CD44 integrates signaling in normal stem cell, cancer stem cell and (pre)metastatic niches

Karin Williams[1], Karan Motiani[2], Premkumar Vummidi Giridhar[3] and Susan Kasper[1]
[1]Department of Environmental Health; [2]Division of Urology, College of Medicine, University of Cincinnati, Cincinnati, OH 45267;
[3]Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA
Corresponding author: Susan Kasper. Email: susan.kasper@uc.edu

Abstract

The stem cell niche provides a regulatory microenvironment for cells as diverse as totipotent embryonic stem cells to cancer stem cells (CSCs) which exhibit stem cell-like characteristics and have the capability of regenerating the bulk of tumor cells while maintaining self-renewal potential. The transmembrane glycoprotein CD44 is a common component of the stem cell niche and exists as a standard isoform (CD44s) and a range of variant isoforms (CD44v) generated though alternative splicing. CD44 modulates signal transduction through post-translational modifications as well as interactions with hyaluronan, extracellular matrix molecules and growth factors and their cognate receptor tyrosine kinases. While the function of CD44 in hematopoietic stem cells has been studied in considerable detail, our knowledge of CD44 function in tissue-derived stem cell niches remains limited. Here we review CD44s and CD44v in both hematopoietic and tissue-derived stem cell niches, focusing on their roles in regulating stem cell behavior including self-renewal and differentiation in addition to cell–matrix interactions and signal transduction during cell migration and tumor progression. Determining the role of CD44 and CD44v in normal stem cell, CSC and (pre)metastatic niches and elucidating their unique functions could provide tools and therapeutic strategies for treating diseases as diverse as fibrosis during injury repair to cancer progression.

Keywords: CD44, CD44v, ESC, CSC, Niche, alternate splicing

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Introduction

CD44, initially identified as the lymphocyte homing receptor,[1,2] is commonly expressed in embryonic,[3] hematopoietic,[4] mesenchymal,[5] epithelial and cancer[6–15] stem cells. Therefore, CD44-based protocols have been used to isolate adult stem cells (ASCs) and cancer stem cells (CSCs) for further characterization. For example, both mammary and prostatic glandular structures could be regenerated from a single isolated $CD44^+$ ASC.[16,17] Similarly, the original histopathology of a prostate tumor, including all of the epithelial lineages within a prostatic gland, could be reconstituted *in vivo* from a single cell-derived $CD44^+$ CSC-like human prostate epithelial cell line isolated from that tumor tissue.[18]

CD44 attracted considerable interest when it was discovered that preparation of the (pre)metastatic niche required the CD44v6-mediated assembly of a soluble matrix that supported exosomes for activating target cells in the (pre)metastatic site.[19] Other studies indicated that CD44 expression was required for maintaining a stem cell phenotype. Knocking down CD44 expression in $CD44^+$ primary breast CSCs resulted in down-regulation of stem cell markers, e.g. Myc and LEF1,[20] a loss of the characteristic long G2/M phase along with a concomitant increased S phase consistent with non-stem cell proliferation[21] and induction of cell differentiation.[20] Unexpectedly, CD44-null mice were viable and fertile[22,23] with only minor hematopoietic,[23] breast and uterine involution abnormalities.[24] Olaku *et al*.[22] reported that c-Met recruited ICAM-1 as a co-receptor to compensate for the loss of CD44 during development. In contrast, transgenic mice expressing an antisense CD44 cDNA driven by the tissue-specific keratin-5 promoter lacked detectable CD44 expression in skin keratinocytes and corneal epithelium.[25] Overall, the loss of CD44 expression resulted in decreased skin elasticity, impaired tissue repair and local inflammatory responses, and delayed hair regrowth.[25] Collectively, these observations suggest that while compensation for the embryonic knockout of CD44 is possible, the loss of CD44 expression in a temporal and spatial manner severely impacts normal tissue development and function.

Here we review the genomic and protein structure of CD44 and its variants and focus on the mechanisms by which they integrate signaling within the stem cell microenvironment, including normal stem cell, CSC and

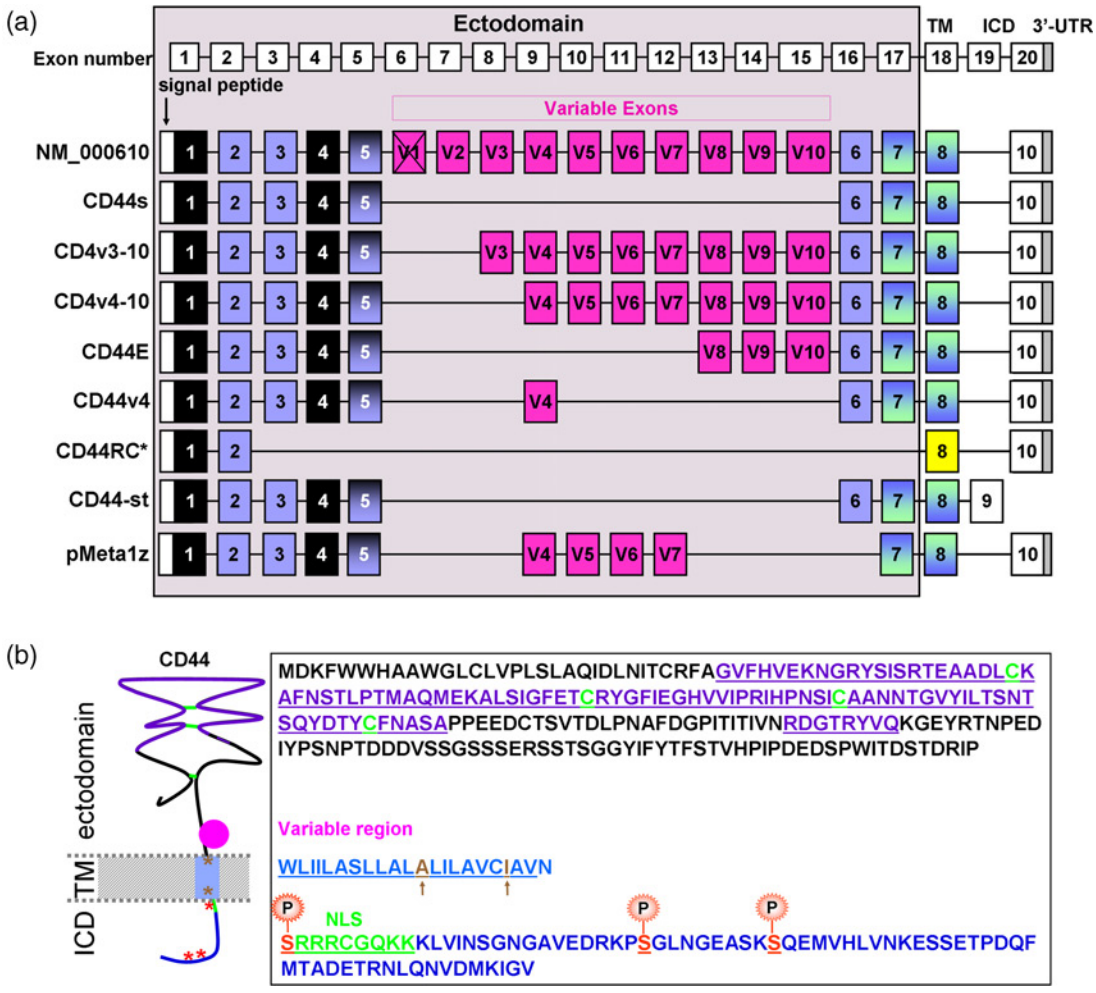

Figure 1 Diagrammatic representations of the CD44 gene and protein structure. (a) Schematic of the genomic structure of CD44. CD44 standard, CD44s; epithelial CD44, CD44E; CD44 variant 10, CD44v10; soluble CD44, CD44RC; shortened-tail CD44, CD44-st. (b) CD44 structural domains and their corresponding amino acid sequences. The modified CD44 molecule line drawing was adapted from Ponta *et al.*[26]

