Received: 4 September 2019 | Revised: 15 May 2020 | Accepted: 19 May 2020

DOI: 10.1002/ijc.33144

MOLECULAR CANCER BIOLOGY

IJC International Journal of Cancer

Cluster of differentiation 44 promotes osteosarcoma progression in mice lacking the tumor suppressor Merlin

Junzhi Ma[1] | Janina Klemm[2] | Monserrat Gerardo-Ramírez[2] | Lucien Frappart[1] | Darko Castven[2] | Diana Becker[2] | Ansgar Zoch[1] | Romain Parent[3] | Birke Bartosch[3] | Kerstin Minnich[1] | Marco Giovannini[4] | Sven Danckwardt[5,6] | Nils Hartmann[7] | Helen Morrison[1] | Peter Herrlich[1] | Jens U. Marquardt[2] | Monika Hartmann[2]

[1]Leibniz Institute on Aging, Fritz Lipmann Institute (FLI), Jena, Germany

[2]First Department of Internal Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[3]Cancer Research Center of Lyon, INSERM U1052 and CNRS UMR5286, University of Lyon, Lyon, France

[4]Department of Head and Neck Surgery, David Geffen School of Medicine at University of California, Los Angeles (UCLA) and Jonsson Comprehensive Cancer Center (JCCC), Los Angeles, California

[5]Center for Thrombosis and Hemostasis (CTH), University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[6]Institute for Clinical Chemistry and Laboratory Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[7]Institute of Pathology, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

Correspondence
Monika Hartmann, First Department of Internal Medicine, University Medical Center of the Johannes Gutenberg University, Langenbeckstr. 1, 55131 Mainz, Germany.
Email: monika.hartmann3@outlook.de

Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Number: 01EO1003; Deutsche Forschungsgemeinschaft, Grant/Award Numbers: HA 7860/1-1, He551, INST 371/33-1, DA89/2-1, MA 4443/2-2, MO 1421/5-1; Jung-Stiftung für Wissenschaft und Forschung; Volkswagen Foundation

Abstract

Merlin is a versatile tumor suppressor protein encoded by the *NF2* gene. Several lines of evidence suggest that Merlin exerts its tumor suppressor activity, at least in part, by forming an inhibitory complex with cluster of differentiation 44 (CD44). Consistently, numerous *NF2* mutations in cancer patients are predicted to perturb the interaction of Merlin with CD44. We hypothesized that disruption of the Merlin-CD44 complex through loss of Merlin, unleashes putative tumor- or metastasis-promoting functions of CD44. To evaluate the relevance of the Merlin-CD44 interaction in vivo, we compared tumor growth and progression in *Cd44*-positive and *Cd44*-negative *Nf2*-mutant mice. Heterozygous *Nf2*-mutant mice were prone to developing highly metastatic osteosarcomas. Importantly, while the absence of the *Cd44* gene had no effect on the frequency of primary osteosarcoma development, it strongly diminished osteosarcoma metastasis formation in the *Nf2*-mutant mice. In vitro assays identified transendothelial migration as the most prominent cellular phenotype dependent on CD44. Adhesion to endothelial cells was blocked by interfering with integrin $\alpha 4\beta 1$ (very late antigen-4, VLA-4) on osteosarcoma cells and CD44 upregulated levels of

Abbreviations: APC, adenomatous polyposis coli; CD44, cluster of differentiation 44; CD44s, CD44 standard isoform; DOX, doxorubicin; ERM, ezrin-radixin-moesin; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen 1; MDR1, multidrug resistance pump 1; NF2, neurofibromatosis type 2; NOD-SCID, nonobese diabetic severe combined immunodeficiency; RTKs, receptor tyrosine kinases; VLA-4, very late antigen-4.

Junzhi Ma, Janina Klemm, Peter Herrlich, Jens U. Marquardt and Monika Hartmann contributed equally to this study.

integrin VLA-4 $\beta 1$ subunit. Among other putative functions of CD44, which may contribute to the metastatic behavior, the passage through the endothelial cells also appears to be critical in vivo, as CD44 significantly promoted formation of lung metastasis upon intravenous injection of osteosarcoma cells into immunocompromised mice. Altogether, our results strongly suggest that CD44 plays a metastasis-promoting role in the absence of Merlin.

KEYWORDS

CD44, Merlin, metastasis, NF2, osteosarcoma

1 | INTRODUCTION

With an annual incidence rate of 3.4 cases per million people worldwide, osteosarcoma ranks among the most common primary malignant bone tumors and is mainly prevalent in children and adolescents.[1] Upon initial presentation, approximately 20% of osteosarcoma patients already show radiographically detectable distant metastases. However, it is estimated that about 80% of patients already have micrometastatic disease by the time of diagnosis.[2] Since the mid-1970s, the overall survival of nonmetastatic osteosarcoma has improved significantly, with the addition of chemotherapy to previous treatment protocols. Yet, metastatic osteosarcoma remains a major challenge and less than 30% of patients with detectable metastases survive 5 years after the initial diagnosis.[1] Thus, identification of molecules causally involved in osteosarcoma progression is highly desirable, both for a better understanding of the underlying processes and for generating new molecular targets that may become relevant for clinical practice; perhaps not for cure, but for delaying metastatic growth or prevention of relapses.

Studies in mice indicate that spontaneous osteosarcoma formation might be initiated by loss of the tumor suppressor protein Merlin (moesin-ezrin-radixin-like protein)[3,4]—which belongs to a larger protein superfamily called Band 4.1 and shares significant sequence homology and similar domain organization as other members of the family, such as the ERM (ezrin-radixin-moesin) proteins[5-7] (Figure SI1). Merlin's tumor suppressor function is primarily referred to as its ability to inhibit cell proliferation in response to adhesive signaling. Deactivation of the gene encoding Merlin (*NF2*) helps neoplastic cells to evade contact inhibition, which is a distinguishing event in cancer.[6]

Germline mutations of the *NF2* gene are associated with the neurofibromatosis type 2 (NF2) human tumor syndrome.[7] NF2 patients have increased susceptibility to developing multiple central and peripheral nervous system tumors, including schwannomas, meningiomas and ependymomas. More recently, loss of Merlin function, either due to mutations or transcriptional inactivation, was detected in other human tumors not directly associated with NF2 disease—these include mesotheliomas, breast, colorectal, skin, thyroid, renal, hepatic and prostate cancers.[7] Of interest, the majority of mutations, both in the germ line of NF2 patients and in sporadically occurring human tumors, have been identified in the region of exons 2 and 3 of the *NF2* gene encoding the so-called *FERM* (4.1 protein, ezrin, radixin, moesin) domain of Merlin, which mediates interactions with membrane-bound proteins.[8] Mutant versions of Merlin that cannot localize to the membrane are unable to inhibit cell proliferation.[9] This indicates that membrane localization of Merlin mediated by the FERM domain might be critical for Merlin's tumor suppressor function. At the cell cortex, Merlin participates in the organization of cell junctions and negatively regulates expression and activation of receptor tyrosine kinases (RTKs) and the activation of downstream pathways, including RAS/RAF/MEK/ERK, RAC/PAK/JNK, PI3K/AKT/JNK, FAK/SRC, mTORC1 and WNT/β-catenin (Figure SI2).[5,9] Moreover, Merlin can translocate to the nucleus, where it attenuates oncogenic gene expression primarily via inhibition of the $CRL4^{DCAF1}$ (DDB1- and Cul4-Associated Factor 1) ubiquitin ligase complex.[5-7] In addition, Merlin acts both at the cell cortex and in the nucleus to regulate the Hippo tumor suppressor signaling pathway (Figure SI2).[6]

Several studies indicate that Merlin exerts its tumor suppressor activity in part by binding a type I transmembrane glycoprotein CD44 and negatively regulating its function (Figure SI3).[7,10-12] CD44 occurs as multiple protein isoforms generated due to alternative splicing of the messenger RNA, encoded by a single gene. In numerous human cancers expression of CD44 is upregulated, suggesting a protumorigenic role.[13,14] CD44 exerts multiple molecular functions (Figure SI3) and thus can influence behavior of tumor cells in several ways. First of all, CD44 modulates cell adhesion by interacting with components of the

What's new?

