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PLASTICITY OF CD44S EXPRESSION DURING PROGRESSION AND METASTASIS OF FIBROSARCOMA IN AN ANIMAL MODEL SYSTEM

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1. ABSTRACT

CD44s (standard isoform), which binds hyaluronan (HA), has been analyzed for its significance during fibrosarcoma progression and metastasis in an athymic nude mouse model using Balb/c 3T3 cells transformed with *ras* or *sis* oncogenes. While *ras* (but not *sis*) transformation leads to upregulated expression of mouse CD44s and HA binding, transfection/overexpression of human CD44s gene (hCD44s) elicited remarkable plasticity of expression during progression and metastasis. In 3T3, hCD44s induced tumorigenesis, HA binding, and micrometastatic competence to lungs and other organs. In tumorigenic (but nonmetastatic) *sis* transformants or *ras*-deleted revertants, it also induced micrometastatic competence. Conversely, large primary tumors and overt metastases lost hCD44s expression and HA binding via hypermethylation of hCD44s gene. Tail vein injections revealed that hCD44s greatly increased the efficiency of colonization of the lung microvasculature at the earliest stages. These studies indicate that hCD44s overexpression and possibly its HA binding are critical for conveying metastatic competence but are antagonistic or selected against during aggressive primary tumor or overt metastasis outgrowth. This remarkable plasticity of expression and its consequences offer an ideal system for dissecting the molecular mechanisms operating during fibrosarcoma progression and metastasis.

2. INTRODUCTION AND BACKGROUND

2.1 CD44 in primary tumors

As primary tumors develop from the original malignantly-converted cell via activation of oncogene(s) and downregulation of specific tumor-suppressor gene(s), it is unclear whether overexpression of CD44 plays any functional role in the progression of the primary tumor from its earliest precursor to a large palpable tumor (1-3; also chapters in this special edition of Frontiers in Bioscience). In select tumor systems where relatively large tumors have been tested, there is some evidence that CD44 levels (specifically the "s" or standard isoform or the "v" variant alternatively-spliced forms) change qualitatively and/or quantitatively. Thus, human neuroblastoma development into more aggressive stages of disease is associated with reduction in CD44v forms (4,5). In our

own studies of mouse fibrosarcoma induced with different oncogenes, overexpression of transfected human CD44s was invariably lost during primary tumor expansion as if this HA(hyaluronan)-binding/lymphocyte-homing form of the molecule was antagonistic to aggressive outgrowth of tumor cells at the subcutaneous site (see sections 4-6 below).

In contrast, upregulation of CD44v forms has been noted in some human tumor classes. This has been noted for breast and colon tumors when compared to normal tissues (6-9). This is complicated by potential variability of CD44v expression in the various epithelial cell classes comprising these complex organs, thereby obviating any assignment of the original cell type of origin for the cancer. However, expression of new isoforms in the tumor is suggestive of a different expression pattern from that of the cell/tissue of origin, as well as possible functional significance for that particular isoform (6,9). This qualitative change in CD44 isoform expression was particularly variable when comparing gastric mucosa with many adenocarcinomas of this tissue, particularly v5 and v6 isoforms and with unusual retention of intron 9 in coding sequences (10,11). In contrast, two different patterns were observed in lung cancers--non-small-cell carcinomas upregulated expression of CD44s (but not CD44v) while small-cell carcinomas had greatly reduced levels of CD44s (12). It is also interesting to note that lung carcinoma cells, possibly via their CD44, can stimulate production of hyaluronan by neighboring fibroblasts, providing a mechanism of synergy between two different cell classes in this organ (13).

In model systems, oncogenes have varying capacities for changing levels of CD44 on target cells. The oncogene Ha-*ras* upregulates CD44s expression in rat embryonic fibroblasts by a transcriptional mechanism; this regulation is "neutralized" by co-introduction of the adenovirus *E1A* gene (14). Similarly, either *ras* or *src* oncogenes upregulate expression of CD44s in rat intestinal epithelial cells (15). Our own studies of Balb/c mouse 3T3/oncogene transfectants demonstrated two different consequences depending on the oncogene used-- *ras* dramatically upregulated CD44s expression while the human *sis* oncogene was without effect (see sections 4-6 below).

2.2 CD44 correlates with metastasis

In contrast to the ambiguity between CD44 isoforms and their levels with primary tumor formation, the arguments for its significance in tumor progression and metastasis are far more compelling. The exception is human neuroblastoma where a negative correlation has been observed (4,5). Gunthert *et al* (16) addressed CD44 function more directly in metastasis by transfecting an overexpressing plasmid harboring CD44s or CD44v isoforms into nonmetastatic rat pancreatic carcinoma cells. While the CD44s gene was without effect in this tumor, CD44v overexpression conveyed metastatic competence to these cells in a mouse model system. Conversely, Sy *et al* (17) observed that transfection of overexpressing CD44s gene (but not CD44v) into nonmetastatic lymphoma cells conveyed metastatic competence. Our own studies in three different mouse fibrosarcoma systems demonstrate acquisition of metastatic competence by overexpressing human CD44s gene (sections 4-6 below). Taken together, these studies indicate distinct functional differences between the standard or variant isoforms in providing metastatic competence--the "v" forms are principally effective in epithelial-derived tumors while the "s" form is effective in lymphoid and connective tissue-derived tumors. Many other systems will need to be tested to confirm these cell-type specificity relationships. These results also suggest that hyaluronan binding by CD44 is very important for lymphoid and connective tissue cells while relatively unimportant (or possibly antagonistic) for carcinoma progression.

Other studies reinforced the interrelationship between overexpression of specific isoforms of CD44 and tumor progression. The v6 isoform is not found in human colon adenomas but becomes prominent in invasive/metastatic colon carcinomas (Duke's class C and D tumors)(18). Other isoforms became evident in metastases of human breast and colon carcinomas when compared to the primary tumors; there were also differences between these two major tumor classes based on their organ of origin (6). A particularly intriguing contrast was made by comparing glioblastomas of the brain with metastases in the brain of other tumor cell types in order to distinguish cell-type and/or tumor-type specificity (19). Glioblastomas are highly invasive but do not metastasize outside the brain; their tumors upregulate CD44s but do not express any CD44v (19). In contrast, most of the metastases of carcinomas originating in other organs were highly expressive of CD44v forms when identified in the brain, consistent with these isoforms as important in spread to this organ (19). *In vitro* studies further dissected the functional importance of CD44s in glioblastoma invasion of Matrigel and adhesion of these cells to several extracellular matrix proteins (20). These comparisons provide compelling evidence for the specific isoform being upregulated in specific tumor classes with its importance in metastatic spread of that tumor class.