(pre)metastatic niches. CD44-mediated regulation of intracellular signaling pathways promoting non-stem cell and cancer cell function have been described in a number of excellent reviews.[26–30]

Genomic structure of CD44 and its variants

In humans, the highly conserved CD44 gene spans approximately 60 kilobases (kb) of genomic DNA on chromosome 11. The full-length CD44 gene is composed of 20 exons and 19 introns and undergoes extensive alternative splicing to generate CD44s (for 'standard' or 'short form') as well as 12 commonly found variants and several less abundant isoforms which are cell type-, disease- or developmental stage-dependent (Figure 1a; Table 1).[2,31–33] The CD44s isoform is generated from exons 1–5 (corresponding to genomic exons 1–5) and exons 6–8 and 10 (corresponding to genomic exons 16–18 and 20). Exon 1 contains the 5′ untranslated region (UTR), the initiating start codon, the signal peptide and part of the extracellular domain. The remaining constant exons express the non-variant stem (exons 16 and 17) and the transmembrane (TM) domain (exon 18). Exons 19 and 20 undergo alternative splicing, resulting in a short-tail (exon 19) or the more common long-tail (exon 20) isoform. All CD44 molecules contain the standard exons 1–5 with the exception of CD44sol which is a soluble non-membrane bound version of CD44.

The CD44 variants arise through splicing events that insert additional exons into the CD44s molecule. The variable exons are typically numbered v1–v10; however, they correspond to the genomic exons 6–15 (Figure 1a). In humans, the v1 exon is not encoded due to an in-frame termination codon; however, it is expressed in non-human mammals.[32] The v1–v10 exons are alternatively spliced and incorporated into the variable region either singly or in combination. While hundreds of combinations could potentially be generated, only 12 isoforms are commonly observed. The modern nomenclature system uses CD44vx–x, where 'x' represents a variable exon number ranging from v1 to v10, to indicate the combination of variable exons expressed in a given isoform. The four most common CD44v isoforms include CD44v4–7, CD44v8–10, CD44v3–10 and CD44v1–10.[26]

In summary, alternative splicing events and post-translational processing modulate the mass of CD44 with

Table 1 CD44 standard and variant accession numbers and reference sequence mRNAs (refseq)

Accession no.	GI	RefSeq for human CD44	Exon usage	Historical nomenclature
NM_001001391	48255940	CD44, transcript variant 4	CD44s	CD44 hematopoietic CD44H
NM_001001392	48255942	CD44, transcript variant 5	CD44RC	
NM_000610	48255934	CD44, transcript variant 1	CD44v2–10	
NM_001001389	48255936	CD44, transcript variant 2	CD44v3–10	CD44 keratinocyte
NM_001001390	48255938	CD44, transcript variant 3	CD44v8–10	CD44E
NM_001202555	321400137	CD44, transcript variant 6	CD44v10	

CD44s ranging from 37 to 80 kDa and CD44v varying from 81 to greater than 250 kDa.[26]

The protein structure of CD44 and its isoforms

The CD44 protein consists of three primary domains, including an extracellular domain (or ectodomain), a TM domain and an intracellular domain (Figure 1b). These functional domains contribute to the complexity of CD44 signaling.

The CD44 ectodomain

The ectodomain senses and interacts with the external microenvironment and is the most diverse region of the molecule, containing four overlapping regions designated as the hyaluronan (HA) binding domain, the Link motif, a basic motif and the stem structure. The HA binding domain encompasses amino acids 20–169 of the CD44 amino-terminal.[34,35] Nested within this region is the Link domain (amino acids 32–120) which contains the HA and glycosaminoglycan binding sites.[36,37] Importantly, four highly conserved cysteine residues form interchain disulfide links which are critical for the stability of the Link motif.[36,38] In addition, the Link motif is flanked on either side by two additional cysteine residues which are required for correct folding and HA binding.[36] The basic motif (amino acids 150–158) is thought to contribute to HA binding.

The CD44 stem forms a stalk-like structure through heavy N- and O-linked glycosylations which are thought to influence the ability of CD44 to bind HA.[26,39] Furthermore, the stem is composed of both non-variable and variable regions. The non-variable region is encoded by exons 5, 6 and 7 and contains protease cleavage sites that span 46 amino acid residues within the membrane-proximal region.[40] The variable region is encoded by exons v1–v10 and alternative splicing increases the stem structure of some isoforms by as much as 381 amino acids. Since the variable region is juxtamembrane, moderating the configuration of the stem structure could determine specificity of binding between different CD44 isoforms in addition to regulating complex formation between CD44, alternate ligands and their cognate receptors. For example, the addition of exon v6 alters CD44 function by binding hepatocyte growth factor (HGF) and presenting HGF to cMet, thereby inducing CD44/HGF/cMet complex formation and activating c-Met signaling (reviewed in Ref.[41]).

The function of the CD44 ectodomain is further diversified by its ability to exist in several forms, including a membrane-bound, cleaved and soluble form. Membrane-bound CD44 binds HA, tethering the cell to the extracellular matrix (ECM) and initiating signal transduction via internal adaptor proteins.[42] Membrane bound CD44 is cleaved by membrane-associated metalloproteases, including membrane-type 1 matrix metalloprotease (MT1-MMP),[43] members of the disintegrin and metalloprotease super family ADAM10 and ADAM17,[44,45] or MMP-9[46] at the membrane-proximal region of the ectodomain. This cleavage is required for regulating CD44:ECM interactions during cell migration.[40,43] The cleaved CD44 ectodomain may interact with membrane bound CD44 to prevent attachment of cells to the ECM or to inhibit tumor growth.[47,48] In contrast, soluble CD44RC enhances binding of membrane-bound CD44 to HA, resulting in increased adhesion, CD44 clustering and signal activation.[49]

The CD44 TM domain

The CD44 TM domain is a single-pass TM domain of 23 amino acids which provides a platform for CD44/co-factor/adaptor protein interactions. Cys^{286} participates in CD44 oligomerization[50] and amino acids Ala^{280}/Ile^{287} are involved in regulating intramembrane proteolysis by γ-secretase which liberates the CD44-ICD.[51,52] The CD44 TM domain associates with Triton X-100 resistant lipid-rich domains in the plasma membrane.[53,54] These lipid-rich domains or lipid rafts function as organizing centers for the assembly of signaling molecules.[55] Tables 2 and 3 summarize the diversity of CD44 and CD44v interactions with niche factors, oncogenes and receptor tyrosine kinases (RTKs), and a number of these interactions will be discussed further in the sections below. Thus CD44 acts as a signaling hub where it heterodimerizes with binding partners to specify cell fate and/or regulate a range of cellular processes, including cell adhesion, cell function, transcription and cell migration.

