Hyaluronidase-1 expression promotes lung metastasis in syngeneic mouse tumor models without affecting accumulation of small hyaluronan oligosaccharides in tumor interstitial fluid

Anja Schmaus2,3 and Jonathan P Sleeman1,2,3

2Medical Faculty Mannheim, Centre for Biomedicine and Medical Technology Mannheim (CBTM), University of Heidelberg, Mannheim 68167, Germany, and 3Karlsruhe Institute of Technology (KIT), Campus Nord, Institut für Toxikologie und Genetik, Postfach 3640, Karlsruhe 76021, Germany

1To whom correspondence should be addressed: Tel: +49-621-383-9957; Fax: +49-621-383-9961; e-mail: sleeman@medma.uni-heidelberg.de

Received 6 August 2014; Revised 26 September 2014; Accepted 26 September 2014

Abstract
Enhanced levels in tumors of hyaluronan, a glycosaminoglycan component of the extracellular matrix, and hyaluronidases such as hyaluronidase-1 (Hyal1) that degrade hyaluronan have both been linked to poor prognosis and metastasis, suggesting that the turnover of hyaluronan might contribute to tumor progression. Small hyaluronan oligosaccharides (sHA) can accumulate in tumor interstitial fluid (TIF), and have been implicated in a number of processes that drive tumor progression, including MMP expression and angiogenesis. The properties of Hyal1 suggest that it might contribute to the degradation of hyaluronan in tumors and the subsequent accumulation of sHA. Accumulation of Hyal1-produced sHA may therefore account for the association between Hyal1 and metastasis. Here we have investigated this hypothesis using mouse syngeneic breast tumor models. Specifically, we modulated Hyal1 expression and activity either in the tumor cells themselves, or in the stromal compartment by using Hyal1 knockout (KO) mice. These approaches did not change sHA levels in TIF, but nevertheless fostered metastasis to the lung in some of the models used in the study. Together, these data suggest that Hyal1 can promote lung metastasis in a manner that is not dependent on altered accumulation of sHA in TIF.

Key words: hyaluronan, hyaluronidase, metastasis, oligosaccharide, tumor

Introduction
Hyaluronidases are endoglycosidases that degrade hyaluronan, a linear, non-sulfated glycosaminoglycan that is a major component of the extracellular matrix. Both hyaluronan and hyaluronidases have been implicated in tumor growth and progression. Increased amounts of hyaluronan have been reported in a variety of tumor types, and high hyaluronan levels often correlate with poor prognosis (Tammi et al. 2008). Paradoxically, expression of hyaluronidases can also correlate with tumor progression (Simpson and Lokeshwar 2008). For example, hyaluronidase-1 (Hyal1) is expressed in different human tumor types and its expression and activity correlates with tumor progression and poor prognosis (Posey et al. 2003; Ekici et al. 2004; Poola et al. 2008; Gomez et al. 2009; Kramer et al. 2011). Hyal1 can influence diverse processes important for tumor growth and progression, including tumor cell proliferation, motility, invasion and angiogenesis (Lokeshwar, Cerwinika, Lokeshwar 2005; Bharadwaj et al. 2009;
Hyal2 and Hyal3, are widely expressed in somatic tissues as well as sequence similarity, but specifically catabolizes chondroitin sulfate (Kaneiwa et al. 2010). The remaining three hyaluronidases, Hyal1, Hyal2, and Hyal3, are widely expressed in somatic tissues as well as in tumors (Csöka et al. 1999; Paiva et al. 2005; Udabage et al. 2003; Simpson and Lokeshwar 2008). In addition to being localized in the lysosomal compartment, Hyal1 can be secreted and is present in extracellular fluids (Lokeshwar et al. 1999, 2001; Patel et al. 2002; Franzmann et al. 2003; Monzón et al. 2008). In vitro the enzyme is able to degrade hyaluronan down to fragments of two or three disaccharides in length (Hofinger et al. 2007). In contrast, Hyal2 is a membrane-bound GPI-linked hyaluronidase whose end products are hyaluronan fragments of 50 disaccharides (Lepperdinger et al. 1998). Moreover, the intrinsic hyaluronidase activity of Hyal3 is controversially discussed, and it may rather be a regulator of Hyal1 stability and activity (Lokeshwar et al. 2002; Harada and Takahashi 2007; Hemming et al. 2008). Thus, the expression of Hyal1 in tumors and its association with poor prognosis, its extracellular localization and its ability to generate HA fragments <25 disaccharides in length make Hyal1 a candidate for producing sHA in the tumor context.

