BBA - Molecular Cell Research 1865 (2018) 379–391

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Distorted leukocyte migration, angiogenesis, wound repair and metastasis in Tspan8 and Tspan8/CD151 double knockout mice indicate complementary activities of Tspan8 and CD51

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ARTICLE INFO

Keywords:
Tspan8
CD151
Knockout mice
Migration
Angiogenesis
Proteases
Metastasis

ABSTRACT

The tetraspanin Tspan8 supports via associated integrins and proteases tumor progression and angiogenesis. To shed light on its activities in non-transformed cells, we generated a Tspan8 knockout (ko) mouse, comparing leukocyte migration, angiogenesis, wound healing and tumor growth with wild type, CD151ko and Tspan8/CD151ko (dbko) mice. CD151ko mice were included as CD151 activities resemble that of Tspan8, and dbko mice to exclude mutual substitution. Tspan8ko and dbko mice show no pathological phenotype. However, delayed type hypersensitivity reactions are mitigated in Tspan8ko mice, angiogenesis is severely impaired in Tspan8ko, CD151ko and dbko mice, with Tspan8 mostly affecting lymphangiogenesis. Distinct contributions of CD151 and Tspan8 to skin wound healing rely on preferentially CD151 anchoring basal keratinocytes and Tspan8 promoting motility. Proliferation of wounded skin keratinocytes is not affected. Metastasis formation of a melanoma and a Tspan8-expressing pancreatic cancer line was impaired in Tspan8ko and dbko mice, pointing towards a contribution of host Tspan8 to tumor progression. In line with the importance of tetraspanins in exosome-mediated intercellular communication, defects became mitigated by Tspan8/CD151-competent serum exosomes, which offers a most promising therapeutic option for chronic wounds and arteriosclerosis.

1. Introduction

Tetraspanins are highly conserved 4-transmembrane proteins with a small and a large extracellular loop. The latter accounts for dimerization and association with non-tetraspanin partner molecules. Palmitoylation of intracellular, juxtamembrane cysteines supports tetraspanin-tetraspanin web formation, protects from lysosomal degradation and provides a link to cholesterol and gangliosides [1,2]. Tetraspanins, associate with a large variety of transmembrane and cytosolic proteins including integrins, proteases, cytoskeleton and cytosolic signal transduction molecules [3–5]. The tetraspanin complexes are located in tetraspanin-enriched microdomains (TEM), which function as a signaling platform [6–8]. Tetraspanins/tetraspanin webs are involved in multiple biological processes, including migration, invasion and fusion [3,5,6,9].

CD151 and Tspan8 are closely related tetraspanins, belonging to group 2, which contains 6 cysteines in the large extracellular domain. However, the two additional cysteines in Tspan8 and CD151 are differently located. By forming S-S bridges, cysteines are important for folding, which affects lateral protein associations of the large extracellular loop with neighboring proteins. Due to different loop formation, some differences in Tspan8 and CD151 TEMs can be expected [10]. The relatedness of Tspan8 and CD151 is also reflected by joint functions. In particular, both tetraspanins consistently promote angiogenesis and metastasis [7,11]. Metastasis-associated activities of CD151 are partly due to the impact of CD151 on MMPs. CD151 allows for focal proteolysis via pericellular MMP activation [5], influences MMP expression and colocalization at the leading edge of lamellipodia [12]. CD151 also is important for proper localization of laminin (LN)-binding

Abbreviations: BMC, bone marrow cells; coll, collagen; dbko, double Tspan8/CD151ko; ECM, extracellular matrix; Exo, exosomes; FITC, fluorescence isothiocyanate; FN, fibronectin; HGF, hepatocyte growth factor; IP, immunoprecipitation; i.m., intramuscular; i.v., intravenous; ko, knockout; LNC, lymph node cells; LN, laminin; MMP, metalloproteinase; OPN, osteopontin; PBL, peripheral blood leukocytes; RTK, receptor tyrosine kinase; S, serum; SADBE, squaric acid dibuthylester; s.c., subcutaneously; SC, spleen cells; SkIL, skin-infiltrating leukocytes; TEM, tetraspanin-enriched microdomains; TIL, tumor-infiltrating leukocytes; UNKC, UNKC6141 tumor line; VEGF, vascular endothelial growth factor; WB, Western blot; wt, wild type

