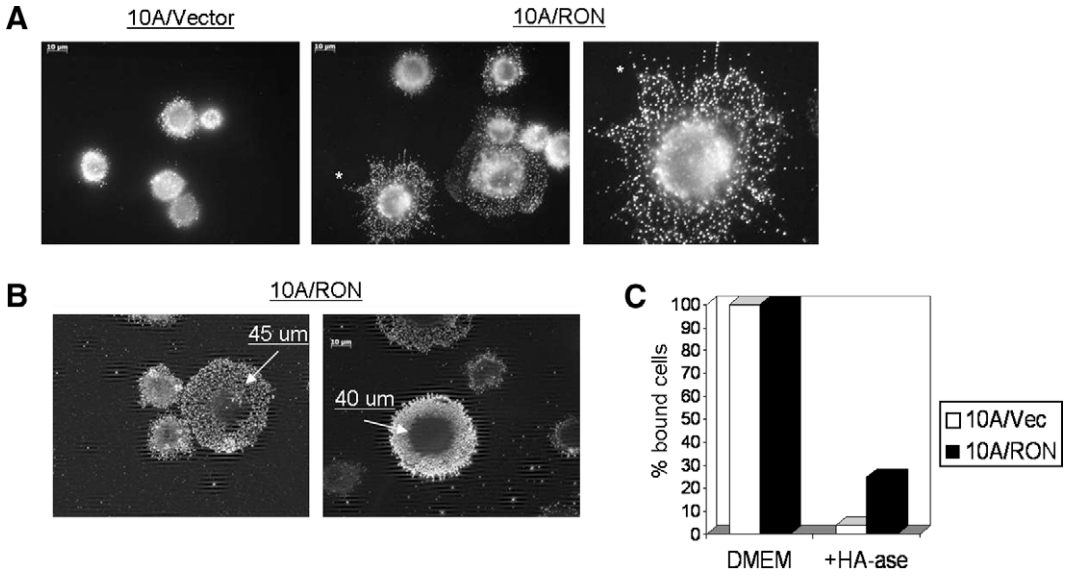

Fig. 1. Chondroitin sulfate proteoglycans are found in microspikes of spreading cells. (A) 10A/Vector and 10A/RON cells were allowed to attach to poly-D-lysine coated coverslips for 10 min. Bound cells were labeled with biotinylated-WFA and detected by Cy3-streptavidin. The asterisk denotes a cell with microspikes and is the magnified image in the 3rd panel. (B) After 10 min attachment, the cells were labeled as in (A). The span of the cell was measured to show that a larger cell span does not necessarily correlate with the amount of CSPGs on the cell surface. (C) The relative number of 10A/Vector and 10A/RON cells was determined after 300 U/ml HAase was added to the cells 10 min prior to the 10 min attachment assay. Shown is a representative experiment.

expression of HAS 2 [27]. As shown in Supplementary Fig. S2, all three cell lines expressed similarly low levels of HAS 1 expression. As expected, MDA-231 cells expressed high levels of HAS 2 compared to either 10A cell line. Both 10A/Vector and 10A/RON cells expressed approximately the same levels of HAS 2 and HAS 3 (Fig. S2), implying that the difference in the amount of HA expression between 10A/Vector and 10A/RON cells was not due to increased expression of HAS proteins on the cell surface of 10A/RON cells. However, there is evidence that HAS proteins must be proteolytically modified in order to secrete HA at the cell surface [26], and RON expression might induce a change in the activation status of HAS isoforms rather than affect expression levels itself.

