RESEARCH ARTICLE

Hyaluronan-CD44/RHAMM interaction-dependent cell proliferation and survival in lung cancer cells†

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Abbreviations: HA, hyaluronan; HAS, hyaluronan synthase; RHAMM, hyaluronan-mediated motility receptor; NSCLC, non-small cell lung cancer; NHLFs, normal human lung fibroblasts; LCAFs, lung cancer-associated fibroblasts; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; LPS, lipopolysaccharide

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Abstract

Although members of the hyaluronan (HA)-CD44/HA-mediated motility receptor (RHAMM) signaling pathway have been shown to be overexpressed in lung cancer, their role in lung tumorigenesis is unclear. In the present study, we first determined levels of HA and its receptors CD44 and RHAMM in human non-small cell lung cancer (NSCLC) cells and stromal cells as well as mouse lung tumors. Subsequently, we examined the role of HA-CD44/RHAMM signaling pathway in mediating the proliferation and survival of NSCLC cells and the cross-talk between NSCLC cells and normal human lung fibroblasts (NHLFs)/lung cancer-associated fibroblasts (LCAFs). The highest levels of HA and CD44 were observed in NHLFs/LCAFs followed by NSCLC cells, whereas THP-1 monocytes/macrophages showed negligible levels of both HA and CD44. Simultaneous silencing of HA synthase 2 (HAS2) and HAS3 or CD44 and RHAMM suppressed cell proliferation and survival as well as the EGFR/AKT/ERK signaling pathway. Exogenous HA partially rescued the defect in cell proliferation and survival. Moreover, conditioned media (CM) generated by NHLFs/LCAFs enhanced the proliferation of NSCLC cells in a HA-dependent manner as treatment of NHLFs and LCAFs with HAS2 siRNA, 4-methylumbelliferone, an inhibitor of HASs, LY2228820, an inhibitor of p38MAPK, or treatment of A549 cells with CD44 blocking antibody suppressed the effects of the CM. Upon incubation in CM generated by A549 cells or THP-1 macrophages, NHLFs/LCAFs secreted higher concentrations of HA. Overall, our findings indicate that targeting the HA-CD44/RHAMM signaling pathway could be a promising approach for the prevention and therapy of lung cancer.

Key words: Hyaluronan, hyaluronan-mediated motility receptor, CD44, Non-small cell lung cancer, lung cancer-associated fibroblasts

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Introduction

Hyaluronan (HA), a prominent component of the micro-environment in most malignant tumors, is a non-sulfated, linear glycosaminoglycan composed of repeating disaccharides of glucuronic acid and *N*-acetyl glucosamine. It is synthesized by transmembrane HA synthases (HAS1, HAS2, or HAS3) [1-3] of a variety of cells, including fibroblasts, epithelial cells, endothelial cells, stem cells, and immune cells, but fibroblasts are the major source.[4] Once released from the pericellular matrix, HA may be subjected to degradation by hyaluronidases, mechanical forces, and reactive oxygen and nitrogen species resulting in low molecular weight-HA (LMW-HA) and HA oligomers.[5] The biological function of HA depends on its size. In general, high molecular weight-HA (HMW-HA) is anti-inflammatory and anti-tumorigenic, whereas LMW-HA is pro-inflammatory and pro-tumorigenic.[5]

HA signals through interaction with several cell surface receptors. Of these, the best characterized ones are CD44, receptor for hyaluronic acid-mediated motility (RHAMM) and Toll-like receptor (TLR4). Binding of HA to CD44/RHAMM on epithelial cells results in a direct or indirect activation of signaling receptors and a variety of downstream signaling pathways regulating cell proliferation, survival and motility.[6,7] Moreover, the interaction between HA and CD44/RHAMM has been shown to stimulate the migration and proliferation of smooth muscle cells, fibroblasts, immune cells, and endothelial cells and cytokine secretion by macrophages.[8] Binding of HA to TLR4 activates innate immune responses and induction of inflammatory gene expression in a variety of immune cells.[9-11]

Compared to normal lung tissue, lung carcinoma contain higher levels of HA.[12,13] Among lung adenocarcinoma patients, higher levels of HA in tumor cells was associated with poor differentiation of the tumor, whereas stronger stromal HA was related to a higher frequency of

recurrence.[13] Also, CD44 and RHAMM are overexpressed in both premalignant[14] and malignant [15-17] lung lesions and high levels of CD44 and RHAMM in tumor tissues have been significantly correlated with poor survival, lymph node metastases and tumor size.[15,17-19] Since lung tumors exhibit high levels of HA, CD44 and RHAMM and aberrant expression of these molecules resulted in enhanced tumor development and progression, it is imperative to understand the role of HA-CD44/RHAMM in lung tumorigenesis. Moreover, members of HA-CD44/RHAMM appear to be promising targets for the prevention and therapy of this malignancy.

In the present study, we assessed (1) levels of HAS1-3, HA, CD44 and RHAMM in non-small cell lung cancer (NSCLC) cells and stromal cells; (2) the role of HA-CD44/RHAMM signaling pathway in the proliferation and survival of NSCLC cells; and (3) the potential of HA in mediating the crosstalk between normal human lung fibroblasts (NHLFs)/lung cancer-associated fibroblasts (LCAFs) and NSCLC cells. We also showed that NHLFs/LCAFs produced by far higher levels of HA than NSCLC cells or THP-1 cells. Silencing of HAS2/HAS3 or CD44/RHAMM in NSCLC cells markedly reduced cell proliferation and survival and exogenous HA partially attenuated these effects. Conditioned media (CM) generated by NHLFs/LCAFs increased the proliferation of NSCLC cells in a HA-dependent manner and CM generated by NSCLC cells, in turn, enhanced HA synthesis by NHLFs/LCAFs and these effects were mediated, at least in part, by cytokines. Moreover, lung tumors from mice treated with the tobacco smoke carcinogen 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and lipopolysaccharide (LPS), a potent inflammatory agent, exhibited higher levels of HA and CD44 than lung tumors induced by NNK only, indicating the role of inflammation in the enhancing HA-CD44 signaling pathway. Overall, our findings indicate that the HA-CD44/RHAMM signaling pathway stands as a compelling target for the prevention and therapy of lung cancer.

Materials and Methods

Cells and reagents

The NSCLC cell lines H520, H2170, H1299, H460, H838, H2009, H2030 and H522 were purchased from ATCC, whereas A549 cell line was obtained from Caliper Life Sciences (Waltham, MA). H1650 and H1975 lines were obtained from Dr. Shujun Liu (Hormel Institute, University of Minnesota). Human THP-1 monocytes were kindly provided by Dr. Schwertfeger (Twin Cities, University of Minnesota). Normal human lung fibroblasts (NHLFs, n=3) and lung cancer associated fibroblasts (LCAFs, n=3) were kindly provided by Dr. Bitterman (Twin Cities, University of Minnesota) and were derived from lung cancer or normal lung tissues adjacent to the tumors of the same patients, respectively.

Upon receiving the cell lines, their authenticity was determined by short tandem repeat analysis technology at MD Anderson`s Cell Line Core Facility. We routinely carry out mycoplasma screening for all of our cell lines. All lung cancer cell lines and THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 incubator at 37°C, whereas NHLFs and LCAFs were maintained in DMEM-low glucose media (Sigma-Aldrich St. Louis, MO) supplemented with 10% fetal calf serum (FCS). All antibodies were obtained from Cell Signaling Technology (Beverly, MA) with the exception of Anti- PARP and anti-RHAMM which were purchased from Santa Cruz Biotechnology (Dallas, TX) and Abcam (Cambridge, MA), respectively. Hyaluronic acid sodium salt (HA, 500 kDa) was purchased from Lifecore Biomedical (Chaska, MN). 4-methylumbelliferone (4-MU) and LY2228820 were purchased from Sigma-Aldrich (St. Louis, MO) and Selleckchem (Houston, TX), respectively. CD44 monoclonal antibody (5F12) was purchased from Thermo Fisher

Scientific (Rockford, IL). CD45-PE, CD31-PE, EpCAM-BV421, and CD44-APC were obtained from Miltenyi Biotec (Auburn, CA). Human recombinant proteins (IL-6, TGF-β, IL-1β and TNF-α) were purchased from R&D systems (Minneapolis, MN). CytoMix-MSC was obtained from Miltenyi Biotec (Auburn, CA).