2.3 Parallels with lymphocyte homing

CD44s and a select few CD44v forms have a lymphocyte-homing domain on sequences proximal to the plasma membrane at its external face(1). The "receptor" for this function has yet to be identified while its significance for lymphocyte homing appears indisputable (studies described more fully in reference (1) and other chapters of this Frontiers in Bioscience special edition). Since metastasis also involves migration and penetration of tumor cells into foreign tissues and through blood vessel walls in ways that may mimic lymphocyte homing, it is reasonable to address the potential relationships between these two complex migratory patterns. A number of studies have begun to do so, including our own studies below (see section 6). However, the roles of CD44v isoforms that have lost lymphocyte-homing and/or HA-binding competence cannot be explained so easily; perhaps, these particular spliced sequences are recognized by counter-receptors on endothelial or other cell types that serve similar functions to lymphocyte-recognition sites.

The comparisons between the two systems were initially drawn by Arch *et al* (21) when they demonstrated that a particular "v" form of CD44, associated with tumor metastasis, also appears transiently on the surfaces of B and T lymphocytes in relatively low concentrations after antigenic stimulation. Antibody recognizing this specific

sequence inhibits B and T cell activation. DeGrendele *et al* (22) showed that CD44s expressed on lymphocytes mediates adhesion and rolling on an endothelium via its HA-binding function. These reactions were subsequently shown important in murine $V_\beta 8^+$ T cells in response to specific antigen stimulation in three different sites in the animal--lymph nodes, peripheral blood, and the peritoneum where an inflammation had been induced(23). These authors concluded that CD44:HA binding could target lymphocytes to specific inflammatory sites(23). These results taken together raise the intriguing question whether metastatic conversion of tumor cells could effect the same relationships between CD44s overexpression and targeted organs (see section 6 on this important issue).

3. CD44S AND GROWTH REGULATION IN THE 3T3/ONCOGENE SYSTEM

3.1 CD44 levels in oncogene transformants and tumors

Our laboratory had been studying an isogenic fibrosarcoma system in which Balb/c 3T3 fibroblasts were transfected with different oncogenes for evaluation of their progression and metastatic capacities to different organs in an athymic nude mouse model system(24-27). Either the Kirsten *ras* or EJ-Harvey *ras* oncogenes convert nontumorigenic 3T3 cells into highly tumorigenic (in any injection site) and metastatic cells targeted to several organs (24-26). In contrast, the human c-*sis* oncogene converted 3T3 cells into tumorigenic cells in the subcutaneous site but they were not metastatic to any organ(26,27). With the findings summarized above on CD44's association with metastatic progression in some tumor systems, our data prompted us to examine a possible relationship between CD44 expression and the differential effects of these oncogenes on the progression properties of 3T3 transformants.

Evaluation of mouse CD44 levels by FACS (fluorescence-activated cell sorting) revealed that 3T3 cells had modest levels of CD44s with no detectable CD44v isoforms, as expected for this fibroblast system (1,28). Exogenous HA bound very poorly to these cells, indicating either that the CD44s was already saturated with endogenously-synthesized HA or that an activation signal was required to induce binding; the former may be unlikely since prior treatment with hyaluronidase failed to change CD44-dependent HA binding. In contrast, either *ras* oncogene greatly elevated the levels of CD44s in these cells, as well as its ability to bind hyaluronan (28). In contrast, revertants of these cells that had lost *ras* expression and c-*sis* transformants maintained the basal level of CD44s observed in parental 3T3 cells (28). A large panel of primary tumors and metastatic tumors of the *ras* transformants were also tested in culture after removal of tumors from nude mice; in all cases, these tumor populations maintained high levels of CD44s and conserved HA-binding capacity (28). These results indicated that *ras*-dependent regulation of CD44 gene expression correlates with progression and metastasis in this 3T3-based "isogenic" fibrosarcoma system; furthermore, the *sis* oncogene is incapable of regulating CD44 levels in such a manner that would lead to metastatic competence.

3.2 CD44 modulation in nonmetastatic systems

The correlates described in section 3.1 led us to speculate whether the *sis* oncogene displays any regulatory control over CD44s expression and/or functions in these cells, particularly since it codes for the PDGF B chain (29). Increasing the concentration of serum in the medium exposed to either 3T3 or *sis*-transformed 3T3 cells resulted in increased levels of CD44s and significant HA binding (29); this serum stimulation was transient and was not observed with *ras* transformants which were constitutively upregulated for CD44s expression. The "serum effect" could be mimicked with addition of PDGF to low-serum medium and subsequently inhibited with anti-PDGF, confirming the specificity of the effect for this growth factor. Our findings are consistent with previous studies showing that the *ras* oncogene regulates transcription of the CD44 gene directly (14,15) and bypasses the requirement for PDGF receptor-mediated signaling in our cells. Furthermore, the changes in CD44s levels upon PDGF treatment of 3T3 cells (or *sis* transformants) closely correlate with dose-responsiveness of chemotactic activity for this growth factor (34); this supports CD44s' role in directed migration by fibroblasts in various pathological situations.

The mechanism by which the *sis* oncogene, and its PDGF B chain product, regulated CD44s levels was investigated further in transformants (30). Increased levels of CD44s protein correlated with increased levels of CD44s mRNA quantitated by RNase protection assay in *sis*-transformed cells. Whether this reflects increased stability of CD44s mRNA or actual increased transcription of this gene in these cells remains to be determined. PDGF secreted into the medium of *sis* transformants was shown to increase the levels of CD44s in parental 3T3 cells, an effect that was blocked with anti-PDGF pretreatment of these "conditioned media" (30); of particular note, the medium of *ras* transformants (or parental 3T3 cells) was without effect in this assay, consistent with the absence of any secretion of this growth factor from these cell types. Therefore, PDGF is operating to regulate CD44s levels in *sis*-transformed cells by an autocrine mechanism while it operates by a paracrine mechanism upon 3T3 cells.