In order to mediate HA-driven cell signaling, cells must express either or both of the HA receptors, CD44 or RHAMM. CD44 and RHAMM also have the potential to tether HA to the cell surface, so that increased expression of these receptors may lead to an increase or change in distribution of HA at the cell surface. We used RT-PCR to detect expression of RHAMM and the standard form of CD44 in our 10A cell lines. Supplementary Fig. S2 illustrates that

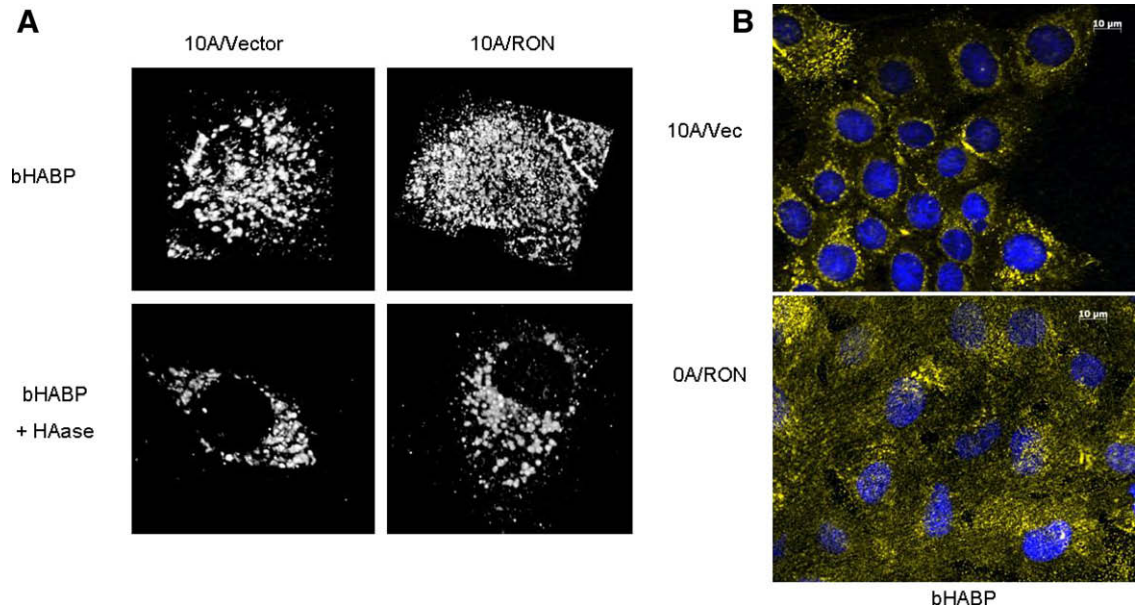

Fig. 2. 10A/RON cells exhibit increased cell-surface hyaluronan expression. (A) 10A/Vector and 10A/RON cells were cultured for 48 h in regular media. Cells were labeled with biotinylated-hyaluronan binding protein (bHABP) and Cy3-streptavidin. Images are 3-D rendered *Z*-stacks taken with Apotome processing of individual cells. The top panel shows cells untreated with HAase, and the bottom panel shows cells that were treated with 300 U/ml HAase for 1 h prior to adding bHABP. (B) Cells were labeled as in (A). Images are 2-D Apotome-processed of representative 10A/Vector and 10A/RON cells. The yellow is a pseudo-color in order to make the HABP staining more visible. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