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https://doi.org/10.1016/j.bbamcr.2017.11.007
Received 21 July 2017; Received in revised form 21 October 2017; Accepted 10 November 2017
Available online 11 November 2017

α3β1 and α6β4, migration and invasion being strengthened by α3β1 co-internalization with CD151 [4,11]. In addition, CD151 associates with growth factor and chemokine receptors as EGFR, c-Met, cKit and TGFβRI. The association of CD151 with receptors activates downstream signaling [11,13], driving mammary cancer induction [14] and skin carcinogenesis [15]. CD151ko mice drive progressive pulmonary fibrosis by reduced adhesion of lung epithelia to the basement membrane [16]. Wound healing is facilitated by keratinocyte CD151-α6β4 binding to LN332 with a suggested engagement of CD151 in fibroblast migration [17]. Increased blood pressure in CD151ko mice induces nephropathy by impaired podocyte-integrin adhesion to the glomerular basement membrane [18]. CD151 expression correlates with microvessel density and thrombus formation/stability in cancer/ischemia [19]. LN111 binding of α6β1-CD151 promotes differentiation of embryonic stem cells into endothelial cells (EC) [20].

Upregulated Tspan8 expression in carcinoma, melanoma and neuroblastoma is associated with poor prognosis [7,21–24]. There is evidence for Tspan8 being engaged in organ-specificity of metastases [25]. Significant inhibition of colon cancer (CoCa) growth by anti-Tspan8 in vivo - without affecting proliferation and apoptosis resistance in vitro - strengthened the engagement of Tspan8 in responding to environmental signals [26]. Tspan8 predisposes to schizophrenia, bipolar disorder [27] and type II diabetes mellitus [28]. Fitting to the latter, Tspan8 expression is upregulated in peripheral occlusive arterial disease [29], defects possibly being due to impaired storage of glucose and gluconeogenesis [30]. Altered Tspan8 expression in acute respiratory distress syndrome relies on miRNA targeting Tspan8 [31]. High Tspan8 expression in distal and collecting tubuli in all nephropathologies points towards engagement in repair mechanisms [32]. Tspan8 overexpression also can induce disseminated intravascular coagulation (DIC), which is exosome (Exo)-mediated [33] and depends on the association of Tspan8 with α4. Instead, Tspan8-associated α6β4 prohibits angiogenesis, but promotes migration and metastasis [34].

Taken together, both metastasis-associated tetraspanins are engaged in motility, repair and angiogenesis, which was confirmed in CD151ko mice. Corresponding studies being missing for Tspan8, we explored the impact of a Tspan8ko in comparison to a CD151ko including a CD151/Tspan8 (db)ko mouse to exclude mutual substitutions.

2. Material and methods

2.1. Tspan8 knockout mouse

A conditional Tspan8^{ko} mouse was generated using the cre/loxp system, floxp sites flanking exon 3 of Tspan8. The target plasmid pLOXP FRT PGK tk/neo FRT [35] has one loxp site and the selection marker neomycin. A DNA fragment containing the Tspan8 gene was isolated from a mouse 129SV genomic library (Biocat). The cloning strategy is detailed in the supplement (Suppl. Methods, Suppl. Table 1A, Suppl. Fig. 1A, B). To induce the Tspan8ko, mice with the floxed Tspan8 allele were crossed with C57BL/6 Cre-recombinase transgenic mice [36]. Heterozygous ko offsprings were crossed to generate homozygous mice. Tspan8/CD151 dbko mice were generated crossing CD151ko mice [37] with Tspan8ko mice.