In this study, we aimed to investigate the connection between Hyal1 expression, sHA levels in TIF and metastasis formation in syngeneic mouse tumor models. In vivo experiments using ectopic expression of Hyal1 in tumor cells and Hyal1 KO animals revealed that Hyal1 expression can promote lung metastasis formation without affecting sHA levels in TIF. These data suggest that while Hyal1 expression may not be sufficient to augment sHA accumulation in the local tumor, it can nevertheless stimulate metastasis formation.

**Results**

Hyaluronidase activity reflects the presence of Hyal1 in conditioned medium from a panel of murine tumor cell lines

To identify appropriate syngeneic mouse tumor models in which to study the contribution of extracellular Hyal1 to tumor progression, we used western blot analysis to determine the presence of Hyal1 in conditioned medium (CM) taken from a panel of 11 cultured murine breast and lung tumor cell lines (Figure 1A). Concomitantly, we examined hyaluronidase activity in the CM (Figure 1B). Varying levels of Hyal1 protein were expressed and secreted by the cell lines analyzed, and with the exception of the RAC10P cell line, the hyaluronidase activity in the CM broadly reflected the amount of secreted Hyal1 protein. We also examined the levels of sHA in TIF derived from tumors grown from these cell lines in vivo. Tumors from only 4 of the 11 models examined were found to contain elevated levels of sHA of between 1 and 4 μg/mL (Figure 1C), levels consistent with our previous findings in human tumors (Schmaus et al. 2014). We also verified the metastatic potential in vivo of the tumor cell lines examined (Figure 1D). Increased volume of the axillary lymph nodes draining the primary tumor was taken as a measure of lymph node metastasis formation. The number of lung nodules and the proportion of animals exhibiting lung metastases was also evaluated.

Ectopic Hyal1 expression in 66cl4 tumors does not increase sHA levels in TIF but enhances lung metastasis

On the basis of the data in Figure 1, we selected the breast cancer cell line 66cl4 as being suitable to study the effect of ectopic Hyal1 expression on sHA accumulation in TIF and metastasis formation. These tumor cells produce very little Hyal1, metastasize to the lung but not to the lymph nodes, and do not produce sHA (Figure 1). The 66cl4 cells were therefore stably transfected with a Hyal1 expression construct or with empty vector DNA. Hyal1 transfectant clones which strongly express Hyal1 and secrete it into the CM were selected. In control clones, Hyal1 was virtually undetectable under these conditions (Figure 2A). To verify enzymatic activity of the secreted Hyal1, we performed hyaluronidase activity assays. As shown in Figure 2B, CM from Hyal1 transfectant clones contained higher hyaluronidase activity compared with control clones.

For in vivo studies, three Hyal1-expressing and three control clones were injected subcutaneously into mice and the tumors were allowed to grow to the legal limit. The animals were then sacrificed and the tumors excised. As shown in Figure 2C and D, hyaluronidase activities were much higher in TIF and tumor lysates in samples from tumors derived from Hyal1-transfected cells compared with samples from control tumors, demonstrating that the ectopically expressed Hyal1 is expressed, secreted and active. Hyal1-expressing 66cl4 tumors grew more slowly than control tumors (Figure 3A). We also...
analyzed sHA concentrations in TIF isolated from these tumors. Although there were strong differences in the hyaluronidase activity between Hyal1 and control tumors, sHA concentrations were not increased in TIF in response to Hyal1 expression (Figure 3B). There were also no differences in the tumor-draining lymph node volumes between the groups, indicating that Hyal1 did not promote lymph node metastasis formation (Figure 3C). Interestingly, however, Hyal1 expression enhanced both the incidence of metastasis to the lung in two of three clones, as well as the number of metastatic lung nodules in one of three clones (Figure 3D).