Preparation of conditioned media (CM)

To prepare conditioned media from NHLFs/LCAFs, cells (3×10^5) were seeded on collagen (Advanced BioMatrix, San Diego, CA)-coated dish and grown in DMEM-low glucose media supplemented with 10% FCS for 24 h. Subsequently, the media was replaced by serum-free DMEM-low glucose, cells grown in this media for 48 h, the media collected, diluted with the same volume of RPMI and used to grow NSCLC cells. For the preparation of CM from A549, cells were cultured in serum-free RPMI for 48 h. Then, the media were collected, diluted with an equal volume of DMEM-low glucose, and used to culture NHLFs and LCAFs. Also, macrophages (THP-1 cells activated with 100 ng/mL PMA) were cultured in serum free RPMI for 48 h, the CM collected, mixed 1:1 with RPMI and used to grow A549 cells or NHLFs/LCAFs.

Cell proliferation assay

Cell proliferation was determined using the methylthiazoletetrazolium (MTT; Biotium, Hayward, CA) as described previously.[20]

Quantitative reverse transcription–PCR (qRT-PCR) analysis

Total RNA was extracted by using the miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction and mRNA levels of different genes were determined as we described earlier.[20] Gene specific forward and reverse primers are shown in Supplemental Table 1.

Measurement of HA levels in mouse lung tumor tissues and culture media

Mouse lung tumor tissues were obtained from previous lung tumor bioassays in which mice were treated with one dose of NNK (100 mg/kg) and given weekly LPS treatment (2 μg/mouse), intranasally for 16 weeks or 44 weeks. Mean tumor multiplicities for the 16 weeks study were 23.2 ± 8.0 tumors/mouse in the NNK+LPS group and 7.0 ± 2.0 in the NNK group. At week 44, tumor multiplicities were 44.0 ± 3.6 tumors/mouse in the NNK+LPS group and 32.3 ± 7.6 in the NNK group. HA levels in normal and tumor-bearing mouse lung tissues as well as in culture media were determined by using a quantitative HA test kit (Corgenix, Broomfield, CO) according to the manufacturer's instruction.

Western blot analysis

For the western blot analysis, about 1×10^6 cells were suspended in the RIPA buffer containing protease and phosphatase inhibitors on ice for 1 h. Subsequently, cell lysates were prepared and Western immunoblotting assays performed as we described earlier.[20]

Flow cytometry analysis

Lung tissues (obtained from the 44 weeks mouse tumor bioassay) were mechanically dissociated and digested with Collagenase/Dispase (1 mg/mL; Roche, Mannheim, Germany) and DNaseI (0.15 mg/mL; Sigma Aldrich, St. Louis, MO) in PBS for 45 min in a 37°C water bath with agitation every 8-10 minutes. Upon completion of digestion, 30 ml of PBS containing 2% BSA was added and the resulting cell suspensions were strained through a 100 μm cell strainer to remove the non-digested tissue. Red blood cells were removed by 1x Red Blood Cell lysis buffer (eBioscience, San Diego, CA). After cells were counted, one million cells per sample were resuspended in 100 μL of staining buffer (PBS including 2% FBS) and stained for 30 minutes on ice with antibodies for the following cell surface markers: CD45-PE, CD31-PE, EpCAM-BV421, and CD44-APC. Data were acquired on a BD LSRII flow cytometer using BD FACSDiva software (BD Bioscience) and analyzed using Flowjo v10 (Flowjo, LLC).

Statistical analysis

All data are reported as mean ± SD of triplicate determinations. Between-group comparisons were performed using one-way ANOVA and two-tailed t-test in Graphpad Prism 5 software (Graphpad, La Jolla, CA). *P*-values < 0.05 were considered significant.

Results

Basal levels of HAS2, HAS3, HA, CD44 and RHAMM in NSCLC cells, lung fibroblasts and macrophages

To determine the status of HA-CD44/RHAMM signaling pathway in lung cancer cells and stromal cells, first we determined the basal levels of the three HAS isoforms (HAS1, HAS2 and

HAS3), HA, CD44 and RHAMM in NSCLC cell lines, NHLFs, LCAFs and macrophages (PMA-activated THP-1 cells). The expression of HAS1 was consistently low in all NSCLC cells, NHLFs, LCAFs and THP-1 cells (CT values > 35, Supplemental Figure 1). The expression of HAS2 was higher in NHLFs/LCAFs than in NSCLC cells or THP-1 cells (Figure 1A), whereas the level of HAS3 was higher in NSCLC cells than in NHLFs, LCAFs or THP-1 cells (Figure 1B). Unexpectedly, the level of HA in NSCLC cells was related neither to the expression of HAS2 nor to that of HAS3. Among the different NSCLC cells, A549, H1299, H1975 and H520 cells produced a relatively higher amount of HA than other cells. On the other hand, in lung fibroblasts and THP-1 cells, the level of HA appeared to be related to the expression level of HAS2 and HAS3, respectively (Figure 1C). Of all the three groups of cells, fibroblasts, in particular LCAFs, produced the highest amount of HA, followed by NSCLC cells, whereas THP-1 cells exhibited the lowest amount of HA. NSCLC cells expressed both CD44 and RHAMM. On the other hand, NHLFs/LCAFs expressed mainly CD44 and the level was much higher than that found in NSCLC cells (Figure 1D). In THP-1 cells, CD44 and RHAMM levels were highly modulated by PMA stimulation, which increased the expression of CD44 but decreased that of RHAMM (Figure 1D).

HA plays an important role in the proliferation and survival of NSCLC cells.

To assess if silencing of HAS2 and HAS3 and the resulting suppression of HA synthesis affects the proliferation and survival of NSCLC cells, five NSCLC cells (A549, H522, H2009, H2030 and H520) were transfected with combinations of specific siRNAs against HAS2 and HAS3 (50 nM of each) for 72 h. Transfection with HAS2 and HAS3 siRNA suppressed the expression of HAS2 and HAS3 by about 70-90% and 50-75%, respectively, with the exception of H2030 cells

in which HAS2 mRNA transcript levels were reduced by 40% (Supplemental Figure 2). Simultaneous silencing of HAS2 and HAS3 reduced the proliferation of A549, H522, H2009 and H520 cells by > 70%, but relatively weaker effects were observed in H2030 cells (cell proliferation was reduced by 34%, Figure 2A). As expected, silencing HAS2 and HAS3 clearly reduced HA synthesis in all cells (Fig. 2B). Overall, the data suggested a clear positive association between HAS2+HAS3 siRNA-induced suppression of HA synthesis and reduction in the proliferation of NSCLC cells. Combinations of HAS2 and HAS3 siRNAs also suppressed levels of CD44, RHAMM, total- and phospho-EGFR, Akt and ERK and induced cleavage of caspase-3 and PARP in A549, H2009 and H520 cells (Figure 2C). To determine if exogenous HA could attenuate HAS2+HAS3-siRNA-induced anti-proliferative effects, A549, H2009 and H520 cells were transfected with the siRNAs (25 nM of each) and exogenous HA (2.5 mg/mL) was added to the culture media. As shown in Figure 2D, HA partially, but significantly, rescued A549, H2009 and H520 cells from the anti-proliferative activity of the siRNAs by 21%, 34% and 20%.

Simultaneous silencing of CD44 and RHAMM suppressed the proliferation and survival of NSCLC cells

In order to assess the role of CD44 and RHAMM in regulating the growth and survival of NSCLC cells, five cell lines were transfected with combinations of siRNAs targeting CD44 and RHAMM (100 and 50 nM, respectively) and modulations of cell proliferation and cell proliferation- and survival-related proteins were determined. Combinations of CD44- and RHAMM-specific siRNAs suppressed the mRNA levels of CD44 and RHAMM by about 85-95% and 70-100%, respectively. Silencing of CD44 and RHAMM reduced the proliferation of

A549, H520, H522 and H2009 cells by about 70%, whereas the proliferation of H2030 cells, in which the protein level of RHAMM was not affected by the siRNAs, was reduced by 30% only (Figure 3A). Unexpectedly, siRNAs targeting CD44 and RHAMM also significantly reduced HA synthesis (50%) in A549 and H520 cells (Figure 3B). In line with the reduction in cell proliferation, CD44 and RHAMM siRNAs suppressed the level of total- and phospho-EGFR, Akt and ERK, and induced caspase-3 and PAPR cleavage although the magnitude of change was cell line-dependent (highest effects in A549 and H520 cells, moderate effects in H2009 and H522 cells and weak effects in H2030 cells, Figure 3C).