4. CD44S OVER-EXPRESSION: ACQUISITION OF METASTATIC POTENTIAL

4.1 Three cell systems under study--transfection of human CD44s gene

The data of section 3 suggested to us that CD44s upregulation could be playing some role (s) in metastatic competence in this 3T3/oncogene system. We decided to test this hypothesis directly by transfecting the human CD44s gene, recognized by monoclonal antibodies that would not recognize mouse CD44s, into three different mouse cell types that were either nontumorigenic or were tumorigenic but not metastatic. The first cell type was *sis* oncogene-transformed 3T3 cells (HUSI cells) that were tumorigenic but not metastatic (31). The second was the *ras*-deleted revertant variant of Kirsten *ras*-transformed cells that had lost *ras* expression and had reverted in their tumorigenic capabilities (25,31). And the third system was the 3T3 cell itself which, of course, is nontumorigenic and nonmetastatic (32). For all three systems, overexpression

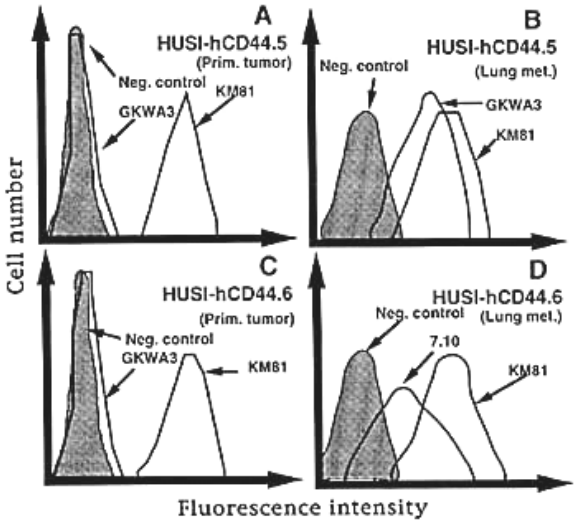

Figure 1. hCD44s protein levels in s.c. primary tumors versus lung micrometastatic tumor cells (31). Tumors were generated as described in the text of section 4.2 in athymic nude mice and their cells recovered into culture. Expression of either mouse CD44s (KM81 antibody) or human CD44s (GKWA3 or 7.10 antibodies) were identified with these specific monoclonal antibodies and FACS analyses. (A) S.c. tumor after injection of HUSI transfectant 5 cells; note the loss of expression of hCD44s while mouse CD44s continues to be expressed in these cells. (B) Lung micrometastatic cells from the same animal as analyzed in (A); note the retention of high levels of expression of hCD44s in these cells. (C) S.c. tumor after injection of HUSI transfectant 6 cells; note the loss of expression of hCD44s. (D) Lung micrometastatic cells from the animal in (C); note the retention of expression of hCD44s. (Reproduced with permission from reference 31).

of the human CD44s gene resulted in the same dramatic phenotypic changes.

4.2 CD44s as HUSI progresses and metastasizes

Several clones of human CD44s-transfected, *sis*-transformed 3T3 cells (HUSI) were isolated and shown to express the usual amounts of mouse CD44s (unable to bind exogenous HA), very high levels of human CD44s (hCD44s), and very high levels of HA binding specific for the human CD44s as determined by FACS analyses (31). These transfectants injected subcutaneously (s.c.) into athymic nude mice generated aggressive primary tumors that, when reisolated back into tissue culture, failed to express much hCD44s or to bind much HA in a CD44-dependent manner (figure 1). For the many large primary tumors examined, human CD44s expression was invariably lost, suggesting that there was a strong selection against its expression (but not that of mouse CD44s (mCD44s)) in these tumors.

4.3 Counter-selection model of progression

For all hCD44s transfectant clones of *sis* transformants, these cells had acquired metastatic competence to the lung and some other organs (31). Lung micrometastatic cells, when reisolated back into culture, continued to express high levels of hCD44s and to bind HA via hCD44s (31) (figure1). This contrasted with loss of hCD44s expression in the animal's primary tumor. Again, many isolates of micrometastatic lung cells displayed this property. This important result indicates positive selection for the human gene product during metastatic spread while there is a *counter-selection* against its overexpression occurring in the primary tumor.

Two alternative mechanisms may explain these important results (31). First, high-hCD44s-expressing cells may escape the primary tumor very early in its initiation at the s.c. site to metastasize to lung and other organs (prior to loss of hCD44s expression in the primary tumor). Alternatively, a very small subset of primary tumor cells, undetectable by our FACS analyses, may persist in their high hCD44s levels and provide the source of micrometastatic cells at all stages of primary tumor formation, even when the vast majority of their siblings had lost this expression.

This model was then challenged with a second round of tumor studies (31). Primary tumor cells from the first-round animals (negative for hCD44s) were injected back into nude mice s.c.. They formed excellent primary tumors that were negative for hCD44s. In contrast, these cells retained metastatic competence to the lung; lung micrometastatic tumor cells overexpressed hCD44s just as effectively as the first-round micrometastatic tumor cells (31). Also, hCD44s-overexpressing lung micrometastatic cells from first-round animals were re-injected s.c. into other animals. Again, all primary tumors had lost hCD44s expression and the ability to bind HA (figure 2). These cells persisted in metastasis to the lung whose micrometastatic cells retained high levels of hCD44s and HA binding (figure2).

Animals injected with these transfectants also displayed overt metastases in the kidney and the gastrointestinal track, as well as the lung. When these tumors were explanted into culture, all three classes of overt metastases had lost hCD44s expression (figure2) (31). These results suggest that hCD44s is important for metastatic spread to various target organs but is selected against during aggressive tumor outgrowth, whether it be the primary tumor or a secondary tumor.

Nicolson in his review of tumor metastasis (35) suggested that there may be gene products that are only transiently expressed in or on cells in order to convey metastatic competence. These molecules would then be down-regulated because they would be counter-productive for aggressive tumor outgrowth. CD44s in our studies certainly satisfies these criteria and demonstrates considerable plasticity in its expression. Furthermore, the interesting issue can be raised as to "why hCD44s and not mCD44s?". It must be remembered that mCD44s fails to bind exogenous HA while the overexpressed hCD44s is very efficient at binding HA. This may indicate that HA-binding function of the molecule is the critical aspect of conveyance of metastatic spread. This can now be tested with mutant forms of CD44s that have lost HA-binding capability because of cytoplasmic deletions (1) or loss of a critical cysteine residue in the transmembrane domain (36).