Ectopic Hyal1 expression in 168FARN cells has no effect on sHA levels in TIF or on metastasis formation

The breast tumor cell line 168FARN endogenously secretes significant amounts of Hyal1, but does not metastasize to the lung, and sHA does not accumulate in the TIF (Figure 1). We used these cells to further evaluate the effect of Hyal1 expression on sHA accumulation and lung metastasis by further augmenting the levels of Hyal1 in these cells through ectopic expression. As before, clones of Hyal1-transfected cells were selected that exhibited increased levels of Hyal1 and increased hyaluronidase activity in their CM compared with control clones (Figure 4A and B). Note that the exposure in Figure 4A does not allow the endogenously expressed Hyal1 in the control clones to be observed.

Hyal1-expressing and control clones were implanted in vivo as before. Although clone-to-clone differences in tumor growth rates were observed, overall there was no significant difference in the growth of Hyal1-expressing tumors compared with control tumors (Figure 5A). TIF and tumor lysates from tumors derived from the Hyal1 clones contained higher hyaluronidase activity compared with those from control tumors (Figure 4C and D). However, ectopic expression of Hyal1 did not result in sHA accumulation in TIF (Figure 5B). Hyal1 expression also had no consistent effect on lymph node metastasis (Figure 5C), and no lung metastases were observed in any of the animals.

Stromal ablation of Hyal1 in 4T1 and 66cl4 tumors decreases lung metastasis without changing sHA concentrations in TIF

Hyal1 is expressed endogenously in a variety of tissues (Csóka et al. 1999; Patel et al. 2002), and thus stromal cells may contribute to...
Hyal1 promotes lung metastasis

Fig. 2. Hyal1 is secreted and active in 66cl4 stable transfectants in vitro and in vivo. Hyal1 plasmids or the empty vector was stably transfected in 66cl4 cells. (A). By western blot, the expression and secretion of Hyal1 was analyzed in CM and cell lysates of several clones using Hyal1- or vinculin-specific antibodies as loading control. Note that the higher molecular weight of Hyal1 in the CM is probably due to N-glycosylation, which is necessary for the secretion of Hyal1 (Goto et al. 2014). (B) CM of the cells was used to compare the hyaluronidase activity in Hyal1 and vector transfectants. (C) (D) Three Hyal1-expressing clones and three control clones were injected subcutaneously in syngeneic mice (n=8 per group) and grown until they reached the legal size limit or until the animals got moribund. TIF (C) or tumor lysates (D) prepared from one tumor of each group were analyzed in hyaluronidase activity assays. In TIF of vector transfected tumors, hyaluronidase activity was not detectable (n.d.) under these experimental conditions.

Hyal1 expression in tumors. To explore the potential role of stromal Hyal1 on sHA production and tumor metastasis, we subcutaneously implanted tumor cells into Hyal1 KO mice (Martin et al. 2008). In this experimental setting, Hyal1 can be expressed by tumor cells but not by the stromal cells that constitute a considerable proportion of the tumor. First, we used 4T1 mammary cells that secrete very low levels of Hyal1, are highly metastatic, and which accumulate biologically active concentrations of sHA in TIF (Figure 1). The growth rate of 4T1 tumors did not differ between KO and wild-type (WT) animals (Figure 6A). Immunoblot analysis and hyaluronidase activity assays using tumor lysates showed that Hyal1 expression and activity are reduced in tumors from KO animals (Figure 6B and C). Nevertheless, sHA levels in TIF were similar in KO and WT mice (Figure 6D). In Hyal1 KO animals, the volume of the tumor-draining lymph nodes was not significantly reduced compared with WT animals (Figure 6E). However, Hyal1 KO mice showed a significant reduction in the number of lung metastases (Figure 6F).