The protein Merlin acts as a tumor suppressor by forming a complex with CD44. Inactivating Merlin appears to allow CD44 to induce metastasis. Here, the authors looked at tumor growth and progression in osteosarcoma-prone mice without functional Merlin protein, either with or without CD44. The loss of CD44 did not affect the formation of primary osteosarcomas, but did reduce the rate of metastasis. They then showed that CD44 regulates integrin α4β1 to enhance attachment of tumor cells to endothelial cells and promote migration and metastasis.

extracellular matrix, including hyaluronic acid (HA) as the major ligand.[13,15] Secondly, certain CD44 isoforms containing amino acid sequences encoded by the variant exon v3 or v6, act as co-receptors for several receptor tyrosine kinases (RTKs) and potentiate mitogenic signaling.[10,15,16] Finally, potentially all CD44 isoforms serve as substrates for proteases and can modulate cellular signaling independently of the RTKs, through the release of physiologically active cleavage products.[17] The cytoplasmic domain of CD44, which is cleaved by γ-secretase, plays a role in transcriptional regulation.[17] These numerous observations led us to expect that CD44, including its alternative CD44 isoforms and cleavage products, may contribute to tumorigenesis and the different steps of the metastatic cascade.

Our study has evaluated the relative contribution of CD44-dependent pathways to Merlin-deficient malignancies with a focus on metastatic osteosarcoma. Our results suggest that upon inactivation of Merlin, CD44 is released from its repression by Merlin which unveils a metastasis-promoting function. The smallest isoform of CD44, so-called standard isoform (CD44s), was identified as sufficient to mediate the addressed metastatic functions, independent of HA-binding.

2 | MATERIALS AND METHODS

2.1 | Sources of reagents

DNA oligonucleotides were purchased from Metabion (Munich, Germany); cell culture reagents, including Dulbecco's modified Eagle's medium (DMEM) were from Gibco (New York, New York); MCDB 131 medium, phosphate-buffered saline, Lipofectamine 2000 and Alexa Fluor 647 Phalloidin from Thermo Fisher Scientific (Waltham, Massachusetts); trypsin/EDTA from PAN-Biotech (Aidenbach, Germany); hydrocortisone, gelatin, L-glutamine, penicillin-streptomycin and adenosine 3′,5′-cyclic monophosphate (cAMP) from Sigma-Aldrich (St. Louis, Missouri); fetal bovine serum (FBS) from PAN-Biotech and Sigma-Aldrich; endothelial cell growth supplement (ECGS) and enzyme-free cell dissociation solution from Merck GmbH (Darmstadt, Germany); dynasore from Enzo Life Sciences (Farmingdale, New York). Other chemicals were from Roth, Qiagen (Hilden, Germany), DakoCytomation (Glostrup, Denmark), Roche (Indianapolis, Indiana) and BD (Becton, Dickinson and Company, Franklin Lakes, New Jersey).

2.2 | Experiments involving animals

All animals used in the present study were housed under constant temperature and humidity conditions, after a 12-hour light/dark cycle. Animals had access to food and water ad libitum. All experiments involving mice were approved by the local authorities (AZ: 23 177-07/G 16-1-032 and 23177-07/G 18-1-016). NOD-SCID (NOD.CB17/*Prkdc*$^{scid/scid}$/Rj) male mice were from Janvier Labs. Generation and genotyping of *Nf2*$^{\Delta/+}$ transgenic mice was described by Giovannini et al.[4] Generation of *Cd44*$^{flox/flox}$ mice (with targeted exon 3) was described by Dhar et al.[18] *Cd44*−/− mice were generated by crossing *Cd44*$^{flox/flox}$ mice with GT(Rosa)26-cre mice (Artemis Pharmaceuticals, Wickford, United Kingdom). *Cd44* knockout was confirmed by genotyping of tissue biopsies using the following primers: *Cd44* forward: 5′-GCTCTCTTGATGT CATACAC-3′; *Cd44* reverse 1: 5′-GCTCTCAAGAGAGTAACAC-3′ and *Cd44* reverse 2: 5′-CAAGAGTCTATCATATCCTTCTTGC-3′ in ratio 2:1:1, respectively. The detected sizes of *Cd44* alleles were: 311 bp (*Cd44* floxed), 217 bp (*Cd44* wild-type) and 139 bp (*Cd44* knockout). Double mutant mice *Cd44*$^{-/+}$; *Nf2*$^{\Delta 2/+}$ were initially interbred onto a mixed C57BL/6 × 129/Sv × FVB genetic background. Brother-sister pairings were then carried out over more than six generations to reduce heterozygosity, allowing comparisons of mice with highly similar genetic backgrounds. Littermates with the following genotypes were analyzed: 29 *Cd44*$^{+/+}$; *Nf2*$^{\Delta 2/+}$ (17 female, 12 male); 35 *Cd44*$^{-/-}$; *Nf2*$^{\Delta 2/+}$ (18 female, 17 male); 22 *Cd44*$^{+/+}$; *Nf2*$^{+/+}$ (15 female, 7 male) and 19 *Cd44*$^{-/-}$; *Nf2*$^{+/+}$ mice (12 female, 7 male). *Nf2*-mutant mice were monitored weekly for occurrence of tumors or decline of health. Latest after 2 years, mice were sacrificed and processed for analysis.

2.3 | Cell lines

2.3.1 | Establishing osteosarcoma cell lines

Osteosarcoma tissue was isolated from *Cd44*$^{+/+}$, *Nf2*$^{\Delta 2/+}$ mice, washed with PBS and minced into 1 to 3 mm^3 pieces with the use of a scalpel. Tissue fragments were plated in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Outgrowing cells were replated on fresh plates. After approximately 12 passages, the cultures presented a macroscopically homogenous population of osteosarcoma cells, negative for *Nf2* (characteristic for tumors).

2.3.2 | Sources and culture conditions of other cells

Generation of *Cd44*-negative mouse embryonic fibroblasts (MEFs) and immortalized human liver endothelial sinusoidal cells (TRP3; RRID: CVCL_W908) has been described previously.[12,19] Osteosarcoma cells and MEFs were grown in DMEM supplemented with 10% FBS. TRP3 cells were grown in MCDB131 culture medium supplemented with 20% FBS, 10 mmol/L L-glutamine, 250 μg/mL cAMP, 50 μg/mL ECGS, 1 μg/mL hydrocortisone, and 1% penicillin-streptomycin. All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. All experiments were performed with mycoplasma-free cells. The human TRP3 cell line has been authenticated using short tandem repeat (STR) profiling within the last 3 years (see Materials & Methods in Supporting Information for details).

2.4 | Statistical analysis

Incidence of primary tumors and metastasis was analyzed by Fisher's exact test. For all quantitative analyses, comparisons between groups

were made with unpaired Student's *t* test unless stated otherwise. Two-tailed *P*-value ≤ .05 was considered as significant (ns: $P > .05$, $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, $^{****}P \leq .0001$).

3 | RESULTS

3.1 | Deletion of the *Cd44* gene affects tumor development and progression in *Nf2*-mutant mice

Double *Cd44*-knockout[20,21] and *Nf2*-mutant mice were generated to study the relevance of the CD44-Merlin interaction in vivo. Exon 2—corresponding to the FERM domain—of the *Nf2* gene was deleted to impair Merlin function and also mimic *NF2* mutations observed in patients.[4,8] As deactivation of both functional alleles of *Nf2* is lethal during embryogenesis,[22] heterozygous $Nf2^{\Delta 2/+}$-mutant mice[4] were used as a model for spontaneous tumor development. Tumor development and growth were compared between $Cd44^{+/+}$; $Nf2^{\Delta 2/+}$ and $Cd44^{-/-}$; $Nf2^{\Delta 2/+}$ animals and the tumors classified based on results from computed tomography and histochemistry (Figures 1A-D and S1). As reported earlier,[3,4] heterozygous mutation at the *Nf2* locus led to development of osteosarcomas at a high frequency (about 40%), as well as of fibrosarcomas and hepatocellular carcinomas at increased frequencies (3/29 and 4/29, respectively). Nearly all *Cd44*-positive osteosarcomas formed distant metastases, primarily in lung and liver. The incidence of osteosarcomas was similar in $Cd44^{+/+}$; $Nf2^{\Delta 2/+}$ and $Cd44^{-/-}$; $Nf2^{\Delta 2/+}$ mice; however, deletion of the *Cd44* gene significantly inhibited metastasis formation of osteosarcomas (Figure 1E,F). We also observed that deletion of the *Cd44* gene inhibited development of hepatocellular carcinomas and fibrosarcomas in the *Nf2*-mutant background (Figure 1G,H). Due to the very low incidence of these tumors in heterozygous $Nf2^{\Delta 2/+}$-mutant mice, their analysis will require generation of additional mouse models.

3.2 | *Cd44* expression is upregulated in osteosarcomas

Osteosarcoma was the predominant and life-limiting tumor type developed by *Nf2*-mutant mice. As the mechanism of osteosarcoma progression remains not well understood, we focused our further studies on this tumor type. In examining whether CD44 could have relevance for osteosarcoma, *Cd44* expression levels and protein localization were measured. Compared to normal bone, osteosarcomas showed significantly increased levels of total *Cd44* mRNA (Figure 2A), as well as of mRNA bearing variant exon v6 sequence and, to a lesser extent, of other isoforms previously implicated in tumor progression.[15,23] Immunofluorescent staining showed typical membrane localization of CD44 in primary and metastatic osteosarcomas (liver metastasis is shown in Figure 2B). In comparison with osteosarcomas, normal bone and liver tissue showed only very weak CD44 staining (Figure 2B). Thus, these in situ results demonstrating increased levels of CD44 in primary and metastatic osteosarcomas are compatible with the role of CD44 in osteosarcoma progression.