2.2. Genotyping and RT-PCR

Genomic DNA was extracted by Gentra Puregene Mouse Tail Kit (Qiagen). Tspan8 genotyping was performed by PCR with one common reverse and two different forward primers (Suppl. material, Suppl. Fig. 1A, Suppl. Table 1B). Wt and floxed alleles are distinguished by primers 1 and 2 amplifying 235 bp and 269 bp fragments. Wt and knockout or heterozygous alleles are distinguished by primers 1, 2 and 3 amplifying 235 bp and 416 bp fragments (Suppl. Fig. 1C). For CD151ko mice genotyping, one common reverse and three different forward primers were used (Suppl. material, Suppl. Table 1C) [37] to distinguish wt, floxed, and deleted alleles.

2.3. RNA extraction followed standard procedures (Rneasy MiNi Kit, Qiagen)

cDNA was synthesized using M-MuLV Reverse Transcriptase (Thermo Scientific). Tspan8 RNA levels were analyzed by RT-PCR (94 °C, 10 min; 94 °C, 30s; 60 °C, 30s; 70 °C, 30s; followed by 29 cycles).

2.4. Cell lines and tissue collection

B16F10 were maintained in RPMI1640/10%FCS and UNKC6141 (UNKC) [38] in Iscove/10%FCS. Minced lung tissue was incubated with collagenase. Dispersed cells were cultured in Isove/20% FCS/1% Nonessential Amino Acids/75 ng EC supplement (Sigma, Munich) [39] to enrich for endothelial cells (EC). 3-methylcholanthrene (MCA)-induced tumors were collected, minced and cultured in RPMI1640/10% FCS/transferrin.

Solid and lymphatic organ tissue was shock frozen or dispersed by meshing through fine gauze. Peritoneal exudates cells (PEC) were collected by flushing the peritoneal cavity with PBS/heparin, Bone marrow cells (BMC) were collected by flushing femora and tibiae with PBS. Heparinized blood was collected by heart puncture. Peripheral blood leukocytes (PBL) were enriched by Ficoll/Hypaque gradient centrifugation. Thrombocytes (TC) were collected after centrifugation of platelet-enriched plasma (centrifugation in Tyrode's buffer) in HEN buffer [40].

2.5. Exosome preparation

Cells were cultured (48 h) in serum-free medium. Cleared supernatants (2 × 10 min, 500*g*, 1 × 20 min, 2000*g*, 1 × 30 min, 10,000*g*) were filtered (0.2 μm), centrifuged (90 min, 100,000*g*) and washed (PBS, 90 min, 100,000*g*). Serum exosomes (S-Exo) were prepared accordingly after a 1:5 dilution of plasma in PBS. After ultracentrifugation, the resuspended pellets were purified by sucrose gradient centrifugation [41].

2.6. Antibodies

HEK293 cells were transfected with Tspan8 cDNA shuttled into modified PCEP4. Culture supernatant was collected and purified via a passage over an anti-cMyc column. Purified protein, mixed with complete Freund's adjuvant, was subcutaneously (s.c.) injected in New Zealand White rabbits. After 3 boost injections in incomplete Freund's adjuvant, rabbits were bled. The IgG fraction was enriched by passing over a proteinG sepharose column. For other antibodies and chemicals see Suppl. Table 2.

2.7. Flow-cytometry of cells and TC followed routine procedures

Where indicated, cells/TC were fixed and permeabilized. Samples were analyzed in a FACSCalibur using the CellQuest program.

2.8. Sucrose density gradient centrifugation and Western blot (WB)

Cell lysates (30 min, 4 °C, HEPES buffer, 1% Lubrol, 1 mM PMSF, 1 mM $NaVO_4$, 10 mM NaF, protease inhibitor mix) in 2.5 M sucrose were overlaid by a continuous sucrose gradient (0.25 M–2 M) and centrifuged (15 h, 150,000*g*), collecting twelve 1 ml fractions [41]. For WB, cell lysates in Laemmli buffer were resolved on 10% SDS-PAGE. After protein transfer, blocking and blotting with antibodies, blots were developed with ECL.

2.9. Bleeding time and blood loss

Bleeding time and blood loss were measured after cutting 0.5 cm of the mouse tail.