In subsequent studies in which we assessed whether exogenous HA could rescue A549, H2009 and H520 cells from the anti-proliferative activities of the siRNAs, we found that HA (2.5 mg/mL) completely abrogated the effect of the siRNAs in A549 cells, whereas partial and non-significant protective effects were observed in H2009 and H520 cells (Figures 3D). Exogenous HA also significantly increased the cell proliferation of the three cell lines treated with scrambled siRNAs. In line with the results of the cell proliferation studies, exogenous HA also modulated the expression of cell proliferation-and survival-related proteins in A549 cells (Figure 3E). Interestingly, in H2009 and H520 cells, exogenous HA induced Akt activation in spite of CD44 and RHAMM silencing, suggesting the presence of other HA receptors mediating HA signaling. Images of NSCLC cells transfected with scrambled siRNA, specific CD44+RHAMM siRNAs, CD44+RHAMM siRNAs+ HA or HA alone are shown in Supplemental Figure 3.

Conditioned media (CM) generated by lung fibroblasts enhanced the proliferation of lung cancer cells and these effects were mediated, at least in part, by HA.

Fibroblasts are considered to be the main source of HA in the tumor microenvironment.[21] To assess if HA produced by fibroblasts modulates the proliferation of lung cancer cells, three NSCLC cell lines were cultured in CM prepared from NHLFs or LCAFs and the effect on cell proliferation was compared to cells grown in normal culture media. Indeed, CM generated by NHLFs increased the proliferation of A549, H2030 and H1650 cells by 217%, 175% and 146%, whereas CM from LCAFs increased the proliferation of the aforementioned cells by 261%, 203% and 147%, respectively (Figure 4A-i). Consistent with this, CM generated by NHLFs and LCAFs increased expression of phospho-Akt in A549 cells (Figure 4A-ii). To determine the role of HA in these effects, first, A549 cells were grown in CM generated by LCAFs transfected with HAS2 siRNA and cell proliferation was compared to cells cultured in normal media or CM generated by LCAFs transfected with scrambled siRNA. As expected, HAS2 siRNA significantly reduced the level of HAS2 and HA synthesis in LCAFs (Figure 4B-i and 4B-ii). Also, compared to A549 cells cultured in normal media, the proliferation of cells grown in CM generated by LCAFs transfected with scrambled siRNA was increased by 230%. However, compared to LCAFs transfected with scrambled siRNA, the growth of A549 cells cultured in CM generated by HAS2 siRNA-treated LCAFs was reduced by 50% (Figure 4B-iii). These results were further corroborated by growing A549 cells in CM generated by LCAFs treated with 4-methylumbellifeorne (4-MU), a potent inhibitor of HASs. Exposure of LCAFs to 0.5 and 1 mM 4-MU reduced the level of HAS2 by 50% and 80% (Figure 4C-i) and that of HA by 50% and 70%, respectively (Figure 4C-ii). Also, the proliferation of A549 cells cultured in CM generated by 4-MU (0.5mM)-treated LCAFs was reduced significantly (from 258% in cells

cultured in CM generated by untreated LCAFs to 148% in cells cultured in CM from 4-MU-treated LCAFs, Figure 4C-iii). Furthermore, we cultured A549 cells in CM generated by LCAFs treated with LY2228820, an inhibitor of p38 MAPK, which is known to control HA synthesis in LCAFs.[22] LY2228820 significantly reduced the expression of HAS2 (Figure 4D-i) and HA synthesis (Figure 4D-ii) in LCAFs. Moreover, the proliferation of A549 cells grown in CM generated by LY2228820-treated LCAFs was decreased in a dose-dependent manner (Figure 4D-iii). In line with the role of the HA-CD44 signaling pathway in the proliferation of NSCLC cells, treatment of A549 cells with CD44 blocking antibody abrogated the pro-proliferative effects of the CM generated by LCAFs. As depicted in Figure 4E-i, compared to the growth of A549 cells grown in normal media and treated with control IgG, the growth of cells cultured in normal media and treated with a CD44 blocking antibody was reduced by 22%, whereas the growth A549 cells cultured in CM generated by LCAFs and treated with a CD44 blocking antibody was reduced by 87% compared to cells cultured under the same condition but treated with a control IgG. The effects of the CD44 blocking antibody were partially suppressed by exogenous HA (Figure 4E-ii). Taken together, our results strongly indicate that HA contained in the CM generated by NHLFs and LCAFs played a key role in the pro-growth activities of the CM in NSCLC cells.

CM generated by NSCLC cells and PMA-activated THP-1 cells enhanced HA synthesis by NHLFs and LCAFs

In the previous section, we showed that HA secreted by NHLFs and LCAFs binds to the CD44 receptor on NSCLC cells and thereby enhancing their proliferation. Here, we assessed if NSCLC cells and THP-1 cells enhance HA synthesis by fibroblasts. First, NHLFs and LCAFs were

grown in CM prepared from A549 cells and changes in the activation status of fibroblasts, as measured by expression of α-SMA and fibroblast activation protein (FAP) and HA syntheses, were determined. CM generated by A549 cells increased expression of α-SMA and FAP in NHLFs and LCAFs although the changes were not uniformly significant (Supplemental Figure 4). As shown in Figure 5A-i, compared to the sum of HA secreted by A549 cells and NHLFs or LCAFs grown in normal media, the level of HA secreted by NHLF1/2 and LCAF1/2 cultured in CM generated by A549 cells was significantly higher. No change was observed in the amount of HA secreted by NHLF3 or LCAF3, probably because of the higher basal level of HA in these cells (Fig. 1C). These results were further corroborated in studies in which the time-dependent increase in HA synthesis was assessed in NHLFs and LCAFs (Figure 5A-ii). To identify some soluble factors that could play a role in the cross-talk between NHLFs/LCAFs and NSCLC cells, first, we assessed if fibroblast-derived CM induces the expression of cytokines and growth factors in A549 cells. These studies showed that IL-6 was markedly increased when A549 cells were incubated in CM generated by LCAFs (Supplemental Figure 5). In further studies, we determined if supplementation of the growth media of NHLFs and LCAFs with different cytokines and growth factors enhances HA synthesis. Among the different agents, only IL-6 (50 ng/mL) and CytoMix (500 pg/mL) significantly increased HA synthesis in LCAFs, whereas only CytoMix showed significant effects in NHLFs (Figure 5B). Also, the amount of HA secreted by A549 cells and NHLFs/LCAFs cultured in CM generated by THP-1 cells was significantly higher than the sum of HA secreted by the individual cells grown in normal culture media (Figures 5C-i,ii and iii).

Mouse lung tumors induced by NNK+LPS exhibited higher levels of CD44 and HA than NNK-induced lung tumors or normal lung tissues

To determine if members of the HA-CD44/RHAMM signaling are differentially modulated during inflammation-associated lung tumorigenesis, mice were treated with vehicle, the pro-inflammatory agent LPS, the tobacco smoke carcinogen NNK, or LPS+NNK, sacrificed at week 16 or 44 and levels of CD44, RHAMM and HA were compared. As compared to the vehicle group, neither the LPS group nor the NNK group showed significant modulation in the expression of CD44 at either week 16 or week 44 time point. On the other hand, treatment with the combination of NNK and LPS induced a trend towards a significant change at week 16 (Fig. 6A-I) and a significant increase at week 44 (Fig. 6A-II). In further studies in which the surface expression of CD44 was determined by flow cytometry in EPCAM-positive epithelial cells isolated from lung tissues of vehicle, -NNK-, or NNK+LPS-treated mice, we observed that NNK+LPS-treated mice (44 weeks tumor bioassay) exhibited significantly higher level of CD44 than vehicle- or NNK-treated mice (Figure 6A-iii and 6A-iv). Unlike CD44, RHAMM level was not increased by NNK or NNK+LPS (Supplemental Figure 6). Next, we compared the level of HA in the different groups of mice and found that, compared to the level in other groups, the level of HA was significantly elevated in the NNK+LPS group at week 16 (6B-i). The level of HA in the NNK+LPS group was even more increased at week 44 (3-fold higher than the levels of HA at week 16).