To further validate the finding that stromal Hyal1 influences lung metastasis independently of sHA accumulation in TIF, we performed additional tumor experiments with 66cl4 tumor cells that produce very little Hyal1, metastasize to the lung and do not exhibit accumulation of sHA in their TIF (Figure 1). Tumor growth was equivalent in Hyal1 KO and WT mice (Figure 7A). Analysis of Hyal1 expression and activity in tumor lysates revealed only a marginal reduction in the KO animals compared with WT animals (Figure 7B and C). Levels of sHA in TIF were very low as expected in both WT and KO animals (Figure 7D). No significant differences in the volume of the tumor-draining lymph nodes were observed between WT and KO animals (Figure 7E). However, fewer metastatic nodules were found on the surface of the lung in Hyal1 KO animals than in control animals (Figure 7F).

Together, data from two different tumor models indicate that the lack of Hyal1 in the stromal tumor compartment does not reduce sHA levels in TIF but decreases lung metastasis.

Discussion

Hyal1 expression has been reported to correlate with poor prognosis and metastasis in human cancer patients, and experimental studies have identified potential mechanisms by which Hyal1 might exert these effects. In particular, the biological properties of Hyal1 suggest it may be involved in the degradation of hyaluronan in the extracellular space and the subsequent accumulation of sHA in the interstitial fluid. In turn, sHA has the potential to stimulate processes associated with tumor growth and metastasis. The augmentation of sHA accumulation by Hyal1 has been proposed to account for the association of Hyal1 expression with tumor progression. Here we have addressed this hypothesis using experimental animal models. Together, our findings suggest that expression of Hyal1 in either tumor cells or tumor-associated stromal cells is not sufficient to stimulate accumulation of sHA in TIF. Nevertheless, Hyal1 expression can be sufficient to promote metastasis formation in the lungs. These data suggest that Hyal1 can contribute to tumor progression independently of any role it may have in the production and accumulation of sHA in primary tumors.

Modulation of Hyal1 expression had no effect on the accumulation of sHA in TIF in any of our experiments. Our data clearly show that biologically active Hyal1—both endogenously and ectopically produced—is secreted into the extracellular space. It could therefore be argued that insufficient hyaluronan was present in the extracellular space for the Hyal1 to degrade into sHA. However, this explanation seems unlikely, as high concentrations of hyaluronan were found in all TIF samples analyzed (Schmaus et al. 2014). As degradation of hyaluronan is thought to occur in a stepwise manner,
between Hyal1-expressing tumors and control tumors could be observed. 

The accumulation of sHA and HMW-HA is a process that continues until the animals got moribund. (Fig. 3A) Upon autopsy, the axillary lymph nodes of tumor-bearing animals were counted on the surface (n = 8). Freshly excised tumors were used for preparation of TIF. 

Fig. 3. Hyal1 expression in 66cl4 tumors decreases tumor growth, does not increase sHA concentrations in TIF but promotes lung metastasis. Hyal1 and vector control clones were injected subcutaneously in mice (n = 8 per group) and tumors were allowed to grow until they reached the legal size limit or when animals got moribund. (A). The tumor volume was determined twice weekly per caliper measurement and is given as mean ± SE. Data collection ceased on day 40 when the first animals had to be killed due to reaching the prescribed legal tumor size limit or because they were moribund. At days 28 and 40 post tumor cell injection statistically significant differences between Hyal1-expressing tumors and control tumors could be observed (P < 0.05). (B). Freshly excised tumors were used for preparation of TIF. For separation of sHA and HMW-HA, ultracentrifugation filtrates of TIF were prepared and analyzed for their sHA concentration by HA ELISA-like assay (n = 3–6). (C) Upon autopsy, the auxiliary lymph nodes of tumor-bearing animals were removed and their size measured (n = 8). (D) In lungs from tumor-bearing animals, metastatic nodules were counted on the surface (n = 8). With Hyal1 clone 3, the mean number of metastatic nodules on the lung surface is significantly increased compared with vector clones (* P < 0.05). In addition, numbers given in the diagram indicate the number of animals with detectable metastatic nodules on the lung surface from the total number of animals analyzed. With Hyal1 clone 2 or 3, all animals (8 of 8) have lung metastases.
The association between Hyal1 expression and poor prognosis indicates that Hyal1 promotes metastasis. Studies by others have reported that Hyal1 can promote lymph node metastasis (Patel et al. 2002; Kovar et al. 2006; Bharadwaj et al. 2009), although in our experiments we did not observe a pronounced effect on metastasis to lymph nodes. The ability of Hyal1 to foster lung metastasis is dependent on the specific tumor context, as Hyal1 expression promoted lung metastasis in three of the experimental models studied here, but this was not the case for Hyal1-expressing 168FARN tumors, despite high levels of expression. Overall differences in Hyal1 levels in tumors growing in either Hyal1 KO or WT mice were relatively small, in accordance with data showing that Hyal1 is mainly expressed by tumor cells rather than stromal cells (Lokeshwar et al. 2001; Posey et al. 2003; Ekici et al. 2004; Kramer et al. 2010). Nevertheless, ablation of Hyal1 expression in the host tissue reduced lung metastasis.