In further dissecting the role of CD44 in tumor-relevant processes, cell cultures of primary osteosarcomas were established from $Nf2^{\Delta 2/+}$ mice. To enable the analysis of CD44-dependent processes in genetically comparable tumor backgrounds, *Cd44* was deleted in *Nf2*-mutated osteosarcoma cells, using the CRISPR/Cas9 approach (Figure S2). Single-cell clones with downregulated *Cd44* expression were isolated by fluorescence-activated cell sorting (FACS) and inactivation of all functional alleles of the *Cd44* gene was subsequently confirmed by Sanger sequencing (Figure S2A). Figure S2B demonstrates fluorescent staining of CD44 in parental osteosarcoma cells and the absence of signal in one of the established *Cd44*-negative cell clones. Effective *Cd44*-knockout was also confirmed by immunoblotting (Figure S2C). Prior to CRISPR/Cas9-mediated *Cd44* knockout, genes encoding firefly luciferase and green fluorescent protein (GFP) were introduced into the cells to enable in vivo monitoring upon injection into mice. Also, expression of specific isoforms and mutants of *Cd44* was reconstituted in *Cd44*-negative clones using lentiviral vectors. Cells with expression levels of CD44 similar to wild-type cells were selected by FACS and tested by immunoblotting (Figure S2D,E). The wild-type osteosarcoma cells and cells with manipulated *Cd44* expression were then used in further functional assays addressing the role of CD44 in osteosarcoma cells.

3.3 | CD44 modulation does not affect tumorigenic properties of osteosarcoma cells

For validation of the osteosarcoma cells, we detected alkaline phosphatase activity (Figure S3A,B), which is considered a highly sensitive and very specific osteosarcoma marker.[24] Both *Cd44*-positive and *Cd44*-negative osteosarcoma cells were positive for alkaline phosphatase (Figure S3A,B). To characterize the tumorigenic potential of the generated cell lines, clonogenic assays in vitro and allotransplantation experiments in vivo were performed. The results demonstrate that CD44 does not affect the ability of osteosarcoma cells to form colonies—neither upon attachment to the cell culture dish (Figure 3A,A′), nor under anchorage-independent culture in soft agar (Figure 3B,B′). Furthermore, the osteosarcoma cells were injected subcutaneously into nonobese diabetic severe combined immunodeficiency (NOD-SCID) mice (Figure 3C,C′). Limited dilution assays demonstrated that 0.5×10^6 *Cd44*-positive osteosarcoma cells were required to initiate tumors in all NOD-SCID mice. At this cell number, *Cd44*-negative osteosarcoma cells were also able to induce tumors in all injected mice. Morphologically, the subcutaneous tumors were identical to the primary osteosarcomas from which the cells had been derived, independent of the *Cd44* gene status (Figures 1C and 3C′) and showed typical for osteosarcoma mineralization, as detected with Alizarin red S stain (Figure S3C). Thus, *Cd44*-positive and *Cd44*-negative osteosarcoma cells did not differ in their ability to form colonies and to establish tumors upon

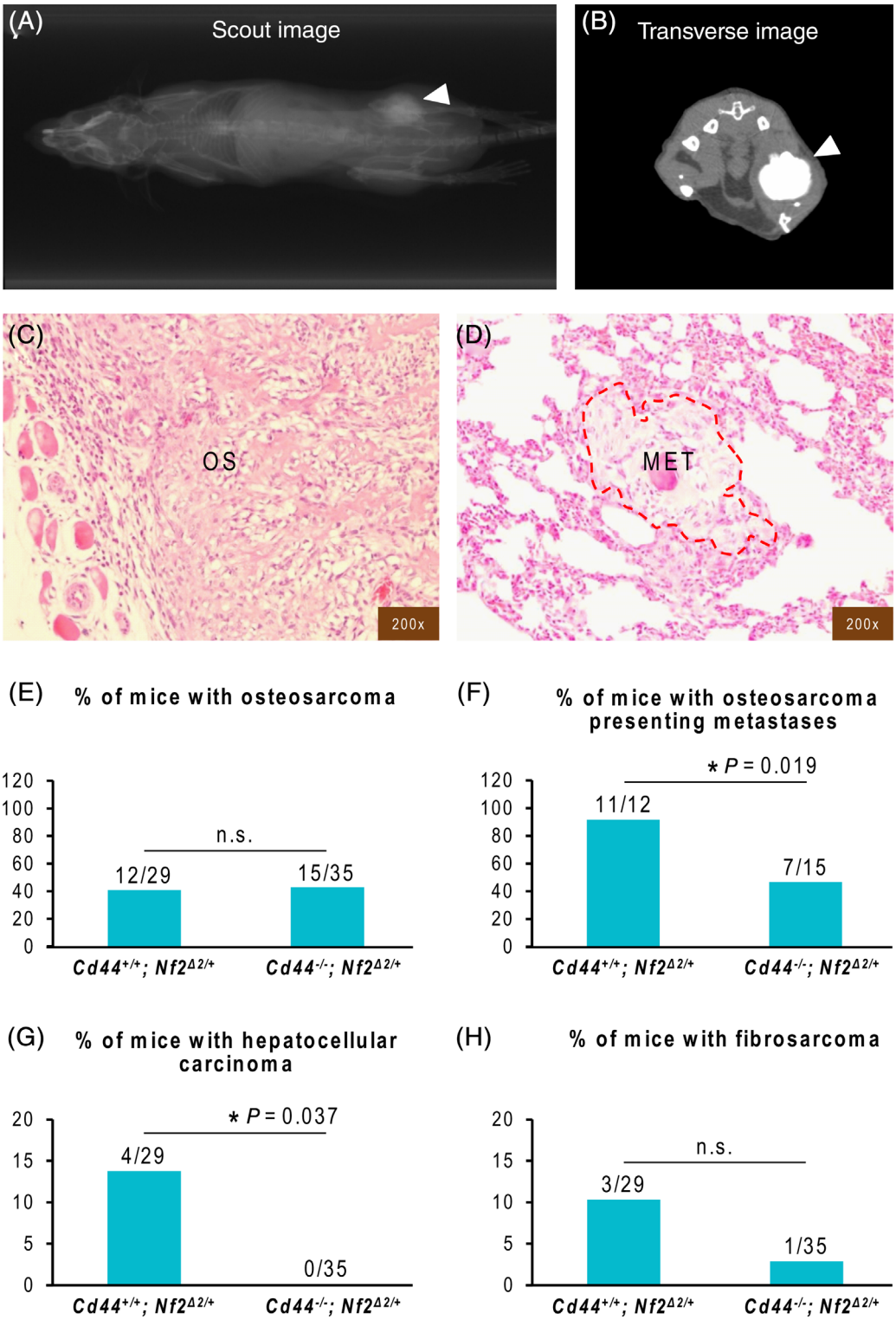


FIGURE 1 Influence of *Cd44* gene deletion on tumor development and progression in *Nf2*-mutant mice. Osteosarcomas were classified based on results from X-ray computed tomography (CT) and histochemistry. Representative scout (A) and transverse (B) images of endogenous tumors detected in $Nf2^{\Delta 2/+}$ mice. Localization of the tumor is indicated with arrowheads. Representative histological sections through primary osteoblastic osteosarcoma (OS; C) and lung metastasis (MET; D) of osteosarcoma stained with H&E. The photographs were taken using an Olympus BX 41 microscope. Comparison of incidence of osteosarcoma (E), osteosarcoma metastasis (F), hepatocellular carcinoma (G) and fibrosarcoma (H) in $Cd44^{+/+}$; $Nf2^{\Delta 2/+}$ and $Cd44^{-/-}$; $Nf2^{\Delta 2/+}$ mice. The numbers above the columns indicate the number of mice bearing tumors vs the total number of mice analyzed (E-G) or the number of mice with osteosarcoma metastases vs the total number of mice with osteosarcoma (F)