Discussion

In the present study, we assessed the synthesis of HA and expression of its major receptors CD44 and RHAMM in human lung cancer and stromal cells as well as mouse tumor tissue, the role of

HA-CD44/RHAMM interaction in modulating the proliferation and survival of NSCLC cells and the cross-talk between lung cancer cells and lung fibroblasts. NHLFs and LCAFs exhibited the highest level of HA followed by NSCLC cells, while THP-1 cells produced negligible amount of the polysaccharide. In NSCLC cells, HAS3 appears to be the main source of HA, but there was no direct relationship between HAS expression and HA synthesis. On the contrary, in NHLFs and LCAFs, HAS2 was the predominant HAS and a higher expression of HAS2 was paralleled by increased HA synthesis. Lung tumors induced by NNK+LPS showed higher levels of HA and CD44 than tumors induced by NNK, indicating that the HA-CD44 signaling pathway plays a crucial role in inflammation-associated lung tumorigenesis. We also showed that the interaction between HA and CD44/RHAMM is highly crucial in the proliferation and survival of NSCLC cells and CM generated by fibroblasts induced the proliferation of NSCLC in a HA-dependent manner. Also, CM generated by A549 cells and THP-1 macrophages increased HA secretion by NHLFs/LCAFs. These findings shed light on the role of the HA-CD44-RHAMM signaling pathway in lung tumorigenesis and could have important implications in the design of preventive and therapeutic approaches targeting both the tumor cells and the pro-tumorigenic functions of the cancerized stroma.

The present studies as well as our previous report[20] showed wide variations in the amount of HA secreted by NSCLC cells and the reasons behind these observations are unclear. Although the expression of HAS enzymes is the most important determinant factor for HA synthesis, there was no parallelism between HAS expression and HA secretion, indicating the existence of additional players in the regulation of HA synthesis in NSCLC cells. The supply of UDP-*N*-Acetylglucosamine (UDP-GlcNAc) and UDP glucuronic acid (UDP-GlcUA), the substrates of HAS enzymes, could also be a limiting factor for HA synthesis.[21,22] Accumulating evidence

indicates that aerobic glycolysis, the hallmark of cancer, increases levels of UDP-GlcNAc[23]and UDP-GlcUA[22] and these effects are under the control of various oncogenic signals. In particular, oncogenic *Kras*, the most common oncogenic event in NSCLC, plays a vital role in the regulation of aerobic glycolysis[24], which suggests that *Kras* mutant cells could overproduce HA. Indeed, *Kras* mutant mouse lung tumors were shown to produce a high level of HA.[25] However, in the present study, not all NSCLC cells with *Kras* mutation secreted high level of HA. For instance, although both A549 and H2009 cells harbor *Kras* mutation, A549 cells secreted about 20 times higher level of HA compared to H1299 cells. Other regulators of HA synthesis that could potentially contribute to the observed differential levels of HA among NSCLC cells are post-translational modifications of HAS proteins and hyaluronidase-mediated degradation of HA. Depending on the stimulus, phosphorylation, the most common form of post-translational modification, could lead to activation or inactivation of HAS proteins. Heregulin-mediated phosphorylation[26] and AMPK-mediated phosphorylation[27] led to activation and inactivation of HAS2, thereby enhancing and suppressing HA synthesis, respectively. Expression of hyaluronidases is critical in controlling HA concentration and the level of HA is generally inversely related to the expression of hyaluronidases.[28,29] However, our preliminary studies on the expression of hyaluronidase 1 and hyaluronidase 2 in NSCLC cells (Supplemental Figure 7) failed to establish an inverse relationship between HA levels and hyaluronidase expression. Future studies are required to identify factors that are responsible for the differential secretion of HA in NSCLC cells.

Analysis of the size distributions of HA generated by different HASs demonstrated that HAS2 produces anti-tumorigenic and anti-inflammatory large sized HA (up to 2,000 kDa), whereas HAS3 synthesizes pro-inflammatory and pro-tumorigenic low molecular weight HA

(100-1,000 kDa).[3, 30] Consistent with these reports, studies in prostate cancer cells showed that HAS2 overexpression slowed the intrinsic growth of the cells, whereas co-expression of HAS2 with hyaluronidase 1 restored the growth of the cells to the control level[31], indicating that overproduction of HA via up-regulated synthesis is insufficient to bestow increased growth properties on cells and that HAS overexpression is tumorigenic only in cell types that also express hyaluronidases.[32] In the present study, NSCLC cell lines predominantly expressed HAS3, whereas NHLFs and LCAFs expressed mainly HAS2, which suggests that NSCLC cells and fibroblasts synthesize low molecular weight HA and high molecular weight HA, respectively. Our observations of high HAS2 levels in CAFs are in accord with previous reports.[25,33] Unlike cancer cells which are endowed with high hyaluronidase expression and activity, normal fibroblasts and CAFs lack hyaluronidases.[34,35] Therefore, in order to induce pro-tumorigenic effects, CAF-generated HA needs to be degraded to low molecular weight HA by hyaluronidases secreted from other cells of the tumor microenvironment. Indeed, Lokeshwar et al (2001) reported degradation of stromal cell HA into angiogenic HA fragments by cancer cell generated hyaluronidase 1, which led to the development of high-grade invasive prostate carcinoma.[35]

Our studies showed that the HA-CD44/RHAMM signaling pathway is indispensable for the proliferation and survival of NSCLC cells as demonstrated by the dramatic reduction in the proliferation of NSCLC cells upon silencing of HAS2/HAS3 or CD44/RHAMM. HAS2/HAS3 siRNAs suppressed not only HA synthesis and expression of cell proliferation- and survival-related proteins, but also CD44 and RHAMM levels, suggesting that HA regulates the expression of its receptors. Interestingly, irrespective of the basal expression of HAS2/HAS3 or CD44/RHAMM, all cell lines but one exhibited similar responses towards the anti-proliferative

effects of the siRNAs. Addition of exogenous HA failed to completely rescue HAS2+HAS3 siRNA-induced cell proliferation defect, highlighting the importance of endogenous biosynthesis of HA in cell proliferation and the differing biological activities of exogenous HA *versus* endogenously synthesized HA. This is consistent with a previous report in which exogenous high molecular weight HA failed to rescue HAS2 siRNA-induced anti-proliferative effects, whereas adenoviral-mediated overexpression of HAS3, which stimulated endogenous HA biosynthesis, was able to do so.[36] Silencing of CD44/RHAMM reduced HA secretion in NSCLC cells with high basal levels of HA, indicating the potential cross-regulation between HAS2/HAS3 and CD44/ RHAMM. In line with this, exogenous HA partially rescued NSCLC cells from CD44/RHAMM siRNA-induced cell proliferation defects, probably through induction of CD44/RHAMM (A549 cells, Figure 3F). Likewise, treatment of glioblastoma cell lines with HA considerably induced CD44 expression, especially when cells were cultured under non-adherent culture conditions.[37] Alternatively, exogenous HA could rescue CD44/RHAMM siRNA-induced cell proliferation defects by binding with alternate HA receptors such as toll-like receptors 2 and 4 (TLR2 and TLR4) which are known to be expressed in NSCLC cells.[38,39] In the absence of CD44, TLR 2 and TLR4 have been shown to mediate macrophage inflammatory gene expression in response to HA fragments.[40]

The lung cancer microenvironment is regulated by a complex bidirectional interplay between the tumor and tumor stroma and cytokines and growth factors have been shown to be key players in mediating these interactions.[41] However, the potential role of HA in mediating this cross-talk between cancer cells and stromal cells is unknown. In the present study, we found that CM produced from NHLFs/LCAFs clearly enhanced the proliferation of NSCLC cells, whereas treatment of NHLFs/LCAFs with HAS2 siRNA, 4-MU, a potent inhibitor of HA synthesis, or