Fig. 4. Hyal1 is secreted and active in 168FARN stable transfectants in vitro and in vivo. Single cell clones were generated from 168FARN tumor cells transfected with either Hyal1 or the empty vector as control. (A). In several clones, Hyal1 expression was analyzed in CM and in cell lysates by western blot. Probing with anti-vinculin antibodies served as a loading control. (B). In CM of transfectant clones, the hyaluronidase activity was determined as described in Materials and Methods. (C and D). Three Hyal1 transfectant clones (clones 1, 2 and 4) and three vector clones (clones 2, 4 and 6) were subcutaneously implanted in mice (n = 5–7 per group). Tumors were excised when they reached the legal size limit. From one or two individual tumors per clone (samples A and B) TIF (C) and tumor lysates (D) were prepared and the hyaluronidase activity was analyzed.

Fig. 5. Influence of Hyal1 expression in 168FARN tumors on tumor growth, sHA levels in TIF and lymph node metastasis. 168FARN Hyal1 and vector transfectant clones were injected subcutaneously in mice (n = 5–7 per group). Animals were sacrificed when tumors reached the legal size limit. (A) Tumor growth was monitored regularly per caliper measurement and is given as mean ± SE. Data collection ceased when the first animal of the group had to be killed due to reaching the prescribed legal tumor size limit. (B) Tumors were excised, TIF was prepared and centrifuged through ultracentrifugation filtrates for separation of sHA and HMW-HA. The concentration of sHA was then determined by ELISA-like assay. Data shown represent the mean of 3–6 samples per clone ± SE. (C) In tumor bearing animals, the volume of the axillary lymph nodes on the ipsilateral and contralateral side of the tumor was assessed (n = 5–7).
Our data indicate that changes in the accumulation of sHA in the extracellular microenvironment of primary tumors cannot account for the effects of Hyal1 expression on lung metastasis that we observed. A number of alternative mechanisms are conceivable to explain these effects. For example, the production and accumulation of mid-sized HA fragments of >25 disaccharides in length would not be detected by the methods used here, and such fragments might contribute to tumor progression through their immunostimulatory function (McKee et al. 1996; Noble et al. 1996). The presence of such HA fragments was recently shown in the skin of Hyal1 transgenic mice (Muto et al. 2014). Furthermore, Hyal1 can increase the invasiveness of tumor cells (Lokeshwar, Cerwinka, Lokeshwar 2005; Lokeshwar, Cerwinka, Isoyama, et al. 2005; Tan et al. 2010, 2011), a key step in the process of metastasis. Moreover, hyaluronan in the circulation has been reported to suppress lung metastasis formation by inhibiting binding of tumor cells to the lung endothelium (Hirose et al. 2012). Increased circulating levels of hyaluronan in Hyal1 KO mice could therefore conceivably reduce metastasis by impeding the binding of tumor cells to the vasculature in the lung. However, we note that serum HA levels in Hyal1 KO mice have been reported to be equivalent to WT mice (Martin et al. 2008). Future work will aim at elucidating how Hyal1 contributes to lung metastasis formation.