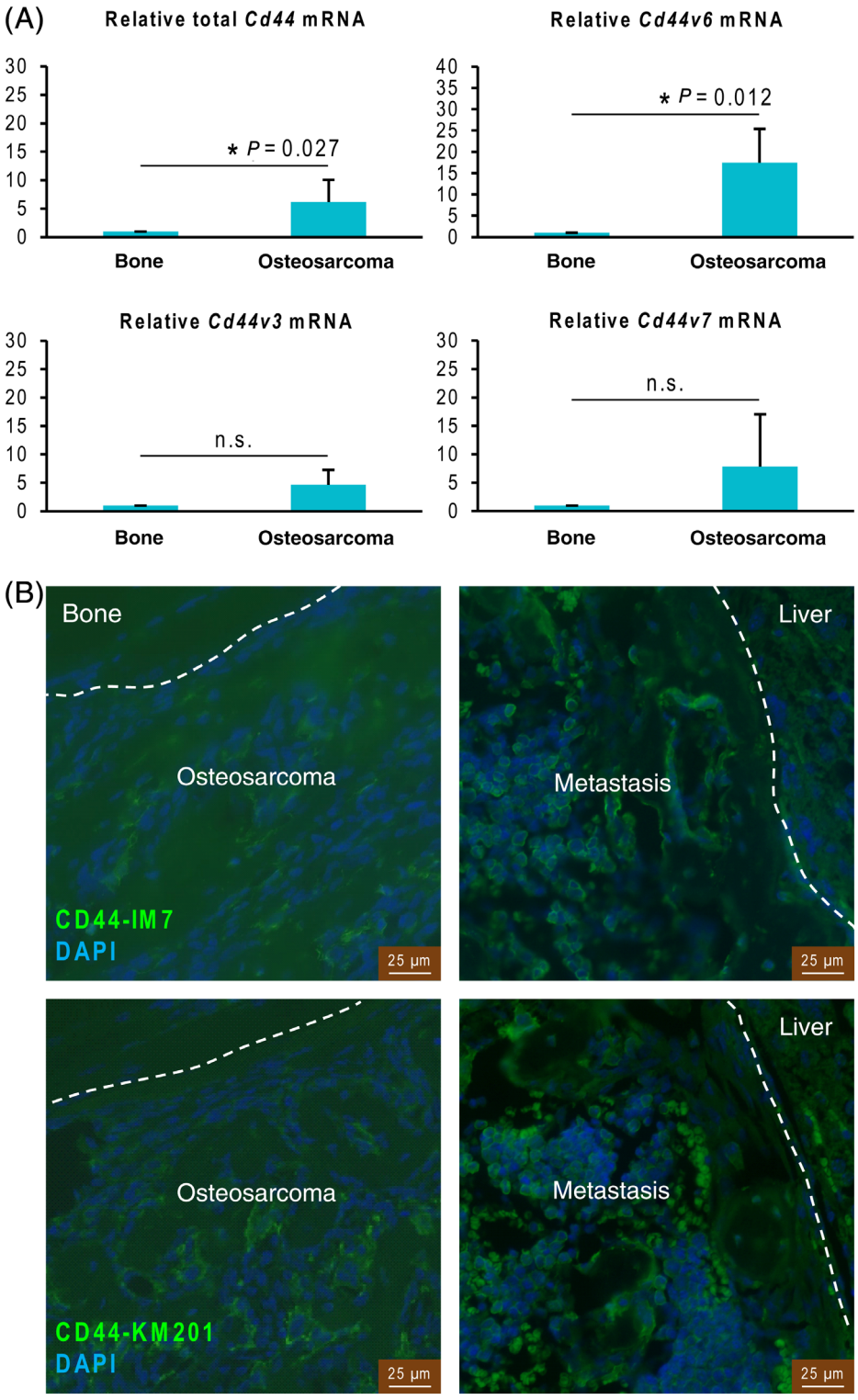
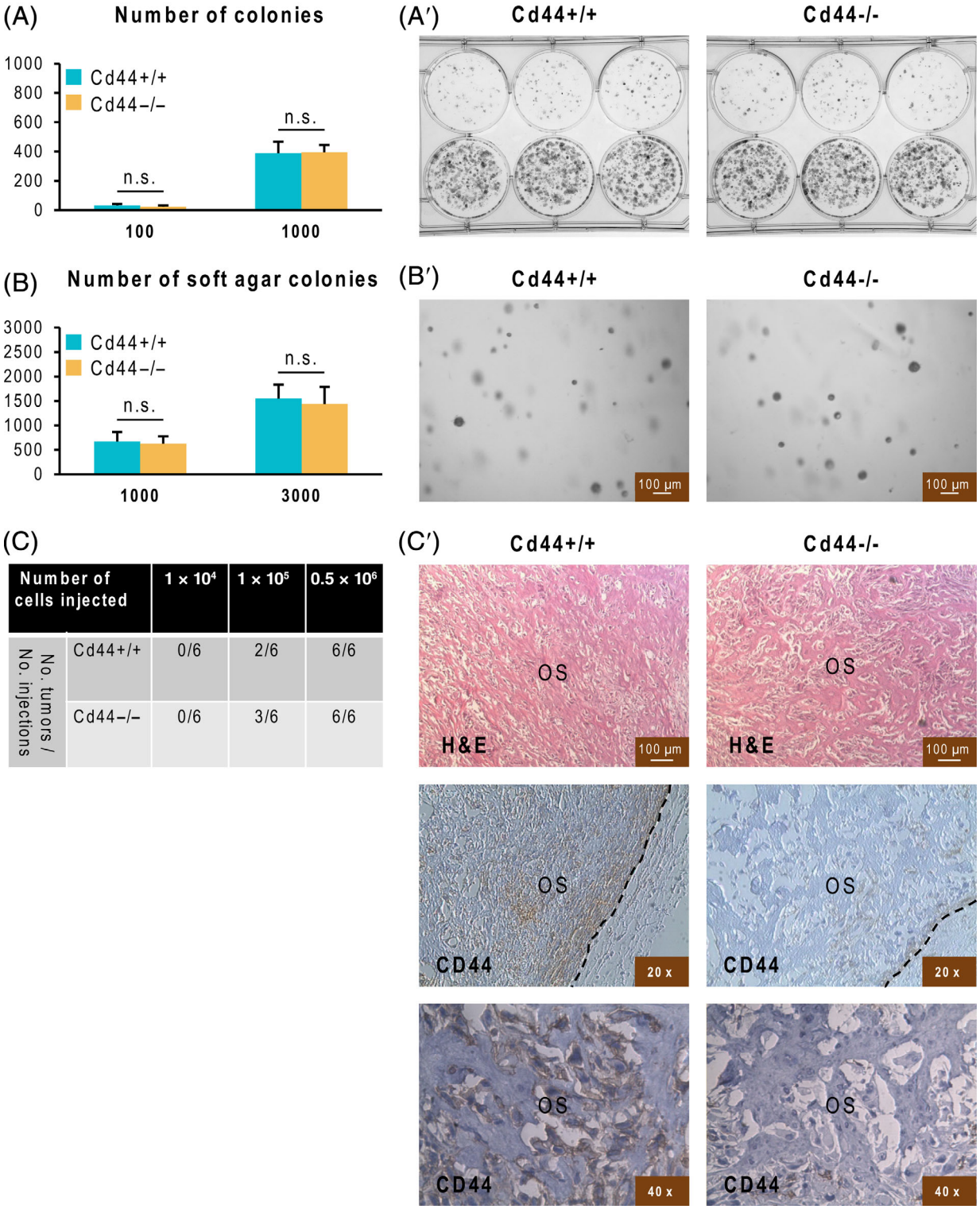


FIGURE 2 *Cd44* mRNA expression and protein localization in osteosarcomas. A, Quantification of mRNA levels in normal bone and osteosarcomas from four independent $Cd44^{+/+}$; $Nf2^{\Delta 2/+}$ mice. Used primers recognize either all *Cd44* transcript variants (total *Cd44* mRNA), or were specific for isoforms carrying exon v3, v6 or v7 sequences. B, Immunofluorescent localization of CD44 in primary and metastatic osteosarcomas. Two different primary antibodies recognizing independent epitopes of CD44 were used: clone IM7 (upper panel) and KM201 (lower panel). A secondary antibody conjugated to Alexa Fluor 488 was used for immunofluorescent detection. Cell nuclei were stained with DAPI. Fluorescent photographs were generated with a Zeiss Axio Imager ApoTome microscope. Scale bar: 50 µM

subcutaneous injection into immunocompromised mice. Moreover, no significant metastasis formation was observed after subcutaneous injection of tumor cells. Selected mice were then injected with higher tumor cell numbers (1×10^6) and monitored over a period of 10 weeks. Even under these conditions, only one of five mice injected with *Cd44*-positive osteosarcoma cells, and none of the three mice who received *Cd44*-negative cells, developed lung micrometastases (data not shown). Although not statistically significant, the result remains suggestive for a role of CD44 in promoting lung metastases. Given the low rate of metastases after subcutaneous injection, we decided to test the metastatic potential of osteosarcoma cells in a lung colonization assay.