4.4 CD44s as revertant or 3T3 transfectants progress and metastasize

hCD44s transfectants of *ras*-deleted revertants, relatively nontumorigenic in nude mice (25), were then

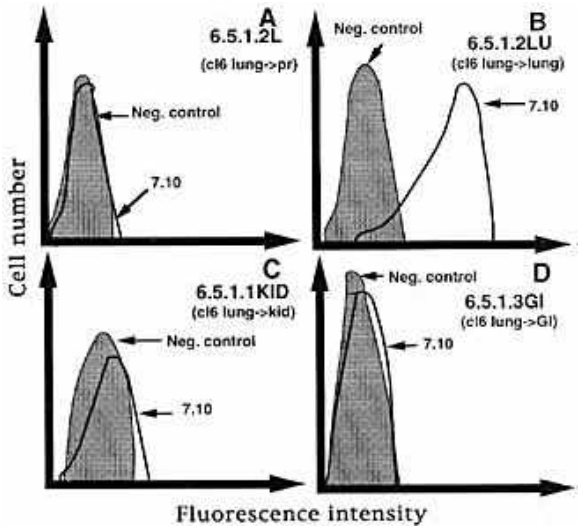

Figure 2. Fate of hCD44s expression in micrometastatic tumor cells re-injected into a second group of animals (31). Lung micrometastatic tumor cells, derived from HUSI transfectant 6 cells and expressing high levels of hCD44s (see figure1), were re-injected into more animals (see section 4.3) and their subsequent primary and metastatic tumor cells analyzed by FACS for hCD44s using monoclonal antibody 7.10 specific for the human antigen. (A) Primary tumor cells from an s.c. injection site; note the loss of expression of hCD44s. (B) Lung micrometastatic tumor cells from the animal in (A); note retention of high levels of hCD44s in these cells. (C) A large overt kidney metastasis was evaluated; it had lost hCD44s from its cell surface. (D) A large overt GI tract metastasis had similarly lost hCD44s from its surface. (Reproduced with permission from ref. 31).

tested in this paradigm (31). Several overexpressing clones (HA-binding) were isolated and injected s.c. into athymic nude mice. They formed aggessive primary tumors, all of which had lost hCD44s from their cell surfaces and the ability to bind HA (31). Furthermore, they were spontaneously metastatic to the lungs of these animals and these micrometastatic cells retained high levels of hCD44s and HA binding (31). Therefore, this gene has made these cells competent for forming primary tumors and for undergoing metastasis to the lungs of these animals in a spontaneous metastasis system.

This led us to test whether the parental Balb/c 3T3 cell could be altered in its tumorigenic properties when transfected with the human gene (32). Indeed, one of three hCD44s-overexpressing clones of 3T3 cells (interestingly, the highest expressor) had acquired tumorigenic capacity in the subcutaneous site and metastasized to the lung and some other organs (32). In this third system, the same expression changes occurred (figure 3). Large primary tumors, explanted into culture, had lost hCD44s and its HA binding abilities while retaining expression of mCD44s (with no exogenous HA binding ability). Lung or pancreatic micrometastatic tumor cells retained high levels of hCD44s while large metastases from GI tract or lymph nodes had lost it (figure3) (32). This third system, therefore, confirms the counter-selection that occurs for or against high levels of this cell surface protein and its ability to bind HA. Furthermore, it would indicate that the hCD44s gene may be considered an "oncogene" under the appropriate circumstances--i.e., it converts nontumorigenic cells into tumorigenic cells much like overexpression of the HA-binding RHAMM gene does (37).

4.5 *In vivo* analyses of CD44s levels

The results of sections 4.2 to 4.4 raised the possibility that explant of tumors into culture may be contributing to loss of hCD44s gene expression in these cells. It was therefore critical that we examine hCD44s levels *in vivo* (32). Using immunohistochemical methods and appropriate fixation procedures for tissues from nude mice, large primary tumors or overt metastases were shown to lack the hCD44s protein while small foci in the lungs, characteristic of micrometastases, were highly stainable for the human antigen (32). No staining was observed in tissues from mice not injected with transfected cells or injected with nontransfected fibrosarcoma cells. In contrast, weak staining for mouse CD44s was observed in all tumor sites, consistent with its persistent expression. Therefore, the analyses using FACS with cultured tumor populations accurately reflect the expression patterns observed by the tumor populations in their respective organ sites (32).

5. CD44S GENE HYPERMETHYLATION: MECHANISM OF EXPRESSION MODULATION

The plasticity of expression of the human CD44s gene in this fibrosarcoma progression system required mechanistic study. Southern blot analyses showed that all hCD44s-negative primary tumors and large overt metastatic tumor populations still retained the human gene at the same dosage and integration site as the original transfectant clone (31). In contrast, RNase-protection assays demonstrated that these tumor populations lacked mRNA for this gene while retaining transcripts of the mCD44s gene. Lung micrometastatic tumor cells had plenty of hCD44s mRNA. Therefore, transcriptional control was presumably operating to regulate this plastic expression.

Recently, more attention has been paid to the role that gene and/or promoter hypermethylation plays in regulating various genes in tumor cells (38,39). This prompted us to examine whether the human CD44s gene, but not the mouse CD44s gene, was undergoing selective hypermethylation to down-regulate its transcription (31). When DNA from these various tumor or transfectant cell populations was tested with the methylation-sensitive restriction enzyme, *Hha*I, very different patterns of fragments were observed on gels (31). A large fragment resistant to breakdown by the methylation-sensitive enzyme occurred at high levels in large primary tumor and overt metastatic tumor cells (figure 4); much lower doses of this band, originating from the mouse CD44 gene, were apparent in the original transfectant cells or in lung micrometastatic tumor cells. Conversely, these latter populations generated a smaller band characteristic of only the human CD44 gene and this band was never observed in large tumors of either class (figure 4). Therefore, the human gene is selectively hypermethylated in the large primary tumor and overt metastatic tumor cells but not in the transfectant population or lung micrometastatic cells. These results correlate precisely with expression levels of hCD44s. These important experiments indicate that hypermethylation is probably the controlling element in