**Materials and Methods**

**Animals and cell culture**

168FARN, 66cl4, 67NR, 4T07, 4T1, Rac5E, Rac10P, Rac34E, 3LL, LLmet and Line1 mouse tumor cell lines were kindly provided by...
Eugene Lukanidin (Copenhagen, Denmark) and have been described previously (Sugiura and Stock 1955; Bahler et al. 1984; Olsson and Forchhammer 1984; Sonnenberg et al. 1986; Aslakson and Miller 1992). 3LL and LLmet cells were cultivated in RPMI supplemented with 10% FCS. All other cell lines were maintained in DMEM, 10% FCS. Mice were maintained under specific pathogen-free conditions. C57Bl/6 and Balb/c mice were bred in-house. Hyal1 KO mice (B6.129X1-Hyal1tm1Stn/Mmcd) were purchased from the Mutant Mouse Regional Resource Center and genotyped as described (Martin et al. 2008). In-house they were crossed onto the Balb/c genetic background for at least six generations. Heterozygous mice were then crossed to obtain WT and KO littermates for tumor experiments.

Transfection and stable selection of Hyal1-expressing tumor cells

The murine Hyal1 coding sequence (NM_008317) was amplified by RT-PCR from mouse tissue and cloned in the pEF6/V5-Topo vector (Life Technologies, Darmstadt, Germany). The following primers were used: 5′-GGCAAGACATGGGGC3′ and 5′-GGTGAGTGGACAGC3′. 66cl4 and 168FARN cells were transfected with vector alone or plasmids encoding Hyal1. Transfections were performed with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Stable transfectants were selected in growth medium containing blasticidin. Individual clones were expanded and screened for Hyal1 gene expression and activity as described in the section Hyaluronidase activity assays.

Fig. 7. Analysis of 66cl4 tumors in Hyal1 KO and WT mice. 66cl4 tumor cells were injected subcutaneously in Hyal1 KO and WT mice (n = 12). Animals were killed when tumors reached the legal size limit or when animals got moribund. (A) Tumor growth was monitored twice weekly and is given as mean ± SE. Data collection for all animals ceased when the first animals had to be killed due to reaching the prescribed legal tumor size limit or because they became moribund. (B). Hyal1 expression levels were analyzed in lysates from four individual tumors of each group by western blot. The blot was probed with Hyal1 or vinculin-specific antibodies as loading control. (C). Hyaluronidase activity was determined in tumor lysates from three individual tumors of WT or KO animals. (D) In ultracentrifugation filtrates of TIF, sHA levels were analyzed by HA ELISA-like assay (WT: n = 9; KO: n = 7). (E). In tumor bearing animals, the volume of the axillary lymph nodes on the ipsilateral and contralateral side of the tumor was assessed (WT: n = 10, KO: n = 10) (F). Shown is the number of metastatic nodules on the lung surface of tumor bearing WT and Hyal1 KO animals (n = 10). Differences between WT and KO animals are not significant (P = 0.079).
Tumor experiments in vivo
All animal experiments were approved by the local authorities. Tumor cells (1 x 10⁶) were injected subcutaneously into one flank of 8- to 12-week-old syngenic mice. Tumor growth was monitored routinely using caliper measurement and the volume was calculated according to the formula of an ellipsoid. The mice were killed when tumors reached the prescribed German legal limit or when the animals became moribund. The primary tumor was excised and the surrounding tissue was removed. Part of the tumor was snap-frozen in liquid nitrogen and homogenized in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 100 mM PMSF and protease inhibitor cocktail) using the TissueLyzer II (Qiagen, Hilden, Germany). This tumor lysate was subsequently clarified by centrifugation. TIF was prepared from the remaining freshly excised tumor using low-speed centrifugation as described (Wiig et al. 2003; Schmaus et al. 2014). To assess lymph node metastasis, the volume of the draining axillary lymph nodes on the ipsilateral and contralateral side of the tumor was determined as described (Schmaus et al. 2014). In brief, TIF was prepared from the remaining freshly excised tumor using low-speed centrifugation as described (Wiig et al. 2003; Schmaus et al. 2014). To assess lymph node metastasis, the volume of the draining axillary lymph nodes on the ipsilateral and contralateral side of the tumor was determined. In addition, the number of metastatic nodules on the surface of the lung was counted.