Number of cells injected		1×10^4	1×10^5	0.5×10^6
No. tumors / No. injections	Cd44+/+	0/6	2/6	6/6
	Cd44−/−	0/6	3/6	6/6

FIGURE 3 Influence of *Cd44* gene deletion on tumorigenic properties of osteosarcoma cells. The ability of osteosarcoma cells to form colonies in conditions of attachment to substratum (A and A′) or in soft agar (B and B′) was measured. The histograms A and B show mean values of colony numbers ±SD from three independent assays. X-axis indicates number of cells seeded per well. Y-axis indicates the number of colonies. A′ and B′, Representative pictures of *Cd44*-positive and *Cd44*-negative colonies. C and C′, Measurement of tumorigenic potential in vivo. Indicated numbers of *Cd44*-positive or *Cd44*-negative osteosarcoma cells were injected subcutaneously into both flanks of NOD-SCID mice. Table C summarizes the incidence of tumor generation by *Cd44*-positive or *Cd44*-negative osteosarcoma cells. C′, Representative cross-sections of subcutaneous $Cd44^{+/+}$ and $Cd44^{-/-}$ tumors stained with H&E (upper panel). In lower panels, immunohistochemical localization of CD44 in subcutaneous osteosarcomas (OS) was determined using CD44 antibody, clone KM201. Photographs were taken using a Leica DFC290 microscope with indicated magnifications

3.4 | Deletion of *Cd44* gene in osteosarcoma cells reduces metastatic potential in vivo

As shown above, constitutive deletion of the *Cd44* gene reduced formation of osteosarcoma metastasis in $Nf2^{\Delta 2/+}$ mice (Figure 1F). To ascertain whether metastasis formation is dependent on CD44 expressed by tumor cells, rather than by the tumor microenvironment, the isolated osteosarcoma cells, with and without modified *Cd44* expression, were injected into the tail vein of *Cd44*-positive NOD-SCID mice and the colonization of lungs evaluated (Figure 4). Histological examination revealed the presence of lung metastases in all NOD-SCID mice injected with *Cd44*-positive cells (n = 6) and in only three of the eight mice injected with *Cd44*-negative cells (Figure 4B,C). In the NOD-SCID mice with detectable metastasis, the total number of metastases per lung section was significantly reduced by *Cd44* deletion (Figure 4D); however, CD44 exerted no influence on the size of metastatic colonies, confirming that CD44 did not affect tumor growth at metastatic sites (Figure 4E). Cells with reconstituted expression of *Cd44* were tested to prove that the observed effects were mediated by CD44. Interestingly, re-introduction of the smallest isoform of CD44—so-called standard isoform (Cd44s)—into *Cd44*-negative osteosarcoma cells was sufficient to reconstitute the ability of the cells to form lung metastases (Figure 4A-D). Our investigations clearly demonstrated that lung colonization depends on *Cd44* expression in the osteosarcoma cells. Moreover, the tail vein injection experiment revealed CD44-dependent steps in the metastatic cascade downstream of blood intravasation (by direct injection into the blood circulation). These CD44-dependent steps could include survival in the bloodstream, attachment to endothelial cells, subsequent transmigration through endothelium of blood vessels, as well as adjustment to the environment and survival at the secondary site. To distinguish between these options, we aimed at reconstituting these processes in vitro.

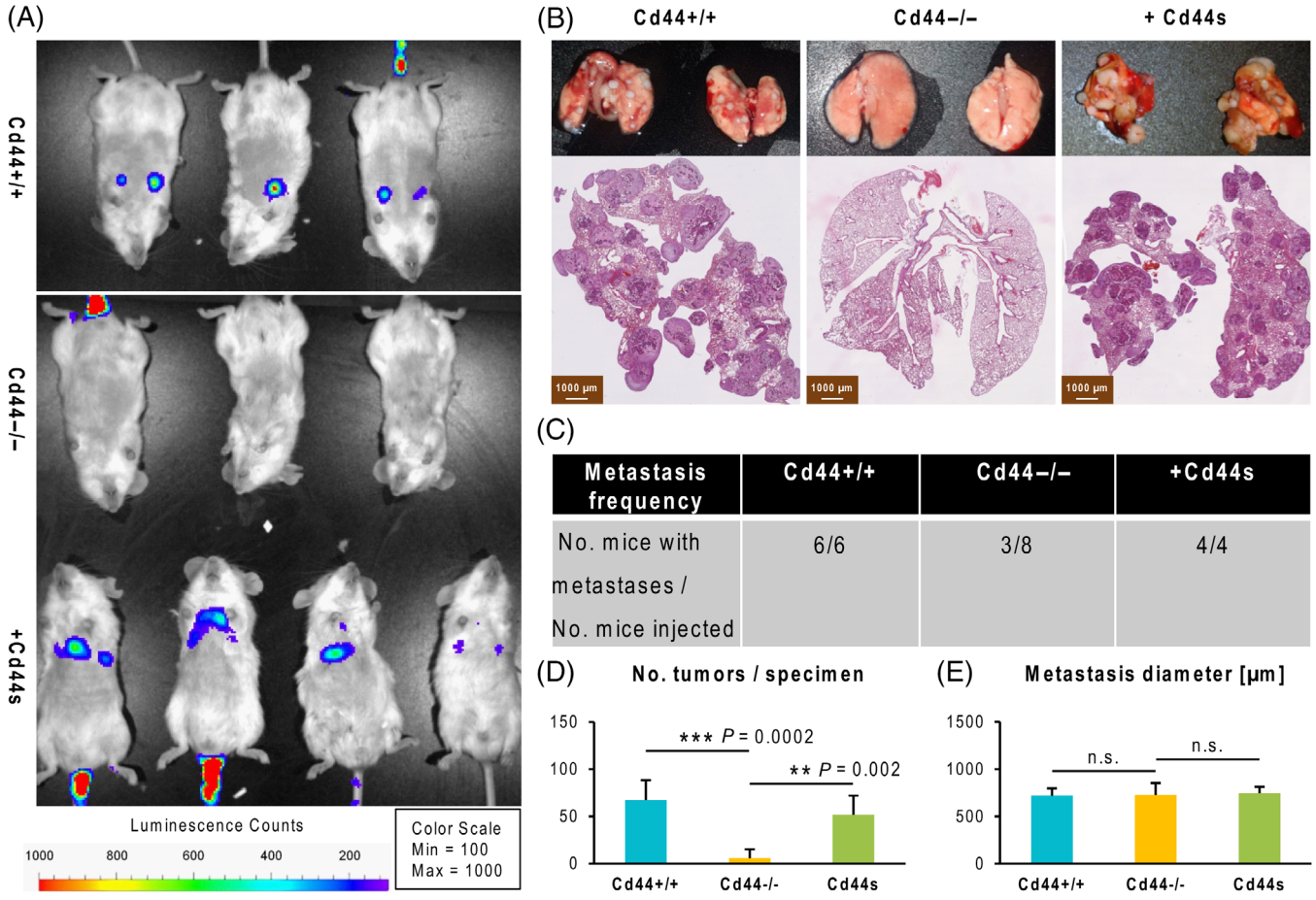


FIGURE 4 Metastatic potential of osteosarcoma cells with modulated *Cd44* expression. Metastatic potential of osteosarcoma cells was measured in vivo upon intravenous injection into NOD-SCID mice. 0.5×10^6 of *Cd44*-negative or *Cd44*-positive cells—wild-type or reconstituted with expression of standard isoform of CD44 (+Cd44s)—were injected intravenously into NOD-SCID mice. A, Development of metastases was monitored using the IVIS Spectrum advanced Imaging System. Measurement of the luminescence counts after 6 weeks is shown. B, Lungs were isolated from NOD-SCID mice and subjected to H&E staining. Representative photographs taken on NanoZoomer 2 OHT are shown. Scale bar is 1000 µm. C, Table summarizes number of NOD-SCID mice presenting metastases detectable on H&E specimens per total number of mice injected. The Fisher's exact test two-tailed *P*-value equals .031. D,E, The histograms show mean numbers of metastases per lung specimen (D) and average metastasis diameter (µm) ± SD (E) from three independent mice

3.5 | Deletion of *Cd44* gene has no influence on survival of osteosarcoma cells

At various times in the metastatic cascade, tumor cells are exposed to stimuli that could induce apoptosis. A large percentage of tumor cells die after entry into the bloodstream or the lymph, due to loss of cell-cell and cell-matrix contacts undergoing a specific type of apoptosis known as "anoikis".[25] In comparing the susceptibility of *Cd44*-positive and *Cd44*-negative osteosarcoma cells to anoikis, we mimicked the respective conditions in vitro by culturing osteosarcoma cells on ultra-low attachment surface plates (Figure S4A). Both cell types formed osteospheres and showed similar viability when grown in suspension—suggesting that CD44 does not influence the ability of osteosarcoma cells to evade anoikis. Another important step in metastasis formation consists of the adjustment to the environment and growth at the metastatic sites. As demonstrated above, the size of metastatic colonies in the lung was not influenced by *Cd44* gene status (Figure 4E). The reduced number of colonies created by the *Cd44*-negative tumor cells could result from an increase in apoptosis within the lung; an in situ TUNEL assay was performed to compare the level of cell death in metastatic colonies in the lung, to explore this possibility (Figure S4B,B′). Here, both *Cd44*-positive and *Cd44*-negative lung metastases displayed a similarly low level of cell death (Figure S4B,B′). Notably, there were no differences in expression of additional apoptosis markers, including cleaved caspase 3 (data not shown). These results support the conclusion that deletion of the *Cd44* gene does not alter the ability of osteosarcoma cells to counteract apoptosis during metastasis.