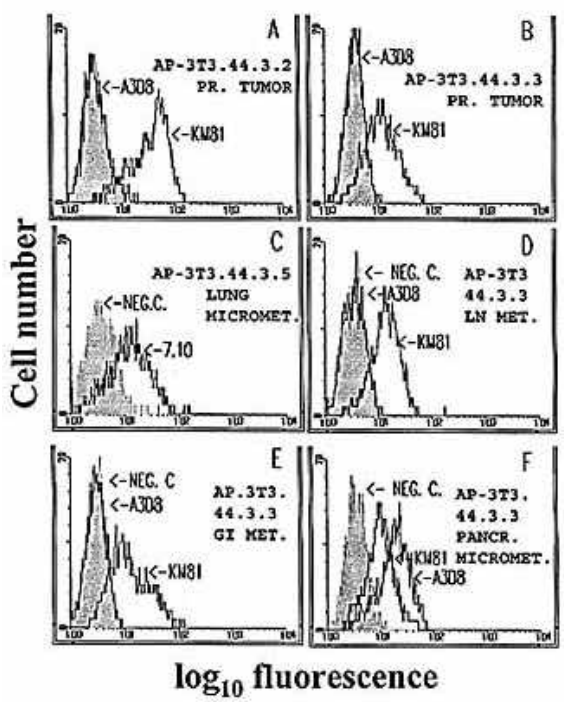

Figure 3. hCD44s protein detected on lung micrometastatic tumor cells but not primary tumor or overt metastatic tumor cells using 3T3 transfectants. hCD44s gene was transfected into Balb/c 3T3 cells and the highest-expressing clones analyzed for tumorigenesis and levels of hCD44s in subsequent tumor populations (32); see section 4.4 for a more thorough description. Mouse CD44s was detected by FACS with monoclonal antibody KM81 and hCD44s with antibody A3D8 or 7.10. (A) Primary tumor cells generated from s.c. injection of transfectant 2 cells; note loss of expression of hCD44s in these cells. (B) Primary tumor cells from s.c. injection of transfectant 3 cells; note again loss of hCD44s in these cells. (C) Lung micrometastatic tumor cells after injection of transfectant 3; note retention of hCD44s on these selected populations. (D) Tumor cells isolated from the local lymph nodes of the animal in (B); note loss of hCD44s in these cells. (E) An overt GI tract metastasis from the animal in (B); note loss of hCD44s in these cells. (F) Micrometastatic tumor cells from the pancreas of the animal in (B); note the retention of hCD44s in these selected cells. (Reproduced with permission from reference 32).

down-regulating expression of this gene in tumor cell subsets, permitting selection pressures for or against this subpopulation being successful or unsuccessful during tumor progression.

These results were confirmed with an alternative approach (31). In this case, tumor cells were exposed to the cytidine analog, 5-aza-2-deoxycytidine, that resists methylation when it is incorporated into DNA sequences, thereby leading to upregulation of hypermethylated genes. Two different primary tumor populations, not expressing hCD44s, were grown in medium with or without the analog for four days; FACS analyses indicated that these cells now express sizable amounts of hCD44s as well as mCD44s (table 1) (31). Furthermore, the levels of hCD44s could also be increased in the original transfectant cells and to some extent in the lung micrometastatic tumor cells with aza-cytidine treatment (table 1), suggesting some methylation of this gene in these "normally-expressing" cells. All of these results taken together strongly suggest that hypermethylation of the human gene is responsible for the transcriptional silencing in selected tumor populations. Whether this hypermethylation is occurring at promoter sequences and/or in coding sequences leading to gene downregulation remains to be determined.

6. FUNCTIONS FOR CD44S IN METASTATIC SPREAD

6.1 Earliest events analyzed with experimental metastasis model

The results of section 4 indicate that overexpressed hCD44s promotes spread of a select subset of primary tumor cells (a very small subset at that) to metastatic target organs (31,32). One approach for dissecting these early events is use of the experimental metastasis assay. In this paradigm, tumor cells are injected into the tail veins of nude mice to evaluate lung colonization directly. This model bypasses tumor cell intravasation into blood vessels at the primary tumor site but considers several steps involved in tumor cell implantation in lung micovessels, extravasation from the blood supply, and colonization of the lung to form micrometastases. We used experimental metastasis in an attempt to dissect some of these very early events in metastatic spread to determine if hCD44s (and its HA binding property) conveyed significant advantage (33). Indeed, this was found to be the case. We took advantage of the drug-resistance markers in these tumor cells to quantitate the number of tumor cells colonizing the lung by their outgrowth in culture plates containing the selective drug; the output for these assays is the number of drug-resistant colonies (33).

6.2 Lung colonization results

While the untransfected *sis*-transformed cells gave significant numbers of cells populating the lungs at 1 or 24 hours when injected into tail veins, both hCD44s transfectants of these cells increased the number of populating cells at all time points (figure 5) (33). At 1 hour, this increase was threefold; by 24 hours when most tumor cell clearing in the lungs had occurred, this advantage increased to tenfold and this advantage was maintained for at least four weeks (figure5). These results indicate that overexpression of hCD44s improves the ability of tumor cells to implant in lung microvessels during their initial minutes of exposure to the endothelium and that it vastly improves the ability of these tumor cells to survive in the lung and form colonies--i.e., penetrate the endothelium and establish small colonies at these sites.

6.3 Hyaluronan binding properties during early events of metastasis

We next examined the hCD44s protein levels and hCD44s-dependent HA binding of these transfectants (33). When transfectant cells were cultured for lengthy periods of time, they lose cell surface hCD44s (figure 6A). In contrast, transfectant cells were reisolated into culture from lungs 1 hour after tail vein injection and enriched during growth in hygromycin medium. They contained very high levels of hCD44s (figure 6B); this expression was conserved in lung-colonizing cells at 24 hours (figure 6C) but was diminished by four weeks (figure 6D),

Table 1. Enhancement/induction of hCD44s expression in transfectants and their s.c. tumor cell lines by inhibition of DNA methylation

	MEAN FLUORESCENCE INTENSITY			
	Without aza-cyt		With aza-cyt	
Cell type	**BG**	**hCD44s**	**BG**	**hCD44s**
HUSI	3.6	5.6*	3.7	5.3*
HUSI.hCD44.5	3.7	30.3	4.2	138.1
HUSI.hCD44.5 PT	14.0	18.1*	14.0	61.5
HUSI.hCD44.6	4.8	67.2	7.2	366.2
HUSI.hCD44.6 PT	6.8	8.5*	8.9	35.6
HUSI.hCD44.6 LU	5.6	19.1	5.7	41.3

Cells were grown in medium with or without 3 uM 5-aza-deoxycytidine (aza-cyt) for 4 days and their hCD44s levels analyzed by FACS as described (31). The results were quantitated using the LYSYS software (Becton-Dickinson); mean fluorescent intensities of cell populations stained with irrelevant antibody (BG) or hCD44-specific antibody (hCD44s) are shown. PT, primary tumor at the subcutaneous site; LU, micrometastatic tumor cells from the lung; *, not significantly different from background. (Reproduced with permission from reference 31).