LY2228820, an inhibitor of p38MAPK-dependent HA synthesis, attenuated these effects. These findings strongly indicate that HA plays a role in the pro-proliferative activities of the CM generated by NHLF/LCAF. CD44 blocking antibody suppressed the pro-growth activities of NHLF/LCAF CM in A549 cells, further indicating the involvement of the HA-CD44 signaling pathway. Our results are in accord with previous studies in which knocking down HAS2 with a specific siRNA or treatment with a HAS inhibitor markedly attenuated CAF-induced invasion and EMT of oral squamous cell carcinoma cells.[34] A549 cell-generated CM in turn enhanced HA synthesis by NHLFs/LCAFs and these effects might be mediated, at least in part, via cytokines since IL-6 and CytoMix enhanced HA secretion by NHLFs/LCAFs. Moreover, cytokines have been shown to be potent inducers of HA synthesis in fibroblasts.[42] Indeed, IL-6 appears to play a central role in the HA-mediated cross-talk between NSCLC cells and NHLFs/LCAFs since CM generated by NHLFs/LCAFs increased IL-6 expression in A549 cells while IL-6-containing CM generated by A549 cells or exogenous IL-6 in turn induced HA synthesis by NHLFs/LCAFs. Similarly, CM generated by PMA-activated THP-1 cells enhanced HA synthesis by NHLFs and LCAFs, which most likely was also mediated via cytokines since activated THP-1 cells secrete substantial quantities of cytokines.[43] Macrophages have also been found to be crucial for the maintenance of HA-rich stromal structures since mice treated with clodrolip, a drug that depletes macrophages, reduced the level of HA in the tumor stroma.[44] HA in turn played a crucial role in mediating macrophage trafficking to the tumor stroma and subsequent polarization of the macrophages in to M2 phenotype, indicating a two-way interaction between macrophages and fibroblasts.

Overall, our findings clearly demonstrated that members of the HA-CD44/RHAMM signaling pathway are expressed at high level in NSCLC cell lines, mouse lung tumors and stromal cells.

The interaction between HA and CD44/RHAMM activated the EGFR/Akt/ERK signaling pathway and thereby enhanced the proliferation and survival of NSCLC cells. Also, HA generated by stromal cells clearly enhanced the proliferation of NSCLC cells and these cells in turn increased HA synthesis by stromal cells. Our present findings provide new opportunities to develop novel approaches for the prevention and treatment of lung cancer. In this regard, drugs that inhibit HA synthesis such as 4-MU and inhibitors of p38MAPK or CD44/RHAMM are promising.

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Figure legends

1. Comparative constitutive levels of HAS2 and HAS3, HA, CD44 and RHAMM in NSCLC cells, NHLFs, LCAFs, and THP-1 cells. NSCLC cells were grown in 10% FBS containing RPMI media to 70-80% confluency. NHLFs/LCAFs were grown in serum free DMEM-low glucose media on collagen-treated dish for 48 h, whereas THP-1 cells were cultured in serum free RPMI media for 48 h. For the studies with NHLFs/LCAFS and THP-1 cells, A549 cells cultured in serum free DMEM-low glucose media and serum free RPMI media, respectively, were included as controls. (A, B) Constitutive mRNA levels of HAS2 (A) and HAS3 (B) in the different cells were measured by qRT-PCR as described in the Materials and Methods section. Fold changes in gene expression was calculated by using the expression in A549 cells as a control. (C) The concentrations of HA in the culture media were measured by ELISA. (D) Protein levels of CD44 and RHAMM in NSCLC cells (i) and NHLFs/ LCAFs and THP-1 cells (ii) as determined by Western immunoblotting using β-actin as a control. For all experiments, at least three independent assays were carried out. $P < 0.05$: *, compared to control (A549 cells); #, compared to NHLF cells.
2. Simultaneous silencing of HAS2 and HAS3 using specific siRNAs reduced the proliferation of NSCLC cells, secretion of HA by these cells and level of cell proliferation- and survival-related proteins and these effects were modulated by exogenous HA. (A) Effects of HAS2+HAS3 siRNA on the proliferation of NSCLC cells. Each cell line was transfected with a mixture of the two siRNAs (50 nM of each) and cell proliferation was determined by MTT assay. (B) Cells were transfected with the siRNAs and the concentration of HA in the culture media was determined by ELISA. (C)

HAS2+HAS3 siRNAs modulated the expression of cell proliferation-and survival-related proteins. NSCLC cells were transfected with the siRNAs, cells harvested, protein samples prepared and modulation of protein expression was assessed by Western immunoblotting. C: scrambled siRNA; S: HAS2+HAS3 siRNAs (D) Exogenous HA (2.5 mg/mL) rescued cells from the anti-proliferative effects of HAS2+HAS3 siRNAs. NSCLC cells were grown in culture media containing 2.5% FBS, treated with scrambled siRNA, HAS2+HAS3 siRNAs (25 nM of each), HAS2+HAS3 siRNAs+HA or HA alone and cell proliferation was determined by MTT assay. For all experiments, at least three independent assays were carried out. $P < 0.05$: *, compared to treatment with scrambled siRNA-treated cells; #, compared to cells treated with HAS2+HAS siRNAs.

3. Simultaneous silencing of CD44 and RHAMM using specific siRNAs reduced the proliferation of NSCLC cells, secretion of HA by these cells and level of cell proliferation- and survival-related proteins and these effects were modulated by exogenous HA. (A) Effects of CD44+RHAMM siRNA on the proliferation of NSCLC cells. Each cell line was transfected with a mixture of the two siRNAs (100 and 50 nM, respectively) and cell proliferation was determined by MTT assay. (B) Cells were transfected with the siRNAs as described above and concentration of HA in the culture media was determined by ELISA. (C) CD44+RHAMM siRNA modulated the expression of cell proliferation-and survival-related proteins. NSCLC cells were grown in culture media containing 10% FBS, transfected with the siRNAs and Western immunoblotting performed. (D) Exogenous HA (2.5 mg/mL) rescued cells from the anti-proliferative effects of HAS2+HAS3 siRNAs. NSCLC cells in culture media containing 2.5% FBS were treated with scrambled siRNA, HAS2+HAS3 siRNAs, HAS2+HAS3 siRNAs+HA

or HA alone and cell proliferation was determined by MTT assay. (E) Exogenous HA attenuated the effects of CD44+RHAMM siRNAs on cell proliferation-and survival-related proteins. NSCLC cells were transfected with scrambled siRNA, specific CD44+RHAMM siRNAs, CD44+RHAMM siRNAs+ HA or HA alone and subsequently expression of the different proteins was determined by Western immunoblotting. All assays were performed in triplicate and repeated three times on different days. $P < 0.05$: *, compared to treatment with scrambled siRNA-treated cells; #, compared to cells treated with CD44+RHAMM siRNAs.

4. CM generated by NHLFs and LCAFs increased the proliferation of NSCLC cells in a HA-dependent manner. (A) Effects of NHLF or LCAF CM on the proliferation (i) and phospho-Akt and Akt expression of NSCLC cells (ii). NHLFs/LCAFs were seeded on collagen-coated dish and grown in DMEM supplemented with 10% FCS for 24 h. Subsequently, the media was changed to serum-free DMEM, cells were grown in this media for 48 h, CM was collected, diluted with RPMI at 1:1 ratio and used in the assay. NSCLC cells were grown in either serum-free RPMI media (control) or CM generated by NHLFs or LCAFs for 48 h and cell proliferation determined by MTT assay. Expression of phospho-Akt and Akt was analyzed by Western immunoblotting. $P < 0.05$: *, compared to NSCLC cells; #, compared to NHLF CM. (B, C, D) Effects of HAS2 siRNA (B), 4-MU (C) or LY2228820 (D) on HAS2 expression (i), HA synthesis (ii) and pro-proliferative activities of LCAF-generated CM in A549 cells (iii). LCAFs were transfected with 100 nM of scrambled siRNA or HAS2 siRNA (B) treated with 4-MU (C) or LY2228820 (D) for 24 h, and CM prepared as described above. Subsequently, A549 cells were grown for 48 h either in control serum-free RPMI media, or CM generated by