3.6 | CD44 promotes transendothelial migration of osteosarcoma cells by regulating binding ability of integrin VLA-4

An in vitro migration assay was conducted to examine a putative effect of *Cd44* gene deletion on cellular migration, an important hallmark of tumor cells during metastasis. The status of *Cd44* expression did not influence migration of osteosarcoma cells into cell-free gaps, or the organization of the actin cytoskeleton in cells migrating into a given gap (Figure S5). In addition to migratory properties, colonization of secondary sites requires efficient extravasation of tumor cells from blood vessels, which relies on efficient adhesion of tumor cells to endothelial cells. Indeed, an in vitro adhesion assay revealed that *Cd44*-positive osteosarcoma cells bound more efficiently to endothelial cells than their *Cd44*-negative counterparts (Figures 5A and S6A). Moreover, loss of *Cd44* significantly diminished migration of osteosarcoma cells through a confluent monolayer of endothelial cells (transendothelial migration; Figures 5B and S6B). As microvascular endothelial cells also express CD44,[26] one could speculate that adhesion of osteosarcoma cells depended on CD44-CD44 interaction, which could be mediated by the CD44 ligand, HA.[26] The role of HA binding was examined by testing *Cd44*-negative osteosarcoma cells stably expressing either the wild-type standard CD44 isoform (Cd44s), or a mutant of this isoform (HA-Mt: Cd44 R41A-Y42F) which is unable to bind HA.[27] Both wild-type and mutant CD44 reconstituted the adhesive properties (Figures 5A and S6A) and the transendothelial migration (Figures 5B and S6B) of *Cd44*-negative osteosarcoma cells equally well, indicating that intact HA binding site and thus HA binding was not required. Introduction of a variant isoform of CD44 (Cd44v4-v7) into *Cd44*-negative cells did not confer any advantage over the standard isoform (Cd44s; Figures 5A,B and S6A,B). The mechanism of CD44-dependent adhesion was further explored by examining the levels of integrin $\alpha 4\beta 1$ (VLA-4) and $\alpha L\beta 2$ (LFA-1) in osteosarcoma cells. Both molecules are major integrins on the surface of leukocytes, involved in adhesion to endothelial cells and subsequent transmigration.[28] Evidence exists that the same integrins contribute to adhesion and transendothelial migration of tumor cells.[29] However, osteosarcoma cells expressed only components of integrin VLA-4 (Figure S6D-F) and lacked expression of integrin αL which is a component of LFA-1 (Figure S6C). Of note, compared to *Cd44*-positive cells, *Cd44*-negative osteosarcoma displayed significantly reduced levels of integrin $\beta 1$ protein (Figure S6E). As expected, blocking antibodies directed against integrin $\alpha 4$ or $\beta 1$ prevented adhesion and transendothelial migration of *Cd44*-positive osteosarcoma cells, but had no impact on the already impaired behavior of *Cd44*-negative cells (Figures 5C,D and S6A,B). Moreover, transendothelial migration of *Cd44*-positive osteosarcoma cells was inhibited by a highly specific inhibitor of integrin VLA-4, BIO-1211 (Figures 5F and S6B). Consistently, compared to *Cd44*-negative osteosarcoma cells, *Cd44*-positive cells (either wild-type or with reconstituted expression of *Cd44s*) bound more efficiently to surfaces coated with the integrin VLA-4 ligand, VCAM-1, suggestive of positive regulation of integrin VLA-4 binding ability by CD44 (Figure 5E). We further dissected the mechanism of integrin VLA-4 regulation by CD44 by also analyzing mRNA levels of integrin $\beta 1$ and could demonstrate they were not different between *Cd44*-positive and -negative osteosarcoma cells (Figure S6G), implying that CD44 regulates integrin $\beta 1$ on the protein level. By inhibiting dynamin—a GTPase responsible for endocytosis in the eukaryotic cell—the levels of integrin $\beta 1$ could be partially re-constituted in *Cd44*-negative cells (Figure S6F). Co-immunoprecipitation allowed us to show that CD44 associated with integrin $\beta 1$ (Figure 5G). These results suggest that CD44 protects integrin $\beta 1$ from endocytosis and possibly from subsequent lysosomal degradation; albeit, further studies are required to reveal the precise molecular mechanism of integrin $\beta 1$ regulation by CD44. Combined, our findings indicate that CD44 acts on integrin VLA-4 expressed on tumor cells, promoting adhesion to and subsequent passage through endothelial cells in vitro (Figure 5H). We do not exclude though, a possible influence of CD44 on additional molecules implicated in transendothelial migration.

3.7 | *CD44* expression is upregulated in human osteosarcomas of metastatic origin

In the mouse model described herein, CD44 promoted osteosarcoma progression. Our group posited whether this might be relevant for