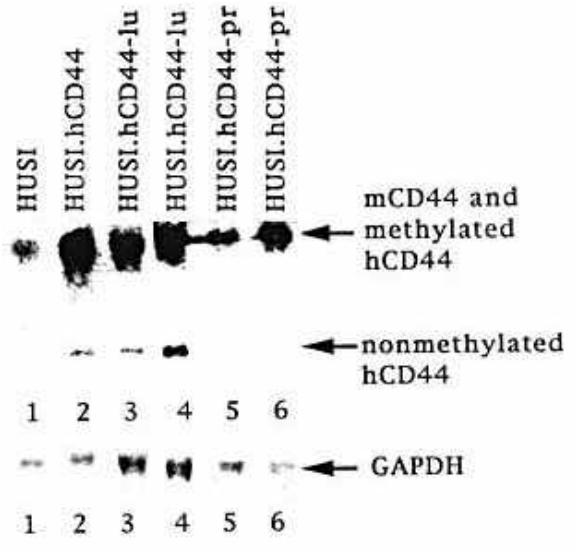

Figure 4. Human CD44s gene is differentially methylated in different tumor populations (31). DNA was isolated from various hCD44s transfectant cells and tumor populations from various sites in athymic nude mice injected with transfectant cells (see section 5 of text for explanation). Southern blot analyses were performed after DNA treatment with the methylation-sensitive restriction enzyme *Hha*I. Position of the 1.4kb HhaI fragment from hCD44s cDNA is indicated; it corresponds to nonmethylated hCD44s DNA. Mouse CD44 and methylated hCD44s fragments are detected at the top of the autoradiogram as indicated. GAPDH--glyceraldehyde phosphate dehydrogenase DNA as a loading control. Lanes are as follows: (1) parental HUSI DNA (cells not transfected with hCD44s); (2) DNA from hCD44s transfectant 6 cells; (3) DNA from lung micrometastatic cells after injection of transfectant 5 cells; (4) DNA from another population of lung micrometastatic cells from hCD44s transfectant 6 cells; (5) DNA from s.c. primary tumor cells after injection of transfectant 6; (6) DNA from another primary tumor after injection of transfectant 6 cells. (Reproduced with permission from reference 31).

demonstrating the strong selective pressure for cells to use this cell surface molecule at the earliest events during lung colonization (33). The lower levels of the human molecule at the 4-week timepoint is again consistent with loss of its expression correlating with outgrowth of overt metastases.

These same cells were then tested for exogenous HA binding by FACS. HA binding was exactly reflective of cell surface levels of hCD44s and was inhibitable with anti-human CD44s but not with anti-mouse CD44s (33). Therefore, this functional capacity is retained in the cells that are selected for during lung colonization.

6.4 Analyses of various tumor classes

This led us to test the various tumor populations derived from these transfectant cells as described in section 4 (31,32). Both hCD44s-negative primary tumor and hCD44s-positive lung-micrometastatic tumor cells were evaluated in this experimental metastasis model (33). The primary tumor cells injected into tail veins gave colonization numbers that were not statistically different from untransfected *sis*-transformed cells at 1 or 24 hours, consistent with their loss of cell surface hCD44s. However, the primary tumor cells populating the lung did display some cell surface hCD44s (although not nearly the levels of the original transfectant or lung micrometastatic cells). This would suggest some upregulation during the first 24 hours of residence in the lung and/or selection for a small subset of primary tumor cells that continue to express the human molecule but are not readily seen by FACS of primary tumor populations. Of particular note as well, overt lung metastases derived from these hCD44s-"negative" primary tumor cells lacked any cell surface hCD44s, consistent with its downregulation upon aggressive outgrowth of tumors.

Then, hCD44s-positive lung micrometastatic tumor cells were injected into tail veins (33). Their lung colonization efficiency was equivalent to that of the original transfectant cells and much higher than that of the hCD44s-nonexpressing primary tumor cells. When reisolated into culture, these lung-colonizing populations at 1 or 24 hours of residence in the lung had very high levels of cell surface hCD44s and HA binding (33). These expression levels