Western blot
Hyal1 expression was analyzed in lysates from cultivated tumor cells and from frozen tumor samples. Secreted Hyal1 was assessed in serum-free medium conditioned by confluent cells for 24 h. For detection of endogenous Hyal1, 1 mL CM was concentrated and analyzed. For detection of transfected Hyal1, unconcentrated medium was used. The samples were subjected to western blot analysis using Hyal1-specific antibodies (Santa Cruz, Heidelberg, Germany). For evaluation of protein loading, the blot was probed with vinculin-specific antibodies (Sigma-Aldrich, Taufkirchen, Germany).

Hyaluronidase activity assays
Hyaluronidase activity was assessed using a modified microplate assay based on the precipitation of undigested hyaluronan with cetylpyridinium chloride (Tung et al. 1994). Hyal1 activity was assayed in 0.3 M sodium acetate buffer, pH 3.7. In brief, 100 µL aliquots of 0.5 mg/mL rooster comb hyaluronan (Sigma-Aldrich)/0.8% agarose dissolved in buffer were pipetted into each well of a 96-well microtiter plate. After polymerization, samples were added in 100 µL of buffer and incubated for 24 h at 37°C. All samples were analyzed in triplicate. The hyaluronan-agarose gels were then washed with H2O and undigested hyaluronan was precipitated for 30 min at room temperature with 10% cetylpyridinium chloride (Sigma-Aldrich) dissolved in H2O. The resulting turbidity was quantified by measuring the absorbance at 595 nm using a plate reader (Bio-Instruments, Jena, Germany). For standard curves, hyaluronidase from bovine testis (Sigma-Aldrich) was used and assayed in 0.3 M sodium phosphate buffer, pH 5.3, using different enzymatic activities (0.03–30 U/mL). The hyaluronidase activity in tumor lysates or in CM (units/mL) was determined relative to the standard curve. The values were then normalized relative to the total amount of protein in the samples and expressed in units per milligram.

Analysis of sHA concentrations in TIF
The concentration of hyaluronan from 6 to 23 disaccharides in TIF was determined as described (Schmaus et al. 2014). In brief, TIF was centrifuged through Amicon ultracentrifugal filters with a molecular-weight cutoff of 10 kDa (Millipore, MA). The sHA concentration in the ultracentrifugation filtrate was assessed using a commercial HA ELISA-like assay according to manufacturer’s protocol (Echelon, UT). Where the volume of TIF was not sufficient for analysis, TIF from different individual tumors was pooled as indicated.

Statistical analysis
Data are expressed as mean ± SE. Statistical significance was determined using the Student’s t-test.

Acknowledgments
The authors gratefully acknowledge the expert technical assistance of Diana Plaumann-Ziegler, Melanie Rothley, Svenja Wagner, Selma Huber, Hella Zabanski, Sabine Müller and Manuela Sauer.

Conflict of Interest statement
None declared.

Funding
This work was supported by a grant to J.P.S. (Deutsche Forschungsgemeinschaft Schwerpunkt Program SPP1190 (tumor-vehicle interface) and by contract research Glykobiologie/Glykomik of the Baden-Württemberg Stiftung. J.P.S. is the “Franz-Volhard-Stiftungsprofessur für Mikrovaskuläre Biologie und Pathobiologie” funded by the Klinikum Mannheim gGmbH.

Abbreviations
sHA, small hyaluronic oligosaccharides; HMW-HA, high-molecular-weight hyaluronic acid; TIF, tumor interstitial fluid; Hyal1, hyaluronidase-1; CM, conditioned medium; KO, knockout; WT, wild-type.

References
Hyal1 promotes lung metastasis