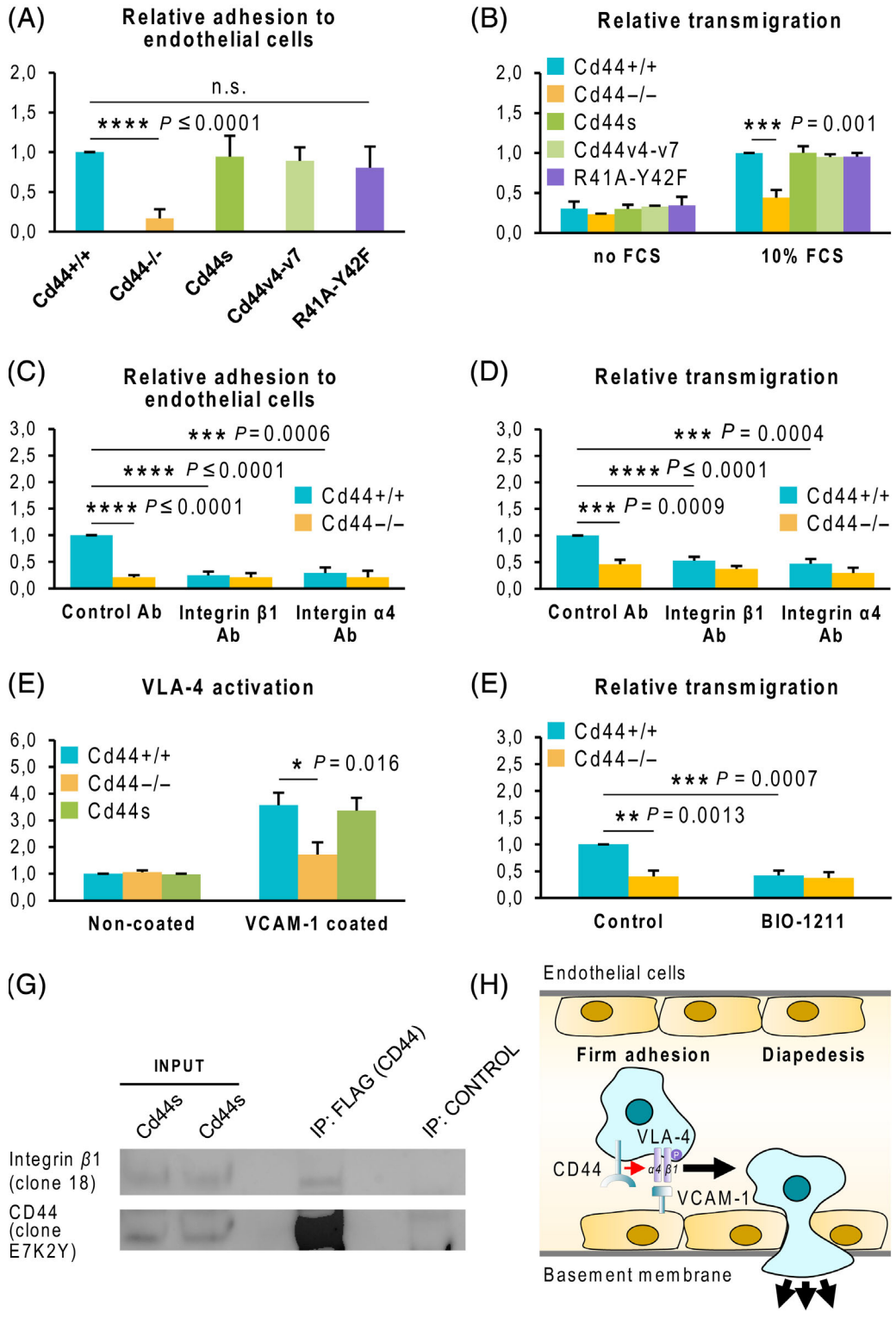


FIGURE 5 Testing adhesion and transendothelial migration of osteosarcoma cells. A and C, Testing adhesion of osteosarcoma cells to endothelial cells. Histograms show mean values of relative adhesion to endothelial cells ±SD from three independent adhesion assays. B, D and F, Transendothelial migration assay. Histograms show mean values of relative transmigration ±SD from three independent transendothelial migration assays. CD44s and CD44v4-v7 represent different CD44 isoforms; Cd44 R41AY42F is a mutant of CD44s unable to bind HA; BIO-1211 is a highly specific inhibitor of integrin $\alpha 4\beta 1$; Ab = antibody. E, Measurement of binding ability of integrin $\alpha 4\beta 1$. The histogram shows mean values of relative adhesion of osteosarcoma cells to surfaces coated with integrin $\alpha 4\beta 1$ ligand, VCAM-1 or noncoated plates ±SD from three independent adhesion assays. G, Integrin $\beta 1$-CD44 association shown by coimmunoprecipitation. Integrin $\beta 1$ was co-immunoprecipitated with FLAG-tagged CD44s. A representative blot out of three independent experiments is shown. H, Model of extravasation of osteosarcoma cells. CD44 upregulates levels of active integrin $\alpha 4\beta 1$ (Very Late Antigen-4, VLA-4) on tumor cells, which then binds to its ligand VCAM-1 on endothelial cells. This VLA-4-VCAM-1 mediated firm adhesion promotes subsequent passage of tumor cells across the endothelial monolayer (diapedesis)

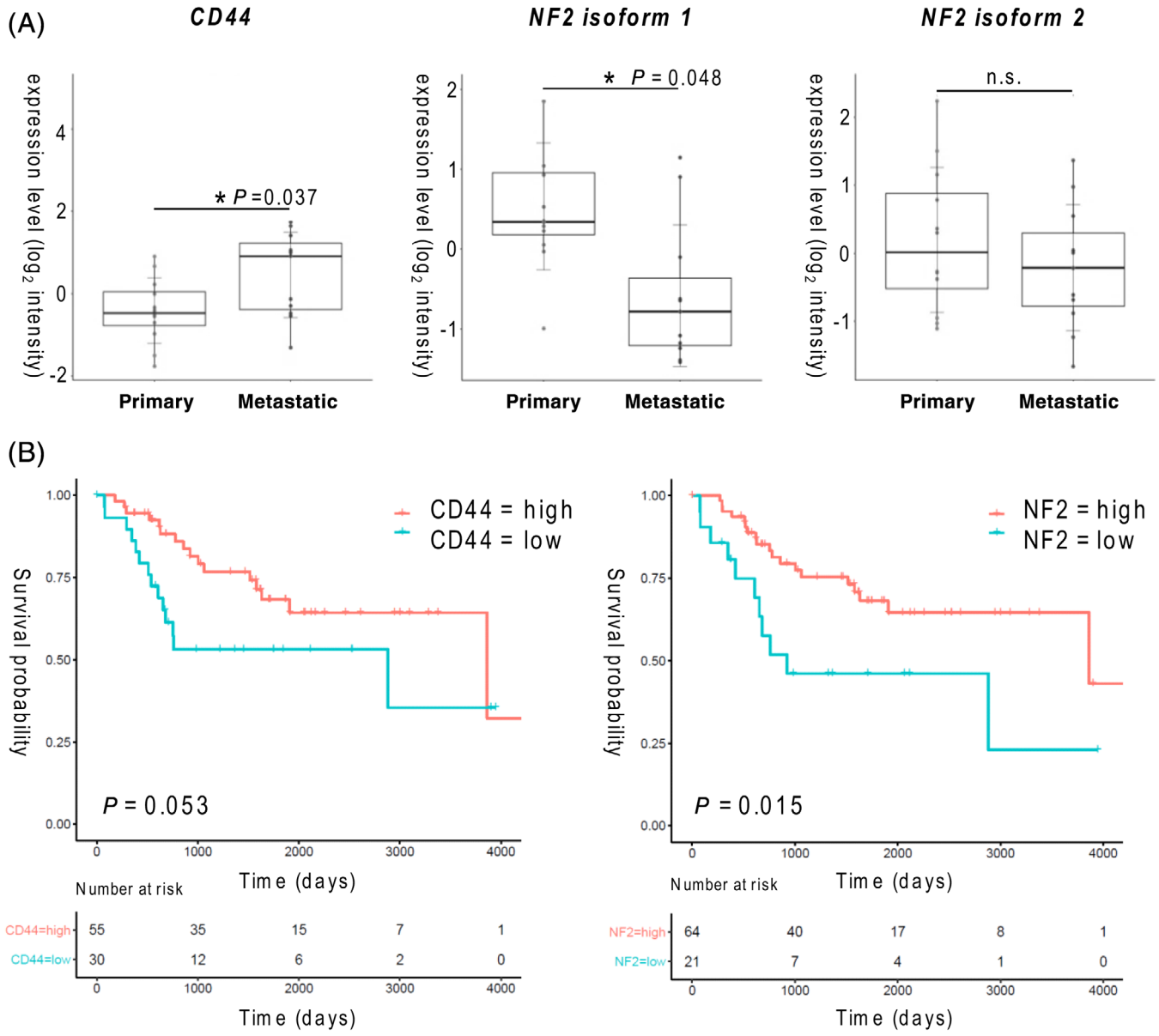


FIGURE 6 Expression of *CD44* and *NF2* in osteosarcoma patients. A, Comparison of *CD44* and *NF2* expression in primary and metastatic osteosarcomas. *CD44* and *NF2* isoforms 1 and 2 mRNA expression in 23 human osteosarcomas of primary and metastatic origin were analyzed using Applied Biosystems Gene Expression Array. The results are represented as Tukey's boxplots where box indicates the first and third quartiles, bar indicates median, whiskers indicate 1.5 interquartile range (IQR) and data beyond the end of the whiskers represent outliers. B, Kaplan-Meier survival curves show the correlation between *NF2* or *CD44* expression in 85 tumor samples and overall survival of osteosarcoma patients. Green: low expression, Red: high expression

human osteosarcoma. So, to obtain a notion on a possible significance for the pathogenesis of human osteosarcomas, expression profiles of *CD44* and *NF2* were analyzed in a panel of 23 human osteosarcoma samples of primary and metastatic origin, using a publically available database (https://www.ncbi.nlm.nih.gov/geo, GSE32981). Consistent with the findings described above, expression of *CD44* was significantly increased in the metastases, while expression of *NF2* isoform 1 and, to a lesser degree, of isoform 2 was decreased in metastases when compared to primary cancers (Figure 6A). In another clinical dataset (https://ocg.cancer.gov/programs/target/data-matrix) with available survival endpoints of patients, high expression of *NF2* in primary tumor significantly correlated with better survival (Figure 6B). Unexpectedly, high CD44 expression in primary tumor also appeared to improve survival. The latter data are not significant, but appear to be counter-intuitive and contradict recent meta-analyses studies.[30] Thus, larger studies are required to resolve this issue.

4 | DISCUSSION

The aim of our study was to evaluate the contribution of CD44 to the onset and progression of Merlin-deficient malignancies. Numerous

NF2 mutations in cancer patients are predicted to perturb the interaction of Merlin with CD44[8] and are expected to interfere with the tumor suppressor activity of Merlin.[9] Our study used *Nf2*-mutant mice as a model of spontaneous osteosarcoma development and progression. We hypothesized that disruption of the Merlin-CD44 association through loss of Merlin unveils putative tumor- or metastasis-promoting functions of CD44. To study the relevance of CD44 for osteosarcoma development and progression, *Nf2*-mutant mice were crossed to *Cd44*-knockout mice. Deletion of *Cd44* did not affect the incidence of osteosarcomas, but did significantly reduce osteosarcoma metastasis formation in the *Nf2*-mutant mice.