The CD44 intracellular domain

The CD44 intracellular domain (CD44-ICD) exists in a long- or short-tail configuration. Long-tail CD44 (CD44-lt, exon 18/20) contains the nuclear localization signal which is located immediately following the TM domain.[56] The ezrin-radixin-moesin (ERM) proteins bind to amino acids 292–300[56] whereas ankyrin binds to amino acids 304–318[57] and a consensus PZD-domain binding motif exists at the CD44 terminal end.[51,58] These binding sites link the intracellular domain of CD44 to the actin cytoskeleton and remodel the actin cytoskeleton during

epithelial–mesenchymal transition, cell migration and invasion.[59–61] Similar to that observed in the generation of Notch-ICD,[52,62] CD44-ICD can be cleaved from the membrane by the presenilin-γ-secretase complex.[63] Upon cleavage, CD44-ICD is internalized and translocated into the nucleus[64,65] where it mediates transcription through 12-*O*-tetradecanoylphorbol 13-acetate-responsive elements[66] and CBP or p300 interactions.[67] Target genes include the CD44 gene itself[64,66,68] and cyclin D1.[66,67,69] Thus, CD44-ICD promotes cell growth via cyclin D1 and increases CD44 signaling through a positive feedback mechanism. Of interest is that full-length CD44 has also been observed in the nucleus; however the mechanism by which this non-cleaved form of the receptor translocates into the nucleus and its contribution to regulation of gene expression is currently unclear.[70]

The short-tail CD44 isoform (CD44HΔ68 or CD44-st [exon18/19]) arises from an in-frame termination signal in exon 19 and contains the three amino acids[292]RRR[295] encoded by exon 18.[33,51,71–73] CD44-st isoforms are generally present in very low abundance compared with the long-tail CD44 isoform.[72] In chondrocytes, CD44-st acts as a dominant-negative receptor to block CD44:CD44 interactions, while inhibition of CD44-st enhances CD44-mediated HA internalization.[73] Furthermore, inhibiting CD44-st reduces pericellular matrix formation, suggesting that CD44-st expression levels modulate the chondrocyte microenvironment.

The CD44 3′-UTR

MicroRNAs are non-coding RNAs which regulate gene expression in a post-transcriptional manner by binding to specific DNA sequences including sites within the 3′-UTR.[74] miR-373 and miR-520c binding to the CD44 3′-UTR down-regulated CD44 expression and promoted prostate cancer (PCa) cell migration and invasion, suggesting that CD44 functioned as a tumor suppressor in PCa.[75] In contrast, miR-199a binding to the CD44 3′-UTR decreased CD44 expression and inhibited cell proliferation, migration and invasion of cells *in vitro* and tumor growth *in vivo*, suggesting that CD44 was a tumor promoter in ovarian cancer initiating cells.[76] In addition, over-expression of the 3′-UTR has been shown to antagonize microRNA function.[77] In a study by Rutnam *et al.*, overexpression of the CD44 3′-UTR increased cell motility, invasion and cell adhesion in the human breast carcinoma cell line MDA-MB-231. This enhanced metastatic phenotype was due, in part, to the ability of the CD44 3′-UTR to bind multiple microRNAs (miR-328, miR-491, miR-671 and miR-512-3p) to repress their inhibitory function and synergistically upregulate collagen type-1a1 and fibronectin type-1 as well as CD44 protein expression.

Taken together, these studies imply that CD44 can modulate the composition of the ECM by regulating the expression of selective ECM components. Furthermore, the function of CD44 as a tumor suppressor or oncogene appears dependent on tumor cell type. Whether the observed differences in microRNA function was due to the stem cell-like characteristics of cancer initiating cells as compared with those of mature tumor cells remains to be determined.

CD44 regulates adhesion, differentiation, homing and migration in the normal stem cell niche

Embryonic, adult and CSCs reside in highly specialized microenvironments or 'niches' (as summarized in Figure 2). The factors within the niche mediate stem cell viability and self-renewal processes and provide signals for differentiation in a developmental and temporal manner. Interactions between stem cells and ECM components are mediated by adhesion molecules present on the stem cell.[78,79] Furthermore, signaling between neighboring stem cells[80] and adjacent differentiated cells[81,82] contribute to moderating stem cell fate.

The principal ligand for CD44 is hyaluronan (a.k.a. hyaluronic acid, HA or hyaluronate), a polysaccharide composed of alternative residues of D-glucuronic acid and *N*-acetylglucosamine (Figure 2a).[83] HA is found at high levels in the stem cell niche[84] where it is produced by oocytes, stem cells and/or stem cell niche support cells.[85–87] Thus, cell culture medium and hydrogels are frequently supplemented with HA for maintaining culturing ACS and human embryonic stem cells in an undifferentiated state.[88–90]

HA/CD44 and HA/CD44v interactions regulate stem cell migration and homing. Studies on the hematopoietic stem cell (HSC) niche provided the first evidence that a specialized CD44 glycoform HCELL (Hematopoietic Cell E-/L-selectin Ligand) promoted HSC homing to the bone marrow (Figure 2b).[96] In response to platelet derived growth factor (PDGF) stimulation, Ap8c3 mesenchymal stem cells (MSCs) expressed high levels of CD44s which facilitated cell migration through extracellular HA.[97] Stromal cell-derived factor 1 (SDF-1) promoted progenitor cell adhesion to HA and formation of actin-containing protrusions with CD44 at their tips, suggesting that SDF-1 dependent trans-endothelial migration (TEM) and anchorage in the bone marrow niche was regulated by HA/CD44 activity.[98] These migratory mechanisms could be critical for recruitment of MSCs during organ development and tissue regeneration, as well as for migration of host MSCs into organ transplants as a wound healing process secondary to rejection injury.[97]

Within the complex milieu of the stem cell niche, HA/CD44 interactions modulate processes including stem cell specification and differentiation. In one study, HA/CD44 induced CD34+ umbilical cord blood-derived progenitor cells to differentiate into mature eosinophils (Figure 2c).[91] Using a long-term bone marrow culture system for the generation of NK cells, other studies have demonstrated that HA/CD44 interactions are required for the proliferation and maturation of pre-NK cells into cytotoxic effector cells.[92] The magnitude of CD44 expression has been shown to be critical for CD34+ precursor differentiation. CD34+ precursors expressing high levels of CD44 differentiated into a mixed population of monocytes and dendritic

cells.[93] Conversely, down-regulation of CD44 expression resulted in differentiation of CD34+ precursors into T-lineage cells, including CD4+/CD8+ thymocytes.[93] High and low molecular weight (MW) HA contribute to the differentiation process. Following tissue injury, peripheral blood monocytes infiltrate the site of injury and differentiate into fibrocytes. High MW HA promoted fibrocyte differentiation while low MW HA inhibited this process.[94]