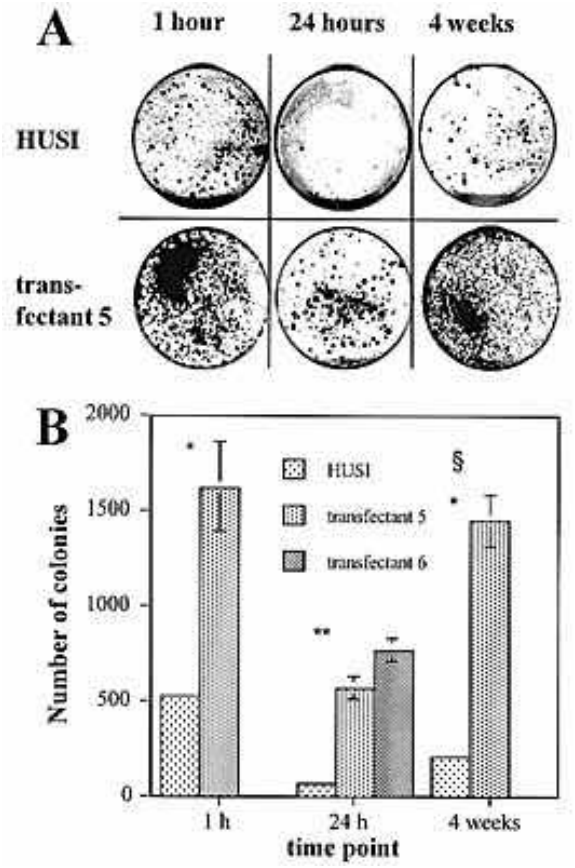

Figure 5. hCD44s overexpression promotes early stages of lung micrometastasis formation (33). Tail vein injections (experimental metastasis assay) were performed with 10^5 cells from various sources (three mice per datum point) as described in section 6.2. Two independent transfectants of HUSI cells with hCD44s cDNA (transfectant 5 or 6) were analyzed. At the indicated times postinjection, mice were killed, their lungs dispersed into culture, and tumor cells were quantitated in a colony growth assay in selective media containing hygromycin B. Colonies were visualized and enumerated with Coomassie brilliant blue (A). Quantitation of colonies were performed in (B)-- *, $P<0.05$; **, $P< 0.005$. (Reproduced with permission from reference 33).

were lost by 4 weeks when overt lung metastases were evident and evaluated after explant into culture.

6.5 Mixtures of two different CD44s classes

Early events in lung colonization promoted by hCD44s (and its HA binding property) may be a result of two general models. First, hCD44s and its ability to bind HA on the surface of another cell could facilitate formation of small aggregates among tumor cells that would "trap" aggregates in the smallest vessels of the lung; this mechanism would involve improved tumor cell:tumor cell adhesion. Alternatively, tumor cell hCD44s could promote more effective adhesion of tumor cells to endothelial cells of the small blood vessels and/or improved interaction with the basement membrane of blood vessels via its HA binding; this would be a heterotypic cell:cell adhesion model.

In an effort to discriminate these and other possibilities, we designed an experiment in which transfectant cells were mixed with a varying ratio of untransfected cells and then the mixtures injected into tail veins to determine the "specific activity" of transfectant cells for colonizing as they compete with untransfected cells (33). Ratios of 80%:20% untransfected:transfected, 50%:50%, and 20%:80% were tested, as wells as homogeneous populations of each cell type as controls. Drug-resistant colonies were assayed from lungs after various residence times, with hygromycin used to select all tumor cells while puromycin was used to select only hCD44s transfectant cells (33). Increasing the proportion of untransfected cells being co-injected failed to decrease the improved efficiency of colonization by transfected cells (again, with a >tenfold increase in efficiency by the transfectant cells). When these lung-colonizing "mixtures" were reisolated into culture and examined for hCD44s levels, all lung populations contained high levels of hCD44s and the proportion of untransfected cells was hard to detect by FACS analyses in the background of these populations (33). These results taken together demonstrate that hCD44s may not function by inducing cell:cell adhesion between transfectant and untransfected cells but rather among transfectant cells selectively (via cell surface HA bound to the hCD44s?) or between transfectant cells and endothelial cells of the microvasculature, again via the HA on endothelial cells bound to overexpressed CD44s on the tumor cells.

The experiments of section 6 demonstrate overwhelmingly that overexpressed and HA-binding hCD44s on tumor cells promotes the earliest processes of metastatic spread into the lung of fibrosarcoma cells (33). hCD44s may either promote intercellular adhesion among this subset of tumor cells and/or promote more effective interaction of this subset with the endothelium in the microvasculature of the lung. There are marked parallels of our own studies of fibrosarcoma metastasis with the lymphocyte homing experiments described in section 2.3 (1,22,23). That HA might target lymphocytes via CD44s binding to endothelial cells at specific inflammatory sites is remarkably similar to the conclusions drawn with our experimental metastasis experiments (33). These two systems open a panorama of interesting and important experiments to dissect the functional significance of CD44s in these events.

7. PERSPECTIVES FOR FUTURE MECHANISTIC STUDIES

7.1 Evaluating other connective tissue cell types

Our studies indicate that overexpressed CD44s provides considerable advantage for a subset of mouse fibrosarcoma cells to undergo metastatic spread to the lung and other organs. In contrast, this overexpression is a distinct disadvantage for aggressive outgrowth of either the primary tumor or overt metastases. It remains to be tested whether any of the variant forms of CD44 could confer this advantage (see sections 7.4-7.5 on this issue).

These studies are consistent with the hypothesis that the standard isoform, not variant isoforms, are important players in progression and metastasis of connective tissue tumors. Human fibrosarcoma systems should now be tested in this model, as well as other tumor

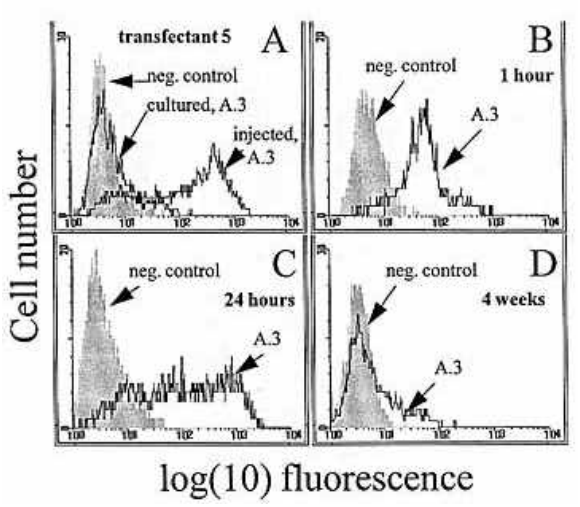

Figure 6. hCD44s overexpression is selected for during early stages of micrometastasis (33). Human CD44s was detected with monoclonal antibody A.3 and FACS analyses using cells recovered from lungs or grown in culture for the indicated times (tumor cells from lungs selected out with hygromycin B); see section 6.3 for further explanations. (A) Injected--transfectant cells prior to their injection into animals; note the high levels of hCD44s on these cells. Cultured--transfectant cells maintained in culture medium for three weeks; note that most of these cells had lost cell surface hCD44s. (B) Micrometastatic cells isolated from the lungs 1 hour after tail vein injection and hygromycin-selected; note the high levels of hCD44s in these lung-colonizing subpopulations. (C) Micrometastatic cells isolated from the lungs 24 hours after tail vein injection and hygromycin-selected; note persistent expression of hCD44s in these lung-colonizing subpopulations. (D) Metastatic tumor cells isolated from the lungs after 4 weeks (hygromycin-selected); overt metastases were forming by this time and these cells had lost much of their hCD44s. (Reproduced with permission from reference 33).

classes derived from connective tissues. Conversely, the mouse CD44s gene should be overexpressed in this mouse fibrosarcoma system to verify that the species origin of this gene is not playing some unknown role. These analyses would include osteosarcoma, chondrosarcoma, and rhabdomyosarcoma tumors to test how broad a spectrum of connective tissue tumors are affected.