untreated or HAS2 siRNA-, 4-MU or LY2228820-treated LCAFs and cell proliferation determined by MTT assay. Levels of HAS2 and HA were determined in scrambled siRNA-treated, siRNA-treated or drug-treated A549 cells as described in Figs. 1 and 2. $P < 0.05$: *, compared to LCAFs treated with scrambled siRNA (B) or DMSO (C and D); #, compared to control (A549 cells); &, compared to LCAF CM. (E) Effects of CD44 blocking antibody on the pro-proliferative activities of LCAF-generated CM. CM was prepared from LCAFs as described above and (i) A549 cells were grown in serum-free RPMI media and treated with control IgG or CD44 blocking antibody (10 μg/mL) or grown in LCAF CM in the presence of control IgG or CD44 blocking antibody. (ii) A549 cells were also grown in 2.5% FBS containing RPMI media and treated with control IgG or CD44 blocking antibody or grown in LCAF CM in the presence of HA (2.5 mg/mL) or CD44 blocking antibody and HA. All assays were performed in triplicate and repeated three times on different days. $P < 0.05$: *, compared to control cells (A549) grown in serum-free media (i) or 2.5% FBS containing RPMI media (ii) and treated with control IgG; #, compared to control IgG-treated cells (i) or HA-treated A549 cells incubated in CM generated by LCAFs (ii).

5. Modulation of HA levels by CM or cytokines in different cells. (A-i) NHLFs and LCAFs were seeded on normal culture dish and cultured for 48 h either under serum-free conditions or using CM generated by A549 cells. Subsequently, HA level in the culture media was determined by ELISA. (A-ii) Cells seeded on collagen-coated dish were grown as described above for 24 h or 48 h and HA levels were determined by ELISA. *$P < 0.05$, compared to the sum of HA secreted by cells grown in serum free media. (B) NHLFs (i) and LCAFs (ii) were untreated or treated with IL-6 (50 ng/mL), TGF-β (50

ng/mL), TNF-α (20 ng/mL), IL-1β (50 ng/mL) or CytoMix (500 pg/mL) for 48 h under serum-free culture condition and HA levels in the culture media were determined by ELISA. *P < 0.05, compared to (i) NHLF control cells and (ii) LCAF control cells. (C) CM was prepared from macrophages (THP-1 cells activated with 100 ng/mL PMA) grown in serum free RPMI for 24 h, the CM mixed 1:1 with RPMI and used to grow A549 cells (i), NHLFs (ii) or LCAFs (iii) for 48 h, and subsequently HA levels in the culture media were measured by ELISA. Cells grown under serum-free conditions were included as controls. All assays were performed in triplicate and repeated three times on different days. *P < 0.05, compared to the sum of HA secreted by the individual cells grown in serum free media.

6. Levels of CD44 and HA increased in lung tumors of mice treated with NNK+LPS. Normal lung tissues (control, LPS) and lung tumors (NNK, NNK+LPS) obtained from 16-week (A-i) or 44-week (A-ii) tumor bioassays were homogenized and used to measure expression of CD44 by qRT-PCR. (A-iii) Representative figure showing FACS-based analysis of CD44 surface expression in EPCAM-positive lung cells of mice treated with vehicle, NNK or NNK+LPS (44 weeks study). (A-iv) Bar graph showing mean ± SD of three measurements. (B) HA levels in lung tissues of untreated mice or mice treated with LPS, NNK or LPS+NNK. B-i: 16 weeks study; B-ii: 44 weeks study. All measurements were performed in triplicate and repeated three times on different days. *P < 0.05, compared to the level in vehicle-, LPS-, or NNK-treated group.