Similar to that reported for CD44, CD44 variants are competent to modulate progenitor cell differentiation (Figure 2d). Treatment of K562 human myeloid progenitor cells with phorbol ester primarily induced expression of CD44v4–10, followed by maturation of K562 progenitors into myeloid cells, suggesting that the preferential expression of CD44v was critical for myeloid lineage specification.[95] Furthermore, expression of CD44v4–10 increased HA binding and adhesion to MS-5 stromal cells. In addition, CD44v4–10 demonstrated a novel function in that it was now able to bind chondroitin sulfate A (CS-A), although this binding did not result in increased cell adhesion.[95]

Using specific antibodies to CD44v6, CD44v7 and CD44v8, Schwärzler *et al.*[99] demonstrated that these isoforms were expressed on $CD3^-CD4^{lo}CD8^-$ stage T cells and that only cells expressing CD44v from fetal liver and adult bone marrow could populate fetal thymic stroma and develop into mature T cells (Figure 2e). Addition of anti-CD44v antibodies to fetal thymic organ cultures inhibited thymocyte development, implying that CD44v expression was necessary for the initial interaction of HSCs with the thymic stroma.[99]

While CD44 primarily binds HA, it interacts with multiple intrinsic niche factors (Figure 2f). ECM is composed of polysaccharides and fibrous proteins and functions as a compressible scaffold to regulate rigidity of tissues and provide a matrix for cell adherence.[100] A common ECM component is heparan sulfate, a linear polysaccharide which associates with proteins to form heparan sulfate proteoglycan. Heparan sulfate acts as a catalyst to cross-link and enmesh heparan sulfate proteoglycan into the ECM.[101,102] Through its heparan sulfate moiety, CD44v3 can bind HGF,[103] FGF2[104] and HB-EGF.[105] Upon binding, the growth factors are presented to their cognate RTK, resulting in CD44/ligand/RTK complex formation and signal transduction.

Cells in the stem cell niche also express integrins, which are cell adhesion receptors that link extracellular adhesion molecules with the intracellular actin cytoskeleton.[106] The role of integrins in stem cells has been reviewed in detail.[107,108] Briefly, there are 24 known integrin heterodimers consisting of one of 18 α subunits and one of eight β subunits. Their expression ranges from human ES cells to hematopoietic, neural and mesenchymal ASCs where they regulate cell–cell adhesion, growth factor receptor signaling, cell lineage specification, differentiation, survival, proliferation and migration.[107] Many of these functions parallel those reported for CD44, suggesting that they could be mediated through integrin/CD44 interactions. Indeed, in T-lymphocytes, interaction between the cytoplasmic tail of CD44 and integrin $\alpha4\beta1$ is required to promote $\alpha4\beta1$-mediated adhesion to the endothelium.[109,110] In B-cell chronic lymphocytic leukemia (B-CLL) cells, integrin $\alpha4\beta1$ binds to a high-MW variant of 190-kDa CD44v to form a docking complex for pro-MMP 9 (proMMP-9). Upon binding, pro-MMP-9 is activated and MMP-9 proteolytic activity inhibits B-CLL cell transendothelial migration and invasion through Matrigel.[111]

Integrins are further linked to CD44 through their interactions with selectins. Homing of hematopoietic stem/progenitor cells to bone marrow requires a coordinated sequence of four steps including: (1) E-selectin receptor/ligand interaction; (2) engagement of co-localized CXCL12 CXCR4, resulting in activation of VLA-4 (a.k.a. integrin $\alpha4\beta1$); (3) VLA-4/$\alpha4\beta1$ adherence to VCAM-1 and (4) transmigration on endothelium,[96] E-selectin, an endothelial C-type lectin, is expressed in a constitutive manner on bone marrow and binds sialofucosylated carbohydrate determinants on its cognate ligands.[112,113] The bone marrow homing receptor is the CD44 glycoform HCELL, a potent human-specific E-selectin ligand expressed on human hematopoietic stem/progenitor cells.[96] In HCELL+ hMSCs, HCELL and VLA-4/$\alpha4\beta1$ associated at baseline levels and E-selectin markedly increased their interaction.[114] Furthermore, VLA-4/$\alpha4\beta1$ activation and adhesive interaction with VCAM-1 was mediated by Rac1/Rap1 GTPase signaling and promoted TEM on stimulated human umbilical vein endothelial cells.[114] Since cytokine treatment was not required for TEM, this study suggested that HCELL expression could bypass CXCL12/CXCR4-mediated signaling in step 2 of the homing process.[114] Indeed, a technology termed Glycosyltransferase-Programmed Stereosubstitution or GPS is currently being investigated for the purpose of selectively modifying CD44 glycans to facilitate their migration to endothelial cells expressing E-selectin.[96] Since CD44 expression is relatively ubiquitous and VLA-4/$\alpha4\beta1$ expression is characteristic of adult HSCs, MSCs and lymphocytes, enforced HCELL expression could be used in stem cell-based regenerative therapeutics and lymphocyte-based immunotherapy.[96]

Finally, CD44 modulates the stem cell niche through matrix assembly (Figure 2g). $CD44^-$ COS-7 cells gained the ability to bind HA and assemble pericellular matrices in the presence of exogenously added HA and proteoglycans when cells were transfected with a CD44 expression vector.[115,116] HA is synthesized by one of the three integral membrane hyaluronan synthase genes-1, -2 or -3 present in stem/progenitor[117,118] and/or niche cells.[117] While HA is not secreted into the extracellular space, it is threaded through membrane pores into the extracellular space where HA-binding proteins anchor HA to the cell forming a platform for the initial matrix.[83] In addition, HA/CD44 interactions promote retention of pericellular matrix.[119] Furthermore, the spatial organization of CD44 at the cell surface could modulate the structure of the pericellular matrix dependent on the HA scaffold.[120] The pericellular matrix is mechanically coupled to cytoskeletal filaments and the nucleus.[121] Therefore perturbations in matrix components would cause physiologically relevant distortion of molecular structures, resulting in alterations in cell shape, intracellular signaling and gene expression.[121]