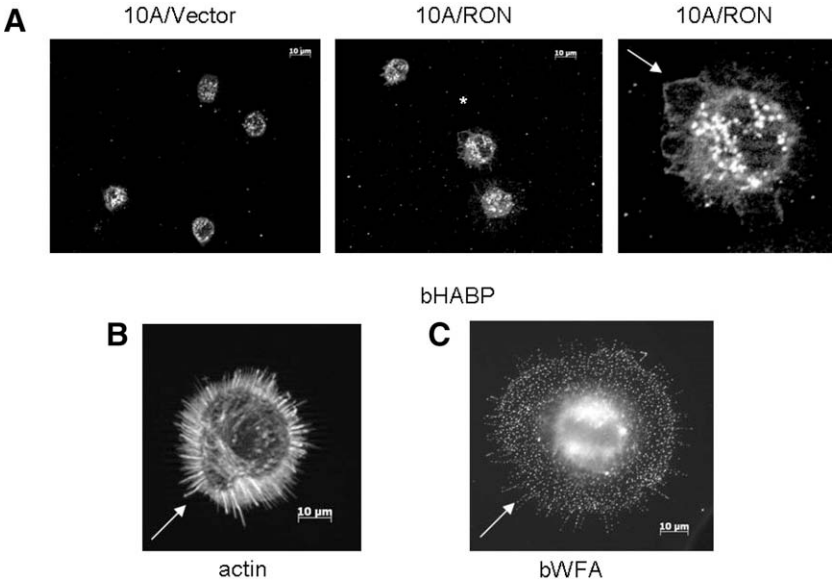

Fig. 3. HA does not localize to actin microspikes. (A) 10A/RON cells were allowed to attach to poly-D-lysine coated coverslips for 10 min before removing unbound cells. Remaining cells were stained with biotinylated-HABP/Cy3-streptavidin. The asterisk denotes the cell that is magnified in the right end panel to show the punctuate deposits of HA present on these cells. (B) 10 min attachment, cells labeled with rhodamine–phalloidin to detect actin. (C) 10 min attachment, cells labeled with biotinylated-WFA to detect CSPGs.

10A/Vector and 10A/RON cells expressed RHAMM and CD44 at approximately similar levels, implying that the increased HA on 10A/RON cells is unlikely due to a greater number of receptors. However, expression of both CD44 and RHAMM suggests that MCF-10A cells have the potential to transduce HA-mediated signals to generate biological responses.

In our earlier studies, the Src kinase was essential for spreading of 10A cells after adhesion but not the initial cell attachment event itself [4]. Using an activation-specific antibody, we found that Src kinase was constitutively phosphorylated to a higher level in 10A/RON cells compared to 10A/Vector cells and was not further activated by addition of MSP (Fig. 4A). We examined whether this constitutive Src activation contributed to the increased deposition of HA in 10A/RON cells using the Src-inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-d]pyrimidine (PP2). When cultured with PP2, both 10A/Vector and 10A/RON cells attached to the tissue culture dish, but did not spread, even after 48 h in culture (Fig. 4B). It was possible that PP2 inhibition of Src blocked spreading because it prevented expression of HA on the cell surface. However, we found that whether the cells were cultured in negative-control DMSO-containing media or PP2-containing media, both 10A/Vector and 10A/RON cells still expressed HA, and again there appeared to be

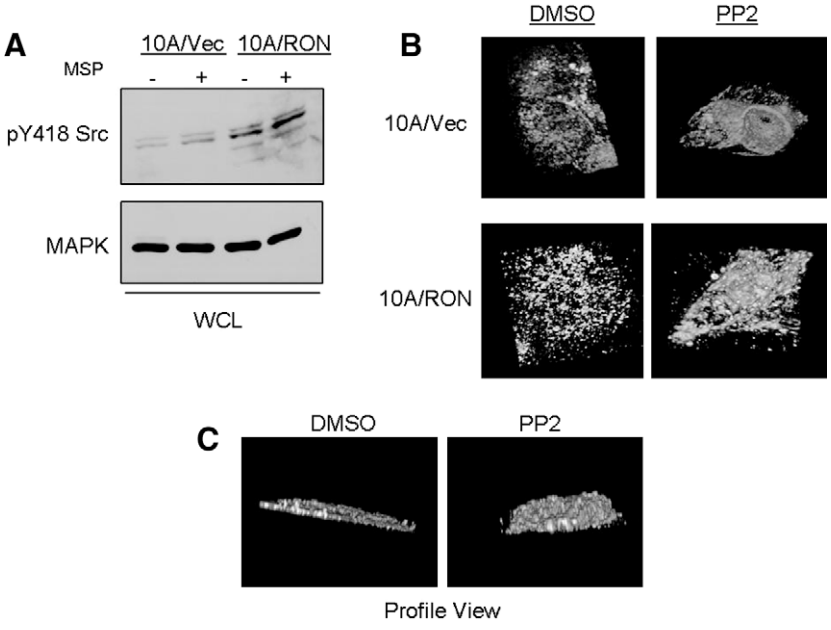

Fig. 4. HA synthesis does not require Src kinase activity. (A) Whole cell lysates (WCL) from 10A/Vector and 10A/RON cells were Western Blotted for pY418 Src, which is an activation-specific form of Src. Prior to lysis, cells were left untreated or treated with 100 ng/ml MSP for 30 min. (B) 10A/Vector and 10A/RON cells were cultured on coverslips for 48 h in the presence of DMSO or PP2. Cells were then labeled with biotinylated HABP/Cy3-streptavidin. Images shown are 3-D rendered *Z*-stacks. (B) Top–down view. (C) Profile view of 10A/RON cells.