Interesting parallels to our results have been observed in mice heterozygotic for the tumor suppressor p53. In this model, deletion of *Cd44* did not affect osteosarcoma incidence, but abrogated formation of metastases.[31] However, Merlin and p53 interfere with CD44 in two unrelated manners—p53 represses CD44 expression by directly binding to its promoter,[32] whereas Merlin inhibits CD44 function by protein-protein interactions.[10-12] In the absence of tumor suppressors, CD44 repression might be released, prompting increased metastasis-promoting functions. Numerous reports have implicated CD44 in the progression of osteosarcomas and other types of cancer.[13,14,31,33-35] The action of CD44 in murine osteosarcoma appears to parallel osteosarcoma development in humans. CD44 is upregulated in metastatic subclones of human osteosarcoma and promotes lung metastasis formation in an allograft assay.[33-35]

As the spontaneous generation of osteosarcoma was similar in $Cd44^{+/+}$; $Nf2^{\Delta 2/+}$ and $Cd44^{-/-}$; $Nf2^{\Delta 2/+}$ mice, CD44 apparently does not influence osteosarcoma tumor initiation. Moreover, CD44 did not influence the general ability of the osteosarcoma cells to induce tumors upon subcutaneous injection into immunocompromised mice, or the colony-forming ability in vitro. The observation that CD44 does not promote osteosarcoma initiation is puzzling, possibly revealing an osteosarcoma specificity. In a number of other tumors, CD44 does play a role in tumor initiation. In the same *Nf2*-heterozygote mice, we found that deletion of *Cd44* inhibited the formation of hepatocellular carcinomas. Reinforcing our results, induction of hepatocellular carcinoma by genotoxic compounds is promoted by the presence of CD44.[18] In primary liver tumors, CD44 was identified as a marker of putative liver cancer stem cells; consistent with its role in liver tumor development.[36] In contrast, as shown by others, osteosarcoma tumor initiation may depend on molecules other than CD44, such as CD133.[35,37,38]

Knockout of *Cd44* reduced the rate of spontaneous osteosarcoma metastasis formation in *Nf2*-mutant mice. The prometastatic function of CD44 was also evident from allotransplantation experiments, wherein isolated osteosarcoma cells with modulated *Cd44* expression were intravenously injected into immunocompromised mice to determine lung colonization. This type of analysis restricts the examined processes, ignoring other putative metastasis-relevant actions of CD44 such as organ specificity of metastasis formation or role of the immune system. This approach, however, identified two important CD44-catalyzed steps relevant for the metastatic cascade—adhesion to endothelial cells and transendothelial migration. Both processes could be blocked by interfering with integrin VLA-4 on osteosarcoma cells. Compared to *Cd44*-negative cells, *Cd44*-positive osteosarcoma cells exhibited higher protein levels of integrin VLA-4 β1 subunit. This correlated with increased binding ability to VLA-4 substrate, VCAM-1, with increased adhesion to endothelial cells and transmigration. Our outcomes echo previous reports showing that CD44 induces integrin-mediated adhesion and transendothelial migration in breast cancer cell line by upregulation of integrin VLA-4.[29] Interestingly, in cell culture of osteosarcoma cells, CD44 improved chemoresistance, for example, doxorubicin resistance (data not shown). We have not pursued this observation further at this time.

Lung colonization upon intravenous injection—as well as endothelial cell adhesion and transendothelial migration in vitro—could be rescued by CD44s, the smallest splice form which does not contain any alternatively spliced exon sequences. Nevertheless, the *Nf2*-deficient osteosarcomas express CD44 splice variants whose expression has previously been correlated with progression of human osteosarcomas.[23,39-41] Thus, we cannot exclude that splice variant of CD44 might act on cancer-relevant processes other than trans-endothelial migration. While Merlin inhibits binding of CD44 to hyaluronic acid (HA),[11] the binding to HA was, unexpectedly,[42] not required for trans-endothelial migration in our osteosarcoma rescue experiments. Indeed, a mutant of CD44s with inactive (mutated) HA binding site rescued adhesive properties and transendothelial migration as efficiently as the wild-type protein. Thus, the smallest CD44 isoform, whose sequences are present in all known CD44 isoforms, mediates the promigratory effects independent of HA binding.

It should be noted that compensatory molecules may overtake some CD44 functions in complete knockout mice.[43] For instance, it has been demonstrated that intercellular adhesion molecule 1 (ICAM-1) replaces CD44v6 in liver regeneration of *Cd44*-null mice.[43] So, we cannot exclude that some of the CD44 functions in osteosarcoma tumors are compensated by other molecules established during embryogenesis. It is possible that additional tumor-relevant CD44 properties might be identified when interfering with CD44 function later in life. Interestingly, one of the earliest reports on *Cd44*-knockout mice showed a defect in myeloid-progenitor and lymphocyte migration/homing,[20,21] suggesting that the CD44-dependent migratory properties cannot be substituted by other factors. CD44 plays a dual role in homing of immune cells. A specially glycosylated form of CD44 (HCELL) serves as a ligand for E-selectin expressed on endothelial cells and participates in initial tethering and rolling of immune cells along the endothelial vessel.[44] However, this function may be compensated by the interaction of E- and P-selectin with their common ligand PSGL-1, and potentially other ligands.[45] In addition, CD44 positively regulates adhesiveness of integrins VLA-4 and LFA-1 on the surface of leukocytes.[28,46] Activated VLA-4 and LFA-1 bind to their respective ligands, VCAM-1 and ICAM-1, on endothelial cells, enabling firm adhesion and passage of leukocytes through endothelial cells.[28,46] Our data and a previous report[29] suggest that tumor cells might use similar mechanisms to pass from the bloodstream into other tissues and to form metastatic colonies. Also, the prometastatic function documented in our study could apparently not be substituted by other molecules in CD44-knockout mice.

The release of the CD44 metastasis-promoting function by loss of *NF2* does not necessarily require an enhanced expression level of *CD44* in primary tumors. Rather, reduction of *NF2* seems to suffice; previous studies demonstrated that the expression of *NF2* mRNA is down-regulated in a large panel of human osteosarcomas.[37] Moreover, as shown here, low *NF2* expression in primary tumors correlates with poor survival in osteosarcoma patients. Unexpectedly, high CD44 expression in primary tumors appeared to even improve survival (Figure 6B). The latter result is not statistically significant and contradictory to recent meta-analyses demonstrating that increased CD44 expression in primary tumors predicts poor patient survival.[30] Nevertheless, it should be taken into consideration that CD44 may participate in both growth and metastasis-promoting or metastasis-suppressive processes, which depend on cellular contexts and environmental conditions.[10,47] As such, it is possible that in the primary tumors CD44 exhibits tumor-suppressive function.[10,47] Nevertheless, analysis of the available database showed that *NF2* expression is decreased and *CD44* expression elevated in human metastatic osteosarcomas, in comparison to primary tumors. These results are compatible with the inhibitory function of Merlin and the promoting role of CD44 in human osteosarcoma progression. This leads us to propose that the data obtained using mouse models likely represent the mechanisms that drive human osteosarcoma progression.

Overall, the studies combining deletion of *Cd44* with mutations of tumor suppressors such as *Nf2* (present study), *Tp53* (24) or *Apc* (adenomatous polyposis coli),[48] suggest that repressing the tumor- and metastasis-related functions of CD44 might be a common mechanism required for tumor growth and progression. In the case of loss of Merlin, CD44 is released to promote transendothelial migration of tumor cells, one of the key steps in the blood-borne dissemination of osteosarcoma.

ACKNOWLEDGEMENTS

Sorting of cells on FACSAria was done by Flow Cytometry Core Facility at IMB, Mainz. Plasmid (pUB6/V5-His A, Invitrogen) encoding firefly luciferase gene was kindly provided by Dr Dennis Strand (University Medical Center of the Johannes Gutenberg University, Mainz, Germany). Plasmids for viral transduction were a gift from Dr Yan Cui. We also thank Monika Herr, Carolin Brandscheid, Birgit Pavelka and Silke Schulz for their excellent technical support and Johannes Peter for help with quantitative RT-PCR. Our study was supported by the Bundesministerium für Bildung und Forschung (BMBF, 01EO1003 to S. D.), Deutsche Forschungsgemeinschaft (HA 7860/1-1 to M. H., He551 to P. H., INST 371/33-1 and DA89/2-1 to S. D., MA 4443/2-2 to J. U. M. and MO 1421/5-1 to H. M.), Jung-Stiftung für Wissenschaft und Forschung (to P. H.) and Volkswagen Foundation Lichtenberg program (to J. U. M.).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA ACCESSIBILITY

Data supporting the findings of our study are available from the corresponding author upon reasonable request.

ORCID

Jens U. Marquardt https://orcid.org/0000-0002-8314-2682
Monika Hartmann https://orcid.org/0000-0001-6074-6961

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Ma J, Klemm J, Gerardo-Ramírez M, et al. Cluster of differentiation 44 promotes osteosarcoma progression in mice lacking the tumor suppressor Merlin. *Int. J. Cancer*. 2020;147:2564–2577. https://doi.org/10.1002/ijc.33144