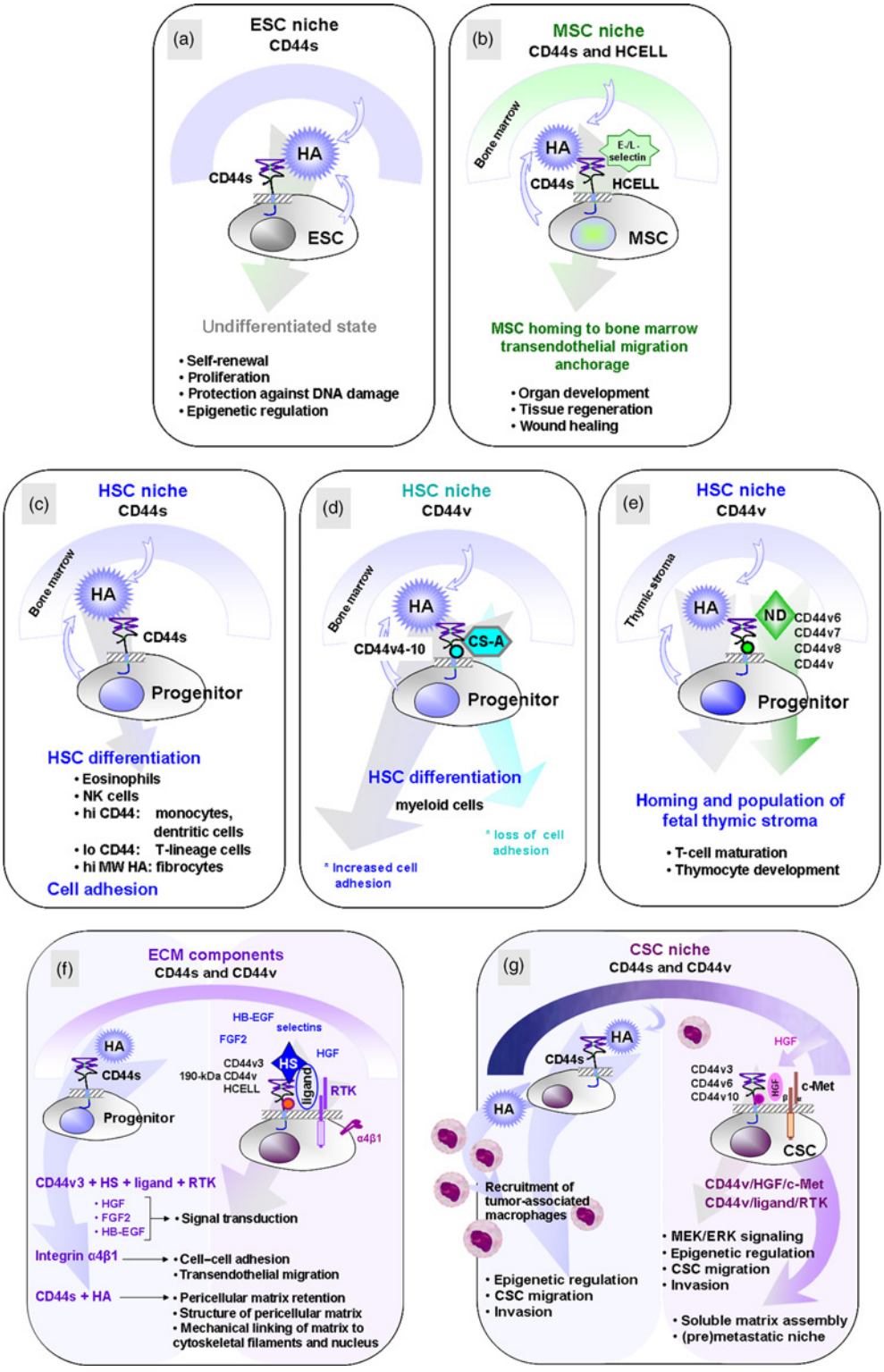

Figure 2 CD44s and CD44v function in discrete stem cell niches. (a) Embryonic stem cell (ESC) niche. The standard CD44 (CD44s) appears to be the primary CD44 isoform in the ESC niche. (b) Mesenchymal stem cell (MSC) niche. In this niche, the two primary isoforms are CD44s and the homing receptor Hematopoietic Cell E-/L-selectin Ligand (HCELL). (c–e) Hematopoietic stem cell niches. These niches represent the functions of (c) CD44s and (d) CD44v in bone marrow and (e) of CD44v in the developing fetal thymus. (f) Overview of extracellular matrix components which modulate HA/CD44s and CD44v/RTK function in the stem cell-like niche. (g) Cancer stem cell (CSC) niche. Both CD44s and CD44v promote tumor progression through similar and unique mechanisms (discussed in more detail in the text). HA, hyaluronan; NK cell, natural killer cells; MW, molecular weight; CS-A, chondroitin sulfate A; ND, ligand not determined; HS, heparan sulfate; RTK, receptor tyrosine kinase; HGF, hepatocyte growth factor; FGF2, fibroblast growth factor 2; HB-EGF, heparin-binding EGF-like growth factor; MEK, mitogen activated kinase kinase; ERK, extracellular signal-regulated kinase

Collectively, CD44 synthesizes matrix molecules and interacts with a multitude of factors to regulate lineage specification, differentiation, stem cell survival, self-renewal, cell migration, cell–cell adhesion and growth factor receptor signaling. Therefore, CD44 appears to be a major regulator in coordinately anchoring normal stem cells in their niche, in regulating stem cell fate and in modulating cell migration into, within or out of the stem cell niche.

CD44 and CD44v interactions in the CSC niche

CSCs synthesize HA and interact with ECM components and adjacent niche cells in a manner similar to that reported for normal ESCs and ASCs. The CSCs synthesize HA to recruit tumor-associated macrophages (TAMs) into the CSC niche.[122] Upon interacting with CSCs, TAMs secrete PDGF isoform BB (PDGF-BB) which binds to its receptor on adjacent stromal cells and recruits them into the CSC niche.

Niche stromal cells secrete numerous growth factors, many of which are known to moderate stem cell function including self-renewal and stem cell fate. CD44s and CD44v bind growth factors sequestered in the ECM to interact with an impressive range of RTKs. Many of the CD44s/RTK and CD44v/RTK interactions have been summarized in Tables 2 and 3. Of particular note is that CSCs frequently express CD44v and these variants initiate alternate signaling pathways. Inclusion of v6 now permits the binding of HGF to the CD44v6 extracellular domain.[19,41] CD44v6 presents HGF to c-Met, resulting in CD44v6/HGF/c-Met complex formation and activation of c-Met, MEK and ERK signaling to promote CSC migration and invasion.[41,123,124] Similar activities have been reported for CD44v3[103] and CD44v10.[125]

A further connection between CD44 and stem cell function is the HA/CD44-mediated induction of Nanog activity

Table 2 CD44/receptor tyrosine kinase (RTK) and CD44v/RTK interactions in mediating stem and non-stem cell activities