Figure 1

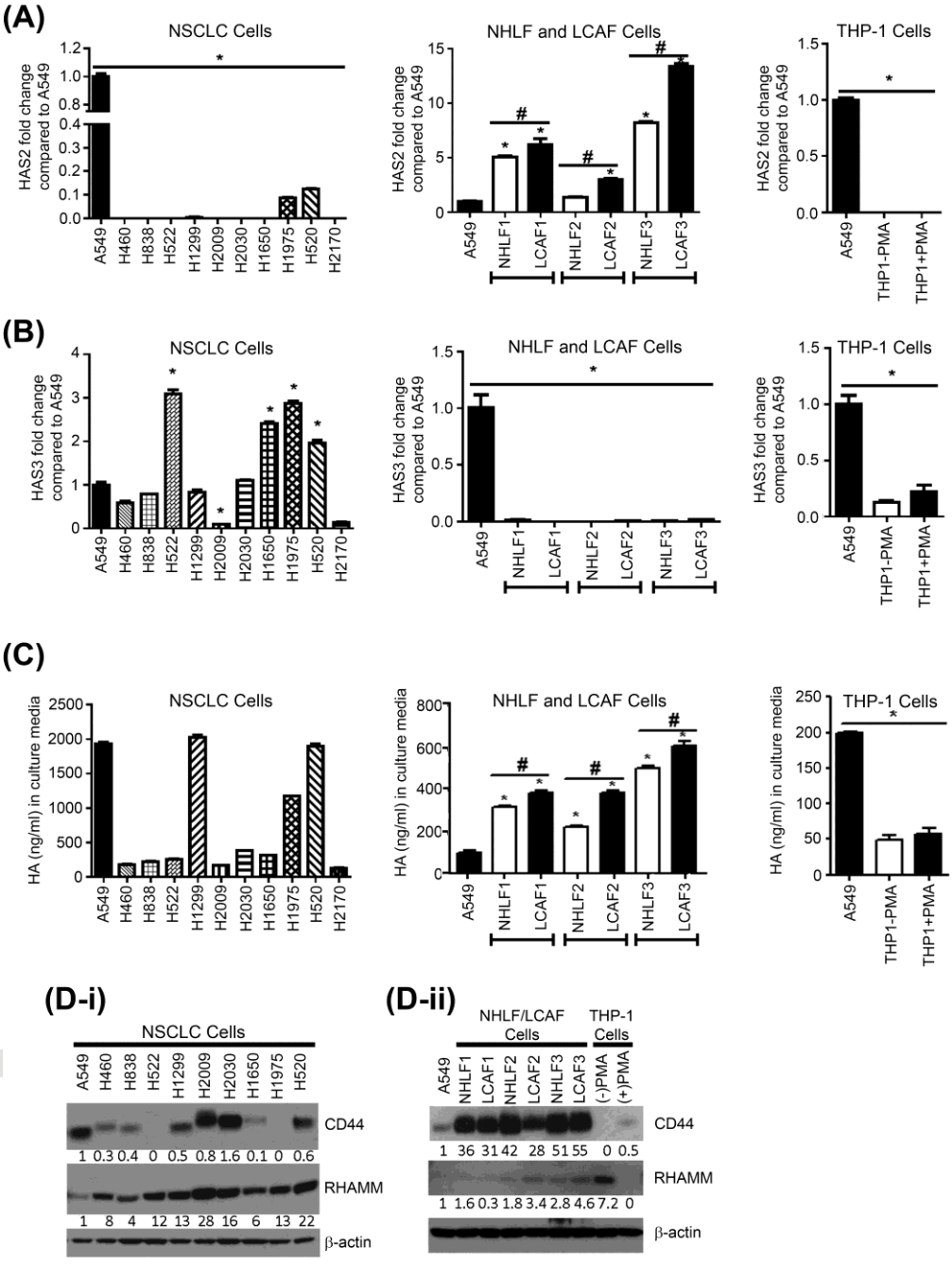

Figure 2

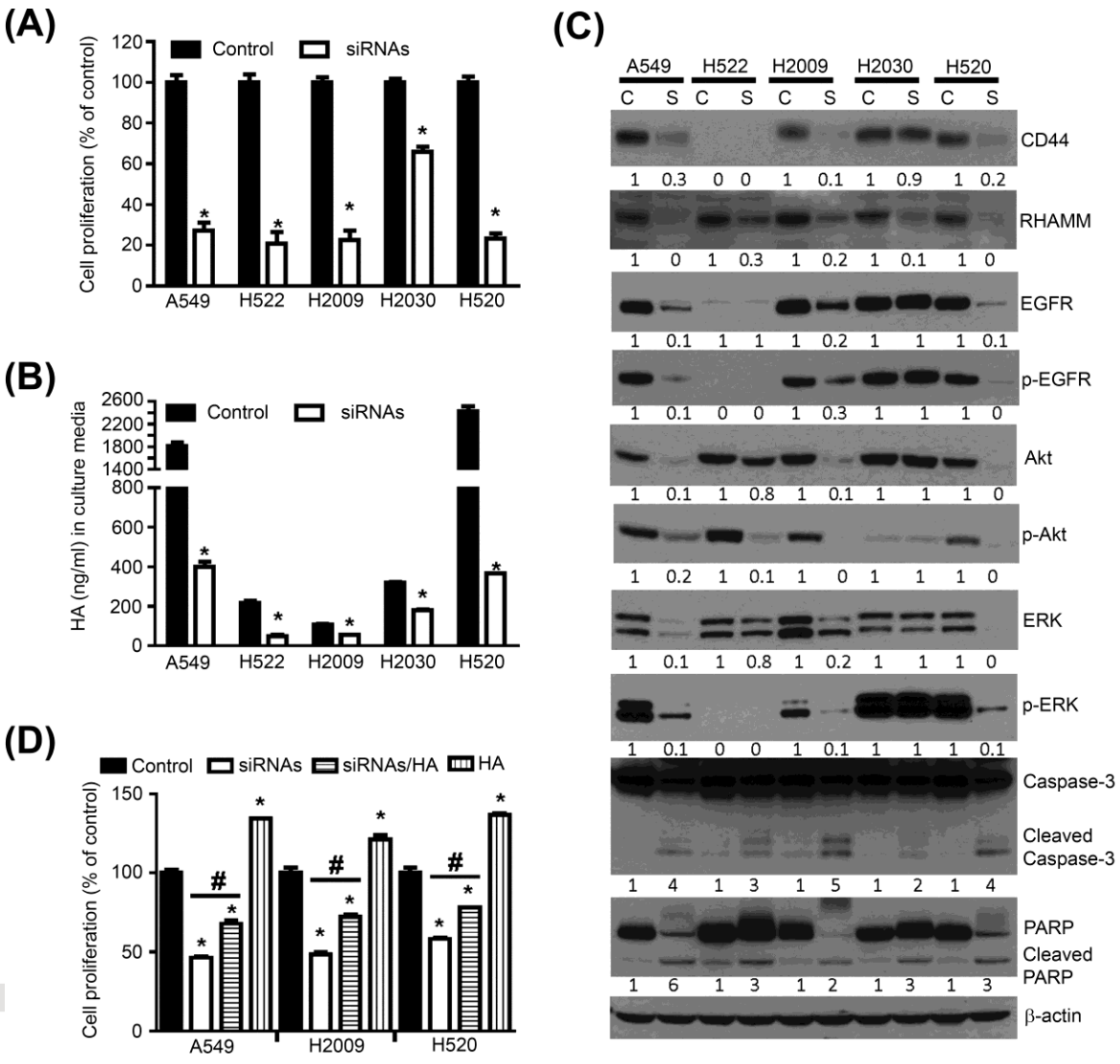

Figure 2

Figure 3

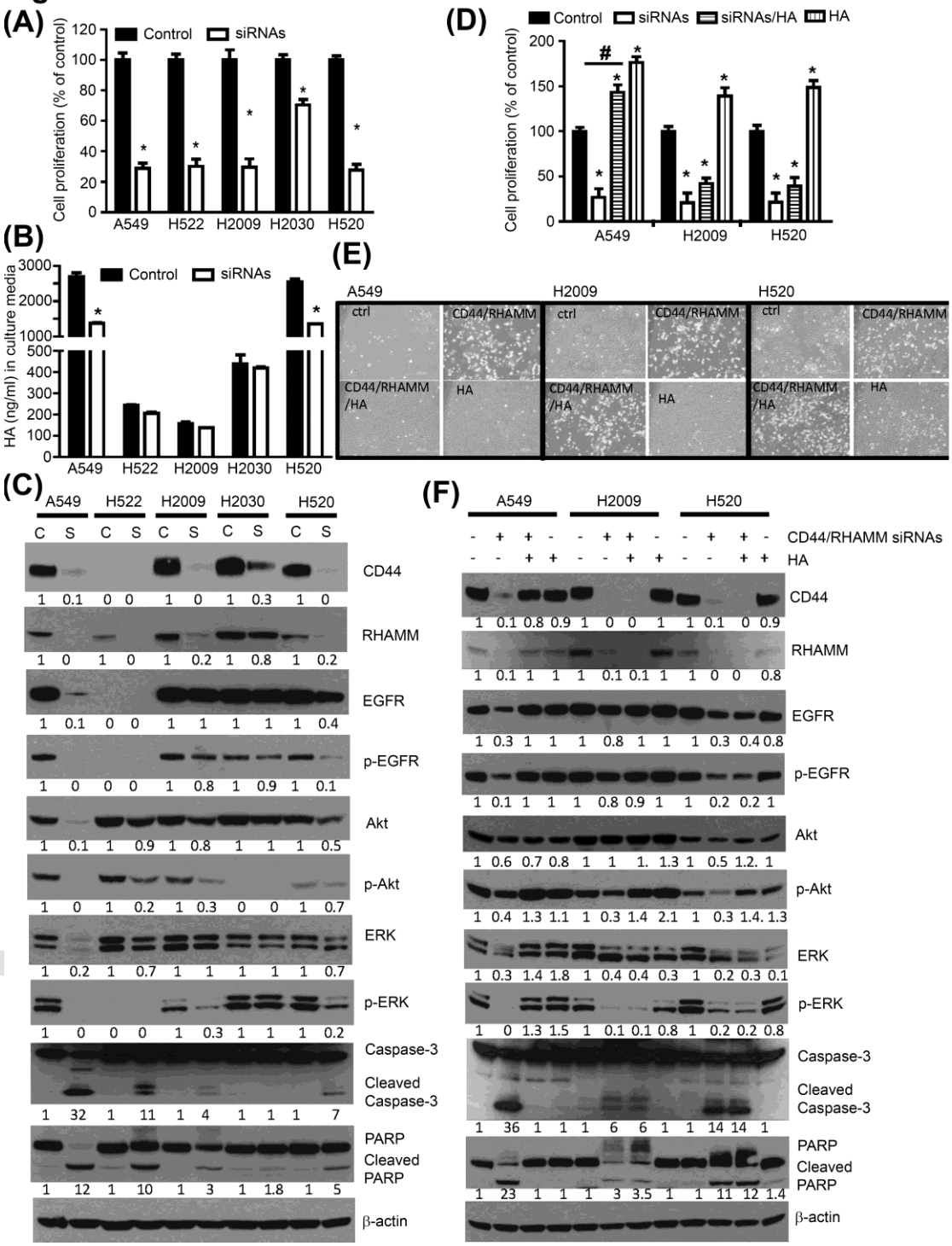

Figure 3