more HA deposited on the surface of the 10A/RON cells (Fig. 4B). The control DMSO-treated cultures exhibited spreading but the PP2-containing cultures did not (Fig. 4B and C). Because of this difference in morphology, it appears that HA has a different distribution in the presence of PP2. This can be seen best in the profile view, in which the PP2-containing 10A/RON cells were much less flat due to the inhibition of spreading and the HA has accumulated on the surface of the cells (Fig. 4C). We also tested to see if inhibition of Src prevented HA secretion following HAase treatment. 10A/Vector and 10A/RON cells were first treated with HAase to remove cell-surface HA, and then treated with DMSO as a control or PP2 to inhibit Src activity. The re-deposition of HA was monitored using bHABP labeling. PP2 did not block the nascent secretion of HA to the cell surface (data not shown). Taken together, these data suggest that Src does not mediate the expression and/or deposition of HA in MCF-10A cells. We conclude that HA is necessary for the early adhesion events in MCF-10A cells, but HA alone is not sufficient to induce cell spreading. Further, because Src is not important for the synthesis of HA, it is also not necessary for the initial attachment event to occur (Fig. 4B and [4]).

Our data suggests that HA mediates the initial binding of MCF-10A cells to poly-D-lysine on glass coverslips, and that because 10A/RON cells exhibit increased deposition of HA on their cell surface, they bind more efficiently than 10A/Vector cells. HA has been shown in other cell systems to mediate attachment to ECM in other breast cell lines [28], consistent with our analysis.

To determine whether HA participates in additional RON-mediated biological events, we examined the contribution of HA to cell migration. 10A/RON cells exhibit increased migration compared to 10A/Vector cells in response to EGF [4], which we hypothesized was due to the increased HA-mediated adhesiveness of 10A/RON cells. 10A/Vector and 10A/RON cells were pre-incubated in HAase prior to adding EGF to the bottom well of the Transwell insert. Supplementary Fig. 3 illustrates that HAase dramatically reduced the number of cells migrating through the filter within 6 h in both 10A/Vector and 10A/RON cells, implying that HA expression was necessary for efficient migration within that time frame. Whether the inhibition of migration was due purely to effects on initial attachment to the filter or whether it was also important for subsequent events that allowed the cells to move through the pores is unclear.

Our current study is the first that we know of to describe a correlation between an increase of RON expression and an increase of HA expression and changes in HA distribution. These results are significant because they point out a potentially novel mechanism by which RON can promote oncogenic behavior in normal epithelial cells in the absence of its ligand, MSP. It is likely that through upregulation of HA, RON contributes to CD44 and/or RHAMM signaling, the two known HA receptors. The role of ECM in controlling cell behavior is now well-established; and the extent of its influence on RTK signaling is still being intensely investigated. The surrounding microenvironment of the cell plays an essential role in determining cancer cell behavior. Through manipulation of the microenvironment, RON expression appears to be capable of providing the necessary cues to promote aggressive and invasive behaviors in human mammary epithelial cells.

Finally, there is mounting evidence that the process of inflammation dramatically contributes to the development of cancers [29]. Interestingly, HA is a key player in inflammatory diseases, including human cancers [30]. Although there is some evidence that RON might be involved in the regulation of pro-inflammatory cytokines when expressed in macrophages [31], the role of RON in the context of inflammation in cancer is poorly-defined. Future studies to examine whether RON signaling contributes to the development of inflammation in the context of cancer development would be interesting and worthwhile.

Conclusions

These results demonstrate that increased RON expression in breast epithelial cells leads to increased cell attachment, which is mediated by the extracellular matrix protein, hyaluronan. Increased RON expression promotes an increase of hyaluronan expression on the cell surface compared to the parental control cells. These results are interesting and significant because of the important roles that hyaluronan plays in inflammation and metastatic progression in human cancers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.080.

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