TRK	Biological activity
BMPRII	• Galectin-9 induces CD44/BMPRII complex formation, Smad phosphorylation and osteoblast differentiation[160]
EGFR/ErbB1/HER1	• EGFR is required for TGF-β1/HA/CD44-mediated cell proliferation[161] • Oxidative stress generated by cigarette smoke or reactive oxygen species (ROS) promote CD44/EGFR interactions and result in EGFR/MAPK activation and MUC5B up-regulation in airway epithelium[162] • $CD44^{high}/EGFR^{low}$ head and neck squamous cell carcinoma cells exhibit EMT and treatment-resistance[163]
ErbB2/Her2/Neu ErbB3	• In glioma cells, CD44 binds ErbB2 in response to HA treatment, resulting in ErbB2 phosphorylation followed by CD44/ErbB2/EGFR complex formation and ERK1/2 phosphorylation[164] • High CD44 expression occurs in early rat neonatal nerves when Schwann cells proliferate. CD44 is constitutively associated with ErbB2 and ErbB3 and enhances neuregulin-induced ErbB2 phosphorylation and ErbB2–ErbB3 heterodimerization[165] • In ovarian tumor cells, HA binding promotes CD44/N-WASP association with ErbB2 and activates ErbB2 kinase activity. This in turn increases phosphorylation of β-catenin and promotes translocation of phosphorylated β-catenin into the nucleus to active TCF/LEF-transcription[166,167] • The interaction of Her2/CD44 up-regulates C-X-C chemokine receptor type 4 (CXCR4) expression via up-regulation of the metastasis-associated protein-1 (MTA1) gene and histone deacetylation of the miR-139 gene[154]
ErbB4/HER4	• CD44v3/CD44HSPG assembles MMP-7, HB-EGF precursor and ErbB4 into a complex, thereby promoting MMP-7-mediated cleavage of HB-EGF and activation of ErbB4. This process supports cell survival and regulates postpartum uterine and mammary tissue remodeling[24]
FGFR	• In chondrosarcoma cells, FGFR binds to CD44s and exogenous Bikunin could abrogate CD44s-FGFR heterodimerization[168] • FGF-2 is required in ES cell culture medium[169] and CD44 is expressed in normal ASCs and CSCs. Thus FGFR/CD44 interactions may play a role in the maintenance of ASCs and CSCs
IGF1R	• The bone marrow microenvironment upregulates IGF1R and CD44v6 which facilitate homing of multiple myeloma cells to bone marrow and adhesion to bone marrow stroma[170]
cMet	• $c\text{-}Met^{+}/CD44^{+}$ pancreatic CSCs exhibit self-renewal and high tumorigenic potential[171] • CD44v6 binds HGF and activates c-Met signaling in primary human melanocytes, primary keratinocytes and breast cancer stem-like cells[122,123,172]
PDGFR	• CD44 suppresses PDGFRβ signaling, thereby inhibiting fibroblast cell migration[24,173]
TGF-βR	• TGB-βRI binds the cytoplasmic domain of CD44, resulting in Smad2/3 phosphorylation, interaction with ankyrin and breast cancer cell migration[174] • CD44/HA interactions modulate TGF-β1 signaling[175,176] • TGF-β1-mediated fibroblast to myofibroblast differentiation is associated with HA pericellular coat assembly[177–179] • HA facilitates TGF-β1-dependent fibroblast proliferation by promoting CD44/EGFR interactions which then promote MAPK/ERK activation and cell proliferation[161,177–179] • CD44/MMP-9 interactions activate latent TGFβ to promote tumor cell survival and metastatic colony formation[180] • CD44s is induced by TGF-β1 and regulates TGF-β-mediated EMT in hepatocellular carcinoma cells[181]
VEGFR	• VEGF promotes survival, colony formation and *in vivo* repopulation of hematopoietic stem cells[182] • In response to VEGF-A, VEGFR2/CD44v6 interactions promote endothelial cell migration, spheroid sprouting and tubule formation, indicating that CD44v6 participates in blood vessel formation[183]

Table 3 Function and biological significance of the CD44 variants (CD44v)

CD44 variant	Biological activity
CD44v1	• CD44v1 generates a truncated protein due to a premature termination codon. If this protein were translated, it would produce a soluble CD44 isoform. This molecule has not been isolated to date[33]
CD44v2	• At present, the function of CD44v2 in stem cells has not been identified
CD44v2–10	• Overexpression of CD44v2–10 is sufficient to confer a metastatic phenotype on non-metastatic SKHep1 cells[184]
CD44v3	• HA treatment promotes CD44v3/Oct4/Sox2/Nanog complex formation in the $CD44v3^{high}ALDH1^{high}$ CSC-like subpopulation isolated from the HSC-3 squamous carcinoma cell line.[153] Subsequently, Oct4/Sox2/Nanog translocates into the nucleus to induce miR-302 transcription, resulting in suppression of epigenetic regulators (AOF1/AOF2, DNMT1) and global DNA demethylation.[153] Furthermore, survival protein (cIAP-1, cIAP-2, XIAP) expression is up-regulated and promotes self-renewal, clonal formation and cisplatin resistance[153] • During limb development, CD44v3–v10 on apical ectodermal ridge cells binds FGF-4 and FGF-8 and presents these ligands to limb mesenchymal cells. Furthermore, specific anti-CD44v3 and anti-CD44 v6 antibodies inhibit FGF-8 presentation and limb bud outgrowth[185] • CD44v3 binds $\alpha M\beta 2$ integrin (CD11b/CD18), resulting in adhesion and migration of polymorphonuclear leukocytes across the intestinal epithelial basolateral surface[186]
CD44v3,v8–10	• CD44v3,v8–10 is detected in >90% of adenomas and colorectal carcinomas and >70% of colorectal liver metastases.[187] Its function is unknown
CD44v4	• CD44v4 mediates breast cancer cell adhesion to endothelial monolayers via binding to E-selectin[188]
CD44v5	• When Ras is activated, the RNA splicing regulator proteins SRm160 and Sam64 (Src-associated in mitosis, 68 kDa) interact and include v5 when producing CD44 isoforms containing v5. Knockdown of CD44v5 reduces HeLa cell migration and invasion *in vitro*[155,156] • In immortalized non-tumorigenic keratinocyte (HaCaT) cells, Sam68 is required for the HGF-induced, MAPK-dependent up-regulation of CD44v5. CD44v5 promotes keratinocyte cell migration[189]
CD44v6	• CD44v3-v10/FGF/FGFR complex formation promotes limb bud outgrowth[185] • CD44v6 supports assembly of a soluble matrix that together with exosomes initiates the formation of a (pre)metastatic niche[190] • The CD44v6-assembled soluble matrix promotes CD44v6/HA-dependent cell motility through c-Met phosphorylation, recruitment and phosphorylation of Ezrin, and phosphorylation of focal adhesion kinase.[191] Furthermore, only cells expressing CD44v6 exhibited CD44v6/HA, c-Met-HGF and $\alpha 6\beta 4$-LN5-mediated induction of cell motility and increased resistance to cisplatin, suggesting that CD44v6 expression was essential for these processes[191] • CD44v6/HGF/cMet interactions promote endothelial cell migration, spheroid sprouting, and tubule formation *in vitro* as well as blood vessels *in vivo*[183] • In brain tumor stem-like cells (BTSC) isolated from glioblastoma multiforme tissue, osteopontin activates CD44v6/AKT signaling to promote BTSC growth[192] • $CD44^{high}$ colon cancer-initiating cells were associated with increased sphere formation and phosphorylation of β-catenin. Furthermore, CD44v6 expression was associated with activating phosphorylation of c-Met in spheroid cells[148] • CD44v6/HGF/c-Met form a complex and promote MEK and ERK phosphorylation[123,193]
CD44v7	• Reciprocal CD44v7 bone marrow cell (BMC) transfer experiments (i.e. $CD44v7^{+/+}$ BMC into $CD44v7^{-/-}$ mice and *vice versa*) demonstrated that $CD44v7^{+/+}$ stromal cells supported hematopoietic progenitor cell homing. Furthermore treatment of $CD44v7^{+/+}$ with anti-CD44v7 antibodies mobilized progenitor cells into the peripheral circulation and promoted proliferation of peripheral blood mononuclear cells[194]
CD44v8–10 epithelial form	• CD44v8–10 interacts with and stabilizes the glutamate-cystine transporter xCT at the plasma membrane and promotes the uptake of cystine for GSH synthesis. Furthermore, CD44v8–10 expression promotes GSH synthesis and cells expressing CD44v8–10 are more resistant to antioxidant treatment. CD44 and xCT expression are required for suppression of ROS-induced p38MAPK activation[147]
CD44v9	• Increased HA stimulates CD44v9 expression and HA/CD44v9 interactions are required for AR gene expression, cell adhesion, and cell proliferation. HGF/CD44v9 interactions increased the phosphorylation of cMet and IGF1R.[195] HGF appeared to induce a lipid raft-associated complex that contained CD44v9, cMet/phosphatidylinositol 3-kinase, HSP90 and androgen receptor[195]
CD44v10	• CD44v10 expressed on bone marrow stromal cells supported hematopoietic progenitor cell adherence and maturation into primarily B-cells. Treatment with CD44v10 blocking antibodies induced efficient mobilization of the progenitor cells and a transient delay in progenitor maturation.[196,197] • In contrast to anti-CD44v10, anti-CD44s inhibited expansion of progenitor cells and repopulation of the thymus and the periphery, demonstrating that CD44s, but not CD44v10, regulates progenitor cell expansion[99]