Figure 4

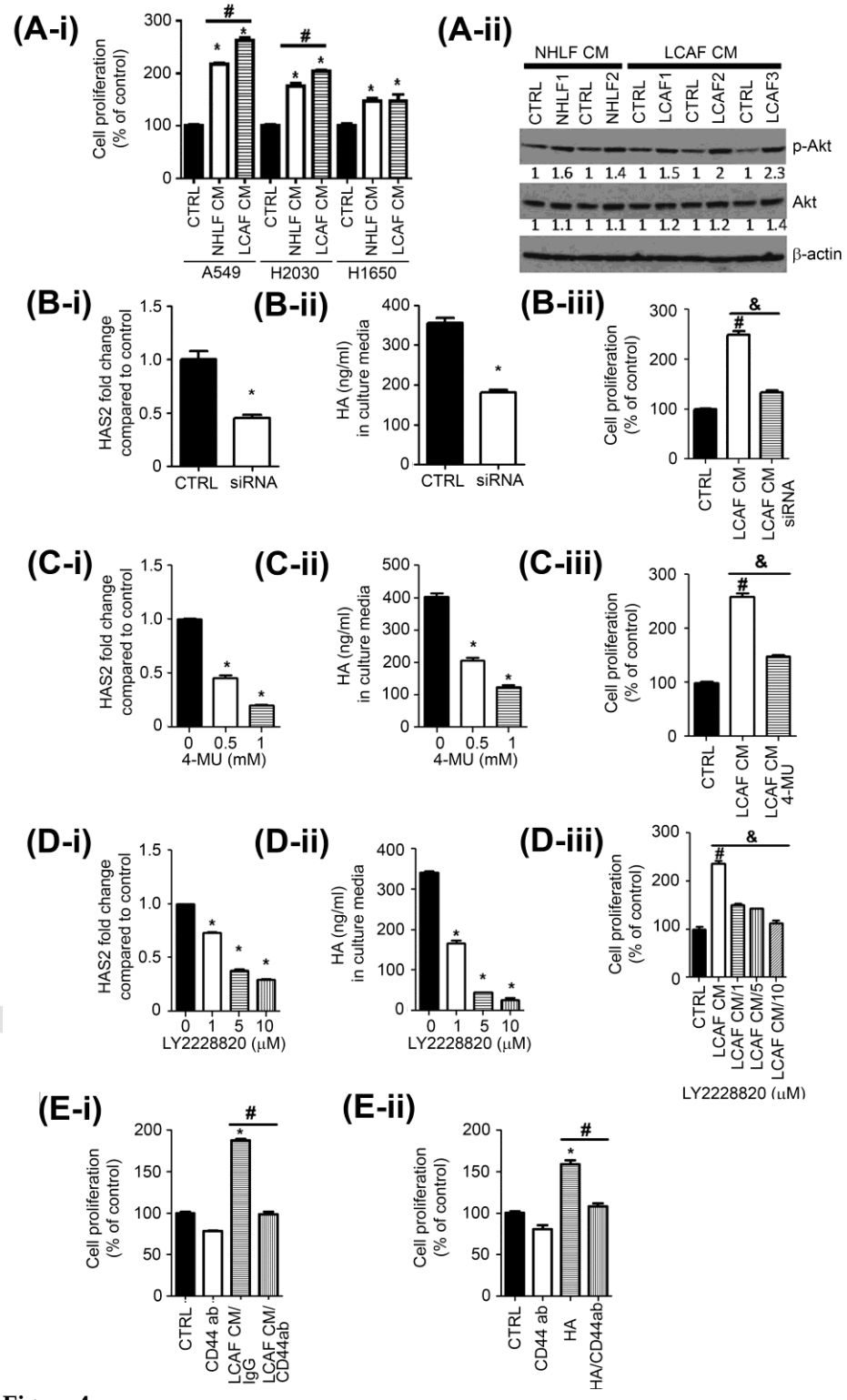

Figure 4

Figure 5

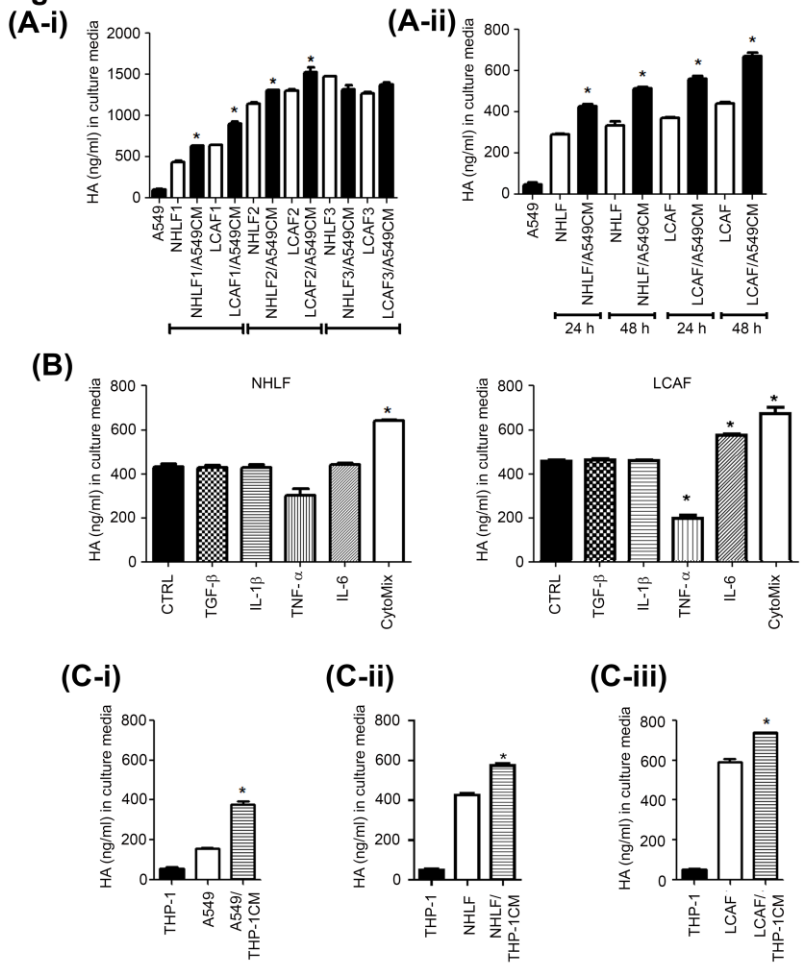

Figure 6

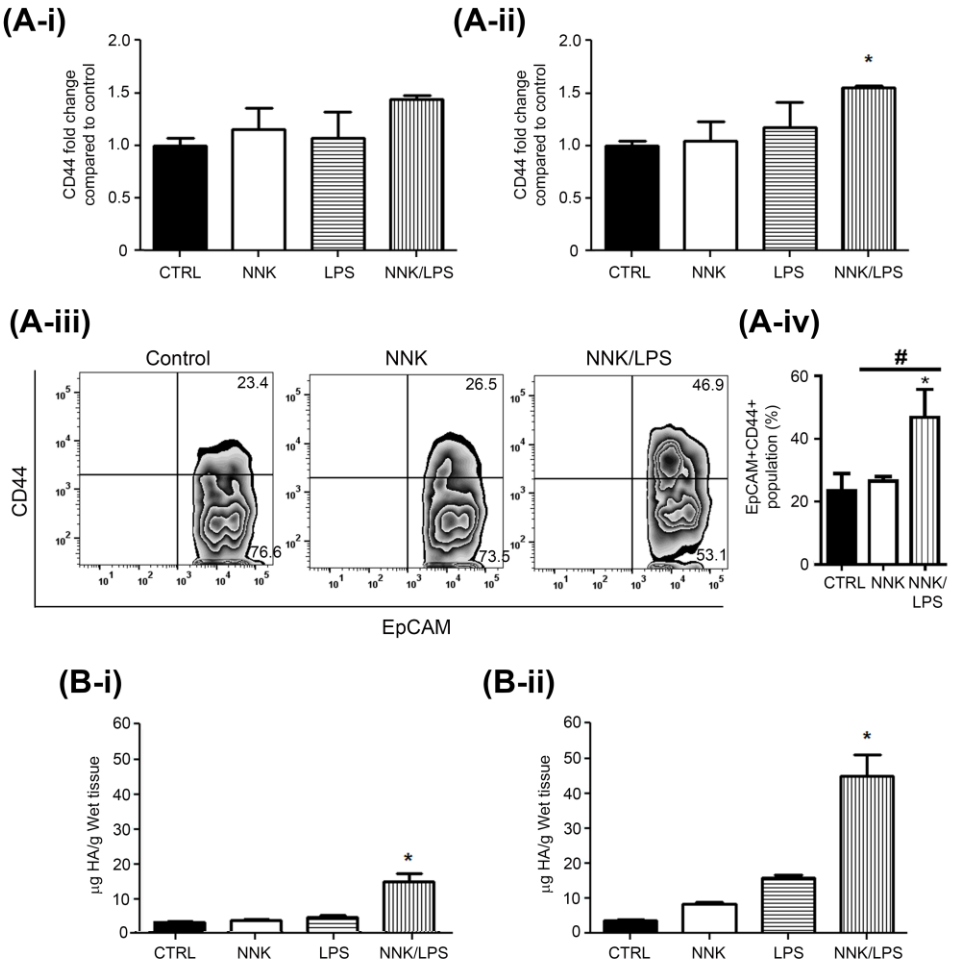

Figure 6