All of the data described in the chapters of this special edition of the Frontiers in Bioscience are consistent with a division of labor among different CD44 isoforms--CD44s is a critical player in lymphomas and connective tissue tumors while CD44v forms (in some case, specific "v" forms while in other cases many "v" forms) are critical in carcinoma progression and metastasis.

7.2 Modulating DNA hypermethylation *in vivo*

The studies reviewed here identify for the first time a molecular mechanism by which a CD44 gene can be regulated--i.e., by hypermethylation (31). Since the gene being transfected in our studies was regulated by the RSV LTR, it is likely that promoter hypermethylation is responsible for its downregulation in primary tumors and overt metastases (the LTR contains a number of CpG islands). This hypothesis can be tested directly by transfecting this gene into the same *sis*-transformed or parental 3T3 cells on a plasmid containing a promoter that is insensitive to hypermethylation. The actin or globin gene promoters are candidates for this experiment. Alternatively, the episomal plasmid derived from Epstein-Barr virus, pREP4, can be used to transfect the hCD44s gene; this episomal-replicating plasmid should be resistant to chromatin-based hypermethylation mechanisms. It will be interesting to determine in these cases if primary tumors and/or overt metastases grow more slowly than tumors bearing an LTR-regulated gene.

It would also be desirable in the system described in sections 4-6 to regulate methylation of DNA *in vivo* more directly for these various tumor systems. Perhaps aza-deoxycytidine could be introduced at the site of primary tumor development in slow-release beads, with subsequent inability of tumor cells to hypermethylate their DNA. Would this result in much slower growing tumors? The problem here is the generalized effects of the aza-cytidine on many different genes of tumor cells. Alternatively, regulation of the hCD44s gene can be manipulated by a regulatable promoter to determine how gene expression modulation affects the parameters described in references 31-33; in this case, the tetracycline regulation system (on or off) would probably be the most effective for *in vivo* analyses (43,44).

With regard to *in vivo* regulation of overexpressed CD44s in our system (31,33), greater study must be dedicated to understanding when and where its regulation changes in various organ sites (32). For example, tagging tumor cells with a histochemical marker gene (*lacZ* or placental alkaline phosphatase (26,27)) has allowed us to analyze the very earliest steps in primary tumor development at the subcutis and the dermis (40,41). Combining this tagging approach with laser-capture-microdissection of individual tumor cells at these sites (42) will allow us to pinpoint the precise environmental conditions under which overexpressed CD44s becomes downregulated. It will be important to determine if different environments apply in the subcutis or dermis versus the microvessels of the lung after tail vein injection or versus the lung tissue itself as an overt metastasis develops.

7.3 Suitable orthotopic model of fibrosarcoma

With regard to tumor-type specificity, the question arises as to the appropriateness of the subcutis since this is an ectopic site for fibrosarcoma development. The dermis may be a more "natural" site (orthotopic) to test this tumor and the use of histochemically-tagged tumor cells has shown us success in this endeavor (40). Perhaps downregulation of hCD44s would not occur in this more orthotopic site of the animal, suggesting that the tissue environment plays a critical role in hypermethylation regulatory mechanisms. Alternatively, downregulation may still be observed, consistent with downregulation occurring whenever and wherever an aggressive tumor begins to grow.

7.4 Significance of HA binding

Plasticity of expression of hCD44s in our studies (31-33) may reflect the importance of HA binding by CD44s isoform. All of our data are consistent with this hypothesis but do not prove it directly. To do so we

would transfect into these cell systems a mutant form of hCD44s unable to bind HA specifically without affecting its lymphocyte-homing ability. Two classes of such mutants have been described (1,36). If these mutant forms of both classes fail to provide metastatic competence to cells and/or are not downregulated, then the HA binding function of the molecule is critical both in conveying metastatic competence as well as providing the selective pressure for downregulation. If these mutant classes are just as effective in altering tumorigenic/metastatic phenotype, then clearly HA binding is not the essential function; perhaps the lymphocyte-homing function is playing a much greater role than we thought.

A complementary approach may also be used in shortterm experiments, such as the lung colonization assay (33). Hyaladherins of the same class as CD44, such as cartilage link glycoprotein or the TSG-6 protein, bind to HA with virtually identical mechanisms and these small proteins (MW <45kD) may be effective hapten inhibitors when adsorbed to tumor cells prior to injection into tail veins. This would test whether an HA-rich coat on the tumor cell surface, bound to overexpressed CD44s, is the important ligand for a target molecule on the endothelium. In contrast, small oligosaccharides of HA (6-10 sugars long) will act as hapten inhibitors of CD44s on the tumor cell surface binding to HA on the endothelium. HA oligosaccharides would be co-injected with tumor cells to test inhibition of colonization, comparable to the inhibition by RGDS synthetic peptides interfering with integrin-dependent adhesion events in the lung.

7.5 Can these results be extrapolated to carcinoma systems?

These data raise the question whether overexpression of CD44s applies specifically to connective tissue (31-33) or lymphoma (17) tumor cells or whether these phenotypic changes could apply to carcinomas as well. From all the evidence summarized in these chapters of this Frontiers in Bioscience special edition, it is unlikely that the CD44s isoform would execute the same changes in epithelioid tumors with regard to metastatic competence. However, it is not unreasonable to think that overexpression of CD44v isoforms in carcinomas may "function" similarly even though they may not have HA-binding or lymphocyte-homing competence. Perhaps, specific "v" isoforms have specific counter-receptors on other cell types that would substitute for the functions in metastasis executed by CD44s. Therefore, breast carcinomas may use specific "v" forms during metastasis that are not useful to prostate carcinoma cells or lung carcinoma cells which might use other "v" forms. To determine whether such relationships exist will require much greater study of the binding domains of the various isoforms, their target ligands, and the consequences of overexpression or downregulation at specific stages of tumor progression.

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