and up-regulation of microRNA-21 (miR-21) transcription.[126] In this signaling cascade, HA/CD44 activated protein kinase Cε which in turn increased Nanog phosphorylation. Phospho-Nanog translocated into the nucleus to interact with RNase III (DROSHA) and the RNA helicase p68, thereby increasing miR-21 expression and down-regulating expression of the tumor suppressor PDCD4 (programmed cell death 4). The decrease in PDCD4 levels promoted IAP (inhibitors of apoptosis proteins) expression, including X-linked IAP and survivin, and increased MDR1 expression, chemoresistance and oncogenesis in MCF-7 breast cancer cells.[126]

Similar to that observed in normal stem cells, integrins interact with CD44 and CD44v to regulate CSC migration. Binding of CD44 to $\alpha 4\beta 1$ activated G-protein coupled signaling and increased human MSC adhesion to, and

transmigration through, vascular endothelium.[127] Association of CD44v6 with $\alpha 6\beta 4$ in colorectal cancer initiating cells (CIC) promoted binding to, and activation of, Src, ankyrin and ERM proteins and down-stream signaling pathways.[128] These interactions promoted CIC maintenance, cell migration and apoptosis resistance.[128] Expression of CD44 and CD44v6 in colorectal cancer is, in part, regulated by the β-catenin/Tcf-4 signaling pathway.[129] While CD44s and CD44v6 expression were restricted to normal crypts, CD44s and CD44v6 were significantly over-expressed in dysplastic crypts and adenomatous polyps from Apc^{+}/Apc1638 mice and in human colon polyp biopsies.[129] Conversely in mice lacking the β-catenin-activated transcription factor Tcf-4, CD44s and CD44v6 expression was absent in the epithelial lining of the small intestine along with an absence of a proliferative stem cell compartment in the crypt regions.[129] This study concluded that CD44 expression was regulated by the β-catenin/Tcf-4 signaling pathway and implied that CD44 was required in the generation and turnover of crypt epithelial cells.[129]

CD44 and CD44v interactions within the (pre)metastatic niche

The importance of CD44v in tumor progression has been supported by the observation that CD44v6 activity assembled a soluble matrix which allowed exosomes to organize a receptive niche in the (pre)metastatic organ.[19] While exosomes themselves were CD44v6-independent, they required CD44v6 to activate c-Met and urokinase-type plasminogen activator receptor and assemble the soluble matrix.[19] In another study, only 12.5% (3/24) rats injected with [CD44v(kd)] ASML cells exhibited visible lung metastasis, indicating that knockdown of CD44v6/v7 produced a matrix which did not support metastatic growth of the highly metastatic rat pancreatic adenocarcinoma cell line AMSL.[130] Furthermore, [CD44v(kd)] matrix could not support homing to lymph nodes *in vivo*.[19] In contrast, CD44s expression does not appear essential in promoting metastasis. Utilizing a gastric cancer xenograft model in which CD97 small isoform was knocked down, Liu *et al.*[131] reported that CD44s expression was down-regulated in early metastatic regional lymph nodes.

HA/CD44-mediated activity in response to oxidative stress

Studies have indicated that low oxygen concentrations can support the long-term culture of pluripotent mouse ESCs and that HA can substitute hypoxia in maintaining ESC pluripotency and reducing their spontaneous differentiation into cardiomyocytes.[132] Both exogenous $oxygen^{2}$ and intracellular reactive oxygen species (ROS) generated as by-products of aerobic respiration contribute to oxidative DNA damage.[133] Therefore hematopoietic and tissue stem cells have available several mechanisms which protect them from the effects of oxidative damage. Firstly, the niche preserves the genomic integrity of the stem cell by maintaining reduced oxygen levels. The hematopoietic niche is one of the most hypoxic, keeping oxygen tensions of $\sim$1%[134,135] to support primitive HSC function.[133,136] In contrast, more differentiated and mitotically active HSC precursors locate to the perivascular region[134] where they begin to differentiate under higher oxygen tension levels.[137–141] While hypoxia did not alter the growth rate of human (h) ESCs, it reduced spontaneous cell differentiation and enhanced formation of embryoid bodies, suggesting that hypoxic culture conditions were required, in part, for maintaining pluripotency.[142] Not all studies have confirmed that hypoxia could maintain hESC pluripotency,[143] suggesting that conditions under which hESCs are cultured may influence their response to hypoxia. Furthermore, while the effects of HA and hypoxia on hESCs have been extensively studied, their effects on ASC and CSC pluripotency and differentiation within the niche remain to be investigated.

The niche is also thought to conserve stem cell pluripotency through ROS-mediated degradation of high MW HA.[133] In WI-38 fibroblasts, constitutive γH2AX phosphorylation (which measures DNA damage caused by endogenous oxidants generated during metabolic mitochondrial activity) was inhibited by HA; with low MW HA being more effective than medium and high MW HA respectively.[144] It was postulated that in cells expressing CD44, HA could be internalized by endocytosis and that this mechanism protected both mitochondrial and nuclear DNA from oxidative damage.[144] Furthermore, association of CD44 with lipid rafts and the subsequent endocytosis of HA[145] provided an intracellular pool of ROS-scavenging HA for decreasing mitochondrial DNA damage.[146] A further study demonstrated that pretreatment with HA prior to genotoxin exposure (peroxynitrite or xanthine oxidase plus hypoxanthine) decreased mitochondrial DNA damage and increased DNA repair capacity in primary chondrocytes cultured from osteoarthritis patients.[146] In addition, cell viability increased while apoptosis was ameliorated.[146] Sperm cells which bound HA also showed chromatin structure with high DNA chain integrity.[133]

Alternatively, stem cells can utilize glutathione (GSH) as a ROS scavenger. CD44 docks and stabilizes the xCT transport system which transports GSH precursors[147] into gastrointestinal[147] and mammary CSCs[148] as well as MSCs.[149] In addition, CD44v8–10 can serve as a docking protein for the xCT transport system.[147]

Taken together, these studies provide evidence that HA/CD44-mediated activity decreases DNA damage, increases DNA repair and enhances cell survival under conditions of oxidative injury. Furthermore, expression of CD44v provides an alternative mechanism for decreasing DNA damage. While the HSC niche has been extensively investigated, many other studies were conducted in cell lines or primary non-stem cell cultures. Therefore, it remains to be established that HA/CD44 interactions are a primary mechanism by which the stem cell niche protects tissue ASCs and CSCs from oxidative DNA damage.

CD44-mediated epigenetic regulation of microRNAs

MicroRNAs have been recognized as key regulators in controlling self-renewal, stem cell fate and transcriptional regulation.[150] An example is microRNA-302 (miR-302) which is associated with maintenance of stem cell self-renewal and pluripotency.[151] In differentiating ESCs, silencing of the miR-302 promoter was accompanied by a decrease in Oct4, Nanog and Rex1 expression, implying that miR-302 expression was restricted to the ESC compartment.[151] Over-expressing miR-302 in human melanoma-derived mirPS-Colo cells resulted in the reprogramming of these cells into ES-like cells through global demethylation.[152] Bisulfite sequencing demonstrated that >90% of the Oct4 gene promoter region was demethylated, suggesting that Oct4 expression was re-activated.[152] Oct4 was recently been shown to interact with HA/CD44v and modulate miR-302 transcription.[153] CSCs derived from human head and neck squamous cell carcinoma (HNSCC) HSC-3 cells express the CD44 variant CD44v3.[153] Upon HA treatment, Oct4, Sox2 and Nanog were recruited to membrane-bound CD44v3 and subsequently, the Oct4–Sox2–Nanog complex was translocated into the nucleus to initiate miR-302 transcription.[153] Upregulation of miR-302 expression suppressed expression of the epigenetic regulators AOF1/AOF2 and DNMT1 and increased expression of the survival proteins cIAP-1, cIAP-2 and XIAP, resulting in HNSCC CSC self-renewal, clone formation and resistance to Cisplatin.[153]

CD44/RTK signaling is a further mechanism by which CD44 modulates miR expression. The binding of HER2 to CD44 upregulated metastasis-associated protein-1 (MTA-1) expression.[154] MTA-1, in turn, induced deacetylation of histone H3 lysine 9 and silencing of the miR-139 promoter region.[154] Consequently, the decrease in miR-139 transcription resulted in the inhibition of C-X-C chemokine receptor type 4 (CXCR4) expression.[154] The over-expression of HER2, CD44 and CXCR4 along with decreased miR-139 expression in gastric cancer could be a mechanism underlying tumor progression and metastasis.

Together, these studies demonstrate that CD44-mediated regulation of miR expression can reprogram cancer cells into CSC-like cells exhibiting stem cell characteristics as well as initiating or repressing miR transcription to promote tumor progression and therapeutic resistance. Therefore CD44 may be a potential therapeutic target for the treatment of cancer by inhibition or elimination of CSCs.

Conclusions and perspectives

CD44 is a central component in the cross-talk between ASCs and CSCs and their respective niches. The complexity of CD44 splicing and the number of isoforms generated reflect the diversity of CD44 function in the niches of many stem cell types, including ESCs, HSCs, MSCs, tissue ASCs and CSCs. In summary, CD44 binds HA to mediate self-renewal, retention of pericellular matrix, proliferation, differentiation and/or homing to bone marrow. CD44/HA interactions are also significant in preserving the integrity of the stem cell genome by decreasing DNA damage, increasing DNA repair and enhancing cell survival under conditions of oxidative stress. CD44/integrin/E-selectin interactions promote TEM and invasion of MSCs into bone marrow. Moreover, CD44 interacts with other ECM components, including heparin sulfate and a range of growth factor ligands, to promote CD44/ligand/RTK complex formation and signal transduction. These signaling pathways regulate matrix production and assembly, development, cell migration, homing and preparation of the (pre)metastatic site. Of particular note is that CD44/ligand/RTK signaling modulates microRNA expression to regulate promoter methylation status and gene expression. Through this mechanism, CD44 participates in reprogramming cells to exhibit a more stem cell-like phenotype. Therefore, reprogramming cancer cells to exhibit a CSC phenotype could be an elemental mechanism in promoting tumor progression and chemoresistance.

The mechanisms that modulate CD44 splicing events in stem cells, normal cell types and cancer cells remain elusive. Studies by Cheng[155] and Matter[156] reported that during alternative RNA splicing, the Ras-activated association of SRm160 and Sam64 promoted inclusion of exon v5 into CD44v. Knockdown of CD44v5 reduced HeLa cell migration and invasion *in vitro*.[155,156] These observations suggest that CD44v5 promotes metastasis and could therefore serve as a target for inhibiting metastatic potential during cancer progression. A further study determined that CD44v4–10 was the primary CD44 isoform expressed during maturation of K562 progenitors into myeloid cells.[95] Thus, identifying the mechanisms by which CD44v4–10 was preferentially expressed could potentially be utilized in developing eradication strategies for chronic myeloid leukemia involving the enforced maturation of self-renewing leukemia stem cells.

To date, it remains difficult to identify and characterize CD44v exon products as well as determine the individual and/or interactive signaling pathways activated by composite CD44v, e.g. CD44v3,v8–10 and CD44v4–10. There is a critical need for CD44v-specific reagents for characterizing the functions/signaling pathways of CD44v exon products. Limited antibodies to the variable exon products are commercially available (e.g. anti v6- and v10-specific antibodies). Moreover, protein size is not conclusive since glycosylation of CD44v is extensive and variable. A further challenge has been in obtaining and isolating sufficient cells from animal model tissues and human biopsy specimens for characterizing non-hematopoietic CD44 function. Therefore as an alternative source, $CD44^+$ side populations with stem cell-like characteristics have been isolated from established cells lines. These studies have provided invaluable insight into CD44 function; yet few have identified the CD44 isoform(s) expressed in these $CD44^+$ side populations. Therefore, our knowledge of the structural and functional diversity of CD44 in solid tissue ASCs and CSCs remains limited.

The similarities and unique differences in CD44 or CD44v function could be used for developing therapeutic strategies to diseases as diverse as preventing fibrosis during injury repair to the treatment of metastatic cancer.[157,158]

Similar activities include HA-mediated regulation of HSC differentiation[91,93,95], MSC migration and homing to bone marrow.[97–99] In addition, loss of CD44s and CD44v6 expression has been associated with loss of the epithelial stem cell specification.[129] However, mounting evidence suggests that CD44 and CD44v also exhibit distinct activities in normal progenitor cell types as well as between normal and CSC populations. One example is CD44v4–10 which can bind CS-A as well as HA; however unlike HA, CS-A does not mediate cell adhesion.[95] A further difference is that CD44s does not bind HGF,[103] whereas several CD44v molecules, including CD44v3[103] CD44v6[159] and CD44v10[125] bind HGF to activate c-Met signaling and promote tumor progression. While CD44s does not appear essential in promoting metastasis;[131] CD44v6 expression is required for the assembly of a soluble matrix that supports preparation of a (pre)metastatic niche.[19] Understanding how ECM composition is modulated by CD44 activities, and how CD44 and CD44v interactions with ECM components influence cancer progression could assist in developing innovative therapeutic interventions that target tumor and (pre)metastatic niches.

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